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Topic Editor
Olivier M. Vanakker

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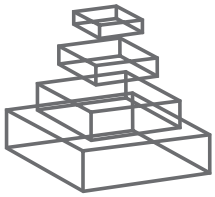

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SOFT TISSUE MINERALIZATION: AN ENLARGING DISEASE SPECTRUM WITH PSEUDOXANTHOMA ELASTICUM AS PARADIGM

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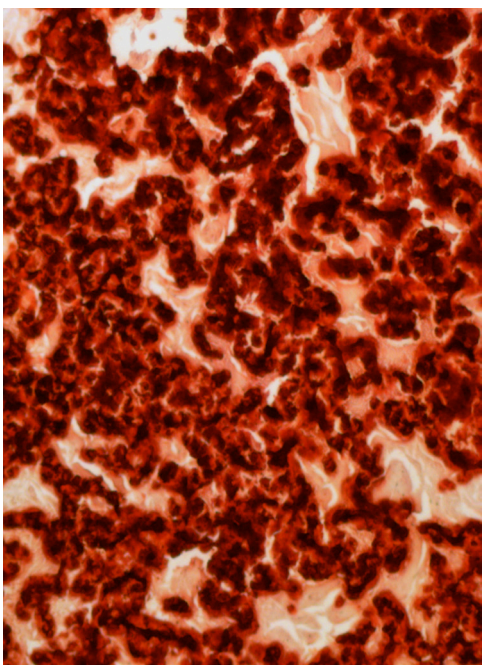

Alizarin red staining of the elastic fibre mineralization in the skin of a PXE patient

Pseudoxanthoma elasticum (PXE, OMIM# 264800) is an autosomal recessive connective tissue disorder in which fragmentation and calcification of elastic fibres results in skin (yellowish papules, skin laxity in flexural areas of the body), ocular (angioid streaks, subretinal neovascularisation and hemorrhage) and cardiovascular symptoms (occlusive artery disease). Caused by mutations in the ABCC6 gene (ATP-binding Cassette C6), PXE has always been regarded a paradigm for soft tissue calcification and ectopic mineralization diseases. An emerging amount of evidence suggests the presence of a disease spectrum encompassing PXE and a number of related phenotypes. Examples include the PXE-like syndrome with generalized cutis laxa and a coagulation defect (OMIM# 610842), caused by mutations in the gamma-carboxylase (GGCX) gene, the PXE phenocopies seen in haemoglobinopathies and acquired phenotypes such as PXE-like papillary dermal elastolysis. The recent descriptions of overlap phenotypes between PXE and PXE-like further strengthen the hypothesis of a disease spectrum.

The PXE phenotype is characterized by significant variability in disease severity. Although it was attempted to delineate genotype-phenotype correlations, it has proven difficult to find a clinically relevant relationship. The impossibility to predict the natural course in an individual patient hampers the use of individualized follow-up and leaves patients and their families with many uncertainties. To meet these limitations, it is assumed that modifier genes may play an

important role in PXE. Unfortunately, to date very few modifier genes with clinical utility have been identified.

Since the identification of ABCC6 as the causal gene for PXE in 2000, more than 300 mutations have been described and progress has been made in the characterization of the ABCC6 transporter. Novel insights have emerged on ectopic mineralization and extracellular matrix homeostasis in PXE and in general. Among the classic theories are the metabolic hypothesis, with vitamin K and vitamin K-dependent calcification inhibitor deficiency and the role of an as yet unidentified serum factor and the cellular hypothesis encompassing the role of mitochondrial dysfunction and chronic oxidative stress. Novel findings and observations - such as the involvement of BMP-related pathways and the potential role of extracellular nucleotides and adenosine - endeavor to bridge the space between the rare ectopic mineralization diseases and ECM calcification in more common disorders such as atherosclerosis or chronic kidney disease.

Despite growing insights in the clinical diversity and pathophysiological mechanisms of this disease spectrum, a significant number of questions remain. Physiologically, the identity of the substrate(s) of the ABCC6 transporter remains, 12 years after its identification, still puzzling; also the relation between deficiency of this transporter and the histological hallmark of PXE - calcification - is a mystery. The cellular mechanisms leading to ectopic mineralization are only just being unravelled and it can be hoped for that pathways relevant for common diseases such as atherosclerosis or age-related macular degeneration may in part also be of interest for the PXE spectrum. Clinically, the delineation of the phenotype of heterozygous carriers of one ABCC6 mutation and the identification of modifier genes are just some of the challenges that lie ahead. Finally, as knowledge increases, it will become more and more important to visualize how the clinical and pathophysiologiacl features of PXE, the related elastinopathies and ectopic mineralization disorders and ECM calcification in common diseases correlate with each other.

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Coordinated orphan disease research: yes, we can!

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Keywords: pseudoxanthoma elasticum, ectopic mineralization, ABCC6, orphan disease, connective tissue diseases, ENPP1

Research in the field of orphan diseases is confronted with several hurdles. Not only lack of knowledge and insufficient awareness for these disorders among the general public, politicians and yes, often also among the medical and scientific community, but also limited funding, dispersed research initiatives and seemingly unrelated datasets usually make it more difficult to progress in the understanding of these disorders. Ectopic mineralization diseases, with pseudoxanthoma elasticum (PXE) as a paradigm disorder, have been no exception to the rule in facing all of these hurdles. But at the same time, PXE has been a prime example of the efforts to overcome these barriers. And to a certain point, they can be overcome as this Special Topic of Frontiers in Systems Biology proves.

Soft tissue mineralization is the result of a delicate interplay of a large number of protagonists—transcription factors, calcification promoting and inhibiting proteins and enzymes, homeostatic cues (Schinke et al., 1999). It is precisely this complexity that makes soft tissue calcification so difficult to comprehend. At the same time, it is the prototype of a pathophysiological mechanism that cries out for a concerted action of different fields of expertise to build our insights into how it functions. Indeed, there are many faces to soft tissue mineralization, several of which are captured in PXE. This rare autosomal recessive connective tissue disorder is characterized by ocular, skin, and cardiovascular symptoms and can present important variability, particularly in the severity of symptoms (Vanakker et al., 2008). The histological hallmark of PXE is mineralization and fragmentation of elastic fibers. PXE was shown to be caused by mutations in the *ABCC6* gene, encoding an ATP-dependent transporter protein, though the physiological role and pathological consequences of ABCC6 remain unclear (Le Saux et al., 2001).

To achieve the ultimate goal of our research, a treatment to stabilize or cure PXE, there is a need to understand the function of the ABCC6 protein which is perturbed in PXE. Despite the considerable progress made in the past years, which is reflected on in this issue, many crucial questions remain unanswered. Concerted action also implies that we can look beyond our own protein of interest to other related proteins and diseases; their story—the difficulties as much as the successes—may give us valuable insights in how we can proceed in understanding our ABC-transporter of interest.

Next to mechanistic insights, the phenotype of patients should be defined in the greatest detail. The importance of this for the follow-up of patients goes without saying but cannot be seen independently of our quest to understand the mechanisms of disease. In this respect, recent findings on the unique characteristics of the PXE vasculopathy and advances in the ophthalmological features of PXE can give us important clues for the mechanisms that lie beneath (De Zaeytijd et al., 2010; Lefthériotis et al., 2011; Campens et al., 2013).

Finally, the molecular biology of these disorders needs be further refined. More than a decade after the identification of *ABCC6* as the causal gene for PXE, the molecular basis of PXE has become increasingly more complex then was initially conceived. Not only have a number of genes been identified which are associated with similar phenotypes, such as *GGCX* or *ENPP1*, but the variability of the PXE phenotype has also triggered the search for modifier genes (Vanakker et al., 2007; Nitschke et al., 2012). Though until now most of the secondary genes with a link to the phenotype should be considered susceptibility factors in stead of true modifiers, prudent progress has been made through the characterization of the role of *VEGF-A* variants in ocular neovascularization (Zarbock et al., 2009).

The drive behind research endeavors aiming to understand a group of rare disorders is the result of the enthusiasm of a group of dedicated scientists but—no less—also of patients and their representing organizations. Despite the use of animal models which can be very valuable for certain aspects of disease, the active involvement of patients in research, comprising clinical studies and experiments on tissues, remains invaluable for the future of PXE research. The advocacy groups of these patients play an important role in translating research findings to the patients but have often also been a thriving force to gather and stimulate researchers and physicians around the world.

In this special topic of Frontiers in Systems Biology, some of the leading scientists and physicians in the field of PXE and ectopic mineralization disorders have summarized the current knowledge in their particular field of expertise and share their thoughts on how further progress can be made. Basic science contributions cover the complexity of ABCC6 transporter function in (patho)physiological mineralization, the role of mesenchymal cells such as fibroblasts and mechanisms of remodeling in calcified vasculopathies. A perspective on the relevance of other ABC transporters for the study of ABCC6 and a methodological review on one of the innovative model organisms, the *Danio Rerio* or zebrafish, provide a balance between current and future endeavors. The molecular etiology is covered by reviews on the transcriptional regulation of the *ABCC6* gene and the role of modifier genes in PXE, while a contribution on *ENPP1* and the overlapping phenotype between PXE and Generalized Arterial Calcification of Infancy (GACI) makes the transition to clinical papers on specific aspects of the PXE retinopathy and vasculopathy. This research topic concludes with a perspective on the

current and future role of advocacy groups in orphan disease research.

The rarity of hereditary soft tissue mineralization disorders require a coordinated effort at all levels mentioned above to obtain faster advances in knowledge of disease pathophysiology and its rapid translation into the clinic. This will inevitably improve health care for patients suffering from these orphan diseases, but may also lead to valuable insights on more common disorders such as atherosclerosis and stroke as well as on the process of aging. Though maybe not always obvious at first sight, there is thus, unquestionably a relation with some of the global health care problems the world is facing today and will continue to face in the future. This Special Topic evidences how the work of several research groups on rare mineralization diseases can be brought together to reflect what is currently living in the field. At the same time, it should be an incentive to work further on a platform to integrate the research findings in these diseases retro- and prospectively. Only by uniting our attempts to gain insights in the complexity of soft tissue mineralization will we be able to get a grip on these diseases.

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The molecular and physiological roles of ABCC6: more than meets the eye

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Abnormal mineralization occurs in the context of several common conditions, including advanced age, diabetes, hypercholesterolemia, chronic renal failure, and certain genetic conditions. Metabolic, mechanical, infectious, and inflammatory injuries promote ectopic mineralization through overlapping yet distinct molecular mechanisms of initiation and progression. The ABCC6 protein is an ATP-dependent transporter primarily found in the plasma membrane of hepatocytes. ABCC6 exports unknown substrates from the liver presumably for systemic circulation. ABCC6 deficiency is the primary cause for chronic and acute forms of ectopic mineralization described in diseases such as pseudoxanthoma elasticum (PXE), β-thalassemia, and generalized arterial calcification of infancy (GACI) in humans and dystrophic cardiac calcification (DCC) in mice. These pathologies are characterized by mineralization of cardiovascular, ocular, and dermal tissues. PXE and to an extent GACI are caused by inactivating *ABCC6* mutations, whereas the mineralization associated with β-thalassemia patients derives from a liver-specific change in ABCC6 expression. DCC is an acquired phenotype resulting from cardiovascular insults (ischemic injury or hyperlipidemia) and secondary to ABCC6 insufficiency. *Abcc6*-deficient mice develop ectopic calcifications similar to both the human PXE and mouse DCC phenotypes. The precise molecular and cellular mechanism linking deficient hepatic ABCC6 function to distal ectopic mineral deposition is not understood and has captured the attention of many research groups. Our previously published work along with that of others show that ABCC6 influences other modulators of calcification and that it plays a much greater physiological role than originally thought.

Keywords: pseudoxanthoma elasticum, ABCC6, calcification, β-thalassemia, generalized arterial calcification of infancy, ectopic cardiac calcification

INTRODUCTION

In the absence of any systemic mineral imbalance, the calcification of soft tissues is defined as ectopic or dystrophic calcification. The latter term specifically distinguishes ectopic calcification that occurs in injured, damaged, and/or necrotic tissues. In contrast, elevated levels of calcium and/or phosphate due to abnormal absorption and/or secretion lead to metastatic mineralization. Whether ectopic or metastatic, abnormal calcification is typically composed of calcium phosphate salts, such as hydroxyapatite and can affect most soft tissues. However, the skin, kidneys, tendons, and cardiovascular tissues are particularly prone to this pathology.

Vascular calcification is a prevalent feature of aging and is also frequently associated with a number of common pathologies that include hyperlipidemia (atherosclerosis), chronic renal insufficiency, and diabetes, as well as certain infrequent genetic conditions. Calcification was long thought to result from passive precipitation of calcium and phosphate but it is now recognized as a complex tightly regulated development involving the osteoblastic differentiation of resident cells such as smooth muscle cells (SMCs), pericytes or adventitial myofibroblasts. It was also viewed as harmless, but calcification is in fact causative in the precipitation of cardiovascular events and mediating chronic damages to these tissues independently of the disease context that brings it about. More importantly, a proliferation of recent data has brought into light the many factors and the complex mechanisms that initiate and promote calcification *in vivo* (Atzeni et al., 2006). Despite the significant improvement of our understanding of calcification processes, how the delicate balance between normal osteogenic signals and ectopic mineralization in soft tissues is altered in pathological conditions is far from being understood.

Based on multiple evidence gathered in the last decade, the ATP-binding cassette (ABC) transporter ABCC6 has joined the list of calcification regulators as a new member. Indeed, reduced levels of ABCC6 protein or loss of ABCC6 function in the liver has been linked to four separate ectopic mineralization pathologies in humans and mice. (1) Pseudoxanthoma elasticum (PXE: MIM 264800) is an autosomal recessive disease characterized by

a slow and progressive ectopic calcification primarily affecting elastic fibers in dermal, ocular, and vascular tissues. Inactivating mutations in the *ABCC6* gene cause PXE (Bergen et al., 2000; Le Saux et al., 2000, 2001). (2) We have also shown, based on results obtained with an animal model, that the calcification phenotype in some β-thalassemia patients of Mediterranean descent (Baccarani-Contri et al., 2001; Aessopos et al., 2002), while not directly caused by *ABCC6* gene mutations (Hamlin et al., 2003), probably results from reduced levels of ABCC6 protein in the liver. (3) Furthermore, generalized arterial calcification of infancy (GACI) is another heritable disorder typically associated with mutations in the *ENPP1* gene. It now appears that a significant fraction of patients diagnosed with GACI are in fact carriers of *ABCC6* mutations while typical PXE manifestations can be associated with ENPP1 mutations in some young patients (Le Boulanger et al., 2010; Nitschke et al., 2012). (4) Recently, two groups of investigators have established that deficiency of the ABCC6 protein is linked to an acute calcification phenotype affecting the myocardium and the media of large arteries in several inbred strains of mice, including C3H/HeJ and DBA/2J (Aherrahrou et al., 2004, 2008; Meng et al., 2007). Because this peculiar phenotype occurs in response to a tissue injury it is referred to as dystrophic cardiac calcification or DCC.

The aim of this review is to summarize present knowledge on ABCC6 function and its possible molecular and physiological roles in various calcification pathologies where there clearly is more than meets the eye with this unique ABC transporter.

THE MOLECULAR CHARACTERISTICS OF ABCC6

ABCC6 IS AN EFFLUX PUMP

ABCC6 is a member of the large ABC gene subfamily C. In this group of transmembrane proteins, in addition to active transporters such as ABCC1, −2, −3, −4, and −5, there are also ion channel-forming proteins and ion channel regulators like ABCC7 (CFTR), ABCC8 and −9 (SUR1 and −2). The work of Ilias et al. (2002) and Belinsky et al. (2002) have shown that ABCC6 is a genuine active efflux transporter that uses ATP to effectively pump a glutathione conjugate of N-ethylmaleimide (NEM-GS) and also leukotriene C4 (LTC4). The affinity of ABCC6 for LTC4 was much lower than for NEM-GS and overall the maximal rate of NEM-GS transport was markedly inferior to two other well-known glutathione conjugate-transporting proteins, ABCC1 and ABCC2. Interestingly, ABCC6 failed to effectively transport 17-β-estradiol-17-β-D-glucuronide that is otherwise a recognized a substrate for ABCC3 (Hirohashi et al., 1999). The same study also found that the most effective inhibitors for ABCC6 were benzbromarone and indomethacin. These *in vitro* observations demonstrated that ABCC6 has a defined perhaps restricted, substrate specificity. Though the actual endogenous substrate(s) for this transporter and hence the contributing factor to ectopic calcification in PXE, β-thalassemia, GACI, and DCC remains unknown.

ABCC6 STRUCTURE

A 3D configuration of ABCC6 was successfully modeled using the X-ray structure of the *Staphylococcus aureus* Sav1866 export pump (Dawson and Locher, 2007). This prokaryote pump has already been used as template to build other homology models for the several human ABCC transporters such as ABCC1 (DeGorter et al., 2008), ABCC4 (Ravna and Sager, 2008), ABCC5 (Ravna et al., 2007), ABCC7/CFTR (Serohijos et al., 2008) as well as ABCB1 (Zolnerciks et al., 2007), ABCG2 (Li et al., 2007). Fülöp et al. have used their model of ABCC6 and the distribution of PXE-causing mutations to demonstrate the strict relevance of the transmission interface (ICL-ABC contacts) as well as the ABC-ABC domain contacts for the function of the transporter (Fulop et al., 2009). For more information on the structure/function relation of ABCC6, see the review of Arányi et al. in this issue.

CELLULAR LOCALIZATION OF ABCC6

ABCC6 protein has thus far been unambiguously associated with the basolateral membrane of hepatocytes in mice, rat, and human samples (Madon et al., 2000; Beck et al., 2003; Le Saux et al., 2011) as well as in the proximal kidney tubules and a kidney cell line (Sinko et al., 2003; Beck et al., 2005). Although a recent publication is unconvincingly attempting to challenge the already well-defined cellular localization of ABCC6 (Martin et al., 2012), it is interesting to note that PXE-causing missense mutations in ABCC6 do lead to defective cellular localization of the protein along with other functional alterations of the translated proteins (Le Saux et al., 2011). Although a relatively small number of mutants were analyzed, two possible outcomes of pathological mutations were described: (1) failure to use ATP causing transport deficiency and (2) the altered folding and/or protein stability leading to intracellular retention and reduced trafficking or a combination thereof. Therefore, the various structural and functional alterations of mutated ABCC6 presumably all result in the loss of physiological function, which provides a reasonable explanation for the observed lack of phenotype–genotype correlation in PXE (Le Saux et al., 2001; Chassaing et al., 2005; Pfendner et al., 2007).

ABCC6 SUBSTRATE(S) CONUNDRUM

Since the identification of *ABCC6* as the causative gene for PXE (Bergen et al., 2000; Le Saux et al., 2000), the question of its substrate(s) has thus far eluded all interested parties, and indeed the identification of an endogenous substrate(s) for an ABC transporter is not an easy task. Our knowledge to date is based on limited experimental data showing ABCC6's ability to use ATP to extrude conjugated metabolites *in vitro* (Belinsky et al., 2002; Ilias et al., 2002). Because the protein rests in the basolateral membrane of polarized cells, the prevailing hypothesis stipulates that the inability of this transporter to secrete its unknown substrate(s) for systemic circulation is the primary cause of the ectopic calcification phenotype of PXE, some GACI, and β-thalassemia patients and for DCC. This has prompted some to describe PXE as a metabolic disorder (Uitto et al., 2001), which could also apply to DCC and the ABCC6-dependent GACI and β-thalassemia cases. The metabolic hypothesis implies that the ABCC6 substrate(s) ultimately acts as an inhibitor of calcification in peripheral tissues. Does it work as a signaling molecule(s) that diffuses from the circulation into connective tissues where it contributes the normal phenotype of resident

cells such as fibroblasts, or SMCs (Quaglino et al., 2000; Boraldi et al., 2003)? Or does it interact directly with the extracellular matrix, collagen fibrils, elastic fibers thereby precluding oxidative stress (Garcia-Fernandez et al., 2008), abnormal assembly/deposition of collagen fibrils (Gheduzzi et al., 2003) and elastic fiber (Le Saux et al., 2006) calcification and fragmentation? As multiple structural and molecular alterations have been noted in PXE notably the so-called "collagen flowers," elastorrhexis along with abnormal glycoaminoglycans depositions (Lebwohl et al., 1993; Passi et al., 1996; Quaglino et al., 2000; Baccarani-Contri et al., 2001; Gheduzzi et al., 2003; Gotting et al., 2005) it is possible that a combination of molecular and cellular processes contribute to all the molecular and structural changes that have been described up to now (**Figure 1**). These interrogations clearly reflect a lack of sufficient data to even speculate as to the nature of the ABCC6 physiological substrate(s). And, in such situations, there are two possible approaches. Either one tests candidate molecules or performs a systematic search.

CANDIDATE ABCC6 SUBSTRATES

Reports by Vanakker and co-workers prompted several laboratories to test one such candidate molecule. The first of two publications described a PXE-like syndrome caused by a deficiency in gamma-glutamyl carboxylase (GGCX) (Vanakker et al., 2007) and a second article reported a deficit in the carboxylation (activation) of Gla proteins, including the calcification inhibitor Matrix Gla Protein (MGP), secondary to reduced vitamin K levels in the circulation of PXE patients (Vanakker et al., 2010). Because the common denominator between both reports is the vitamin K, the speculation that this co-factor could be an ABCC6 substrate was proposed (Borst et al., 2008) and tested in both indirect and direct experiments. The indirect approaches focused on phenotype correction in animal models of PXE, namely the calcification of vibrissae, with diets enriched in phylloquinone (vitamin K_1) and menaquinones (vitamin K_2). Three independent studies were carried out and all obtained negative results (Brampton et al., 2011; Gorgels et al., 2011; Jiang et al., 2011). These results were later confirmed by the direct observation that ABCC6 failed to

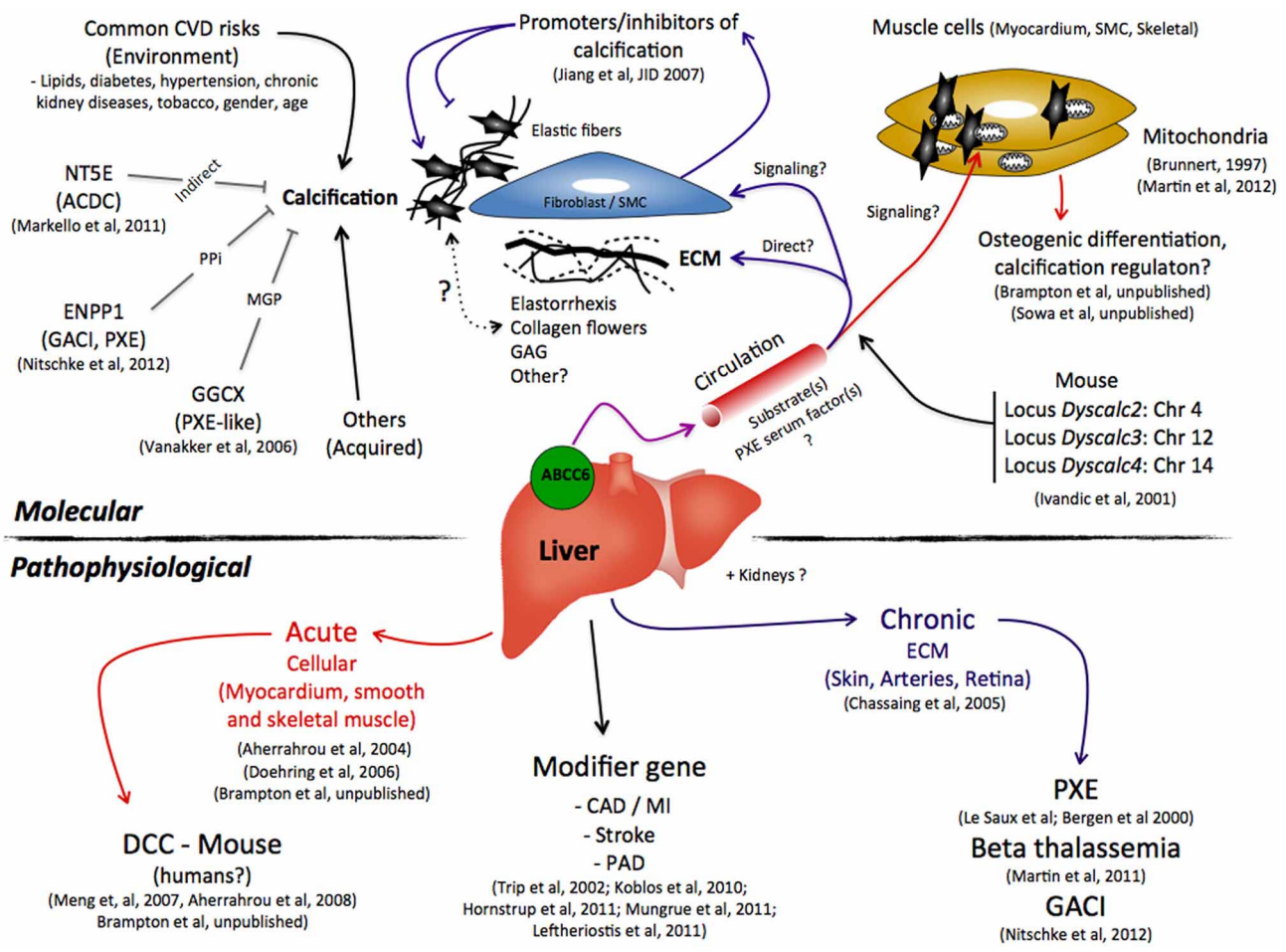

FIGURE 1 | A summary of the possible molecular and pathophysiological roles that ABCC6 plays in the ectopic calcification as seen in pseudoxanthoma elasticum, β-thalassemia, generalized arterial calcification of infancy, dystrophic cardiac calcification, and other cardiovascular diseases. Abbreviations: CAD, Coronary Artery Diseases; CVD, Cardiovascular Diseases; ECM, extracellular matrix; GAG, Glycoaminoglycans; MGP, Matrix Gla Protein; MI, Myocardial Infarction; PAD, Peripheral Arterial Disease; PPi, pyrophosphate; SMC, Smooth Muscle Cells.

effectively transport a glutathione conjugate of vitamin K_3, unlike ABCC1 (Fulop et al., 2011). Vitamin K_3 is an intermediate generated during the conversion of the dietary vitamin K_1 to the most abundant form vitamin K_2.

More recently, another candidate molecule was tested on the premise that adenosine has a key role in the etiology of arterial calcification due to deficiency of CD73 (ACDC), a rare condition that has phenotypic similarities with PXE (Markello et al., 2011). The ACDC mineralization primarily affects the large lower limb arteries (femoral, popliteal, and tibial arteries) and cartilage tissues but spares the main arterial vessels of the upper body. ACDC results from mutations in the *NT5E* gene that encodes the 5′ exonucleotidase CD73. This protein is a glycosyl phosphatidylinositol-anchored plasma membrane protein that generates extracellular adenosine, downstream of ENPP1 as part of the extracellular degradation pathway from ATP to adenosine and inorganic phosphate. The calcification in ACDC patients derives from an increase tissue non-specific alkaline phosphatase (TNAP) expression as a consequence of the lack of adenosine signaling. The partial overlap between the PXE and ACDC phenotypes prompted Markello et al. to suggest that adenosine might be an ABCC6 substrate (Markello et al., 2011). However, the limitations of this theory were quickly apparent as CD73 contributed to calcification only in specific arterial territories without dermal or ocular involvement (Leftheriotis et al., 2011b). Subsequent *in vitro* testing showed that indeed ABCC6 did not efficiently transport adenosine (Szabo et al., 2011).

THE SYSTEMATIC SEARCH FOR ABCC6 SUBSTRATE(S)

As for systematic approaches, at present three groups of laboratories are reportedly undertaking experiments using animal or human tissue extracts and fluids that potentially contain the ABCC6 substrate(s). Though no publications or public reports has been made thus far, two of these groups have based their technical approaches on the principle described by Krumpochova et al. (2012) which use of a combination of inverted membrane vesicles from *Spodoptera frugiperda* (Sf9) insect cells overproducing ABCC6 and liquid chromatography/mass spectroscopy-based metabolomics to determine the compounds transported into the vesicles (Personal communications, O. Le Saux, A. Varadi, and P. Borst). The third group of laboratories actively seeking the ABCC6 substrate(s) uses the methodology reported by Van et al. (2008): NMR spectroscopy with animal and/or human urine and serum (Personal communications M. Dean).

THE TIP OF THE ICEBERG

As of today, the sum of current publications only paints a relatively fragmented picture of the pathophysiological role of ABCC6 that seem to favor a signaling/hormone-type of activity for ABCC6 substrate(s) toward a variety of tissues, including but not limited to, the connective tissue. For example, the observations that Fetuin-A, MGP (Hendig et al., 2006, 2008b), vitamin K (Vanakker et al., 2010), osteogenic makers, and oxidative stress (Pasquali-Ronchetti et al., 2006; Garcia-Fernandez et al., 2008; Hendig et al., 2008a) are altered in the circulation of PXE patients points indirectly to unbalanced homeostasis of multiple organs and tissues. More direct evidence have shown the presence of pathological metabolite(s) in the serum of PXE patients and *Abcc6*$^{-/-}$ mice that promoted elastic fibers structural alterations and ectopic calcification (Le Saux et al., 2006; Jiang et al., 2007). Further, we have also described a significant expansion of the lymphatic vessel network in *Abcc6*$^{-/-}$ mice (Le Corre et al., 2012) that emphasize the systemic and global nature of the physiological changes that accompany and/or lead to the mineralization phenotype linked to ABCC6 deficiency. This calcification has always been presented as the hallmark of PXE and the major pathological development DCC and GACI, but it might in fact be only the visible tip of the iceberg.

THE PATHOPHYSIOLOGIES ASSOCIATED WITH ABCC6 DEFICIENCY

Little is known about ABCC6 other than it is a member of the large ABC gene subfamily C (multi drug resistance proteins) and encodes a transmembrane protein that uses ATP hydrolysis to export organic anion transport across cellular membranes. Work to characterize the physiological role of ABCC6 has first relied upon tissue expression profiles. ABCC6 is found primarily expressed in liver and the kidneys but is also in the intestine, the retina and to a much lesser extent in most other tissues including the lung, skin, and vasculature (Bergen et al., 2000; Beck et al., 2003, 2005). Although it is unclear if ABCC6 has a unique function distributed in multiple tissues or tissue-specific roles, its deficiency was first and foremost linked to calcification activation in PXE, β-thalassemia, and GACI in humans and DCC in mice. As off today, only two studies point to the liver as the tissue with the most relevance with respect to the calcification phenotype (Martin et al., 2011; Brampton et al., 2012).

PSEUDOXANTHOMA ELASTICUM

PXE (MIM 264800) is a recessive disease affecting the connective tissue and is defined by the calcification and fragmentation of elastic fibers (Chassaing et al., 2005). The PXE clinical signs primarily involve the skin, the Bruch's membrane of the eyes and cardiovascular system resulting in skin sagging and redundancy, visual impairment caused by retinal hemorrhages and peripheral arterial disease (PAD) associated with gastrointestinal bleeding and intermittent claudication. The localization of the transmembrane ABCC6 protein into the basolateral plasma membrane of hepatocytes and proximal kidney tubules cells is very relevant to its physiological function. It is its inability to secrete its unknown substrate toward the circulation that is the most likely cause of the ectopic calcification in PXE, which describes this disease as metabolic rather than a connective tissue disease.

β-THALASSEMIA

The second calcification phenotype linked to ABCC6 is in fact a phenocopy of PXE. β-thalassemia (MIM 141900) is a monogenic disorder caused by mutations in the β-globin gene that leads to the underproduction of β-globin chains. The stoichiometric excess of α-chains unbound to β-globin is unstable and precipitate in red blood cell precursors forming inclusion bodies. These are responsible for the intramedullary destruction of the erythroid precursors and the ineffective erythropoiesis

that characterize the β-thalassemias. Ineffective erythropoeisis in thalassemia major and certain intermedia patients results in considerable marrow expansion causing bone deformities and iron overload that is further exacerbated by frequent blood transfusions (Thein, 1998). The β-thalassemias are widespread throughout the Mediterranean, Africa, the Middle East, the Indian subcontinent, and Southeast Asia. In the past decade, it has become apparent that a large number of Mediterranean patients affected by β-thalassemia or sickle cell anemia also develop manifestations similar to PXE (Aessopos et al., 2002). β-thalassemia and PXE are distinct genetic disorders yet, the ectopic mineralization phenotype of seen in β-thalassemia patients is clinically and structurally identical to inherited PXE (Baccarani-Contri et al., 2001; Cianciulli et al., 2002; Farmakis et al., 2003, 2004). As we have established that the PXE-like mineralization in β-thalassemia patients arises independently of *ABCC*6 mutations (Hamlin et al., 2003), we hypothesized that the expression of the *ABCC*6 gene or the biological properties of its product could be disrupted in liver and/or kidneys as a secondary consequence of the hemoglobinopathy. We have tested this possibility by following the synthesis of ABCC6 in the liver and kidneys of a β-thalassemia mouse model ($Hbb^{th3/+}$). We found a progressive liver-specific downregulation in the *Abcc*6 gene expression and the corresponding protein levels. This downregulation became significant at 6 months of age and stabilized at older ages at ∼25% of the wild type protein levels. Studying the transcriptional regulation of the *Abcc*6 gene revealed that the main cause of the downregulation resided with the absence of a single transcription factor, the erythroid-specific NF-E2 from the *Abcc*6 promoter. Coincidentally, NF-E2 is a major transcription factor for the expression of several hemoglobin-related genes (Andrews, 1998). $Hbb^{th3/+}$mice did not develop spontaneous calcification as seen in $Abcc6^{-/-}$ mice probably because the ABCC6 protein decrease occurred late in life and/or was insufficient to promote mineralization in the $Hbb^{th3/+}$ mouse with the DCC-resistant C57BL/6J genetic background (Martin et al., 2011). Nevertheless, as the transcriptional regulation of the mouse and human *ABCC*6 genes is similar (Aranyi et al., 2005; Douet et al., 2006, 2007; de Boussac et al., 2010; Ratajewski et al., 2012), it is likely that the human β-thalassemia phenotype could induce comparable molecular changes leading to a suboptimal endowment in ABCC6 and increased susceptibility to ectopic mineralization in a PXE-like manner.

GENERALIZED ARTERIAL CALCIFICATION OF INFANCY

The third disease related to the ABCC6 deficiency is GACI, a rare autosomal-recessive disorder characterized by severe pathologic calcifications in the arterial media with intimal proliferation leading to vascular occlusion. GACI is associated with biallelic mutations in *ENPP1* and affected patients suffer from hypertension, severe myocardial ischemia and congestive heart failure. Most affected patients die within the first 6 months of life. The obvious overlapping mineralization phenotype between GACI and PXE led to a recent study that correlated the phenotype to genotype in GACI and PXE patients (Nitschke et al., 2012). This work found clinical manifestations unique to PXE in GACI patients carrying *ENPP1* mutations including angioid streaks and identical skin lesions. Additionally, mutations in *ABCC*6 accounted for a significant subset of GACI patients where no *ENPP1* mutation was found. The authors concluded that PXE and GACI are in fact diseases with overlapping characteristics reflecting a spectrum of severity in ectopic calcification rather than two distinct entities (Nitschke et al., 2012). However, the clear resemblances between the GACI and PXE phenotypes rather suggests an underlying convergence of ENPP1 and ABCC6 molecular pathways toward a common inhibition of mineralization somewhere upstream of the phenotypic manifestations, i.e., calcification because *ENPP1* deficiency leads to elastic fiber alterations typical of PXE in the vasculature, the skin, and ocular tissues (**Figure 2A**).

A somewhat similar convergence exists between the PXE-like syndrome in which *GGCX* mutations lead to insufficient MGP carboxylation (activation) and the classic inherited PXE (Gheduzzi et al., 2007; Vanakker et al., 2010). Unlike the PXE/GACI connection, the similarities between PXE and GGCX-associated syndrome represent a convergence of phenotypes rather than merging pathways (**Figure 2B**). The similarities are probably only due to the involvement of MGP deficiencies in both diseases. The patterns of elastic fiber mineralization are structurally different and the clinical evolution of the PXE-like syndrome diverges from PXE notably with a much greater laxity of the skin (Vanakker et al., 2007). A similar paradigm exists for other diseases such as *cutis laxa*, which can be either inherited through mutations in several genes related to elastic fibers or acquired through other processes affecting the structural integrity of these elastic fibers (Berk et al., 2012).

THE MURINE DYSTROPHIC CARDIAC CALCIFICATION

DCC

In recent years, two groups of investigators have established that ABCC6 deficiency is linked to an acute dystrophic calcification phenotype affecting the myocardium of several inbred strains of mice, including C3H/HeJ, 129S1/SvJ, and DBA/2J (Doehring et al., 2006; Meng et al., 2007; Aherrahrou et al., 2008). This murine phenotype is designated DCC. It is an autosomal recessive trait that was described several decades ago in animal models (Eaton et al., 1978; Everitt et al., 1988). It corresponds to a condition affecting cardiac tissues that can either occur spontaneously over the long-term or be initiated by specific dietary regime. Importantly, DCC can develop into an acute phenotype if triggered by a severe injury including surface freeze-thaw injuries (Ivandic et al., 2001; Aherrahrou et al., 2004) or ischemia (Brampton et al., 2012). In addition to cardiac tissues, the vasculature, notably the aortic artery (SMCs) as well as skeletal muscles, are also susceptible to developing calcification in response to the same type of severe injuries (Brunnert, 1997; Doehring et al., 2006). The major locus controlling this trait was first mapped to chromosome 7 (Ivandic et al., 2001) and subsequently was linked to a single *Abcc*6 gene mutation in C3H/HeJ mice that leads to a large constitutive decrease in ABCC6 protein levels in the liver (Aherrahrou et al., 2008). The same mutation is present in 129S1/SvJ and DBA/2J mouse strains while it is absent in C57BL/6J mice that are DCC-resistant. There are three additional

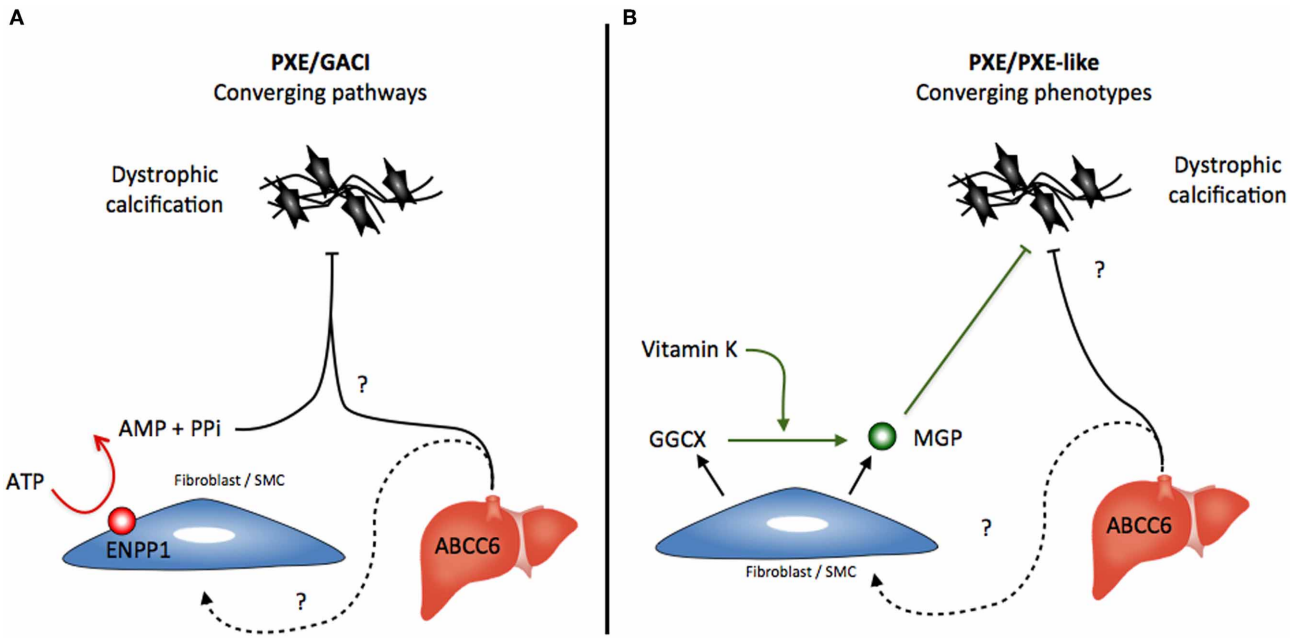

FIGURE 2 | Possible scenario for the converging (A) pathways that lead to the calcification phenotypes caused by ENPP1 and ABCC6 deficiencies and (B) the converging of the dissimilar PXE and GGCX-dependent PXE-like syndrome. Abbreviations: PPi, pyrophosphate; MGP, Matrix Gla Protein.

minor loci affecting the penetrance and the expression of the DCC phenotype that were mapped to chromosomes 4, 12, and 14 (Ivandic et al., 2001), though no specific genes have been identified as yet.

DCC-susceptible C3H/HeJ mice develop an attenuated version of the murine PXE phenotype as compared to the *Abcc6*$^{-/-}$ animals, while the DBA/2J mice present little or no manifestations (Smolen et al., 2012). It is interesting to note that the murine PXE manifestations recently reported in KK/H1J mice are remarkably severe and somewhat more extended than those of the *Abcc6*$^{-/-}$ mice (Li et al., 2012). More interesting is that all these strains of mice carry the exact same *Abcc6* gene mutation, which clearly underlines the influence of the genetic background in dystrophic calcification and thus the synergistic convergence of multiple gene-encoded pathways toward a common end-result that is the pathological mineralization of soft tissues (**Figures 1** and **2**).

In a study that we have now submitted for publication, we specifically explored the role of ABCC6 in the calcification response to cardiovascular insults. We used two different models of infarction, the non-ischemic freeze-thaw (cryoinjury) and coronary artery ligation. We first confirmed the propensity to cardiac mineralization of *Abcc6*$^{-/-}$ mice backcrossed into the DCC-resistant C57BL/6J background and thus the primordial role of ABCC6 in acute calcification. Furthermore, we have successfully modulated the calcification response to cryoinjury by varying the expression levels of ABCC6, either using heterozygous *Abcc6*$^{+/-}$ mice or the transient expression of the human ABCC6 protein in the liver of *Abcc6*-null mice. Moreover, the levels of *ABCC6* correlated with the amount and distribution of the regulators of mineralization osteopontin (OPN) and MGP but not osteocalcin (OC) clearly indicating that ABCC6 regulates cardiac calcification in conjunction with the local regulators of mineralization (Brampton et al., 2012).

Mitochondrial calcification

In 1997, Brunnert reported for the first time the precipitation of amorphous calcium within and around swollen mitochondria, a few hours after myocardial damage in the DCC-susceptible C3H/HeJ and DBA/2J mice. Subsequently, these electron-dense calcium deposits grew larger encompassing the entire cytoplasm and eventually the surrounding myofibrils (Brunnert, 1997). These findings prompted the author to hypothesize that dystrophic calcification may actually result from altered mitochondrial function. A suggestion that the recent report from Martin et al. has partially corroborated by showing that mitochondria in cardiac, liver, and renal tissues of *Abcc6*$^{-/-}$ mice were structurally altered and presented decreased respiratory capacities (Martin et al., 2012). Later, one of us (Aherrahrou, Z.) as well as others confirmed the formation of hydroxyapatite in cardiomyocytes mitochondria of C3H/HeJ mice (Aherrahrou, 2003). And interestingly, hydroxyapatite crystals in the mitochondria of cardiomyocytes were present equally in both C3H/HeJ mice and the DCC-resistant C57BL/6 animals in the first 24 h after cardiac injury. That changed in the following days when the growth of calcium crystal continued to spread in the C3H/HeJ mice while it subsided in C57BL/6J (Aherrahrou, 2003). These data suggested that in both strains, the mitochondria were first be able to sequester and concentrate calcium salts beyond solubility in the injured cells. And indeed, the key role that the ATP-dependent mitochondrial calcium sequestration exerts on intracellular calcium stores during cell death processes has largely

been documented (Chakraborti et al., 1999; Raha and Robinson, 2001). However, the runaway formation of hydroxyapatite crystals in C3H/HeJ mice, which also requires the involvement of phosphate ions, appears to be linked to an ABCC6-dependent deficiency of a calcification inhibition from within the mitochondria. It is unclear what this calcification inhibition might be and whether the rapid progression of crystal formation in C3H/HeJ mice is connected to the abnormal respiration function of mitochondrial as noted by Martin et al. (2012). Though, one would wonder if vitamin K_2, which has recently been described as an electron carrier in mitochondria (Vos et al., 2012), participates in this acute calcification phenotype especially in the light of the large discrepancy we previously described in the levels of circulating vitamin K_1 and K_2 between *Abcc6*$^{-/-}$ mice and C57BL/6J controls animals fed an enriched diet (Brampton et al., 2011).

DCC vs. PXE

One must distinguish the fundamental differences that exist between the induced calcification phenotype of DCC mice and the mineralization seen in the prototypic PXE disease. The latter phenotype is characterized by a long-term chronic and passive development of calcification that primarily affects the extracellular matrix (elastic fibers) over a period of time counted in years for humans and in months for mice. In contrast, the DCC phenotype is acute when induced and develops over a very short period of no more than 72 h, seemingly affecting only non-elastic muscular tissues. Moreover, the induced DCC calcification is intracellular, occurring within mitochondria. Both chronic and acute molecular pathways leading to calcification share the same molecular origin, i.e., ABCC6 deficiency. However, their mechanism of initiation and progression are clearly different which indicate that ABCC6 signaling (from the liver) has much broader ramifications toward a variety of cellular and molecular processes than we originally thought. Of note, the DCC phenotype has not (yet) been described in human PXE patients, though cardiac calcification occurring within cardiomyocytes mitochondria is not uncommon after severe myocardial damages following an ischemic event (Bloom and Peric-Golia, 1989; Lockard and Bloom, 1991).

VITAMIN K AND MGP-DEPENDENT INHIBITION OF CALCIFICATION

As discussed above, vitamin K or one of its derivatives is not a substrate transported by ABCC6, though the depleted levels of circulating vitamin K in PXE patients was thought to have a direct consequence in the carboxylation (activation) status of the calcification inhibitor MGP and the susceptibility to chronic calcification in PXE (Gheduzzi et al., 2007; Vanakker et al., 2007, 2010). We have shown that increasing the availability of vitamin K_1 or K_2 in peripheral tissues of *Abcc6*$^{-/-}$ mice did not significantly affect the MGP carboxylation status in the calcified capsule of vibrissae (Brampton et al., 2011). And going further, Boraldi et al. (2012) have now established that dermal fibroblasts isolated from PXE patients were able to uptake and use vitamin K_1 or K_2 for overall protein carboxylation as efficiently as healthy fibroblasts but not for MGP, which remained specifically undercarboxylated. As described above, we found that variable ABCC6 expression levels in the liver modulated the amounts of undercarboxylated MGP in calcified cardiac tissues (Brampton et al., 2012). Taken together these results suggested that MGP or the regulation of its carboxylation process and possibly OPN correlate with ABCC6 signaling and/or the ectopic calcification status.

ABCC6 AS A PHENOTYPE MODIFIER GENE

Infarct size

A recent study by Mungrue et al. (2011) suggested a relationship between ABCC6 function and infarct size under short-term ischemia reperfusion conditions (under an hour). In their studies, the authors noted the absence of any calcification in the myocardium of *Abcc6*-null mice suggesting that only a sustained cardiac injury lead to significant tissue necrosis and calcification in the absence of ABCC6 function (**Figure 1**).

Susceptibility to common artery diseases

The independent report by Köblös et al. as well as Trip and coworkers have both suggested that human heterozygous carriers of *ABCC6* mutations are more likely to develop complications resulting from cardiovascular incidents than the general population (Trip et al., 2002; Koblos et al., 2010). However, this is not without controversy as a much larger study based on 66,831 individuals has found no risk for ischemic heart diseases associated with the ABCC6 p.R1141X mutation (Hornstrup et al., 2011). Stroke is also a vascular-related condition frequently reported in PXE patients (Aessopos et al., 1997; van den Berg et al., 2000) but it could well be that strokes etiology in certain PXE individuals might not be related to ABCC6 deficiency as Hornstrup et al. could not statistically link cerebrovascular diseases with the most frequent ABCC6 mutation (p.R1141X). The occurrence of PAD in PXE (**Figure 1**) is less contentious as its precise characteristics are being carefully studied in a French cohort (Leftheriotis et al., 2011a). For more details on the prevalence and the peculiar presentation of PAD in PXE, see the review of Leftheriotis et al. in this issue.

CONCLUDING REMARKS

For many years, *ABCC6* was considered to have little more relevance than the causative gene for a rare heritable disease, PXE. However, we and others have now assembled a large body of data that clearly demonstrates that ABCC6 is far more important for cardiovascular health, in aging, and multiple diseased states than was initially thought. ABCC6 deficiency not only increases directly the susceptibility to connective tissue (elastic fibers) calcification in PXE, it also contributes to and aggravates the pathology of a significant fraction of GACI and β-thalassemia patients. This protein is the root-cause of an acute mineralization phenotype that when triggered dramatically affects the intracellular calcium homeostasis in muscle tissues. ABCC6 is now a fully fledged inhibitor of calcification that works at a systemic level (through the circulation) as part of a larger ensemble of local and general regulators of calcification. But, the loss of ABCC6 function also leads to various physiological changes other than calcification that we have only begun to describe and undoubtedly, more is yet to come.

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Fibroblast involvement in soft connective tissue calcification

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Soft connective tissue calcification is not a passive process, but the consequence of metabolic changes of local mesenchymal cells that, depending on both genetic and environmental factors, alter the balance between pro- and anti-calcifying pathways. While the role of smooth muscle cells and pericytes in ectopic calcifications has been widely investigated, the involvement of fibroblasts is still elusive. Fibroblasts isolated from the dermis of pseudoxanthoma elasticum (PXE) patients and of patients exhibiting PXE-like clinical and histopathological findings offer an attractive model to investigate the mechanisms leading to the precipitation of mineral deposits within elastic fibers and to explore the influence of the genetic background and of the extracellular environment on fibroblast-associated calcifications, thus improving the knowledge on the role of mesenchymal cells on pathologic mineralization.

Keywords: fibroblasts, PXE, PXE-like disorders, elastin, extracellular matrix, ectopic calcification, mesenchymal stromal cells

CALCIFICATIONS IN SOFT CONNECTIVE TISSUES

For long time, unwanted calcification, as that occurring in arterial calcification and in nephrolithiasis, has been considered as a passive, physical–chemical phenomenon representing a degenerative, irreversible process often associated with aging (Shroff and Shanahan, 2007). Many recent investigations, however, pointed out that calcium and phosphate precipitation are the result of complex and highly regulated series of events in which the balance between calcification inducers and inhibitory mechanisms may become severely deranged locally and/or systemically.

The deposition of calcium and phosphate in soft connective tissues can be classified into three major categories: metastatic calcification, dystrophic calcification, and calcinosis (Black and Kanat, 1985). Metastatic calcification occurs when calcium–phosphorous levels are elevated mainly due to metabolic/hormonal alterations and/or to tumor-associated complications. Dystrophic calcification takes place in the presence of damaged or necrotic tissue as in atherosclerosis. Calcinosis is generally associated to hypovascularity or hypoxia, it may involve a localized area or it may be widespread, causing secondary muscle atrophy, joint contractures and skin ulceration, with recurrent episodes of inflammation or infection (Boulman et al., 2005).

In most cases, mineral deposition develops in the extracellular environment without being localized on specific matrix components/structures. A typical example is represented by "calciphylaxis," a rare disease in which a generalized calcification is associated with thrombotic cutaneous ischemia and necrosis, thus causing a mortality rate ranging from 60 to 80% due to wound infection, sepsis, and subsequent organ failure (Arseculeratne et al., 2006; Hoff and Homey, 2011).

As clearly shown by several experimental findings and clinical observations, calcification may also occur in a number of genetic diseases, in metabolic disorders, such as uremia, hyper-parathyroidism, and diabetes, or in areas without adjacent inflammation or atherosclerosis. Due to the heterogeneity of factors contributing to the development of calcifications, many studies have been carried out in order to find common pathogenetic mechanisms and to identify possible druggable targets (i.e., single molecules and/or signaling pathways). Within this framework, numerous proteins have been identified to be involved in bone calcification as well as in ectopic mineralization. It has been suggested that an active and dynamic balance of pro- and anti-calcifying mechanisms occurs in both physiological and pathological calcification (Abedin et al., 2004) and that mesenchymal cells are key players, not only because they synthesize most of the mineral regulatory proteins, but also because they are responsible for the qualitative and quantitative characteristics of the extracellular environment, where apatite ectopic deposition arises.

ROLE OF PRO- AND ANTI-CALCIFYING FACTORS IN ECTOPIC CALCIFICATION

The role of calcitropic hormones, namely catecholamines, parathyroid hormone (PTH), and vitamin D or $1,25(OH)_2D_3$ on calcium metabolism is well-known (Rizzoli and Bonjour, 1998). However, in the last decade, a growing number of evidence is highlighting the importance of many other molecules as part of a composite network that, on the basis of common structural components, exhibits peculiar interactions and/or undergoes different regulatory mechanisms depending on the tissue [e.g., osteoprotegerin (OPG) or matrix Gla protein (MGP) in bone and vascular tissue; Kornak, 2011] and on the environmental context. In addition, these molecules can be produced and locally secreted by mesenchymal cells, or can diffuse from circulation to

peripheral tissues, where they may exert different effects on local calcium/phosphate homeostasis (**Figure 1**).

The mechanisms of calcification in skeletal and dental tissues have been under investigation since long time. One common feature to almost all physiological mineralization mechanisms seems the involvement of small (20–200 nm) membrane particles, called matrix vesicles (MVs). They bud off from the plasma membrane of mineralizing cells and are released into the pre-mineralized organic matrix serving as a vehicle for the concentration of ion or ion-enriched substrates, which are required for the activity of membrane-bound enzymes triggering mineral deposition at specific sites.

The observation that MV-like membranes are present in a number of ectopic calcification processes supports the concept that the mechanisms of vascular calcification are similar to those seen in normal skeletal development (Golub, 2011).

However, soft connective tissue calcifications activate a number of common pathways, but, at the same time they may exhibit local specific variations (e.g., in different tissue/body regions), possibly depending on the genotypic/phenotypic peculiarities of each mesenchymal cell type/subtype. Mineralization of dermal constituents, for instance, has been never associated with MVs, indicating that fibroblasts, differently from smooth muscle cells, can be responsible for mineral deposition, even in the absence of MVs. It could be, therefore, hypothesized that the role of mesenchymal cells in ectopic calcification may differ depending on the ability of the cell type to acquire a bone-oriented phenotype.

To further increase the complexity and the heterogeneity of mechanisms regulating pathologic calcification there are studies demonstrating that factors promoting or inhibiting ectopic calcifications are under the control of different genes, as in the case of extracellular pyrophosphate (PPi), a small molecule made of two phosphate ions, linked by an ester bond, that regulates cell differentiation and serves as an essential physiologic inhibitor of calcification by negatively interfering with crystal growth (Terkeltaub, 2001). The amount of extracellular PPi is regulated by two different gene products, as it originates either from the breakdown of nucleotide triphosphates by the ectonucleotide pyrophosphatase/phosphodiesterase (PC-1/ENPP1) or from the PPi transport by the transmembrane ankylosis protein homolog (ANKH). Consistently, either mutations or knockdown of these genes can induce hyper-mineralization of aorta (i.e., generalized

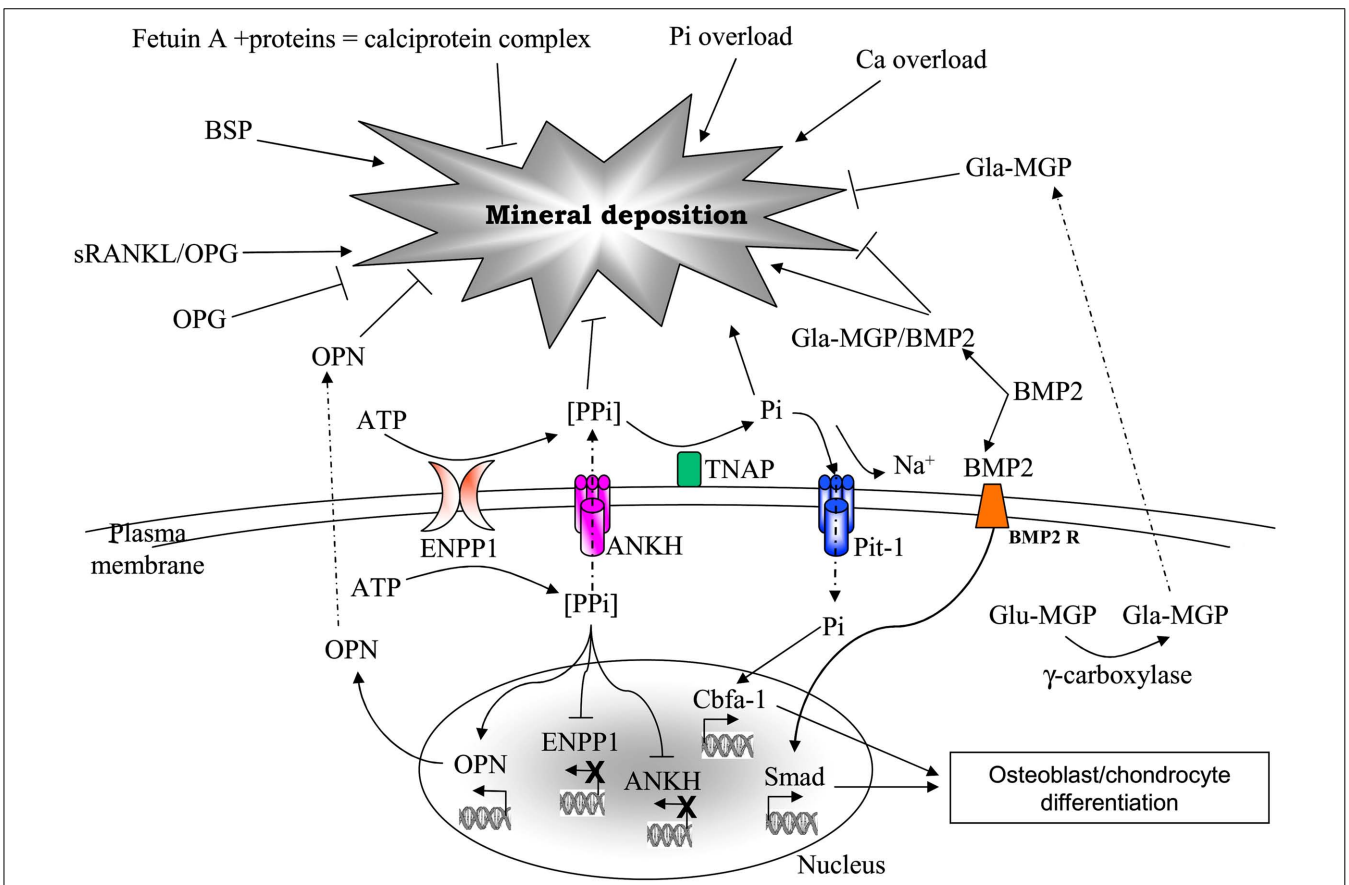

FIGURE 1 | Major factors involved in mineral deposition. AMP, adesosine monophosphate; ANKH, ankylosis protein homolog; ATP, adenosine triphosphate; BMP2, bone morphogenetic protein-2; BMP2R, bone morphogenetic protein-2 receptor; BSP, bone sialoprotein; Ca, calcium; ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase; Glu- and Gla-MGP, uncarboxylated- and carboxylated-matrix Gla protein; OPG, osteoprotegerin; OPN, osteopontin; Pi, inorganic phosphate; Pit-1, phosphate transporter-1; PPi, pyrophosphate; RANKL, receptor activator of nuclear factor kappa-B ligand; TNAP, tissue non-specific alkaline phosphatase.

arterial calcification in infants or GACI) and of ligaments and articular cartilage (i.e., chondrocalcinosis) in humans and mice, respectively (Okawa et al., 1998; Ho et al., 2000; Pendleton et al., 2002; Rutsch et al., 2003).

In the extracellular space, phosphate levels are directly controlled by tissue non-specific alkaline phosphatase (TNAP), a cell membrane-bound ecto-enzyme that increases inorganic phosphate availability by releasing it from a variety of phosphate-enriched substrates and, at the same time, reduces the levels of calcification inhibitors, promoting the hydrolysis of PPi and the dephosphorylation of osteopontin (OPN; El-Abbadi et al., 2009; Orimo, 2010). As a consequence, expression and activity of TNAP are associated either with physiological and pathological calcifications, although changes in enzyme activity may not be directly proportional to the level of mineralization, which is actually the result of the activity of many genes/proteins (Mendes et al., 2004). Consistently, increased TNAP expression and activity have been observed in CD73 deficiency, a disorder that, due to mutations in NT5E (a gene encoding for the membrane-bound ecto-enzyme that cleaves adesosine monophosphate (AMP) to adenosine and inorganic phosphate), is characterized by tortuosity and calcification of lower limb arteries and by mineralization of hand and foot joint capsules (StHilaire et al., 2011).

Alkaline phosphatase, similarly to other osteogenic genes, as type I collagen, osteocalcin (OC), and bone sialoprotein (BSP), can be transcriptionally regulated by bone morphogenetic protein 2 (BMP2; Kim et al., 2004), a powerful cytokine that, by activating Smad signaling pathways, promotes differentiation of mesenchymal cells into osteoblasts *in vitro* and induces bone formation *in vivo* (Rosen, 2009). Consistently, in fibrodysplasia ossificans progressiva endochondral ossification is triggered by BMP signaling in muscle cells (Shen et al., 2009). It has been demonstrated that treatment of smooth muscle and bone cell cultures with BMP2 (i) promotes osteogenic phenotype transition of smooth muscle cell (SMC; i.e., up-regulation of Runx2/Cbfa1 and down-regulation of SM22 expression), (ii) enhances elevated phosphate-induced calcification, but does not induce calcification under normal phosphate conditions. These results clearly indicate that phosphate transport via Pit-1 is crucial in BMP2-mediated calcification and in cell phenotype modulation (Suzuki et al., 2006; Li et al., 2008). Pit-1 is a type III sodium-dependent phosphate co-transporter that, through the activation of the Erk 1/2 signaling pathways, promotes calcification and favors changes of vascular smooth muscle cell (VSMC) toward an osteochondrogenic phenotype. Moreover, it has been shown that Pit-1 may exert effects also at the endoplasmic reticulum level. Studies on VSMC revealed that, when these cells are treated with platelet-derived growth factor (PDGF), they exhibit increased Pit-1 expression and it has been hypothesized that Pit-1 may regulate anti-calcification proteins (such as MGP), as well as kinases able to phosphorylate secreted matrix proteins (such as OPN; Villa-Bellosta et al., 2007). In addition, recent evidence has been provided that Pit-1 have other unexpected functions in cell proliferation and embryonic development (Lau et al., 2010), thus emphasizing the regulatory importance of phosphate in cell behavior.

Another protein favoring calcification is BSP, originally identified in bone and at sites of ectopic calcification in blood vessels, heart valves, and skeletal muscle. It is involved in the early stages of mineralization and bone desorption, since it is immobilized on collagen fibrils where the poly-glutamic acid sequences of BSP act as possible nucleation sites for hydroxyapatite crystals. BSP, together with another bone phosphoprotein named OPN, can modulate crystal shape by adsorption on a specific face of the crystals (Ganss et al., 1999).

Osteopontin is in fact a highly phosphorylated and glycosylated secreted protein originally discovered in bone, but identified also in calcified vascular lesions (Giachelli et al., 1993), where it may counteract apatite deposition by physically inhibiting crystal growth (Boskey et al., 1993) and/or by up-regulating the expression of genes, as carbonic anhydrase II, favoring mineral absorption, mainly through the activation of macrophage activities (Rajachar et al., 2009). These properties depend on the level of OPN phosphorylation as well as on the targeted tissue (i.e., bone or soft connective tissues; Jono et al., 2000). Recent evidence puts forward that OPN is actually a multi-functional protein able to interact with several integrin receptors, thus playing a role in activation, adhesion and migration of many cell types, not only in tissue mineralization and tumor growth, but also in inflammation (Jahnen-Dechent et al., 2008). These broad biological activities underlie the presumed role of OPN in the pathogenesis of cardiovascular diseases, including atherosclerosis and abdominal aortic aneurysm (Giachelli and Steitz, 2000), thus paving the way toward the clinical use of OPN plasma levels as biomarker of inflammation and as predictor of the risk for cardiovascular complications (Cho et al., 2009).

Another interesting protein is OPG that serves as a decoy receptor for the receptor activator of nuclear factor κB-ligand (RANKL) and acts as an inhibitor of osteoclastogenesis and osteoclast activation by blocking RANK activation (Boyle et al., 2003; Van Campenhout and Golledge, 2009). As demonstrated in the KO animals, the absence of OPG is associated with osteoporosis as well as with calcifications of aorta and renal arteries (Bucay et al., 1998). Therefore, within the vasculature, OPG may exert a protective role toward ectopic calcification down-regulating alkaline phosphatase activity (Van Campenhout and Golledge, 2009). Consequently, serum OPG levels have been significantly associated with the presence of coronary artery disease (Jono et al., 2002), suggesting that OPG may represent a strong risk factor for mortality in dialysis patients (Morena et al., 2006).

Matrix Gla protein belongs to a large family of proteins whose maturation requires vitamin K-dependent carboxylation of glutamyl residues (Schurgers et al., 2007; O'Young et al., 2011). It is considered the most active anti-calcifying agent in vessels (Shanahan et al., 1998; Price et al., 2006), but it is actually produced by several cell types, among which VSMCs, osteoblasts, and fibroblasts (Davies et al., 2006; Park et al., 2006). The phenotype of *MGP−/−* mice is characterized by arterial calcification and by arterial-venous malformations (Yao et al., 2011), suggesting that MGP has roles in connective tissue development and homeostasis, as well as in preventing ectopic calcification. The corresponding human disorder is Keutel syndrome (Munroe et al., 1999), characterized by enhanced mineralization of the growth plate cartilage leading to reduced longitudinal growth and osteopenia, as well as calcification of the elastic lamellae in the arterial wall due to

chondrocyte transformation of VSMCs. This aberrant cellular differentiation could be related to the ability of MGP to act as a regulator of BMP2 in a dose-dependent manner. Low levels of MGP relative to BMP2 may result in mild enhancement of BMP2 activity, whereas intermediate levels would inhibit and high levels strongly increase BMP2 activity (Zebboudj et al., 2002). These findings clearly demonstrate the complexity of the mechanisms regulating ectopic calcifications, which are dependent not only on the presence/absence of specific proteins and on their activity (due, for instance, to post-translational modifications as phosphorylation and carboxylation), but also on the ratio among different molecules.

A similar vitamin K-dependent carboxylated protein is OC that, synthesized by osteoblasts, is deposited into bones, where it controls the size and the speed of crystal formation and acts as a chemo-attractant for osteoclasts (Roach, 1994). Moreover, it is released into circulation, where it is also used as a biomarker of bone metabolism and vitamin K status. The increase of undercarboxylated OC (ucOC) levels in the aging population led to the hypothesis that vitamin K insufficiency might be related to the calcification paradox (namely age-dependent bone loss associated to vascular calcification), however, clinical trials did not provide support to the hypothesis that vitamin K supplementation will reduce bone loss or fracture risk. Very recent results from *in vitro* and *in vivo* experimental models indicate that ucOC is an active hormone with a positive role on glycemia. If this hypothesis will be proved also in humans, vitamin K supplementation, by decreasing ucOC, might exert unknown, possibly detrimental, effects on glucose metabolism (Gundberg et al., 2012). This hypothesis sustains the importance to perform broad and extended investigations when diet regimens, supplemented with even physiological/endogenous components, are used as therapeutic tools. Interference with a specific molecule may in fact have "domino" consequences on many other, apparently unrelated, pathways.

Finally, a novel γ-carboxyglutamate (Gla)-containing protein, named Gla-rich protein (GRP) due to its high content in Gla residues, has been identified in association with chondrocytes and bone cells. Although its molecular function is yet unknown, the high content of Gla residues and its accumulation at sites of pathological calcification in skin, vascular system and breast cancer tumors suggest that GRP modulates calcium availability, regulates cartilage matrix organization and influences matrix stability being associated with fibrillar collagens (Cancela et al., 2012).

Although not synthesized by mesenchymal cells, being secreted from hepatocytes into the circulation, never the less, fetuin A exerts its biological role in the periphery, where it inhibits calcification by the transient formation of soluble colloidal spheres (Heiss et al., 2003). It binds calcium phosphate and calcium carbonate with high affinity and, although with lower efficiency, magnesium phosphate (Schinke et al., 1996). In rat sera fetuin A is present in high molecular weight complexes, termed "calciprotein" particles, which contain calcium, phosphate and matrix Gla protein. They act as inhibitors of mineralization in solution and of cell-mediated mineralization by inhibiting the *de novo* formation of calcium phosphate without dissolving preformed minerals (Schlieper et al., 2007).

The complexity of the mechanisms regulating pathologic calcification is further highlighted by the involvement of apparently unrelated gene products, as it was noticed for Klotho and multidrug resistance protein 6 (MRP6), just to mention few of them. Klotho is a transmembrane protein with an extracellular (β-glucosidase domain that can be shed from the plasma membrane by Adamts proteases and, in addition to its enzymatic function, binds directly to fibroblast growth factor (FGF)23 acting as an essential FGF-coreceptor. In the kidney, FGF23 signalling leads to a down-regulation of the sodium/phosphate co-transporter (NaPi) and of the vitamin D 1α-hydroxylase. Therefore, Klotho deficiency, in spite of high FGF23 levels and of high 1,25-dihydroxy vitamin D3 and calcium concentrations, leads to osteopenia, hyper-phosphatemia, and consequently widespread vascular and soft tissue calcifications (Moe, 2012). By contrast, dysfunction of the ATP-binding cassette (ABC)-transporter *ABCC6* (coding for the transmembrane protein MRP6 highly expressed in liver and kidney) causes pseudoxanthoma elasticum (PXE), a rare disease characterized by mineralization and degeneration of elastic fibers within soft connective tissues, thus causing skin laxity, cardiovascular complications, and visual impairment in a setting of normal levels of circulating calcium and phosphate and without bone abnormalities (see ahead for further details; Quaglino et al., 2011).

ROLE OF THE EXTRACELLULAR MATRIX IN ECTOPIC CALCIFICATION

Changes in the characteristics of the extracellular matrix and in the ratio between matrix constituents influence not only the mechanical properties of connective tissues, but significantly contribute to modulate cell phenotype by altering integrin expression, focal adhesions, cytoskeletal organization and consequently intracellular signaling pathways.

As a consequence, it has been shown that osteogenic differentiation of calcifying VSMCs was promoted by type I collagen and fibronectin, but it was inhibited by type IV collagen. By contrast, valvular interstitial cells (a heterogeneous population of fibroblasts, with a small percentage of myofibroblasts and smooth muscle cells ranging from 5 to 30% in physiological or pathological conditions, respectively) when grown on type I collagen or fibronectin remain in a quiescent fibroblastic state, whereas those cultured on fibrin surfaces exhibit a myofibroblast phenotype and rapidly form calcified aggregates (Chen and Simmons, 2011). These data further highlight the complex interactions between cells and between cells and matrix.

Therefore, beside alterations in the balance between pro- and anti-calcifying factors, changes in the extracellular matrix may significantly contribute to mineral deposition. It is noteworthy to mention that in soft connective tissues, if mineralization is not triggered by necrotic cell debris, elastic fibers seem to represent the selected target of pathologic mineralization, possibly due to their low turnover and/or susceptibility to calcium ion-binding (Pugashetti et al., 2011).

Purified elastin has been demonstrated to have calcium-binding capabilities (Molinari-Tosatti et al., 1968; Cox et al., 1975; Long and Urry, 1981). Moreover, addition of elastin peptides to cultured SMC enhances Von Kossa positive calcium precipitates in the phosphate model of *in vitro* calcification (Hosaka et al., 2009).

In accordance with these data, elastin degradation due to the up-regulation of matrix metalloproteinase (MMP)2, MMP9, and cathepsin S has been shown to increase arterial calcification in the uremic mice model of ectopic calcification (Pai et al., 2011) and to favor calcification of native heart valves (Perrotta et al., 2011). It has been therefore suggested that peptides or fragments derived from elastin degradation, due to their high hydrophobicity and coacervation properties, may enhance abnormal mineralization (Abatangelo et al., 1975; Long et al., 1975) leading to the formation of abnormal complexes with high calcium-binding capabilities (Bell et al., 1974; Tamburro et al., 1977; Urry et al., 1982). These findings sustain the association between inflammation and ectopic calcification, especially in the vascular compartment (Shao et al., 2010).

Beside elastin itself, elastic fibers are made of several components whose exposure, with age and in pathological conditions, further contributes to the preferential localization of ectopic calcifications on elastin and on elastic fiber-associated molecules. For instance, calcium-binding sites have been found on specific domains in fibrillin I (Handford, 2000), one of the principal elastin-associated proteins. Therefore, not only elastin *per se*, but also elastic fibers, as a whole, could function as nucleation center for calcium precipitation (Starcher and Urry, 1973). Beside fibrillin, proteoglycans (PG), glycosaminoglycans (GAGs), and other glycoproteins present inside elastic fibers could also represent additional calcium-binding sites.

We have repeatedly demonstrated that GAGs are present inside elastic fibers, possibly regulating the mechanical properties and stability of these fibers (Pasquali-Ronchetti et al., 1984; Contri et al., 1985). Changes in the type or ratio of GAGs, as those occurring with age, in the course of pathologic conditions or depending on tissue or on specific physiological requirements (Berenson et al., 1985; Cherchi et al., 1990; Passi et al., 1997; Qu et al., 2011), may influence the characteristics of elastic fibers and of the whole extracellular matrix, as demonstrated for instance in the vasculature where connective tissue molecules follow a gradient depending on the distance from the heart (Madhavan, 1977). Moreover, we have also demonstrated that calcified elastic fibers exhibit peculiar type and localization of PG/GAGs such as heparan sulfate, putting forward the hypothesis that GAGs have a role in elastic fiber homeostasis as well as in the calcification process (Passi et al., 1996; Gheduzzi et al., 2005).

Furthermore, it was shown that, in cartilage, PG/GAGs act as calcium-concentrating agents promoting calcification, but they also behave as inhibitors of hydroxyapatite formation functioning as a cation-exchanging calcium reservoir (Hunter, 1991). Consistently, decorin, a small leucine-rich PG containing one dermatan sulfate or chondroitin sulfate chain, beside its regulatory role on transforming growth factor (TGF)-β activity and collagen fibrillogenesis, binds to hydroxyapatite (Boskey et al., 1997; Rees et al., 2001; Mochida et al., 2009) and colocalizes with areas of calcification in skeletal tissues, in the adventitia of blood vessels, and in the skin (Hocking et al., 1998).

A further link between GAGs and the calcification process is the ability of BMPs to bind to heparin and to induce osteoblast differentiation of mesenchymal cells. Moreover, heparan sulfate and dextran sulfate enhanced BMP2 activity serving as ligands to their signaling receptors on cell membranes (Takada et al., 2003).

ROLE OF MESENCHYMAL CELLS IN SOFT CONNECTIVE TISSUE CALCIFICATIONS

PERICYTES

The presence of perivascular cells closely associated with capillaries was described more than 100 years ago, although their origin remained elusive for many decades (Díaz-Flores et al., 1991). Some studies proposed that they may derive from the neural crest, whereas other studies suggested that pericytes derive from smooth muscle cells, fibroblasts, endothelial cells, and bone marrow and that they exhibit a multi-lineage potential being capable of differentiating into a variety of cell types including osteoblasts and chondrocytes, as demonstrated, both *in vitro* and *in vivo* experimental models. On the basis of these observations, it was suggested that pericytes play a role in mediating ectopic calcification (Collett and Canfield, 2005).

There is now good evidence that angiogenesis regulates ectopic calcification in several ways: (i) angiogenic factors are mitogenic for mesenchymal cells and osteoblasts and enhance bone formation *in vivo*; (ii) cytokines as BMP2 and BMP4, released by endothelial cells, induce both the differentiation of osteoprogenitor cells and calcification *in vitro* and *in vivo*, although this effect is context-dependent (Shin et al., 2004); (iii) new vessels serve as a conduit for osteoprogenitor cells that may derive from the circulation or from pericytes themselves. Consistently, the association between angiogenesis and ectopic calcification has been noted in several cases, as in ductal carcinoma *in situ*, in calcifying fibroblastic granuloma, in choroidal osteoma and in the calcifications of the retina.

When cultured in standard growth medium, pericytes undergo a process of growth and differentiation characterized by the formation, within approximately 8 weeks, of large multi-cellular nodules that, similarly to the matrix found in calcified vessels, contain type I collagen, OPN, matrix Gla protein and OC and hydroxyapatite crystals with a calcium to phosphate ratio analogous to that of bone (Doherty and Canfield, 1999; Abedin et al., 2004).

VASCULAR SMOOTH MUSCLE CELLS

Studies in human lesions and mouse models of arterial calcification as well as *in vitro* calcification models of human and bovine VSMC support the concept that mesenchymal-derived vascular cells participate in mineral deposition by mimicking bone formation, since they exhibit several hallmarks of endochondral ossification (Liu and Shanahan, 2011).

It has been clearly demonstrated that VSMC (1) undergo osteoblastic differentiation with loss of smooth muscle-specific gene expression and gain of osteoblast-like properties, including expression of the osteoblast differentiation factor Cbfa-1; (2) activate the mineralization process in the presence of high concentrations of extracellular phosphate; (3) may require a sodium-dependent phosphate co-transporter function to calcify (Giachelli, 2001; Vattikuti and Towler, 2004). The complexity of VSMC phenotypic changes associated to ectopic calcifications has been recently clearly outlined by a whole-genome expression array approach in uremic rats fed on a high phosphate diet. It was in fact

demonstrated that the transition from "muscle-related" to "bone-related" gene expression involved the deregulation of at least 53 genes (Román-García et al., 2010) and the activation of Erk1/2 and Wnt pathways (Lau et al., 2010).

Interestingly, it has been shown that, in appropriate culture conditions, approximately 10–30% of VSMC have the capacity to express osteoblast differentiation markers and to retain this phenotype through *in vitro* passages (Boström et al., 1993). In agreement with these *in vitro* data, a variety of bone matrix proteins and regulatory factors have now been demonstrated in human calcified plaque, including OC, BSP, osteonectin, collagen I, alkaline phosphatase, Msx-2, and Cbfa-1.

FIBROBLASTS

Many studies performed on VSMC demonstrated that mesenchymal cells, whether locally producing pro- and anti-calcifying factors or being involved in extracellular matrix synthesis and degradation, are involved in the mineralization of soft connective tissues. Never the less, a key question is whether all mesenchymal cells behave similarly, or if differences in their tissue-specific differentiation may be associated to a different susceptibility of connective tissues to mineralize.

For instance, skin seems to be only rarely affected by ectopic calcification in contrast to the vascular system. Very few studies have been done on fibroblasts and especially on dermal fibroblasts, although in a number of disorders there is a clear evidence for their close association to ectopic calcifications (**Figure 2**).

Among the few reports on fibroblast-associated calcifications are those showing that a human gingival fibroblast cell line may exhibit both intracellular and extracellular ectopic mineralization starting within round and irregularly shaped vesicles contained in large cytoplasmic vacuoles. This may suggest that mineral accumulation and transformation of amorphous mineral into crystalline structures take place within cellular vesicular structures like MV (Yajima et al., 1984).

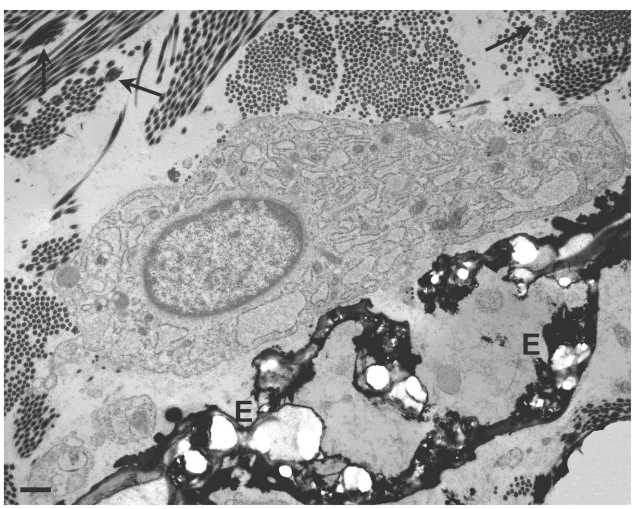

FIGURE 2 | Transmission electron microscopy of a dermal biopsy from a patient affected by pseudoxanthoma elasticum (PXE). Deformed calcified elastic fibers (E) are present in close proximity to large fibroblasts with abundant and dilated cisternae of the endoplasmic reticulum. Collagen fibrils occasionally organized into collagen flowers (arrows) are also visible. Bar = 1 μm.

By contrast, MV have been never observed within or around dermal fibroblasts in areas of matrix calcification *in vivo* nor in the high phosphate-calcification model *in vitro* (personal observations). This finding indicates the occurrence of different phenotypic characteristics between dermal and gingival fibroblasts, but at the same time demonstrates that mineral deposition can be observed also independently from MV.

In the attempt to understand the interactions between cells from hard and soft connective tissues and to unveil the complexity of the mechanisms involved in fibroblast-associated calcification, Yu and coworkers performed a cDNA microarray analysis on fibroblasts from spinal ligaments cultured in the presence of conditioned media of osteoclast-like cells. In this environment fibroblasts exhibited high levels of alkaline phosphatase and mineral deposition, but more interestingly, microRNA expression profiles revealed a significant down-regulation of a group of microRNAs known to negatively interfere with genes associated with osteogenic differentiation (e.g., BMP2, OC, Runx2). In the light of these data, it has been hypothesized that osteoclasts might induce the osteogenic differentiation of fibroblasts *in vitro* and that miRNA may play an important role in the regulation of cell–cell interactions between osteoclasts and fibroblasts (Yu et al., 2011).

An additional demonstration of the ability of fibroblasts to modulate their phenotype in response to specific environmental characteristics has been provided by studies on rat dermal fibroblasts cultured in the presence of elastin degradation products and of TGF-beta1. Mineralization was preceded by up-regulation of alpha-smooth muscle actin, type I collagen and MMP2, which are characteristic features of myofibroblasts. Thereafter, osteogenic markers as OC, alkaline phosphatase, and osteoprotegerin increased their expression and, after 21 days, multi-cellular calcified nodules were observed. It was proposed that elastin-associated mineralization might result from defective/unbalanced dynamic remodeling events similar to those occurring during the repair process (Simionescu et al., 2007).

Therefore, it is important to note that, irrespective of the cell type, a specific environment is required for calcification to occur, *in vivo*, but especially *in vitro*. All cultured mesenchymal cells, in fact, are dependent for their growth on a variety of cytokines and adhesive molecules as those easily provided by addition of fetal/calf bovine serum. However, the amount of "serum factors" significantly higher compared to physiological "*in vivo*" concentrations, provide cells of a number of other components that, depending on the characteristics of the serum, directly influences the mineralization process or may regulate cell behavior and, as a consequence, the expression of specific gene/proteins. Among these factors, serum fetuin A, that is usually present in cell culture media, represents a powerful inhibitor of the calcification process making cells unable to mineralize in standard cell culture conditions. To overcome this problem, it is possible to utilize serum-free media (i.e., media with chemically defined components and supplements) and/or to add to standard cell cultures [in Dulbecco's modified Eagle's medium (DMEM) plus serum] high concentrations of

phosphate (that can easily precipitate as soon as it forms complexes with calcium) or of phosphate donor substrates (that require an active involvement of cells for the enzymatic release of inorganic phosphate from substrates). Human skin-derived fibroblast precursor cells, for instance, can acquire an osteoblast-like behavior and start to mineralize the newly deposited extracellular matrix only if cultured in a pro-osteogenic medium (supplemented with ascorbic acid, beta-glycerophosphate, and dexamethasone); as a result induced expression of alkaline phosphatase, BSP and OC leads to mineral deposition (Buranasinsup et al., 2006).

A further difference, between mesenchymal cell types is the time required *in vitro* to obtain a calcified matrix, which may be taken as predictive of the pro-osteoblastic potential of the cells. In osteoblast and VSMC cultures, mineralization can be obtained after 3–5 or 8–10 days in culture, respectively (Uchimura et al., 2003; Li et al., 2004). By contrast, mineralized matrix becomes clearly evident only after 2–3 weeks in dermal fibroblast cultures (Boraldi et al., in press; **Figure 3**).

On the basis of these results it could be hypothesized that dermal fibroblasts are rather resistant to be converted into osteoblast-like cells, in agreement with the uncommon occurrence of dermal calcifications.

Interestingly, ectopic soft tissue calcification is a well-known symptom in Werner syndrome (WS), an autosomal recessive progeroid disorder caused by mutations in RecQ DNA helicase. Cultured fibroblasts from WS patients undergo spontaneous mineralization *in vitro* at normal phosphate concentration, and overexpress Pit-1 at mRNA and protein levels. Both calcification and Pit-1 up-regulation have been also detected *in situ* in the skin of patients (Honjo et al., 2008), supporting the concept that dermal fibroblasts mimic and retain *in vitro* at least some of the pathologic characteristics they have *in vivo*, thus representing a valuable model to investigate the pathogenetic mechanisms of diseases.

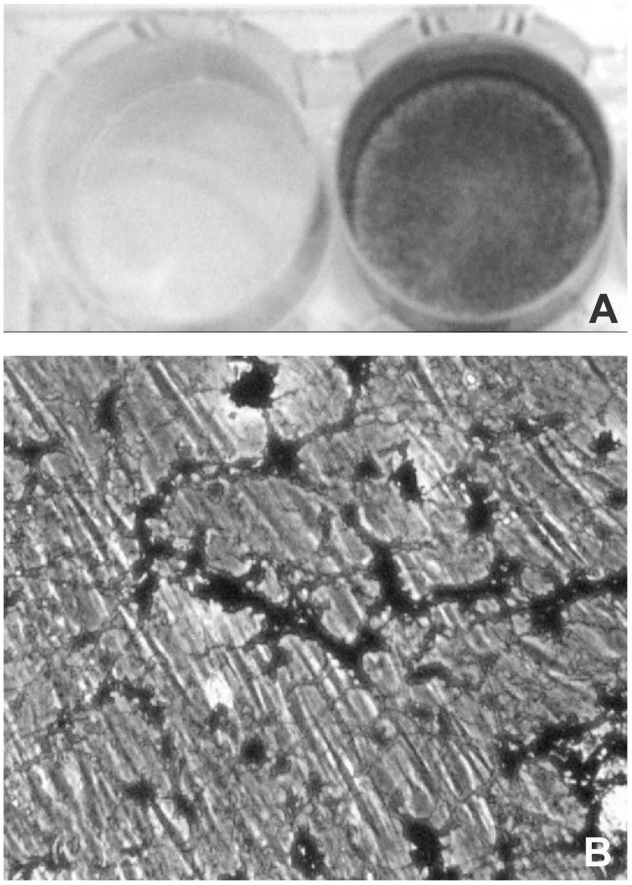

FIGURE 3 | Light microscopy of dermal fibroblasts cultured for 3 weeks in the presence of DMEM (A) or of DMEM supplemented with ascorbate, beta-glycerophosphate, and dexamethasone (B). In the presence of the calcified medium, dermal fibroblasts exhibit areas of mineralization that can be clearly visualized upon Von Kossa staining as dark deposits (lower panel; see also Boraldi et al., in press, for methodological details).

PXE AND PXE-LIKE DISORDERS AS MODELS FOR INVESTIGATING THE ROLE OF FIBROBLASTS IN SOFT CONNECTIVE TISSUE CALCIFICATIONS

PSEUDOXANTHOMA ELASTICUM

Pseudoxanthoma elasticum is a rare genetic disorder characterized by skin papules on the neck, axillae, and groin, often associated with skin redundancy and laxity, by retinal alterations as angioid streaks and neovascularization, and by middle sized artery narrowing up to occlusion. All these alterations depend on the deposition of calcium minerals inside or associated with elastic fibers (Truter et al., 1996; Gheduzzi et al., 2003; **Figure 2**). The phenomenon is rather peculiar as calcifications affect only elastic fibers, whereas collagen does not calcify; moreover, this abnormal mineralization occurs in the absence of increased calcium and phosphate levels, in the total absence of inflammation, cell necrosis, apoptosis.

Pseudoxanthoma elasticum has been associated to mutations in the *ABCC6* gene, a member of the ABC family of membrane transporters (it encodes for the membrane transporter MRP6), that is mainly expressed in liver and kidney (Bergen et al., 2000; Le Saux et al., 2000; Ringpfeil et al., 2000), whereas its expression is surprisingly low in tissues specifically involved in the clinical manifestations of PXE. In this context, it has been suggested that a still unknown circulating metabolite released (or not released) by the liver in *ABCC6* deficiency may directly affect elastic fiber formation, stability, and calcification (Le Saux et al., 2006).

Actually, in a setting of normal calcemia and phosphatemia, several abnormalities have been documented in the circulation of PXE patients, from PGs and enzymes involved in their synthesis (Götting et al., 2005; Schön et al., 2006; Volpi and Maccari, 2006), to protein and lipid abnormalities indices of oxidative stress (Garcia-Fernandez et al., 2008), to high levels of MMP2 and MMP9 (Diekmann et al., 2009) and of elastin-derived peptides (Annovazzi et al., 2004; **Table 1**).

Moreover, low levels of fetuin A and of vitamin K have been measured in the circulation of PXE patients and in the PXE animal model (Jiang et al., 2010; Vanakker et al., 2010). Low levels of fetuin A could be explained by the augmented capture of this molecule by peripheral mineral precipitates (Price et al., 2004; Hendig et al., 2006), although it cannot be excluded that PXE fibroblasts may

Table 1 | Genes/molecules involved in the regulation of elastic fiber calcification in PXE.

Regulators of mineral formation/deposition	PXE findings (reference)
Circulating ion levels	Normal Ca and P (Boraldi et al., in press)
Phosphate homeostasis	Normal serum alkaline phosphatase (ALP; Boraldi et al., in press)
	↑ TNAP in fibroblasts (Boraldi et al., in press)
Calcification inhibitors	↓ Gla-MGP (Gheduzzi et al., 2007; Li et al., 2007; Hendig et al., 2008b)
	↓ Fetuin-A (Hendig et al., 2006; Jiang et al., 2010)
	Polymorphisms of OPN (Hendig et al., 2007)
	Mutation of ENPP1 (Nitschke et al., 2012)
Extracellular matrix components	↑ MMP2, MMP9 in serum (Diekmann et al., 2009)
	↑ MMP2 in fibroblasts (Quaglino et al., 2005)
	Polymorphisms of MMP2 (Zarbock et al., 2010)
	↑ Elastin-derived peptides (Annovazzi et al., 2004)
	Different ratio PG/GAGs (Tiozzo-Costa et al., 1988; Passi et al., 1996; Maccari and Volpi, 2008)
	↑ Circulating selectins (Götting et al., 2008)
	↑ Serum intercellular adhesion molecule (ICAM; Hendig et al., 2008a)
	Different expression of integrins (Quaglino et al., 2000)
	↑ Serum XT-I activities (Götting et al., 2005)
	Polymorphisms of XT-I (Schön et al., 2006)
Redox balance	↑ ROS in serum (Garcia-Fernandez et al., 2008)
	↑ ROS in fibroblasts (Pasquali-Ronchetti et al., 2006; Boraldi et al., 2009)
	Polymorphisms of antioxidant genes (Zarbock et al., 2007)

sequester this inhibitor (Boraldi et al., 2007) as a consequence of higher intracellular uptake that may prevent fetuin A from exerting its regulatory role in peripheral tissues. The recent finding that in the mouse model of PXE ectopic calcification can be significantly reduced by overexpressing fetuin A (Jiang et al., 2010) may suggest that in PXE the role of this inhibitor should be further investigated.

Nevertheless, it is unlikely that all these plasma abnormalities in PXE patients would directly depend on the deficiency of the membrane transporter MRP6. It would seems more reasonable that inherited *ABCC6* deficiency, along the years, would induce a series of metabolic adjustments in several tissues possibly epigenetically involving a network of different genes and leading to the complexity and heterogeneity of PXE alterations. Moreover, since each patient has a different genetic background, the consequences of these "metabolic adjustments" would be different in each individual thus explaining the extreme variability of clinical manifestations among patients.

Therefore, clinical and experimental data strongly suggest that elastic fiber calcification in PXE is not a passive process merely due to the presence or absence of one or more abnormal plasma components, but the result of activities mediated by local cells. At least in skin, fibroblasts should be considered the principal candidates for several reasons.

First of all, if elastic fiber calcifications in PXE are a passive process due to the infiltration of plasma molecule(s), all elastic fibers should calcify; on the contrary, skin elastic fiber calcification is present only in peculiar regions of the body. Moreover, also in areas prone to calcification, not all elastic fibers mineralize. This is in agreement with the overgrowing evidence of the diversity of skin fibroblasts at different anatomical sites, as these cells display distinct and characteristic transcriptional patterns for a large number of genes depending on the body region they come from (Chang et al., 2002; Lindner et al., 2012). Therefore, skin fibroblasts may be considered differentiated cell types that, depending on their location, maintain their positional identities even when isolated and cultured *in vitro* (Rinn et al., 2008) and probably react in different ways to abnormal exogenous stimula, such as those present in the circulation of PXE patients.

A second evidence for the involvement of fibroblasts in skin abnormalities in PXE is, beside elastic fiber calcification, the documented presence of huge aggregates of PGs and of various extracellular matrix proteins in the affected areas of the dermis (Pasquali-Ronchetti et al., 1986; Tiozzo-Costa et al., 1988; Baccarani-Contri et al., 1994; Passi et al., 1996), consistent with a significant increase of the total amount of GAGs in the skin of patients (Maccari and Volpi, 2008; **Table 1**) and with the observed decreased susceptibility of GAG-associated elastin to pancreatic elastase (Schwartz et al., 1991). Such structural and chemical alterations, very likely responsible for skin redundancy and laxity in the affected areas, must be under the local control of fibroblasts, which are responsible for the synthesis of the extracellular milieu in soft connective tissues.

An indirect indication that also in the vessel wall fibroblasts are probably involved in the early calcification of elastic fibers is the observation that calcification in PXE vessels is often present within the elastic fibers close to the adventitia, in the absence of any osteoblast-like phenotype of the adjacent cells, that in fact maintain a fibroblast-like appearance (Gheduzzi et al., 2003).

Finally, several studies by our and other groups have shown that fibroblasts isolated from the dermis of PXE patients have and maintain *in vitro* a metabolic behavior different from fibroblasts isolated from the same body areas of gender and age-matched normal subjects. Actually, it has been demonstrated that PXE fibroblasts suffer from an oxidative stress condition (Pasquali-Ronchetti et al., 2006), produce highly sulfated GAGs (Tiozzo-Costa et al., 1988; Passi et al., 1996), exhibit abnormal proteoglycanase (Gordon et al., 1978, 1983) and higher metalloproteinase activities (Quaglino et al., 2005), are unable to properly carboxylate MGP (Gheduzzi et al., 2007; Boraldi et al., in press; **Table 1**), and have a different protein profile (Boraldi et al., 2009) indicating that their

metabolic behavior is genetically or epigenetically different from control fibroblasts and is maintained when cells are cultured *in vitro* (Rinn et al., 2008).

In the light of these observations, dermal fibroblasts from PXE patients can be considered a valuable and very informative model to better understand the contribution of these cells to soft connective tissue calcifications. Therefore, beside the role of fibroblasts in regulating the characteristics of the extracellular environment and, as a consequence, the different susceptibility of tissues and of elastic fibers to calcify, a key question remains whether the same osteoblast-related pathways, as those described in SMC, are also involved in the phenotype of fibroblasts prone to calcify (i.e., PXE fibroblasts).

Recent evidence from our laboratory indicates that TNAP activity, although within normal range in the circulation of patients, is higher in PXE fibroblasts compared to control cells and that these differences are further amplified when cells are grown in a calcifying medium. The process is rather complex, however, data suggested that the local increase of phosphorus in the extracellular milieu together with the reduced amount of anti-calcific molecules, such as carboxylated MGP, may favor hydroxyapatite formation (Boraldi et al., in press).

Interestingly, low levels of MGP have been found in the circulation of PXE patients (Hendig et al., 2008b) and in the *Abcc6*−/− mice model of PXE (Li et al., 2007). In accordance, low levels of carboxylated MGP are produced by skin PXE fibroblasts *in vitro*, indicating that the local synthesis of the mature protein is of paramount importance in preventing elastic fiber calcification (Gheduzzi et al., 2007) and that fibroblasts are likely involved in the local secretion of this important anti-calcific protein. Since MGP γ-carboxylation is a vitamin K-dependent process (Theuwissen et al., 2012), it was suggested that PXE calcification could be due to vitamin K deficiency (Borst et al., 2008). Although the level of vitamin K is low in PXE patients (Vanakker et al., 2010), nevertheless the availability and the cellular utilization of vitamin K do not seem responsible for MGP under-carboxylation. Both in PXE fibroblasts and in two different *Abcc6*−/− mice models, addition of vitamin K did not improve MGP carboxylation (Boraldi et al., in press) nor prevented calcification in spite of the high serum concentration of vitamin K upon treatments (Brampton et al., 2011; Gorgels et al., 2011; Jiang et al., 2011). Therefore, low vitamin K does not seem to play a pivotal role in MGP carboxylation nor in elastic fiber calcification in PXE. Moreover, in PXE, carboxylation of proteins involved in coagulation or in bone calcification seems adequate, as no defects in coagulation or in bone mineralization have been described in patients. Therefore, both vitamin K availability and the carboxylase system do not seem directly involved in PXE mineralization. Recent data from our laboratory seem to suggest that the low carboxylation rate of MGP by PXE skin fibroblasts, even in a setting of high vitamin K concentration, might depend on the intrinsic ability of MGP to be carboxylated (Boraldi et al., in press)

In addition, evidence has been provided through the years that the PXE phenotype can be obtained through pathways other than those caused by *ABCC6* mutations. An indirect proof of this is that PXE-like clinical and histo-pathological manifestations have been described in a number of patients affected by beta-thalassemia (Aessopos et al., 1992, 1998; Baccarani-Contri et al., 2001; Cianciulli et al., 2002) and other hemoglobinopathies (Goldberg et al., 1971; Nagpal et al., 1976; Aessopos et al., 2002; Fabbri et al., 2009), in subjects treated with penicillamine (Rath et al., 2005), in cases of γ-carboxylase gene (*GGCX*) and *ENPP1* mutations (Vanakker et al., 2007; Le Boulanger et al., 2010; Li et al., 2012; Nitschke et al., 2012; see further for additional data) and, more recently, in a few cases of liver transplantation where the impossibility to examine the DNA from all donors and recipients made not clear if the transplanted liver harbored or not *ABCC6* mutations (Bercovitch et al., 2011).

PXE-LIKE DISORDERS

Beta-thalassemia

As already mentioned, the metabolic complexity at the basis of elastic fiber calcification could, at least partially, explain the phenotypic similarities of the skin lesions in inherited PXE and in a number of different unrelated disorders, such as in patients affected by beta-thalassemia (Aessopos et al., 1992; Baccarani-Contri et al., 2001).

More than 60 years ago, elastinopathies similar to that in PXE were documented in sickle cell anemia (Paton, 1959; Suerig and Siefert, 1964) and, later, in a series of hemoglobinopathies (Nagpal et al., 1976), among which β-thalassemia (Aessopos et al., 1992, 1997). Subsequent studies better defined the almost identical clinical and histo-pathological alterations in PXE and in a relevant number of β-thalassemia patients (Aessopos et al., 1998). In both these unrelated genetic disorders, angioid streaks (Gibson et al., 1983; Kinsella and Mooney, 1988; Aessopos et al., 1989, 1992; O'Donnell et al., 1991), arterial elastorrhexis, and calcification (Aessopos et al., 1998; Tsomi et al., 2001; Cianciulli et al., 2002) as well as coalescent skin papules on the posterior/lateral aspect of neck, axillae, and groin with elastic fiber calcification (Aessopos et al., 1992; Baccarani-Contri et al., 2001) have been repeatedly described.

Skin abnormalities in genetic PXE and in beta-thalassemia patients with clinical PXE-like manifestations (β-thal/PXE) have been carefully analyzed. It was observed that both disorders had identical elastic fiber calcifications, "collagen flowers" abnormalities, as well as cell and matrix alterations, suggesting that similar metabolic changes could be involved in both disorders as final consequence of mutations in apparently unrelated genes.

In agreement with this hypothesis are data from experiments aiming to verify if the similarities of clinical and histo-pathological features in genetic PXE and in β-thal/PXE patients could be sustained by analogous similarities in the metabolic behavior of cultured fibroblasts.

Fibroblasts from healthy subjects, from PXE patients as well as from individuals affected by beta-thalassemia exhibiting (β-thal/PXE) or not (β-thal) ectopic calcification, were investigated for their ability to accumulate and to extrude calcein-AM (acetomethoxy derivate of calcein; Boraldi et al., 2008), a chemical widely used for determining cell viability. The non-fluorescent calcein-AM enters living cells where it is hydrolyzed by intracellular esterases into the strongly fluorescent green anion calcein that can be retained in the cytoplasm or actively extruded. The accumulation and the extrusion of fluorescent calcein can be easily

visualized by confocal microscopy and quantified by flow cytometry (Boraldi et al., 2003). Within few minutes of incubation with calcein-AM (0.1 μM) dermal fibroblasts accumulate the fluorescent molecule into discrete granules in the cytoplasm. After a 30-minute incubation, calcein accumulation is much higher in PXE and in β-thal/PXE cells than in controls and in β-thal fibroblasts. Furthermore, also calcein efflux from β-thal/PXE fibroblasts is significantly different compared to controls ($p < 0.05$), whereas it is identical to that of fibroblasts from patients with inherited PXE (**Figure 4**). Therefore, *in vitro* dermal fibroblasts from β-thal/PXE patients, in the absence of *ABCC6* mutations (Hamlin et al., 2003), exhibit functional alterations similar to those of fibroblasts isolated from patients with inherited PXE. In this specific case, the calcein assay is defective in fibroblasts isolated from subjects with identical elastic fiber calcification (i.e., PXE and β-thal/PXE), whereas it is normal in fibroblasts from β-thalassemia patients without PXE-like clinical alterations. Therefore, these findings further support the hypothesis that PXE-like clinical manifestations described in some β-thalassemia patients might derive from metabolic alterations occurring in this particular sub-group of patients and that similar pathways may be at the basis of elastic fiber calcification in inherited PXE and in β-thal/PXE patients (Boraldi et al., 2008).

In β-thalassemia patients an abnormal oxidative stress induced by the iron overload derived from repeated transfusions, by

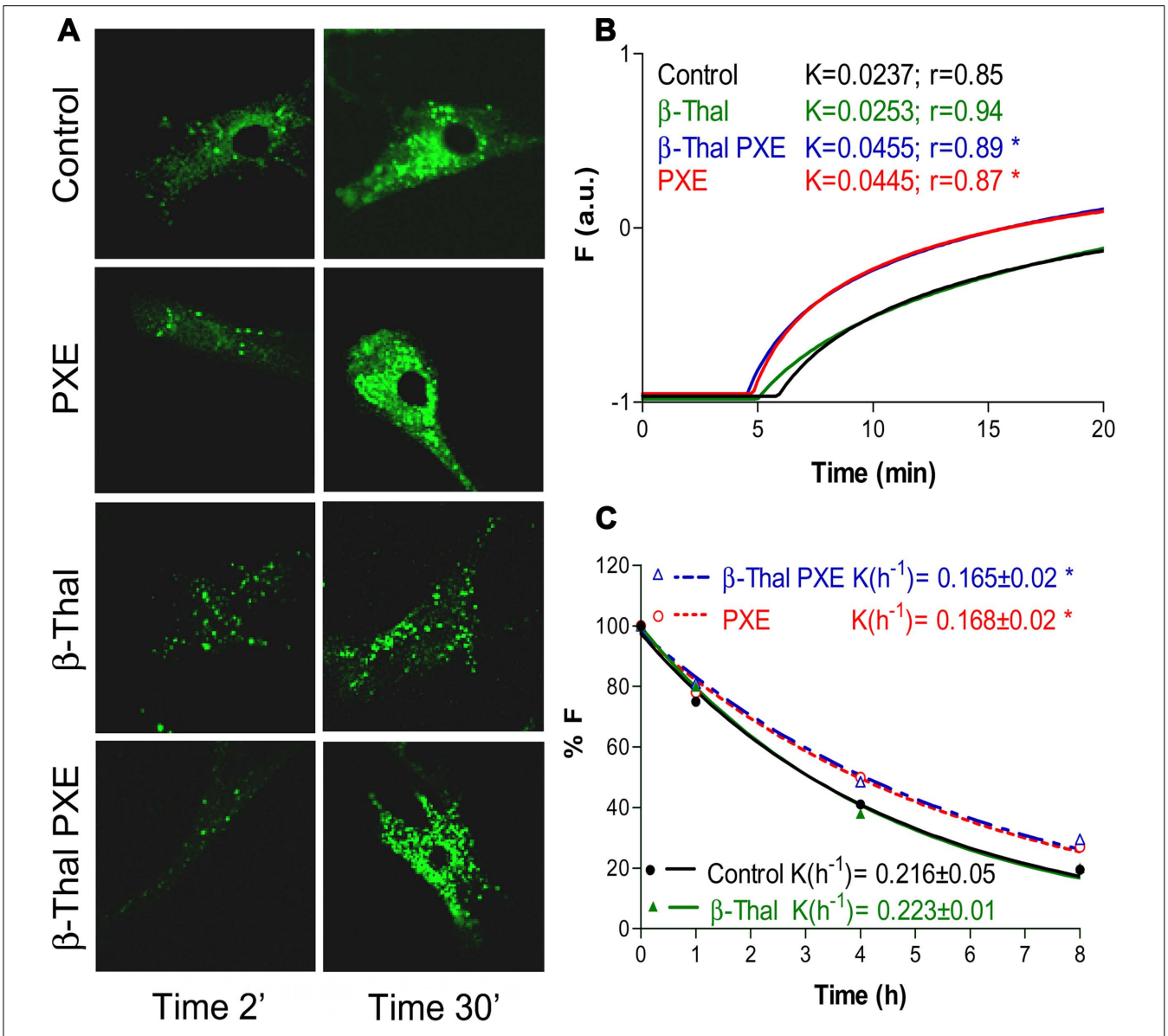

FIGURE 4 | Calcein uptake and extrusion in cultured fibroblasts from healthy individuals (Control), PXE patients (PXE), beta-Thalassemic patients with (βThal/PXE) and without (βThal) PXE-like manifestations. Intracellular calcein accumulation is shown after 2 and 30 min by confocal microscopy **(A)**. Calcein uptake **(B)** and extrusion **(C)** have been measured by flow cytometry and are shown in panels on the right (see also Boraldi et al., 2003, for methodological details).

unpaired alpha-globin chains (Livrea et al., 1996; Hershko et al., 1998; Cighetti et al., 2002) and by deficient oxygen transport to peripheral tissues (Chan et al., 1999; Meral et al., 2000) has been described. How oxidative stress interferes with connective tissue metabolism is still largely unknown. It has been shown that iron overload and the consequent oxidative stress affect the synthesis of elastin by human dermal fibroblasts *in vitro* (Bunda et al., 2005) and that genetic factors as well as environmental oxidative stress may deeply influence the extracellular matrix and the behavior of cells *in vivo* (Hanley and Repine, 1993). It could be suggested that clinical and histological alterations in β-thal/PXE patients could be due, at least in part, to chronic oxidative stress that, similarly to inherited PXE (Garcia-Fernandez et al., 2008), is not adequately compensated due to a peculiar genetic/epigenetic background. Interestingly, the introduction of oral iron chelators markedly increased the survival of β-thalassemia patients (Borgna-Pignatti et al., 2004) and reduced the level of oxidative stress, in agreement with the observation that PXE-like clinical manifestations are never found in properly treated new β-thalassemia patients (personal observation).

Interestingly, $Hbb^{th3/+}$ mice are characterized by a significant liver-specific decrease of mrp6 production, due to failure of the NF-E2p45 transcription factor to bind to the *Abcc6* proximal promoter. Even though this animal model of thalassemia is not characterized by soft connective tissue mineralization, never the less it demonstrates that *Abcc6* gene expression can be modified by environmentally-induced changes in transcription factor activity (Martin et al., 2011) and that oxidative stress could play a relevant role. In this context, it is worthwhile to mention that there are data in the literature in favor of a relationship between NF-E2p45 and Nrf2 transcription factors, as independent groups have shown the role hemin in stimulating the expression of antioxidant heme oxygenase 1 (Li et al., 2011) as well as in inducing beta-globin gene expression through the functional intervention of p45NF-E2 transcription factor (Moore et al., 2006). Moreover, in favor of a negative effect of oxidative stress on the expression of *ABCC6* are data showing that vitamin K3 and oxidant agents induce down-regulation of *ABCC6* expression in HepG2 cells (De Boussac et al., 2010).

Deficit of vitamin K-dependent gamma-carboxylase system

The vitamin K-dependent gamma-carboxylation system is composed of the gamma-carboxylase and the warfarin-sensitive enzyme vitamin K(1) 2,3-epoxide reductase (VKOR), which are located in the endoplasmic reticulum where they interact with other proteins like calumenin and protein disulfide isomerase, negative and positive regulators of the vitamin K cycle, respectively (Wajih et al., 2004; Wallin et al., 2008). Different expression of these two regulatory proteins has been demonstrated on *in vitro* cultured fibroblasts to be probably involved in the pathogenesis of PXE and of PXE-like calcifications (Boraldi et al., 2009).

During vitamin K-dependent post-translational gamma-glutamyl carboxylation, vitamin K hydroquinone is oxidized to the epoxide form (K>O) that, in turn, is reduced by the enzyme VKORC1 (vitamin K epoxide reductase complex component 1) to complete the vitamin K cycle.

The demonstration that the enzyme VKORC1 is the target for the anti-coagulant drug warfarin and that patients treated with this drug develop extensive vascular calcifications (Palaniswamy et al., 2011) sustain the importance of the vitamin K-dependent regulatory mechanisms of calcification. In particular, VKORC1 appears to be a rate-limiting step in the biosynthesis of functional vitamin K-dependent proteins.

Interestingly, skin lesions due to elastic fiber calcification almost identical to those in PXE have been described in cases of mutations of *GGCX* (Vanakker et al., 2010). As already mentioned, this enzyme is necessary for the γ-carboxylation of a number of proteins some of which are involved in ectopic calcification (Shanahan et al., 1998; Price et al., 2006). Therefore, mutations in the *GGCX* gene are at the basis of an autosomal recessive disorder characterized by a generalized deficiency of the Vitamin K-dependent clotting factors as well as mineralization and fragmentation of elastic fibers leading to thickened, inelastic skin and limited retinopathy, associated to accumulation of uncarboxylated Gla proteins (MGP and OC) in plasma, serum and dermis, in the presence of normal serum levels of vitamin K (McMillan and Roberts, 1966). Even though, the deficient carboxylation of coagulation proteins can be restored by vitamin K administration that increases the level of the electron-donor hydroquinone form of vitamin K available for GGCX, never the less, 1 year treatment with vitamin K did not ameliorate skin lesions nor elastic fiber calcification in one patient affected by *GGCX* mutations (unpublished observations). It could be suggested that carboxylase is essential for maturation of MGP, but that the electron donor level of vitamin K does not influence the performance of MGP carboxylation. These data and those from other laboratories showing that vitamin K supplementation does not increase the level of circulating carboxylated MGP in a case of Keutel syndrome (*MGP* mutations; Cranenburg et al., 2011) seem to indicate that MGP carboxylation is under a complex control, only partly dependent on vitamin K, in agreement with recent results obtained on PXE fibroblasts treated *in vitro* with vitamin K supplementation (Boraldi et al., in press).

To further enlarge the spectrum of ectopic calcification disorders which are clinically and/or pathogenetically related to PXE, there is a recent report describing a patient, bearing two *ABCC6* mutations and a gain of function single-nucleotide polymorphism (SNP) in the *GGCX* gene, who was characterized by both classic PXE (papules, retinopathy, and calcifications) and by a PXE-like syndrome (cutis laxa beyond the flexural areas; Vanakker et al., 2011).

Mutations in the *GGCX* or *VKORC1* genes are associated with a hereditary deficiency of the vitamin K-dependent clotting factors as well as a clinically relevant dependency of anti-coagulants (Brenner, 2000; Vanakker et al., 2010). Besides these enzymatic defects, a deficiency of vitamin K has been described in association with coagulation, bone (osteoporosis, osteoarthritis) and vascular (arteriosclerosis) disorders resulting from insufficient carboxylation of Gla proteins (Neogi et al., 2006).

Generalized arterial calcification of infancy

Generalized arterial calcification of infancy is associated with mutations in the *ENPP1* gene and is characterized by mineralization of the internal elastic lamina of large and medium-sized

arteries and stenosis due to myointimal proliferation. Although survival to adulthood has been reported, most patients die within the first 6 months of life (Rutsch et al., 2003).

Features of PXE have been recently described in patients with homozygous missense mutation of the *ENPP1* gene (Li et al., 2012). Cutaneous calcification was never been previously described in ENPP1 deficiency and this finding is a clear demonstration of the role of PPi as a critical anti-calcific agent in PXE and PXE-like disorders.

It is therefore noteworthy the occurrence of a clinical and genetic overlapping between PXE and GACI as clearly highlighted by a recent study on two brothers born from to unrelated parents, showing that the elder developed a PXE condition bearing *ABCC6* mutations, whereas the younger died at 15 months of age of a condition clinically reminiscent of GACI, although it appeared independent of *ENPP1* mutations (Le Boulanger et al., 2010).

The finding that MGP and fetuin A are involved in both conditions further sustain the hypothesis that *ABCC6* mutations account for a significant subset of GACI patients, and *ENPP1* mutations can also be associated with PXE lesions in young children, thus reflecting two ends of a clinical spectrum of ectopic calcifications, possibly through the involvement of common physiological pathways (Nitschke et al., 2012).

CONCLUSION

In spite of the extreme complexity and still incomplete knowledge of the various actors involved, we have reported evidence supporting the importance of mesenchymal cells, and of fibroblasts in particular, in the occurrence and development of soft connective tissue calcifications. Within this context, fibroblasts from PXE and PXE-like disorders offer a valuable model to better understand the complex pathways that end up with elastic fiber mineralization. It can be argued that not all mesenchymal cells behave in the same way and that morpho-functional characteristics of tissues as well as composition of the extracellular matrix and exogenous agents should be taken into account for understanding the susceptibility/resistance to calcification of different body regions in physiological conditions, in aging and in both genetic and acquired disorders.

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Mechanisms of arterial remodeling: lessons from genetic diseases

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Vascular disease is still the leading cause of morbidity and mortality in the Western world, and the primary cause of myocardial infarction, stroke, and ischemia. The biology of vascular disease is complex and still poorly understood in terms of causes and consequences. Vascular function is determined by structural and functional properties of the arterial vascular wall. Arterial stiffness, that is a pathological alteration of the vascular wall, ultimately results in target-organ damage and increased mortality. Arterial remodeling is accelerated under conditions that adversely affect the balance between arterial function and structure such as hypertension, atherosclerosis, diabetes mellitus, chronic kidney disease, inflammatory disease, lifestyle aspects (smoking), drugs (vitamin K antagonists), and genetic abnormalities [e.g., pseudoxanthoma elasticum (PXE), Marfan's disease]. The aim of this review is to provide an overview of the complex mechanisms and different factors that underlie arterial remodeling, learning from single gene defect diseases like PXE, and PXE-like, Marfan's disease and Keutel syndrome in vascular remodeling.

Keywords: arterial remodeling, calcification, genetic disease, vitamin K, vitamin K-antagonists

INTRODUCTION

Arterial remodeling refers to the myriad of structural and functional changes of the vascular wall that occur in response to disease, injury, or aging. Although arterial remodeling can be regarded as a mechanism that naturally occurs with aging, early arterial remodeling is associated with significant hemodynamic changes and cardiovascular morbidity and mortality. Arterial remodeling is set into motion by a variety of complex pathophysiological mechanisms that are closely interrelated, and that influence both the cellular and non-cellular components of the vascular wall. Mechanisms involved in arterial remodeling include fibrosis, hyperplasia of the arterial intima and media, changes in vascular collagen and elastin, endothelial dysfunction, and arterial calcification. Migration and proliferation of vascular smooth muscle cells (VSMCs) contribute to thickening of the arterial intima. Differentiation of VSMCs from their contractile to a secretory or osteogenic phenotype may lead to increased vascular tone, and promotes extracellular matrix (ECM) calcification. Additionally, alterations in the activity of vitamin K-dependent proteins may affect the progression of vascular remodeling, including the induction of calcification. Because of this complexity, it is difficult to study to what extent a single mechanism contributes to arterial remodeling. Monogenetic diseases such as pseudoxanthoma elasticum (PXE), PXE-like syndrome, Marfan's syndrome or Keutel syndrome are characterized by a clinical phenotype that is similar to that of arterial remodeling, but are caused by a specific defect that affects only one or several pathophysiological mechanisms of arterial remodeling. Lessons learned from these relatively rare diseases may therefore ultimately provide insight in more common, multifactorial cardiovascular diseases such as hypertension, diabetes mellitus, and chronic kidney disease as well as in normal vascular aging.

GENERAL FEATURES OF ARTERIAL REMODELING

Arterial remodeling is thought to reflect adaptation of the vessel wall to mechanical and hemodynamic stimuli (Nichols and O'Rourke, 2005). Arterial remodeling is characterized by alterations in the structure and function of the vascular wall and can be divided into atherosclerosis and arteriosclerosis. Whereas atherosclerosis is characterized by a focal inflammatory process in the intima initiated by accumulation of lipids in plaques, arteriosclerosis is a more diffusely localized alteration of the medial arterial vascular wall (Libby, 2002). Arteriosclerosis is associated with aging and generalized cardiovascular, metabolic, or inflammatory disease. Macroscopically, different types of arterial remodeling can be distinguished, depending on the type and localization of the vessel (**Figure 1**) (Mulvany et al., 1996). Arterial remodeling can be either inward or outward and can be hypertrophic (thickening of the vascular wall), eutrophic (constant wall thickness), or hypotrophic (thinning of the vascular wall) (Mulvany et al., 1996). Changes observed in arteriosclerotic arterial remodeling are mainly seen in large central elastic arteries. They are characterized by increased vessel diameter and thickened intimal and medial layers of the vascular wall (outward hypertrophic remodeling) (O'Rourke and Hashimoto, 2007). On the other hand, remodeling of muscular peripheral vessels is more often inwardly eutrophic or hypertrophic, probably reflecting sustained vasoconstriction of vessels (Mulvany, 2008).

Thickening of the arterial wall is caused by intimal hyperplasia, medial hypertrophy and hyperplasia of VSMCs, and deposition

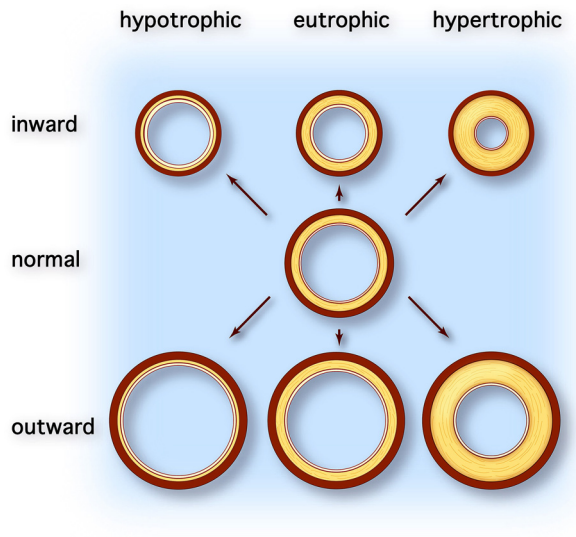

FIGURE 1 | Types of vascular remodeling. Adapted from Mulvany et al. (1996). Different types of arterial remodeling can be distinguished: hypotrophic (left column), eutrophic (center column) and hypertrophic (right column). In addition remodeling can be either inward or outward. Hypotrophic remodeling results in a relative thinner wall and a lower wall-to-lumen ratio. Conversely hypertrophic remodeling is characterized by thickening of the vascular wall due to cellular hyperplasia and/or hypertrophy or deposition of extracellular matrix material and results in increased wall-to-lumen ratio. When the diameter of the vessel changes but the wall-to-lumen ratio remains the same it is called eutrophic remodeling. All types of arterial remodeling can occur in cardiovascular disease, depending on the underlying pathophysiology (e.g., aneurysm or hypertensive arterial stiffening) and arterial site (e.g., central elastic arteries vs. peripheral resistance arteries).

of ECM material including minerals (Virmani et al., 1991; Safar et al., 1998; Schwartz et al., 2000). The normal composition and lay-out of ECM of the vascular wall is disrupted in arterial remodeling. In the media of the normal arterial wall, elastic fibers are arranged in parallel, concentric, fenestrated layers, alternating with layers of VSMCs anchored to the elastic fibers and structural fibers by glycoproteins and integrins (Dingemans et al., 2000; Nichols and O'Rourke, 2005). These structures, termed elastic lamellae, enable the vessel to expand and buffer the systolic blood pressure pulse, while simultaneously maintaining structural stability. Elastic fibers provide passive elastic buffering, whereas VSMCs dynamically redistribute tensile stress across fibers due to their ability to contract and relax (Rachev and Hayashi, 1999). With arterial remodeling the layered architecture of elastic lamellae is lost as they become progressively fragmented and fibrotic (Farand et al., 2007). At higher levels of blood pressure, vessels dilate which results in increased tensile stress on the vascular wall, in accordance with LaPlace's Law of circumferential wall tension (Nichols and O'Rourke, 2005). Thickening of the arterial wall occurring with arterial remodeling reduces tensile stress. VSMCs of adults do not synthesize new elastin but mainly non-elastic collagen resulting in stiffening of the vascular wall (Greenwald, 2007). Closely related to the degradation of ECM, the deposition of calcium minerals further contributes to stiffening and remodeling of vascular tissue (Blaha et al., 2009; Sekikawa et al., 2012).

In addition to structural changes, endothelial function plays an important role in arterial remodeling. Blood flow and shear stress stimulate endothelial cells to produce nitric oxide (NO), which in turn influences contraction and relaxation of VSMCs. Endothelial function decreases with age and endothelial dysfunction is common in many cardiovascular diseases. Moreover, in response to pathological conditions, such as altered shear stress or inflammation, endothelial cells produce cytokines and growth factors that influence the homeostasis of the vascular wall (Csiszar et al., 2009; Urschel et al., 2012). Endothelial cells produce transforming growth factor-beta (TGF-β) and bone morphogenetic proteins (BMPs) which stimulate VSMCs and vascular pericytes to proliferate, to differentiate and to deposit ECM matrix (discussed in more detail below) (Simionescu et al., 2005; Boström et al., 2011).

PATHOGENESIS OF ARTERIAL REMODELING

Arterial remodeling is driven by numerous, highly regulated and interrelated processes. Processes that are of particular importance as they are central in arterial remodeling include: (1) VSMC proliferation and differentiation, (2) degradation and fracture of elastin fibers, and (3) calcification and deposition of ECM material (**Figure 2**). Genetic diseases with a phenotype resembling vascular disease all affect one or several of these key processes and may thus provide more insight in the mechanisms of vascular disease (**Figure 3**).

VASCULAR SMOOTH MUSCLE CELL PROLIFERATION AND DIFFERENTIATION

VSMCs are key regulators of vascular tone and health and insight into their function is of utmost importance for our understanding of the causes of arterial remodeling. In normal arteries, VSMCs in the tunica media regulate vessel tone and diameter in order to maintain hemodynamic balance (Alexander and Owens, 2012). To fulfill this regulatory function, VSMCs need to have a contractile phenotype. Contractile VSMCs are characterized by a number of phenotype-specific marker proteins such as smooth muscle 22-alpha (SM22α), alpha-smooth muscle actin (αSMa), and smoothelin (Iyemere et al., 2006; Eys et al., 2007). Although the majority of VSMCs in the vascular wall display a contractile phenotype, studies have shown that a specific subset of medial VSMCs has the ability to differentiate into a synthetic phenotype which can be further subdivided into a migratory-proliferative phenotype, a secretory phenotype or an osteogenic phenotype (Gerthoffer, 2007). Phenotypic flexibility of VSMCs is necessary to deal with the varying conditions of vascular tissue. Stress signals switch gene expression that will modulate VSMC phenotype to adapt. This process of differentiation is termed phenotype switching and is considered to be a key mechanism in arterial remodeling (Iyemere et al., 2006; Alexander and Owens, 2012).

Phenotype switching occurs in response to vascular injury or stress and is characterized by reduced expression of genes which are specific for contractile VSMCs and cellular morphology (Alexander and Owens, 2012). Although the precise mechanisms are still not fully understood, many different stimuli have been identified, some of which are summarized in **Table 1**

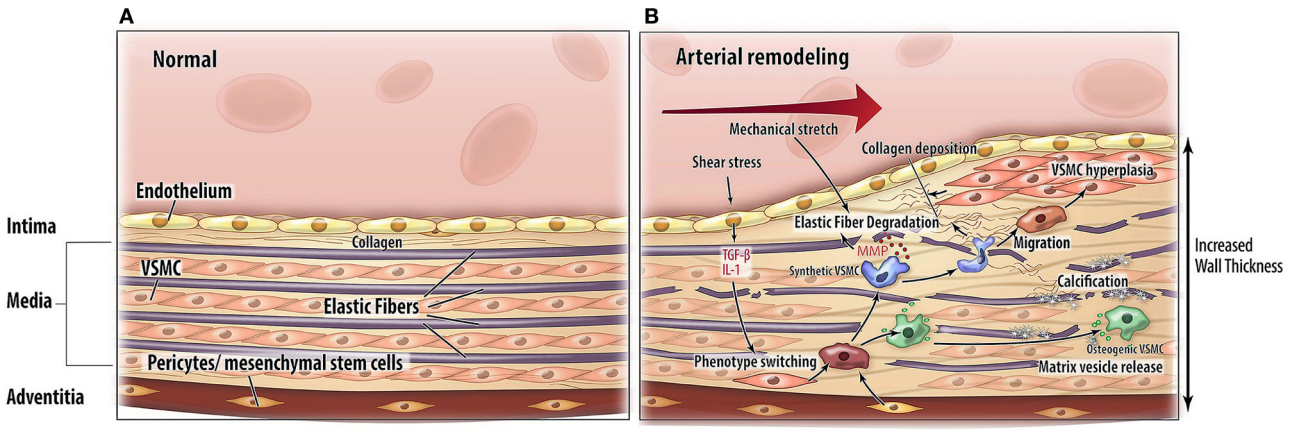

FIGURE 2 | Pathophysiological mechanisms of arterial remodeling. Cross sectional schematic view of the arterial wall. **(A)** Normal situation. **(B)** Arterial remodeling. Arterial remodeling is characterized by thickening of the wall. Elastic fiber degradation, extracellular matrix calcification and collagen deposition lead to adaptation of the vascular wall. Abbreviations: TGF-β, transforming growth factor-beta; IL-1, interleukin 1; MMP, matrix metalloproteinases; VSMC, vascular smooth muscle cell.

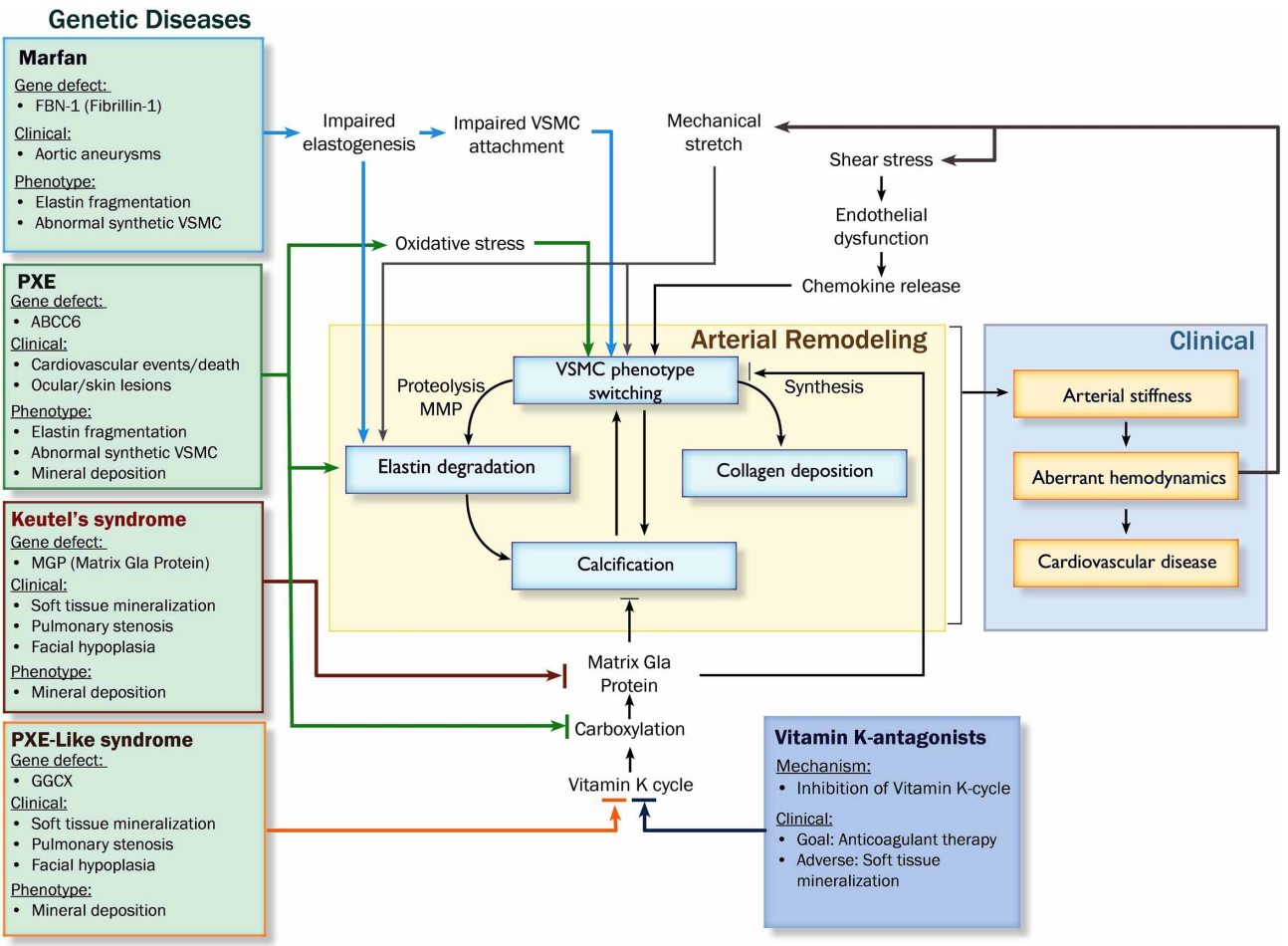

FIGURE 3 | Pathophysiological pathways leading to arterial remodeling in genetic and cardiovascular disease. Abbreviations: PXE, pseudoxanthoma elasticum; MMP, matrix metalloproteinases; VSMC, vascular smooth muscle cell; GGCX, gamma glutamyl transferase.

Table 1 | Stimuli for vascular smooth muscle phenotype switching.

Inflammation
Oxidative stress
Hemodynamic shear stress
Mechanical stretch
Advanced glycation end products (AGE)
Increased calcium-phosphate product
SYSTEMIC HORMONAL
Angiotensin II (Ang II)
Aldosterone
PARACRINE STIMULI
Transforming growth factor-β (TGF-β)
Fibroblast growth factor (FGF)
Endothelial growth factor (EGF)
Platelet derived growth factor (PDGF)
Matrix metalloproteinases (MMP)

(Alexander and Owens, 2012). Migratory stimuli, for instance, alter the cytoskeleton of VSMCs. As a consequence, cell adhesion molecules are detached from the ECM and surrounding vascular cells. Lamellipodia protrude from the leading edge of the cell due to actin polymerization, enabling it to move through the ECM toward a chemotactic stimulus (Willis et al., 2004). This migration contributes to intimal VSMC proliferation and hyperplasia, which is an important cause of arterial wall thickening.

Synthetic VSMCs produce elastolytic enzymes (matrix metalloproteinases; MMPs), which facilitate migration by detaching cells from the basement membrane and ECM. Indeed, upregulation of MMPs coincides with the migration of VSMCs (Willis et al., 2004). A genetic disorder that is associated with VSMC phenotype switching is Marfan's disease. It is characterized by abnormal synthesis and function of elastic fibers (Kielty, 2006). Patients with Marfan's disease suffer from abnormal growth, skeletal disorders, ocular problems and increased tendency to develop aneurysms. The gene defect underlying Marfan's disease is a mutation of the fibrillin-1 (FBN-1) gene, which encodes the glycoprotein FBN-1. FBN-1 is essential for maintaining structural stability of elastic fibers, as well as attaching VSMCs to the elastic fibers (Bunton et al., 2001). Because of defective synthesis, elastic fibers are prone to early mechanical fragmentation and therefore disruption of elastic laminae. However, additional studies on the pathophysiological mechanisms in Marfan's disease showed that, preceding elastic fiber degradation, impaired binding of VSMCs-induced differentiation into a synthetic proteolytic phenotype (Galis et al., 1994; Bunton et al., 2001; Galis and Khatri, 2002). The resulting production of MMPs damages the already weakened vascular wall (Pratt and Curci, 2010). These patho-mechanistic changes in Marfan's disease help to understand underlying mechanisms leading to general vascular disease. Indeed, Goodall et al. showed that VSMCs from inferior mesenteric veins of patients with aortic aneurysms display increased MMP-2 production and an increased number of migratory VSMCs (Goodall et al., 2002). Bendeck et al. demonstrated that inhibition of MMP activity inhibited VSMC migration in rats (Bendeck et al., 1996). Moreover, VSMCs are important for atherosclerotic plaque stability. VSMCs and myofibroblasts in the fibrous cap provide stability to atherosclerotic plaques if they deposit collagen. On the contrary, if a significant part of these VSMCs display a proteolytic phenotype, degradation of fibrous cap material may facilitate plaque rupture (Johnson, 2007). Therefore, the role of VSMCs in maintaining atherosclerotic plaque stability largely depends on VSMC phenotype, stressing out the importance to find therapeutic agents that are able to modify the VSMC phenotype (Orr et al., 2010).

Osteogenic VSMC phenotype

Under specific stimuli such as sustained high extracellular levels of calcium and phosphate or in the absence of inhibitors of calcification, VSMCs can differentiate into an osteogenic phenotype in which VSMCs acquire features usually observed in chondrocytes and osteoblasts (Shanahan et al., 1994; Iyemere et al., 2006). Osteogenic VSMCs are characterized by down regulation of mineralization inhibitory proteins, upregulation of alkaline phosphatase and release of matrix vesicles (MVs) (Shanahan et al., 2011). *In vitro*, culturing VSMCs with elevated phosphate concentrations results in up-regulation of osteogenic markers (Runx2, osterix, and alkaline phosphatase) and down-regulation of VSMC lineage markers (SMa actin, SM22a) (Shanahan et al., 2011). Downstream, bone morphogenetic protein-2 (BMP-2) induces an osteogenic differentiation of VSMCs. BMP-2 has been shown to be expressed in human atherosclerotic lesions (Boström et al., 1993). The phenotypic switch of VSMCs to chondrocyte- and osteoblast-like cells by BMP-2 is limited by calcification inhibitory proteins such as matrix Gla-protein (MGP). In MGP knock-out mice, the absence of MGP results in heavily calcified elastic fibers, and loss of VSMCs which are differentiated into chondrocytic VSMCs (Luo et al., 1997). Additionally, MGP deficiency in VSMCs results in decreased smooth muscle markers which is accompanied by an up-regulated expression of the bone-specific transcription factor cbf1a/Runx2 and the osteogenic protein osteopontin (Speer et al., 2002). The ability of MGP to keep VSMCs in the contractile phenotype may be accomplished by binding BMP-2 (Wallin et al., 2000; Zebboudj et al., 2003).

Tanimura and co-workers were the first to report an association between small membrane encapsulated particles, MVs, and vascular calcification (Tanimura et al., 1983). Vesicular structures have been found in both intimal and medial layers and were likely derived from VSMCs (Kim, 1976; Bennett et al., 1995; Hsu and Camacho, 1999). The release of vesicle bodies from VSMCs was first described as a rescue mechanism against calcium overload trying to prevent apoptosis of VSMCs (Fleckenstein-Grün et al., 1992). VSMC-derived MVs have been identified in human arteries in association with atherosclerosis and hypertension (Kim, 1976; Kockx et al., 1998). *In vitro*, MV from VSMCs form the nidus for calcification (Shanahan et al., 1999).

DEGRADATION AND FRACTURE OF ELASTIN FIBERS

Elastin

Elastic fibers consist of polymers of tropoelastin cross-linked to fibrillin-rich microfibrils. In the vasculature, elastin is mainly produced during the fetal and neonatal period by (secretory)

VSMCs. Above we discussed the importance of elastin for maintaining arterial wall stability and VSMC homeostasis in Marfan's Disease. Additionally, elastin is also an important nidus for calcification. This is illustrated in PXE disease and its accompanying clinical features. PXE is characterized by extensive calcification that mainly occurs along elastic fibers. Although cutaneous manifestations are primarily of cosmetic concern, presence of characteristic skin lesions signifies risk for development of vascular calcification with considerable morbidity and occasional early mortality (Uitto et al., 2010).

Even in the absence of diseases which directly affect elastin structure and function, similar processes can be observed in vascular aging and aortic stiffening (Smith et al., 2012). The question remains, what causes disruption of elastic fibers associated with aging? Initially, it was hypothesized that elastin degradation was predominantly the result of material fatigue caused by cyclic stretching of elastic fibers with every heart beat (O'Rourke, 1976; Nichols and O'Rourke, 2005). Diseases such as (systolic) hypertension would accelerate this process, since increased pulse pressure (PP) exerts greater tensile stress on the vascular wall and increased stretch on fibers. In support of this hypothesis, structural alterations in elastin have been demonstrated to be inversely associated with total number of heart beat cycles *in vitro* (Avolio et al., 1998). However, there are no *in vivo* studies supporting mechanical fragmentation of elastin.

CALCIFICATION AND DEPOSITION OF ECM MATERIAL

Both VSMC phenotype switching and ECM degradation result in enhanced and accelerated vascular calcification. Initially, vascular calcification was regarded as passive mineral deposition. However, this view has been abandoned since overwhelming evidence exists that vascular calcification actually is a highly regulated process. Soft tissue calcification is thought to result from an imbalance between calcification-promoting and -inhibiting factors (**Table 2**). Calcification is the hallmark of patients with genetic diseases like Keutel's syndrome, PXE, and PXE-like syndrome (Ziereisen et al., 1993; Munroe et al., 1999; Vanakker et al., 2007; Rutsch et al., 2011). Keutel's syndrome is caused by a mutation in the gene encoding MGP, which is considered to be the most important inhibitor of vascular calcification. MGP is a 14 kD protein which requires vitamin K-dependent carboxylation to become biologically active. Clinically, lessons learned from the mechanisms underlying Keutel's disease can help understanding vitamin K-antagonist-induced vascular calcifications (discussed below) (Rennenberg et al., 2010; Weijs et al., 2011; Schurgers et al., 2012).

In PXE, the underlying genetic defect is a loss-of-function mutation of the abcc6 gene. This gene encodes a transmembrane transporter protein (Multi Drug Resistant Protein 6; MDRP-6). The substrate of the MDRP-6 is not known, and the exact mechanisms by which this mutation leads to elastin calcification are not yet fully understood. Recent studies have pointed toward calcification being stimulated by phenotype switching of VSMCs, oxidative stress, and interference with carboxylation of MGP (Pasquali-Ronchetti et al., 2006; Garcia-Fernandez et al., 2008; Boraldi et al., 2009; Li et al., 2009b; Rutsch et al., 2011). Similarly, in PXE-like syndrome a mutation in the γ-glutamylcarboxylase (GGCX) gene causes elastic fiber calcification as is observed in vitamin K-antagonist-induced vascular calcification (Gheduzzi et al., 2007; Vanakker et al., 2007; Rennenberg et al., 2010; Weijs et al., 2011; Schurgers et al., 2012). The GGCX mutation is associated with increased bleeding tendency due to impairment of vitamin K-dependent coagulation factors (Vanakker et al., 2007; Li et al., 2009a). This has led to the concept that vitamin K-dependent proteins are of importance in inhibiting vascular elastin calcification. The GGCX mutation results in decreased activity of MGP and subsequently an impaired inhibitory potential for calcification, similar to the situation in Keutel's syndrome in which MGP is absent (Schurgers et al., 2008; Vanakker et al., 2010). In a similar manner, treatment with vitamin K-antagonists may also induce an increased tendency for calcification (**Figure 2**) (Price et al., 1998; Schurgers et al., 2007; Rennenberg et al., 2010; Chatrou et al., 2012). Since vitamin K-antagonists work by inhibiting the Vitamin K cycle and by reducing carboxylation of MGP, these findings confirm the important central role of MGP in the regulation of calcification. Therefore, it is highly probable that in these diseases, MGP also plays an important regulatory role in calcification (Shanahan et al., 1999; Schurgers et al., 2007).

Table 2 | Calcification regulating factors.

FACTORS PROMOTING CALCIFICATION
Bone morphogenetic protein 2 (BMP-2)
↑ Calcium-phosphate product
Tumor Necrosis Factor α (TNF-α)
Interleukin 6 (IL-6)
Receptor activator of nuclear factor κB (RANK) ligand (RANKL)
Insulin-like growth factor I (IGF-I)
Insulin
↑ Glucose
↑ Parathyroid hormone
Matrix metalloproteinases (MMP)
Elastin degradation
Hydroxyapatite crystals
FACTORS INHIBITING CALCIFICATION
Fetuin-A
Matrix gla protein (MGP)
Osteoprotegerin (OPG)

CLINICAL ASPECTS OF ARTERIAL REMODELING

Since the normal function of vessels is to maintain adequate perfusion of organs and tissues and to buffer oscillating blood pressures, arterial remodeling results in changes in this function. At first, these are compensatory (i.e., reducing wall tension). However, in later stages these compensatory mechanisms become detrimental and initiate a vicious cycle of pathophysiological aberrations.

ARTERIAL REMODELING, ARTERIAL STIFFNESS AND DAMAGING HEMODYNAMICS

Fragmentation of the elastic lamina, hyperplasia and hypertrophy of VSMC, loss of contractility of VSMC, deposition of collagen, and arterial calcification lead to stiffening of arteries.

Many studies have shown that arterial stiffness, which is clinically measured as the carotid-femoral pulse wave velocity (cfPWV), is independently associated with cardiovascular risk and mortality (Laurent et al., 2001, 2012; Mitchell et al., 2010; Vlachopoulos et al., 2010). In addition, arterial stiffness is independently associated with, and predictive of target organ damage of the heart, kidneys, and brain (Laurent and Boutouyrie, 2005). Arterial stiffness reflects the degree of remodeling in large arteries and is used as a parameter for cardiovascular risk stratification next to traditional cardiovascular risk factors (Nurnberger et al., 2002). The mechanism linking arterial stiffness to an adverse outcome is thought to involve a pathological hemodynamic profile in large, central arteries such as the aorta (Mitchell, 2009). This pathological hemodynamic pattern consists of an increased systolic blood pressure (SBP; i.e., systolic hypertension) and decreased diastolic blood pressure (DBP) resulting in an increased PP. The pressure waveform in the aorta is composed of a forward traveling wave generated by contraction of the left ventricle of the heart, and a backwards traveling wave generated by reflection from peripheral arteries (**Figure 4A**). This reflected wave is generated at vascular bifurcations and at sites where the elastic conduit arteries transition into muscular resistance arteries (Mitchell, 2004). At this site the difference in impedance of the vascular wall causes the forward traveling wave to be reflected. The shape of the aortic pressure waveform is largely determined by timing and speed with which the pulse wave propagates through the arteries. With arterial stiffening the speed of both the forward and backward traveling wave is increased. Remodeling of arteries causes an earlier wave reflection. As a result of different timing of both waves, the forward traveling wave and the reflected wave are summated, leading to an augmented systolic peak and a relatively low DBP (**Figure 4B**), generating a highly pulsatile flow in aorta and branching arteries. It is this blood pressure pulsatility that is thought to have damaging effects on sensitive target organs as well as on vascular function, and to contribute to the vicious cycle of arterial remodeling.

High blood pressure pulsatility leads to increased mechanical vascular wall stress. With high central PP, the amplitude in which the arterial wall expands and contracts with each consecutive heartbeat is increased. This leads to higher stretch on elastic and collagen fibers in the arterial wall and this in turn may contribute to material fatigue, fracture, and degradation. Additionally, cyclic stretching of VSMC has been demonstrated to stimulate phenotype switching and arterial remodeling (Williams, 1998). Secondly, pathological blood pressure pulsatility adversely affects endothelial function since structure and function of the endothelium are modulated by hemodynamic forces (Gimbrone and García-Cardeña, 2012). In hypertensive patients, a high pulse-pressure is associated with endothelial dysfunction, which can be measured as the vasodilator response to acetylcholine (Ceravolo et al., 2003). In the normal situation, a laminar blood flow pattern and cyclic shear stress maintain proper endothelial function such as: NO-mediated regulation of vascular tone, maintaining a non-thrombotic and non-inflammatory state, preserving ECM metabolism, and regulating vascular permeability (Vita and Mitchell, 2003; Gimbrone and García-Cardeña, 2012). In arteries with remodeling, blood flow becomes increasingly oscillatory with peaked systolic flows as well as stasis and even flow reversal during diastole (Domanski et al., 1999; Mitchell, 2004). The ensuing turbulent flow and locally altered shear stress patterns cause endothelial dysfunction, which is characterized by impaired NO synthesis and upregulation of pro-inflammatory and pro-atherogenic factors, increased oxidative stress, as well as vasoconstriction (Keulenaer et al., 1998; Blackman et al., 2002; Gimbrone and García-Cardeña, 2012). In addition, altered flow and increased pressure pulsatility have been shown to activate the endothelium and induce production of osteogenic factors such as BMP-2 and BMP-4 (Qiu and Tarbell, 2000; Sorescu et al., 2003;

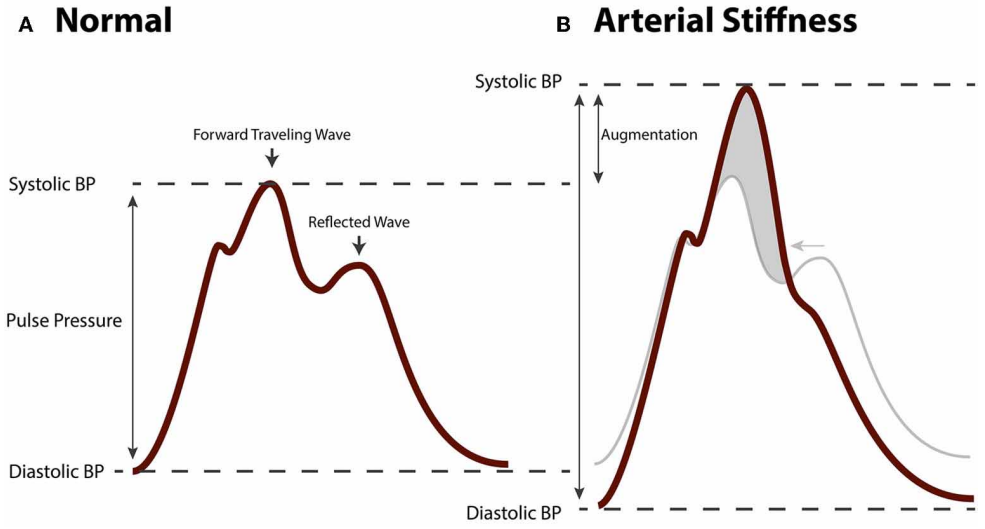

FIGURE 4 | Hemodynamic changes in arterial stiffening. (A) Aortic blood pressure waveform of a healthy, normotensive person. The forwards traveling wave precedes the (backwards traveling) reflected wave. **(B)** Aortic pressure waveform of a person with arterial stiffness. Due to increased pulse wave velocity, the forward traveling wave and reflected wave are summated leading to augmented pulse pressure.

Boström et al., 2011). Indeed, BMP-2 transgenic apoE$^{-/-}$ mice display increased calcification of atheromatous lesions, whereas MGP transgenic apoE$^{-/-}$ mice have less atherosclerotic mineralization, suggesting a key role for MGP in suppressing BMP-2-induced vascular mineralization (Nakagawa et al., 2010; Yao et al., 2010).

Arterial stiffness and endothelial function not only stimulate the development of atherosclerotic plaques but also further promotes arterial media remodeling. In this way, arterial stiffness may explain the interrelationship of arteriosclerosis and atherosclerosis.

Finally, the pathological hemodynamic patterns due to arterial stiffness lead to damage of susceptible organs such as kidneys, brain, and heart. It has been established that arterial stiffness and chronic kidney disease are closely interrelated (Safar et al., 2004). Patients with primary kidney disease have accelerated arterial remodeling and calcification due to altered homeostasis of calcium and phosphate, high degrees of inflammation and oxidative stress, uremia, altered cholesterol metabolism, and an activated renin-angiotensin system (RAS) (Safar et al., 2004). Conversely, increased arterial stiffness and pressure pulsatility induce renal damage (Verhave et al., 2005; Ford et al., 2010; Briet et al., 2011; Chen et al., 2011). Blood pressure pulsatility has been put forward to be able to cause renal damage. Although kidneys are normally protected against high blood pressure by an effective autoregulation, abnormal blood pressure pulsatility has been shown to blunt the renal myogenic response (Bidani and Griffin, 2004; Bidani et al., 2009; Hultström, 2012), exposing the vulnerable glomerular microcirculation to damaging pressure oscillations (Safar et al., 2012).

CALCIFICATION AS CARDIOVASCULAR RISK FACTOR AND POSSIBLE THERAPEUTIC TARGET

In PXE, PXE-like syndrome as well as in Keutel's syndrome, arterial calcification is an important feature of the clinical phenotype. Besides these, arterial calcification is also observed in more common disorders such as diabetes, hyperparathyroidism, and chronic kidney disease as well as in vascular aging. In addition, vascular calcification may be induced by drugs that adversely affect the regulatory balance between factors inducing or inhibiting calcification. For instance, chronic treatment with vitamin K-antagonists (such as warfarin) is associated with peripheral artery calcification (Rennenberg et al., 2010). Calcification occurs in both arteriosclerosis and atherosclerosis. Aortic medial calcification has been demonstrated to contribute to arterial stiffness in different populations (Odink et al., 2008; Cecelja et al., 2011; Sekikawa et al., 2012). Moreover, the presence of aortic calcification is predictive of coronary artery disease (Jang et al., 2012). Calcification of coronary arteries predominantly reflects atherosclerosis and can be measured and quantified by computed tomography (CT) using the calcium-score. The calcium score (expressed as Agatston units) has been used as a sensitive tool for risk stratification and decision-making regarding coronary revascularization and diagnostic angiography. A negative calcium score indicates that the presence of atherosclerotic plaque is very unlikely, whereas a high calcium score is associated with significant cardiovascular risk (Budoff et al., 2006). The importance of calcification with respect to cardiovascular outcome is further stressed by the fact that rapid annual progression of the calcium score is independently associated with outcome (Raggi et al., 2004). For this reason, the calcification process may become an important therapeutic target. The challenge is that an intervention should be aimed at a modifiable factor in the pathophysiological process. As can be learned from PXE, PXE-like syndrome and Keutel's syndrome, MGP and the vitamin K cycle are among the most important known regulators of calcification and VSMC phenotype switching. As described above, MGP requires vitamin K mediated carboxylation to be biologically active. Therefore, treatment with vitamin K would theoretically inhibit or possibly reverse arterial calcification and slow down the development of arterial stiffness. Indeed, our group demonstrated that calcification could be reversed in rats that had extensive calcification due to warfarin treatment, by subsequently administering vitamin K (Schurgers et al., 2007). In humans, the 3-year daily supplementation of 500 mcg vitamin K on top of a multi-vitamin resulted in hold on progression of vascular calcification (Shea et al., 2009) In the observational Rotterdam study, high dietary intake of vitamin K was associated with better cardiovascular outcome and reduced coronary artery calcification (Geleijnse et al., 2004; Gast et al., 2009). Also, in post-menopausal women, treatment with vitamin K resulted in improved markers of vascular stiffness (Braam et al., 2003). Furthermore, a recent study by Westenfeld et al. showed that vitamin K2 supplementation reduced plasma levels of inactive, undercarboxylated MGP (Westenfeld et al., 2012). Since vitamin K has no reported adverse side effects, it might be a promising treatment for calcification. Clinical trials investigating the effects of vitamin K supplementation on calcification and arterial remodeling are currently in progress.

ARTERIAL REMODELING AS POTENTIAL THERAPEUTIC TARGET

In addition to calcification, other pathophysiological pathways of arterial remodeling such as arterial stiffening, fibrosis, or elastin degradation may also be potential candidates for intervention. However, finding suitable, modifiable candidates has proven to be a challenge. Although most existing antihypertensive drugs may reduce arterial stiffness to some extent, it is difficult to determine whether this effect is mainly due to blood pressure reduction or represents a true effect on ECM remodeling (Boutouyrie et al., 2011). Since the RAS plays an important pro-fibrotic role in arterial remodeling it has been suggested that beneficial effects of RAS antagonists are (partly) due to their anti-fibrotic action, independent of their effects on blood pressure. Indeed, Tropeano et al. showed that treatment with 8 mg perindopril was associated with lower carotid stiffness independently of the effects on blood pressure, whereas a dose of 4 mg did not have such an effect (Tropeano et al., 2006). Similar blood-pressure-independent destiffening effects have been reported for selective aldosterone antagonists such as eplerenone (White et al., 2003), supporting possible effects of RAS system inhibition on ECM remodeling. Especially in diabetes, advanced glycation end-products (AGE) contribute to arterial stiffness by creating cross-links between elastic and collagen fibers. Therefore, the AGE crosslink-breaker alagebrium has received attention as potential de-stiffening drug

(Zieman et al., 2007). This α-Aminoguadine compound improved aortic stiffness and improved peripheral arterial endothelial function in hypertensive patients, independently of blood pressure (Kass et al., 2001; Zieman et al., 2007). However, further research is required to properly assess the effects and safety of this class of drugs.

CONCLUSION AND FUTURE PERSPECTIVES

Studying genetic diseases such as PXE, PXE-like syndrome, Keutel's syndrome and Marfan's disease increase our knowledge about pathophysiological mechanisms underlying arterial remodeling (summarized in **Figures 2** and **3**). Single gene defects of these specific diseases affect major regulatory pathways such as VSMC phenotype switching, matrix degradation, and calcification that are also involved in common cardiovascular disease and aging. Lessons learned from PXE, PXE-like syndrome and Keutel's syndrome have given attention to the major calcification regulatory protein MGP and has provided a possible new target for intervention. In this way, the continued study of these relatively rare genetic diseases may ultimately provide us with potential new targets for therapeutic intervention above and beyond traditional cardiovascular risk management and treatment of risk factors. Conceivably, since VSMC phenotype switching has such an important regulatory role in arterial remodeling, specifically targeting the direction of VSMC phenotype switching may prove to be promising. Ultimately, these novel concepts learned from studying specific genetic diseases can be applied to general cardiovascular medicine.

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The ABCC6 transporter: what lessons can be learnt from other ATP-binding cassette transporters?

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ABC transporters represent a large family of ATP-driven transmembrane transporters involved in uni- or bidirectional transfer of a large variety of substrates. Divided in seven families, they represent 48 transporter proteins, several of which have been associated with human disease. Among the latter is ABCC6, a unidirectional exporter protein primarily expressed in liver and kidney. ABCC6 deficiency has been shown to cause the ectopic mineralization disorder pseudoxanthoma elasticum (PXE), characterized by calcification and fragmentation of elastic fibers, resulting in oculocutaneous and cardiovascular symptoms. Unique in the group of connective tissue disorders, the pathophysiological relation between the ABCC6 transporter and ectopic mineralization in PXE remains enigmatic, not in the least because of lack of knowledge on the substrate(s) of ABCC6 and its unusual expression pattern. Because many features, including structure and transport mechanism, are shared by many ABC transporters, it is worthwhile to evaluate if and to what extent the knowledge on the physiology and pathophysiology of these other transporters may provide useful clues toward understanding the (patho)physiological role of ABCC6 and how its deficiency may be dealt with.

Keywords: pseudoxanthoma elasticum, ABCC6, ABC transporters, substrate identification, clinical variability, integrated approach, systems biology, modifier genes

In this paper, we will summarize relevant knowledge and methods for analysis on ABC transporters which may be useful for the further study of ABCC6.

Pseudoxanthoma elasticum is an autosomal recessive disorder resulting from aberrant mineralization and fragmentation of elastic fibers in the extracellular matrix of the skin, the eyes and the cardiovascular system. It is characterized by papular skin lesions, a retinopathy prone to hemorrhage due to subretinal neovascularisation, and premature occlusive vessel disease, with considerable inter- and intra-familial variability of severity (Vanakker et al., 2008). Thirteen years after the discovery of the causal relation between *ABCC6* mutations and PXE, significant progress has been made in the characterization of the *ABCC6* gene and the transporter it encodes (Bergen et al., 2000; Le Saux et al., 2000). Nevertheless, ABCC6 remains surrounded by a number of unsolved enigmas, including its substrates and physiological functions as well as the mechanisms by which it is connected to ectopic elastic fiber mineralization. Further, the significant variability in clinical severity of the PXE phenotype cannot be explained by the *ABCC6* mutations, as demonstrated by numerous negative genotype-phenotype correlation studies (Meloni et al., 2001; Gheduzzi et al., 2004; Chassaing et al., 2005; Pfendner et al., 2007). This suggested the involvement of modifier genes, influencing particularly the severity of PXE symptoms (Zarbock et al., 2009).

Despite marked structural resemblance with other ABC transporters, ABCC6 has been considered the odd man out, due to its unique relation with a connective tissue disease affecting tissues in which it is hardly expressed. Drawing analogies between ABCC6 and the other ABC transporters has been done in the past, particularly to gain insights in substrate interactions. While, after more than a decade, the identification of this substrate is being further pursued, it may be a good time to evaluate if information on other ABC transporters as well as the difficulties that were and are still encountered in their study may point out potential pitfalls, concepts and approaches on how to deal with the challenges in the field of PXE.

THE PHYSIOLOGICAL FUNCTIONS OF ABCC6

Perhaps one of the most intriguing questions surrounding ABCC6 is the nature of its substrate and hence the physiological function of the transporter. Following unsuccessful comparisons, it is doubtful that the function of other members of the ABC transporter family will imply anything concerning the actual function of ABCC6 (Madon et al., 2000). It has been previously demonstrated in plant ABC transporters that – despite phylogenetic similarities and domain homologies – related ABC transporters can in fact serve diverse physiological functions. In humans, this was endorsed through the comparison of the cystic fibrosis transmembrane conductance regulator (ABCC7 or CFTR, the gene for cystic fibrosis) and P-glycoprotein (ABCB1 or MDR – multidrug resistance protein; Luckie et al., 2003). This however does not imply that nothing can be learned from previous experiences with functional dissection of ABC transporters. Awareness has risen for several of these that the complexity of (patho)physiological mechanisms related to the wild type and mutant transporter is much higher than anticipated; in this respect and based on our current knowledge, ABCC6 will probably not be an exception to the rule. This enlarging complexity began with the changing concept of

substrate specificity. For several ABC transporters either one or a small number of closely related substrates are known. However, there have been reports of other transporters, such as ABCC1, ABCC2 or ABCB1, for which multispecificity for a diverse range of substrates has been shown (Jemnitz et al., 2010; He et al., 2011). The diversity of (patho)physiological observations in PXE – oxidative stress, the PXE serum factor, vitamin K deficiency, the relation with *ENPP1*, does not make it unthinkable that they result from aberrant transport of more then one substrate, thus influencing more than one physiological process (Le Saux et al., 2006; Pasquali-Ronchetti et al., 2006; Vanakker et al., 2010; Nitschke et al., 2011). For many ABC transporters – including ABCC6 – the specific sequences responsible for substrate recognition have not yet been identified, which may add to the complexity of substrate identification (Glavinas et al., 2004). Nevertheless, for some transporters such as ABCC2 and Pgp, binding-site models have been proposed usingrelatively high accuracy *in silico* methods. Such models include the presence of one large binding site and multiple smaller ones, primary and secondary binding sites or three distinct sites (Hirono et al., 2005; Borst et al., 2006; Pedersen et al., 2008; Ferreira et al., 2013).

These *in silico* approaches such as molecular docking, though insufficiently reliable to pinpoint with certainty the physiological substrates, may also have the advantage to generate a list of potential substrates in a cost- and time-efficient manner. Subsequently, these predictions need to be validated *in vivo*. Several approaches can be applied to identify physiological compounds transported by ABC proteins, such as *in vivo* hepatobiliairy elimination studies in mutant animal models, and using membrane vesicles or functional assays based on mass spectrometry (Ci et al., 2007; Katona et al., 2009; Jemnitz et al., 2010). Though murine model organisms are commonly used for such experiments and the *Abcc6* knockout mouse model largely recapitulates the clinical features of PXE, there have been reports emphasizing the differences in the physiological mechanisms between rodents and humans, which may slow down substrate identification (Ivan et al., 2013). This has been the case for the ABCG2 protein, recently discovered to be an urate transporter (Nakayama et al., 2011). It should remind us to be cautious to extrapolate findings (positive or negative) in other species to the human disease. Remembering the adage that humans remain the most optimal model to search for substrate(s), an interesting approach for substrate screening has been reported for ABCC2 (Krumpochova et al., 2012). To determine the extent of its substrate spectrum, a variant on the classic vesicular transport experiments has been applied to extract substrates from body fluids. Classic vesicular transport studies have as a drawback that an upfront hypothesis about the transported compounds is necessary and that only one substrate can be evaluated at a time. Certainly, the necessity of an upfront hypothesis is a major disadvantage with respect to ABCC6. By incubating the vesicles in body fluids (in the case of *Abcc2* murine urine) and analyzing the vesicle content by LC/MS, several novel compounds were identified. To apply this technique for ABCC6 would imply the use of human plasma and/or hepatocytes. This transportomics technique has great potential with several advantages including a reduced number of experimental animals and would mean that identification of compounds is not a prerequisite to study their transport in vesicular transport experiments as one can fish for new substrates. Combining transportomics with untargeted metabolomics analysis would further increasethe range of potential substrates that can be identified. Disadvantages of the technique include that it is less suitable for identifying hydrophobic substrates. The complex composition of body fluids may require. fractionation to limit the effect of regulators of the transporter which could mask transport of some substrates (Krumpochova et al., 2012).

After several years of tranquility, the expression profile of *ABCC6* has again been the subject of debate. ABCC6 is predominantly expressed at the basolateral side of liver and kidney cells, though the transporter also has differential expression in the gut and gastro-intestinal tract (Sinkó et al., 2003; Mutch, 2004; Pomozi et al., 2013). Recently, a supposed intracellular location in the mitochondria-associated membrane (MEM) – part of the ER complex – has been described (Martin et al., 2012). Though the shift in the paradigm linking the expression and function of ABCC6 to the hepatic and renal plasma membrane (PM), which is declared in this paper, should be reviewed with skepticism – the amount of evidence locating ABCC6 in the PM is after all overwhelming – the concept of an additional intracellular localization is potentially interesting and may further increase the complexity of the pathophysiological enigma at hand. This issue has been the topic of debate in recent papers by respectively Martin et al. (2012, 2013) and Pomozi et al. (2013). Demonstrating again the PM localization of ABCC6, the latter group could not confirm the MEM localization while the former called on methodological arguments to defend their findings (Martin et al., 2013; Pomozi et al., 2013). Challenging the cellular localization of proteins is not unprecedented in the ABC superfamily. Recently, the position of the ABCB6 transporter, originally thought to function in mitochondrial porphyrin metabolism, was challenged and extensively documented with experimental and literature data (Kiss et al., 2012). Despite the critical review and convincing evidence of the true physiological function of ABCB6, the authors did not completely exclude a contribution of ABCB6 to porphyrin metabolism and appealed for further and thorough study of the true pathophysiological function(s) of this transporter (Kiss et al., 2012). The knowledge on the subcellular localization of a native protein is of critical importance to model and understand the pathophysiology of any disease. Therefore, unity should be achieved regarding the PM localization of the ABCC6 transporter, but as with ABCB6 I would at this point not completely reject the idea of an additional intracellular localization of ABCC6. In view of the current contradictory results on the potential MEM localization, further work should be done to clarify this issue as the abnormal mitochondrial morphology and membrane potential in PXE – mentioned by Martin et al. (2012) as supporting evidence is insufficient; indeed, it has been demonstrated for the renal ABCB1 transporter that morphological abnormalities of the mitochondria can occur in the absence of a mitochondrial localization, possibly due to accumulation of toxic products (Huls et al., 2007). It cannot be excluded that ABCC6 may move between different membrane compartments under particular conditions, which theoretically may explain the contradicting findings for MEM localization. This has been shown for the PM ABCA1 transporter which, in the absence of extracellular Apo-AI, can be sequestered on intracellular membranes or

degraded (Tang et al., 2009). As one particular protein can serve strictly different functions in separate parts of the same cell – in view of the complexity of what we know on PXE, this might not even be so unexpected – a validated intracellular localization of ABCC6 may help us further to understand the disease (Kumar and Snyder, 2002).

Though much attention has been paid to the expression of the ABCC6 transporter in the liver, its presence in the kidney, gut and the intestinal tract are less frequently the focus of attention. It has been shown for ABC transporters such as ABCC2 that the ABC protein may have different organ-specific functions (Jemnitz et al., 2010). Where in the liver it functions in biliairy transport, in the kidney it is involved in the excretion of small organic anions. A similar tissue-dependent function was shown for ABCA1 and ABCG1 (Tarling et al., 2013). Specifically addressing these extra-hepatic ABCC6 proteins is necessary as it is not unconceivable that for example the mucosal involvement in PXE may depend on or be modulated by the intestinal ABCC6 transporter.

REGULATION OF ABCC6

Distinct pathways are involved in the regulation of ABC transporters, comprising genetic, epigenetic and nuclear-receptor mediated mechanisms, as well as post-transcriptional target repression by microRNAs (miRNAs), which can be triggered by hormones, growth factors and exogenous factors. Several examples exist of ABC transporters, such as Pgp, MRP4, BCRP, whose regulation depends on a combination of mechanisms (Masereeuw and Russel, 2012). Nuclear factor regulation and methylation dependent epigenetic regulation has been described for ABCC6 (Arányi et al., 2005; de Boussac et al., 2010; Ratajewski et al., 2012). So far, no correlation with the PXE phenotype, its variability or potential therapeutic approaches has been attempted. The influence of epigenetic changes, such as promotor DNA methylation, on disease variability has been shown for ABCA1 and coronary artery disease and various drug transporters such as ABCB1and MDR1 (Baker et al., 2005; Reed et al., 2008; Guay et al., 2012). Therapeutic consequences may include the use of histone deacytelase inhibitors, as reported for MDR1 (Jiang et al., 2009).

MicroRNA is a family of short non-coding RNAs involved in the negative regulation of gene expression at the posttranscriptional level (He and Hannon, 2004). With a few hundred miRNAs identified, it is estimated that 50% of the protein-coding genes are regulated by them. Among ABC transporters, miRNA dependent regulation has been documented for *CFTR*, *MRP2* and *4*, *PgP* and *BCRP*, either directly or indirectly through nuclear factors (Borel et al., 2012; Oglesby et al., 2013). In the *CFTR* gene, variants have been described that may influence the miRNA target sites in the 3′ UTR (Amato et al., 2013). It has been suggested that certain single nucleotide polymorphisms (SNPs) can influence the affinity for inhibitory miRNAs and may explain the differences in clinical expression between patients with an identical genotype. Mutations in the 3′ UTR have also been described in the *ABCC6* gene, so the involvement of miRNAs in PXE pathophysiology may not be purely theoretical. Identification of such miRNAs does not only open the possibility of using modulators, such as chemically engineered oligonucleotides called antagomirs, with the goal of influencing mechanisms that underlie disease initiation or progression (Masereeuw and Russel, 2012). Of interest is that miRNAs have been demonstrated useful biomarkers in kidney disease (Amato et al., 2013; De Guire et al., 2013). The development of an accurate biomarker set for PXE, which does not yet exist, will enable clinical studies to determine whether a compound has a clinically significant effect on the PXE phenotype.

CLINICAL VARIABILITY

The clinical variability of the PXE phenotype remains a challenge for patients and physicians, making an individualized approach at this moment nearly impossible. Because of the lack of correlations between the patients phenotype and *ABCC6* genotype, the possibility of modifier genes has been suggested (Hendig et al., 2007; Zarbock et al., 2010, 2009). The number of potential modifier genes in PXE is currently still limited, which is not totally unexpected; for many ABC transporters the identification of clinical modulators remains challenging. The clinical variability in PXE shows similarities with the variability in cystic fibrosis (CF), the clinical course of which is also difficult to predict using the *ABCC7* mutations. Consequently, the quest for modifier genes has started and several modifiers of pulmonary outcome in CF have been described (Blaisdell et al., 2004; Boyle, 2007; Weiler and Drumm, 2012; von Kanel et al., 2013). The search for CF modifiers has lead to several recommendations which are equally valid for ABCC6. First, the importance of an in depth, unambiguous and universal definition of the phenotype has been deemed extremely important. Indeed, the description of the clinical features of the PXE phenotype is often inconsistent in different reports, even when tools such as the Phenodex® are available (Pfendner et al., 2007). A more standardized definition of the phenotypic features will allow a more reliable identification and comparison of modifiers. Further, the limitations of association studies where the relationship between phenotype and polymorphisms in candidate modifier genes is examined have become clear, with the possibility of false positive studies or the causal effect of other genes which may travel with the candidate gene(s) (Nadeau, 2001; Accurso and Sontag, 2003). Finally, a study in twins and siblings in CF indicates that functions directly related to CFTR, membrane ion transport and/or intracellular trafficking of mutant protein are subject to modifier effects (Bronsveld et al., 2000, 2001). Identification of modifiers for functions directly related to the ABC transporter may also yield further insights in the pathophysiology of PXE and provide novel therapeutic targets. Next generation sequencing (NGS), a revolutionizing sequencing technique enabling parallel sequencing of multiple genes and whole exome sequencing is also an opportunity to identify modifier genes and variants, particularly in rare disorders in which large cohorts are often difficult to gather. By combining analysis of extreme phenotypes with pathway analysis, significant power can still be obtained, as was demonstrated by using NGS in CF (Emond et al., 2012).

Modifier genes may not be the only mechanism involved in the variability of PXE. For several ABC transporters, compensatory mechanisms have been described. These include upregulation of MRP3 expression in Dubin-Johnson syndrome, thus compensating for the impaired ABCC2 function (Masereeuw and Russel, 2012). Such compensatory mechanisms have also been suggested for PXE. Gene expression profiling of ABC transporters in dermal

fibroblasts revealed increased expression of seven genes, including *ABCC2* and several members of the A-subfamily (Hendig et al., 2008). The latter was further explored in hepatocytes of a knock-out mouse model where tissue specific upregulation of *Abca4* was demonstrated in the liver (Li and Uitto, 2011). However, no further studies have investigated other potential compensatory mechanisms, though they may be of significant importance in understanding PXE, for aiming a more personalized follow-up of patients and for the introduction of novel therapeutic approaches.

AN INTEGRATIVE APPROACH FOR PXE RESEARCH

Pseudoxanthoma elasticum is one of the diseases where, through the dedicated work of a relatively small group of researchers, a large number of data and observations are gathered. To take on the challenges that we are facing in the field of PXE, integration of all these data and findings may ultimately be the most difficult though imperative step to move forward efficiently. One interesting initiative in this respect is the Clinical and Functional Translation of CFTR (CFTR2) project, which presents a novel approach to clinical and functional annotation of mutations in the *CFTR* gene. Within this project, clinical and molecular data are gathered from CF registries and centers, in a standardized way and under the control of data managers. Further, data on functional assessment of mutations was added (Castellani and CFTR2 team, 2013). Though valuable initiatives have been taken to establish mutation database for *ABCC6*, and linking these molecular data to phenotypical characteristics, no such comprehensive database is currently available. A comprehensive database would improve our ability to identify biomarkers and interpret underlying mechanisms of disease variability in PXE. This database would ideally incorporate information on functional mutation data, potential or established modifier variants, exome sequencing data, serum measurements of patients, fibroblast observations, proteomics, metabolomics and other -omics in clinically well-characterized patients. This is expected to enhance diagnostics, carrier testing and screening, genotype-phenotype correlations, modifier analysis and insights into pathogenesis and therapies. It can be concluded that the other members of the ABC transporter family can provide us with valuable information and useful precedents for further characterizing the ABCC6 transporter. Perhaps the most important lesson to incorporate in current PXE research is the concept that only an integrative approach will finally enable us to elucidate this disease completely. To this purpose, it is therefore imperative that joint initiatives can be outlined to merge and integrate past, present and future research data.

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Zebrafish models for ectopic mineralization disorders: practical issues from morpholino design to post-injection observations

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Zebrafish (ZF, *Danio rerio*) has emerged as an important and popular model species to study different human diseases. Key regulators of skeletal development and calcium metabolism are highly conserved between mammals and ZF. The corresponding orthologs share significant sequence similarities and an overlap in expression patterns when compared to mammals, making ZF a potential model for the study of mineralization-related disorders and soft tissue mineralization. To characterize the function of early mineralization-related genes in ZF, these genes can be knocked down by injecting morpholinos into early stage embryos. Validation of the morpholino needs to be performed and the concern of aspecific effects can be addressed by applying one or more independent techniques to knock down the gene of interest. Post-injection assessment of early mineralization defects can be done using general light microscopy, calcein staining, Alizarin red staining, Alizarin red-Alcian blue double staining, and by the use of transgenic lines. Examination of general molecular defects can be done by performing protein and gene expression analysis, and more specific processes can be explored by investigating ectopic mineralization-related mechanisms such as apoptosis and mitochondrial dysfunction. In this paper, we will discuss all details about the aforementioned techniques; shared knowledge will be very useful for the future investigation of ZF models for ectopic mineralization disorders and to understand the underlying pathways involved in soft tissue calcification.

Keywords: zebrafish, embryos, morpholino, mineralization, osteogenic pathways

INTRODUCTION

Mineralization is an essential step in the process of skeletal tissue formation, which needs to be rigorously controlled and restricted to specific regions. Though incompletely understood, multiple factors – both agonists and inhibitors – have been shown to work synergistically in achieving physiological mineralization of bone and dental tissue (Hosen et al., 2012). In many cases, ectopic mineralization results from a disturbance of the complex interplay between mineralization propagators and antagonists set out to regulate this process (Shanahan et al., 2011). Although most soft tissues can undergo calcification, certain ones – including skin, kidneys, cartilage and tendons, eyes, and vasculature – are considerably more prone. Pathologic mineralization in any of these tissues may result in disease with significant morbidity and mortality (Viegas et al., 2009). The mechanisms contributing to physiological calcification not only play a pivotal role in the pathophysiology and clinical manifestations of these diseases, but also in the prognosis and possible therapeutic options for rare as well as more common calcification disorders.

Traditionally, mice have been the preferred models of human diseases, often through the development of Knockout (KO) animals by targeted ablation of the corresponding genes. While the KO mice often show remarkable similarity to the human phenotype both at the genetic, gross morphologic, histopathologic, and ultrastructural level, this model system knows considerable limitations (Lieschke and Currie, 2007). It may take several years to develop a KO mouse and in some cases, development of a KO mouse of the corresponding human disease is not feasible due to the absence of the corresponding gene in the mouse genome such as the human SMAD9 gene (Li et al., 2007). These considerations, together with cost containment, the large space needed to keep the animals and low number of progeny, has prompted the search for alternative model systems to study mineralization-related disorders.

The zebrafish (ZF, *Danio rerio*), a freshwater vertebrate belonging to the teleosts, has become an important model for the study of basic pathogenetic mechanisms in different human diseases. The popularity of the ZF as a model organism is due to some important properties including their easy maintenance in the laboratory at low cost, the production of large numbers of synchronous developing embryos per mating, the short reproductive cycle with external fertilization and rapid rate of development, the optical transparency of the embryos, and the possibility to conduct high resolution *in vivo* imaging, availability of a wide range of molecular techniques such as large-scale genome mutagenesis

and over-expression/knock-down approaches, and freely available web based resources, i.e., ZFIN, Zebrafish International Resource Center (ZIRC), Trans-NIH ZF initiative, ZF-Health, etc.

The ZF genomic sequence, which was completely mapped in 2009 (Bhartiya et al., 2010), demonstrates about 70% homology with the human genome, suggesting the evolutionary conservation of a large number of genes and genetic pathways. Due to an ancient genome duplication event that occurred after divergence of actinopterygian and sarcopterygian ancestors, a substantial number of ZF genes are present in two or more copies (Woods et al., 2000; Lu et al., 2012). Although the genome duplication can complicate gene function analysis, in some cases, this resulted in the partitioning of ancestral gene functions between duplicated descendants. This role-separation by paralogs can provide a unique opportunity to study the sub-functions of an individual human gene (Postlethwait et al., 2004).

To explore physiological and aberrant (soft tissue) mineralization in ZF, till now the bone, skin, scales, and vasculature have been intensively studied. ZF have been proposed as a model to study osteogenesis, bone metabolism, and remodeling based on study of their scales, although these structures are strictly speaking tooth- and not bone-related (Sire and Akimenko, 2004; Pasqualetti et al., 2012). Bone mineralization is an essential and well-orchestrated process in vertebrates in which crystals of calcium phosphate are orderly arranged into hydroxyapatite crystals in very close association with collagen fibers to build the bone mineralized matrix (Kawasaki et al., 2009). The ZF skeleton shows high similarity with human bones in terms of cells, matrix proteins, and molecular signaling pathways involved. The latter include Notch, Wnt, and TGF beta/bone morphogenetic proteins (BMP) signaling pathways (Rusanescu et al., 2008).

The skin of ZF consists of two layers: the epidermis and the dermis. At 1 day post fertilization (dpf), two different layers representing the epidermis and the dermis can be recognized. At 6 dpf, a two cell layer epidermis can be observed, clearly separated from the underlying connective tissue stroma (Li et al., 2011). Despite several structural differences (lack of keratinization, presence of several unicellular glands, etc.), the epidermis of the ZF shows high homology with the human epidermis, and several similar molecular genetic factors and mechanisms – including the retinol-binding protein 4 (RBP4) and apolipoprotein Eb (APOEB) – were reported in both species in the process of (epi)dermal development and homeostasis (Tingaud-Sequeira et al., 2006). However, the underlying molecular mechanisms of scale calcification which occur during ZF development are still incompletely understood. The scales of ZF, represent a significant reservoir of calcium and are subjected to a specific mineralization pattern. In elasmoid scales, deposition of mineralization-related proteins occurs from the epidermis into the dermis (Sire et al., 1997a,b; Hong et al., 2011). Besides detailed morphological studies, characterization of the molecular events involved in scale development has been initiated, revealing the role of Sonic Hedgehog (Shh), BMP2, 4, osteocalcin, or osteopontin (Sire and Akimenko, 2004; Hong et al., 2011).

Because of the transparency of the embryonic stages, *in vivo* observation of the heart rhythm as well as the vasculature and circulation in ZF is possible and does not require physical intervention. The heart of ZF embryo starts beating within 26 hour post fertilization (hpf; Baker et al., 1997) and undergoes looping by 2 dpf (Stainier et al., 1996). A fully functioning vascular tree is present by 3 dpf (Sehnert and Stainier, 2002). At 4 dpf, cardiomyocyte proliferation thickens the ventricular wall (Antkiewicz et al., 2005) and by 5 dpf the heart has developed valves (Forouhar et al., 2004). To characterize the large number of cardiovascular mutants in ZF, a comprehensive array of cellular, molecular, physiological, and genetic techniques has been developed (Warren and Fishman, 1998; Xu et al., 2002). The vital nature of the heart poses a challenge for studying its function at a molecular level. Although targeted inactivation of mouse genes can provide a wealth of information, inactivation of heart-specific genes is frequently hampered by the early embryonic lethality it creates. By contrast, the ability of ZF embryos to survive on diffused oxygen for several days without a functioning cardiovascular system is an important distinguishing feature. Using ZF, forward genetic screens have been conducted, identifying more than 100 genes required for heart formation and function (Chen et al., 1996; Stainier et al., 1996). Studies in ZF, mice, and humans indicate that Notch works in conjunction with other angiogenic pathways to pattern and stabilize the vasculature (Shawber and Kitajewski, 2004). Recent progress in cardiovascular research has suggested that arterial and valve calcification is the result of an active process of osteogenic differentiation, induced by pro-atherogenic inflammatory response. At the molecular level, the calcification process is regulated by a network of signaling pathways, including Notch, Wnt, and TGFβ/BMP pathways, which control the master regulator of osteogenesis CBFA1/Runx2 (Rusanescu et al., 2008).

One of the most substantial features of ZF in their use as a model system to study ectopic mineralization is that the expression of specific genes can be easily knocked down by injection of 1–4 cell embryos with morpholino (MO)-based anti-sense oligonucleotides (Eisen and Smith, 2008). After MO injection and analysis of its efficacy, a larger number of embryos and larvae can be comprehensively screened for phenotypic manifestations using light microscopy. Once the phenotype has been characterized, the embryos are readily amenable to further investigation (**Table 1**) at the molecular and cellular level, including calcification-specific stains [i.e., calcein staining, Alizarin Red (AR-S) Staining, Alcian blue-Alizarin red double staining], the use of mineralization specific transgenic lines, and analysis of ectopic calcification-related marker gene expression by quantitative real-time polymerase chain reaction (qPCR) or western blot analysis, respectively. Also specific pathophysiological mechanisms involved in aberrant mineralization, such as apoptosis and mitochondrial dysfunction can be assessed [e.g., using transferase dUTP nick end labeling (TUNEL) and MitoTracker staining]. Hitherto, the number of reports applying MO injection to study (ectopic) mineralization processes and diseases is relatively scarce. Li et al. (2010) were able to KO about 84% of the abcc6a gene [a homolog gene of human ABCC6, causing pseudoxanthoma elasticum (PXE)] and suggested that abcc6a may also have a developmental role. Hughes et al. (2004) knocked down the otop1 (otopetrin) gene required for the formation of otoliths – large extracellular biomineral particles involved in transducing sound into neuronal signals – in the ZF ear, and with more than 96% of otop1 morphants failing to develop otoliths, demonstrated that

Table 1 | Overview of different methods that can be applied in zebrafish models for ectopic mineralization.

Methods	Application	Stages of application in MO approach
MO injection	To evaluate the gene function by injecting synthetic anti-sense nucleotide oligomers	1–4 Cell stage embryos
Light microscopic observation	Phenotypic screening after injection (see **Table 2**).	Post-injection to morphant death
RNA rescue experiment	Validation of gene specificity by co-injection of MO and mRNA (encoding protein from the targeted locus of other species	1–4 Cell stages of embryos
Western blotting	Validation of the efficiency of TB MOs	After phenotypic confirmation, 1–4 dpf, until when effect of MO can be observed
PCR	Expression profiling of targeted gene Validation of the efficiency of SJ MOs	From 0- different time points After phenotypic confirmation, 1–4 dpf, until when effect of MO can be observed
Quantitative real-time PCR	Expression profiling of targeted gene Validation of the efficiency of SJ MOs	From 0- different time points After phenotypic confirmation, 1–4 dpf, until when effect of MO can be observed
Calcein staining	Fluorescent chromophores specifically bind to the calcified skeleton of live ZF embryos	5 dpf to morphant death
Alizarin red S	To identify calcium in tissue sections or whole mount embryos	4 dpf to morphant death
Alcian blue-Alizarin red double staining	Alcian blue stains cartilage blue and is used as a counterstaining to AR-S to distinguish cartilage and bone Alizarin red stains as red in calcified matrix (calcified cartilage, bone)	4 dpf to morphant death
IHC	To detect presence and localization of (mineralization-related) protein in tissue sections or whole mount embryos	0 hpf to morphant death
μCT imaging	Useful for skeletal analysis, used to understand developmental processes of three-dimensional embryos, embryo phenotyping, and quantitative modeling of development	5 dpf
ISH	To assess gene expression profiling in wild-type embryo and differential gene expression in morphant	0–4 dpf of embryos, as until 4 dpf effect of MO can be observed
MS	To analyze differential protein expression by measuring the mass-to-charge ratio	0–4 dpf, until MO effect can be observed
2D gel electrophoresis	To assess differential protein expression, where proteins are separated in the gel according to their isoelectric point	0–4 dpf, until MO effect can be observed
Microarray	Used to identify genome-wide expression of genes. In morphant differential expression of different gene can be identified	0–4 dpf, until MO effect can be observed
Transcriptome analysis	More sensitive compared to microarray, used to identify differential expression of transcripts. By this method closely homologous genes can be distinguished, alternatively spliced transcripts and non-coding RNAs can be characterized, and rare transcripts which are undetectable in microarray analysis can be detected	0–4 dpf, until MO effect can be observed
TUNEL staining	To assess *in situ* cell death in the whole mount embryo. TUNEL labels degraded DNA products enzymatically or by a fluorescent probe and stains apoptotic bodies	30 hpf–4 dpf, until MO effect can be observed
CMH2DCF staining	Used to determine oxidative stress or level of ROS in live embryos	0–4 dpf, until MO effect can be observed
MitoTracker Red CM-H2XRos	Used to determine mitochondrial membrane potentiality in live embryo	0–4 dpf, until MO effect can be observed
Chemical screening	Used to identify small chemicals which can rescue the morphant phenotype and can be predicted as a potential drug	0–4 dpf, until MO effect can be observed

MO, morpholino; PCR, polymer chain reaction; IHC, immunohistochemistry; ISH, in situ hybridization; MS, mass spectrophotometry; 2D, 2 dimensional.

otopetrin 1 has a conserved role in the timing and shaping of otolith formation. In this paper, we highlight several experimental procedures which can be used to assess ectopic calcification and its related processes in ZF, illustrated with current knowledge on ZF mineralization.

GENE KNOCK-DOWN APPROACHES

Zebrafish has already been proven a good model for forward (phenotype driven) genetic approaches, where mutagens are employed to produce random changes in the DNA followed by phenotypic evaluation. The unbiased nature has made this approach very powerful (Lieschke and Currie, 2007). Less attention has been given to reverse (candidate gene-driven) genetic approaches, though these have become robust tools when forward genetics is less feasible or to investigate redundant gene function. The ability to make precise targeted changes to a genome has long been the holy grail to the reverse genetic approach. ZF genomic sequencing made it possible to analyze its genes function in a systematic way by inactivating protein-coding genes by targeted or random mutation (Varshney et al., 2013a). The main obstacles of these reverse approaches include the need for available knowledge of candidate genes and the focus on a single gene or a small group of genes. Recently Varshney et al. (2013b) performed proviral insertions coupled with high-throughput sequencing and succeeded to widely mutagenize genes in the ZF genome. To facilitate such studies, the Zebrafish Insertion Collection (ZInC)[1] have generated a genome-wide KO resource that targets every ZF protein-coding gene (Varshney et al., 2013a). All mutants from ZInC are freely available through the ZIRC. The most frequently used reverse genetic technique is morpholino-induced knock-down. The specificity of the observed phenotypic effects have always been a concern using this technology and can be addressed by running a second or more experiments, applying other reverse genetic approaches as detailed below.

ANTI-SENSE APPROACHES

Morpholino-induced knock-down

Among many reverse genetic approaches, the most commonly used method is a gene knock-down approach by injecting so-called morpholinos (MOs). The ease of use and exciting results within few hours made this approach increasingly popular for gene function analysis. MOs are synthetic anti-sense nucleotide oligomers used to block proper gene expression by binding to complementary sequences of RNA. Instead of degrading the target molecules, the knock-down effect is achieved by preventing cells from making the targeted proteins (Summerton, 1999; Nasevicius and Ekker, 2000). Among all gene knock-down reagents, MOs are the only molecules which have all properties of stability, nuclease-resistance, efficacy, comparatively long-term activity, water solubility, low toxicity, and specificity. MOs consist of standard nucleic acid bases (**Figure 1**) but, contrary to nucleic acids, those bases are bound to morpholine rings and linked through phosphorodiamidate groups (Summerton and Weller, 1997). To date, translational blocking (TB) and/or Splice Junction (SJ) MOs are commonly used. TB MOs can be used to target sequences around or slightly upstream from the translation initiation codon, which interfere with the formation of the ribosomal initiation complex from the 5′ cap to the start codon (AUG) to prevent translation of the coding region of the targeted transcript (Draper et al., 2001). MOs targeted to SJs will modify pre-mRNA splicing or can block the binding sites of splice-regulatory proteins (Bruno et al., 2004).

To obtain ZF embryos for MO injection, adult males and females can be put in breeding tanks and kept separated to control the laying time, by using a separator in the tank. After the divider in the breeding tank has been removed, the fish will usually begin to lay eggs within approximately 30 min, though this period may be extended. To get the most prominent effect of the MO, injection should be done in the 1-cell stage (which allows best distribution of MO in each cell and avoid mosaic phenotypic features). However, MO injection can be done between the 1- and 8-cell stage and still give ubiquitous delivery (Nasevicius and Ekker, 2000). Rapid delivery of MO into embryos is done by MO injections using a glass micro-needle and a micromanipulator with air pressure. An optimal concentration (<6 ng/nl) of MO is preferred to obtain the most specific phenotype (Rikin et al., 2010).To overcome possible redundancy between different paralogs, combined MO injection targeting the different paralogs can be necessary to obtain the expected phenotype (Bill et al., 2009; Bedell et al., 2011). For polygenic disorders, simultaneous injection of different MOs may allow the inactivation of more than one gene at the same time, which represents a paramount advantage, compared to any mammalian assay available and can be exploited for the identification of novel gene(s) (Nicoli and Presta, 2007).

Different procedures are available for the maintenance of injected and wild-type embryos (Lawrence and Mason, 2012). In our lab, embryos are maintained in an E3 medium (containing methylene blue) in Petri dishes and kept in an incubator at 28°C (Kimmel et al., 1995) until 9 dpf for normal growth. Every day, half of the E3 medium is replaced by fresh E3 medium and from 6 dpf on the embryos are fed twice a day with 50 μM solid dry granules.

To avoid melanin formation in the skin, which can hide some internal structures from microscopic observation [phenylthiourea (PTU), a tyrosinase inhibitor commonly used to block pigmentation] can be added starting after gastrulation (around 10–12 h) and before 24 hpf of embryonic life (Renaud et al., 2011). PTU affects early embryonic development, therefore it is important not to treat the embryos with PTU before the end of gastrulation (Thisse and Thisse, 2008).

RNA INTERFERENCE

RNA interference (RNAi) is a powerful approach to knock-down gene function in different model systems. However, success of RNAi in ZF is not obvious due to ineffective penetrance or non-specific effects (Zhao et al., 2008). Simple small-hairpin RNA (shRNAs)-mediated knock-down approaches also appear ineffective in ZF (Wang et al., 2010). Micro-RNAs (miRNAs) are endogenous ∼21–23 nt RNAs which can also regulate gene expression. Recently, miR-shRNAs were reported to be (12-fold) more efficient in ZF gene knockdown (De Rienzo et al., 2012). However,

[1] http://research.nhgri.nih.gov/ZInC/

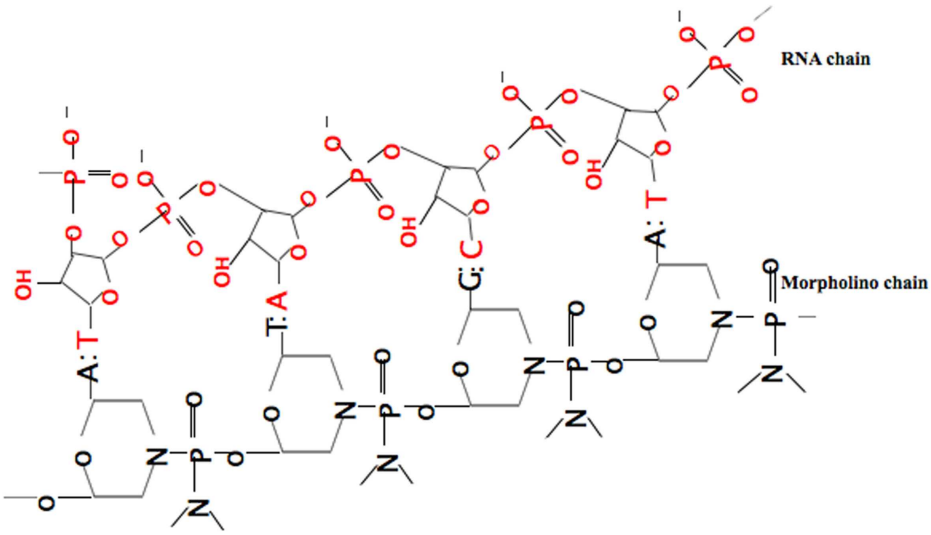

FIGURE 1 | Binding of the anti-sense morpholino chain to the RNA chain. MOs have standard nucleic acid bases bound to morpholino ring, which are linked through phosphorodiamidate groups, while RNA has ribose rings which are linked with phosphates.

the capability of germ-line transmission is very poor. Therefore, the potential of RNAi for stable and conditional gene knock-down in ZF remains uncertain.

ENGINEERED NUCLEASES

Transcription activator-like effector nucleases

Transcription activator-like effector nucleases (TALENs) are a powerful and robust approach for efficient inactivation of a targeted gene. TALENs are artificial restriction enzymes generated by fusing a TAL effector (derived from plant pathogenic bacteria Xanthomonas) DNA-binding domain to a DNA-cleavage domain or *Fok*I nuclease. These restriction enzymes are introduced into cells to edit the genome *in situ*. When fused to the *Fok*I nuclease domain, TAL effectors recognize specific DNA sequences using a straightforward DNA base recognition code. *Fok*I cleaves only when present as a dimer and the binding of two TAL effectors to DNA, which allows *Fok*I to dimerize, will thus create a break in the nucleic acid strand. Because of the specificity of this DNA-binding, TAL effectors can bind virtually any DNA sequence (Cermak et al., 2011). TAL effectors possess several advantages, including ease of design and easier optimization, shows few off-target effects and are thus potentially more reliable compared to other reverse genetic approaches (Clark et al., 2011).

Zinc finger nucleases

Another nuclease-based technology for efficient inactivation of a targeted gene in ZF (Sander et al., 2010, 2011) is the use of zinc finger nucleases (ZFNs). These are artificial restriction enzymes generated by fusing engineered zinc finger DNA-binding domain to a DNA-cleavage domain, and function as dimmers to introduce targeted DNA double-strand breaks. ZFN treated ZF can transmit mutant alleles (as if they were heterozygous carriers) and cause minimal collateral damage to the genomes of treated ZF. Moreover, it is possible to also generate targeted knock-ins in ZF using ZFNs. However, injected nucleases can be toxic to the embryo (Doyon et al., 2008; Meng et al., 2008) and together with the gaps in our understanding of sequence-specific DNA recognition by zinc fingers can still restrict the ability to construct ZFNs targeting any desired site in a genome (Lawson and Wolfe, 2011).

TARGETING INDUCED LOCAL LESIONS IN GENOMES

Targeting induced local lesions in genomes (TILLING) is a quick, reliable, and increasingly popular method to identify chemically induced point mutations in ZF (Wienholds et al., 2003). The TILLING method combines mutagenesis (by a chemical mutagen) with a sensitive DNA screening-technique that identifies single base mutations in a target gene. The TILLING method relies on the formation of DNA heteroduplexes between the wild type and mutant PCR fragment, formed when multiple alleles are amplified by PCR and are then heated and cooled. These heteroduplexes are then incubated with celery derived CelI endonuclease, which recognizes and cleaves mismatches in the heteroduplex DNA generated by small nuclear polymorphisms (SNPs) and point mutations (Oleykowski et al., 1998). Labeled digested fragments are separated and visualized on slab gel sequencers. Fragments generated due to the presence of SNPs will be present in all animals. This approach holds great promise for the rapid identification of large numbers of mutant alleles from mutagenized libraries. However, the need for screening large number of F1 fish to find a lesion in a gene of interest (Wienholds et al., 2002), the requirement of specialized equipment, and a significant investment in computational resources and personnel represent the initial bottleneck of this approach. To date, the "Welcome Trust Sanger Institute" has successfully provided 8402 genes (including abcc6) with mutations under the "Zebrafish Mutation Project (ZMP) KOs for disease model" by using the TILLING approach[2].

[2]http://www.sanger.ac.uk/Projects/D_rerio/zfm_DAS_conf.shtml

RETROVIRAL AND TRANSPOSON MEDIATED MUTAGENESIS

Insertional mutagens retrovirus or transposon can be utilized to identify modified alleles of a target gene. The main advantage of these systems is readily identifiable tag that simplifies screening for carriers of a particular disrupted allele within the library (Jao et al., 2008). It is also possible to generate conditional alleles in ZF by including recombination sites flanking these gene-breaking elements (Petzold et al., 2009). A limitation of these insertion mutagenesis techniques is the inability to generate full null alleles in most instances.

DOMINANT NEGATIVE APPROACH

Here, a mutant gene product is used to adversely affect the wild-type gene product within the same cell to get a reduced level of gene activation. Two main Dominant negative (DN) approaches are: (1) mutation in a transcription factor that removes the activation domain, which can block the wild-type transcription factor to bind with the DNA-binding site, resulting in a reduction of gene activation, and (2) overexpression of a constitutively active protein (CAP) with mutation in/manipulation of the regulatory domain, leading to diminished opportunity for the regulatory subunit of the wild-type protein to bind with its receptor, and binding of a malfunctioning protein domain to the receptor will decrease the wild-type protein expression (Concordet et al., 1996). For example, in a protein which is functional as a dimer, a mutation leading to removal of the functional domain while retaining the dimerized domain would cause a DN phenotype. Lanham et al. (2011) successfully used aryl hydrocarbon receptor 2 (Ahr2) as a DN approach in ZF to protect developing ZF from dioxin toxicity by removing the C-terminal transactivation domain or replacing it with an inhibitory domain. CAP also been used to explore the function of various components of signal transduction pathways. The main advantage of DN approach over anti-sense RNA strategies is the possibility of producing null mutations. The disadvantages of DN approaches are that the technique is not applicable to all genes, has a relatively low throughput, and that it is usually impossible to fully understand the true endogenous function of the molecule *in vivo* (Niwa and Slack, 2007).

PHARMACOLOGICAL APPROACHES

Pharmacological approaches (PA) are not true reverse genetic approaches, because they do not depend on specific knowledge of an individual gene of an organism. Once the treatment with a reagent reveals phenotypic consequences, this can be utilized to understand interacting molecular pathways. Many pharmacological reagents are used in the ZF model system including, e.g., cyclopamine – an inhibitor of Shh signaling pathway – (Cooper et al., 1998) or SU5614 and SU1498-an inhibitor of VEGF/Flk-1 tyrosine kinase signaling (Liang et al., 2001). Biochemical characterization of the pharmacological reagents is highly recommended before applying them in the model system. Exert pleiotropic effects is the bottleneck of using pharmacological reagents which can result an unspecific phenotype (Skromne and Prince, 2008).

INDUCIBLE SYSTEMS

Most of the aforementioned techniques used in the ZF gene-knock-down process are only suitable to evaluate phenotype and gene function in early embryonic stages, as severe malfunction of embryo leads to early death. So, gene function(s) at late stages are missed, which can be overcome by inducible systems (IS) including heat shock promoters orgal4-UAS system. Application of IS can be done by regulating the activity of the protein or regulating the expression of the corresponding gene. In ZF, downstream gene expression can be induced throughout the body by raising the temperature from 28.5 to 38°C (Shoji and Sato-Maeda, 2008). Heat shock protein (hsp) promoters have been used to regulate exogenous gene expression in a variety of studies. The hsp promoter can be used to search cis-acting transcriptional elements during ZF embryogenesis. Hsp:egfp (enhanced green fluorescence protein) can be injected into fertilized eggs with distal DNA fragments for tissue/cell type specific activation (Islam et al., 2006). One such way for inducing heat shock response in targeted cell is by using a laser microbeam under the microscope. The main advantage of this technique is visualization of a whole cell due to stable and longtime expression of the GFP protein, however laser ablation can be sensitive to the cells.

POST-INJECTION FOLLOW-UP

EVALUATION OF THE PHENOTYPE

Phenotypic screening is mainly done based on classic criteria (Haffter et al., 1996), which can provide important information on gene function as well as the molecular events that underlie a biological process. Such screening focuses primarily on morphological landmarks by dissecting microscopy. A significant number of phenotypic traits can be screened for, as summarized in **Table 2**. The phenotypic screening is conducted at different time points corresponding to the embryonic developmental stages (**Figure 2**). Due to transient character of MO-induced gene knockdown, with efficient knockdown up to 4–5 dpf, phenotypic screening is preferably done during this time frame (Nasevicius and Ekker, 2000; Bill et al., 2009).

Morpholino mediated knockdown to study gene function in ZF offers many advantages, including ease of delivery, a high efficacy, and rapid phenotypic screening. However, because of their dilution in rapidly growing embryos, the effect of MOs is temporary, preventing gene function analysis during the entire life cycle of ZF. Moreover, many genes – e.g., the macrophage stimulating protein Msp (recently found to have a role in the regulation of calcium homeostasis of adult ZF) (Huitema et al., 2012) – which are expressed during the adult period and some organs such as the skeleton which have only fully matured after 2–3 weeks cannot be studied sufficiently after MO knockdown. Altogether, this emphasizes the usefulness of permanent mutants in addition to MO-induced models.

MORPHANT VALIDATION

Every MO-induced phenotype must be validated to confirm that it is due to gene-specific effects. Besides the application of an independent knock-down approach as described above, several MO-specific validation steps need to be addressed. First, to assess the phenotypic variation and effect of the injection procedure, commercially available standard control MO needs to be injected in parallel with the active MO. Secondly, for each targeted gene, the

Table 2 | Phenotypic traits which can be screened in ZF embryos beyond early development.

System	Phenotypic trait
Body axis	Dorsilisation (48 hpf) Ventrilisation (48 hpf) Prechordal plate and hatching (24 hpf) Tail (24–48 hpf)
Mesoderm	Notochord formation, differentiation, and degeneration (24 hpf) Somite formation and patterning (24 hpf)
Central nervous system	Forebrain (24 hpf) Midbrain (24 hpf) Hindbrain (24 hpf) Neural tube – spinal cord (24 hpf)
Organs	Vasculature (e.g., aortic arches, dorsal aorta, common/posterior cardinal vein, blood island; 24–48 hpf) Heart: morphology, beating (48 hpf) Liver, kidney, gut (larva stadium) Eye (24 hpf) Ear (48 hpf) Otoliths (48 hpf)
Pigmentation	Cell number and pattern (48 hpf) Melanin pigmentation (48 hpf)
Motility	Muscles (48 hpf) Pectoral and caudal fin (48 hpf – larva stadium) Reduced motility (48 hpf)

For each trait, the timing when evaluation can start is mentioned. From this timing on, serial evaluations in time are usually performed.

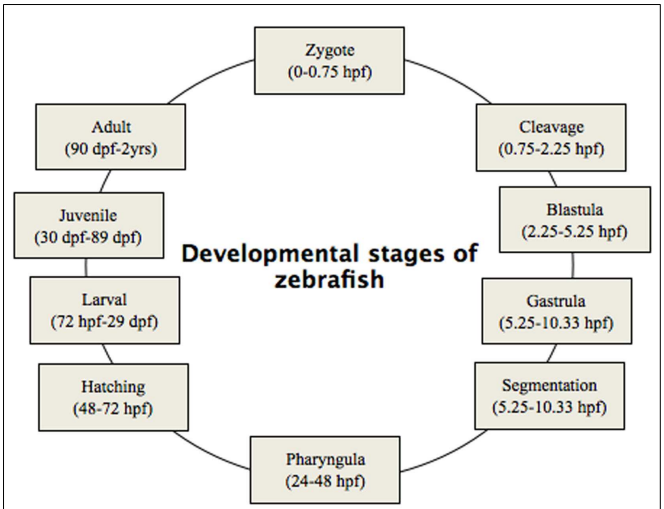

FIGURE 2 | Developmental stages of zebrafish, from zygote to adult. Zygote: the newly formed fertilized egg after completion of the first zygotic cell cycle. Cleavage: zygotic cell cycles 2–7 occur rapidly and synchronously. Blastula: rapid and metasynchronous cell cycles (8, 9) occur, which give way to lengthened, asynchronous ones at the midblastula transition, then epiboly begins. Epiboly is the first coordinated cell movement in zebrafish embryos and begins before gastrulation. Gastrula: morphogenetic movements of involution, convergence, and extension from the epiblast, hypoblast, and embryonic axis through the end of epiboly occur. Bud-100% epiboly is the stage where epiboly completely covers the yolk plug. Segmentation: Somites (after completion of epiboly and initial appearance of the tail bud, first the somatic furrow forms and makes a boundary, between what will become the first and second somites), pharyngeal arch primordia, and neuromeres develop, primary organogenesis and earliest movements take place, and the tail appears. Pharyngula: phylotypic stage of embryo, body axis straightens from its early curvature around the yolk sac; circulation, pigmentation, and fins begin development. Hatching: completion of rapid morphogenesis of primary organ systems, cartilage development in head and pectoral fin, hatching occurs asynchronously across individuals. Larval: swim bladder inflates; food-seeking and active avoidance behaviors.

injection of both TB and SJ MO is recommended, to reveal the role of either the entire protein-coding region of the gene, or of certain exons. MOs appear to have non-target-related phenotypes including overall developmental delay and cell death due to activation of p53-mediated apoptosis (Ekker and Larson, 2001), which can be overcome by using stage matched (rather than age matched) control embryos and co-injection of an anti-p53 MO along with the experimental MO. Injection of SJ MO generally causes skipping of the targeted exon or retention of the adjacent intron while TB MO blocks gene transcription. These molecular effects can be validated by conducting western blotting in case of SJ MO's and RT-PCR for TB MO's. Further evidence of the specificity of the gene knockdown can be obtained by rescuing the phenotype by mRNA injection.

RNA rescue experiments

mRNA rescue is the most reliable approach to examine the specificity of the effects of MO knockdown. To rescue the gene-specific phenotype, co-injection of the MO of interest and synthetic mRNA encoding the targeted protein can be done at the 1–4 cell stage of the embryo (Hyatt and Ekker, 1999; Bedell et al., 2011). many genes, knockdown by MOs followed by ubiquitous mRNA delivery rarely results in truly rescued phenotypes (Bedell et al., 2011).The most important issues to consider in RNA rescue experiments are: (1) achievement of appropriate levels of injected MO and mRNA by co-injecting different concentrations of both components (Little and Mullins, 2004), (2) making sure that injected synthetic mRNA does not include the MO target sequence (Eisen and Smith, 2008). For TB MOs against the 5′ UTR sequence, the open reading frame can be cloned by PCR into a standard transcription vector (Hyatt and Ekker, 1999). For MOs that target part of the open reading frame, the rescue constructs can be engineered to change the nucleotide sequence without altering the encoded protein through degradation of the genetic code (Bill et al., 2009). Another frequently used approach for rescuing TB morphant is co-injection of mRNA from a different species. In their ZF model for PXE, Li et al. (2010) injected full-length mouse mRNA together with the MO; this co-injection completely reversed the phenotypic effects of the MO and the rescued embryos showed essentially the same morphology as controls. Co-injection of SJ MO and full-length mRNA (whose sequence is non-homologous to the SJ MO) can rescue a SJ morphant (Cline et al., 2012).

Qualitative and quantitative validation methods

The effect of TB MOs can be validated by western blotting, which correlates reduced protein levels of the targeted gene with an observed phenotype (Hutchinson and Eisen, 2006). The main obstacle for performing western blot in ZF is the currently limited availability of antibodies that are specifically generated to recognize ZF proteins, although a number of already available antibodies from other origins show cross-reactivity with ZF proteins.

When using SJ MO, the phenotype can be validated with PCR. Qualitative evaluation of exon skipping or intron retention can be done by performing RT-PCR with primers located in exons upstream and downstream of the MO-targeted sequence showing smaller and larger band respectively in addition to the original band (**Figure 3**). Intron retention or exon skipping can result in a frameshift and consequently nonsense-mediated decay and reduced transcript levels can be assessed quantitatively by using quantitative real-time PCR (qRT-PCR).

CHARACTERIZATION OF (ECTOPIC) MINERALIZATION PHENOTYPES

CALCEIN STAINING

Calcein ($C_{30}H_{26}N_2O_{13}$) is a fluorescent chromophore that specifically binds to calcium. As the skeletal system contains calcified structures, calcein has been used to label bone structures and to study bone growth (Ducy et al., 2000). During calcein staining, fluorescent chromophores of calcein rapidly penetrate into ZF embryos and specifically bind to the calcified skeleton (**Figure 4**). Calcein staining can be used to follow the development of skeletal structures in ZF embryos. Calcified skeletal structures appear in a progressive fashion from head to tail. First appearance of calcein signals, observed at ∼5 dpf are restricted to the head, followed by the axial skeleton in the trunk (Du et al., 2001). Du et al. (2001) observed that the axial skeleton calcified in two domains. This was later confirmed by AR-S staining (Bensimon-Brito et al., 2012). The first domain consists of three anterior vertebral centra (centra 3–5), whereas the second domain consists of the remaining abdominal and caudal centra which develop in an anterior-to-posterior direction. This confirms the sensitivity of calcein staining for visualizing mineralized structures in developing ZF embryos and its effectiveness for detecting defective bone structures and mineralization.

ALIZARIN RED S STAINING

Alizarin Red S is an anthraquinone derivative used to identify calcium in tissue sections or whole mount embryos, where tissue calcium forms an AR-S-calcium complex in a chelation process, producing a birefringent end product (Nejati-Yazdinejad, 2006). The reaction is not strictly specific for calcium, since magnesium, manganese, barium, strontium, and iron may interfere, but these elements usually do not occur in sufficient concentration to interfere with the staining (Lievremont et al., 1982). AR-S staining can be performed on both live embryos and adult ZF as well as on fixed tissues. In live embryos, AR-S is used as a vital stain which labels mineralized matrix and is a very sensitive method for detecting bones (Kimmel et al., 1995; Walker and Kimmel, 2007). On the other hand, in fixed embryos or adult fish, the fixation itself is a very critical step for good AR-S staining. As PFA negatively affects bone staining, fixation with 4% PFA should be restricted to 2 h (**Figure 5**; Huitema et al., 2012) demonstrated an ectopic mineralization phenotype in dragon fish (dgf) mutants by Alizarin red staining, which exhibit ectopic mineralization in the craniofacial and axial skeleton and encode a loss-of function allele of ectonucleotide pyrophosphatase phosphodiesterase 1 (enpp1).

Besides AR-S staining, an Alcian Blue-AR-S double staining can be used to distinguish cartilage and bone. Walker and Kimmel (2007) developed an acid-free alcian blue-AR-S staining method which is now widely used to stain cartilage and bone simultaneously in ZF larvae.

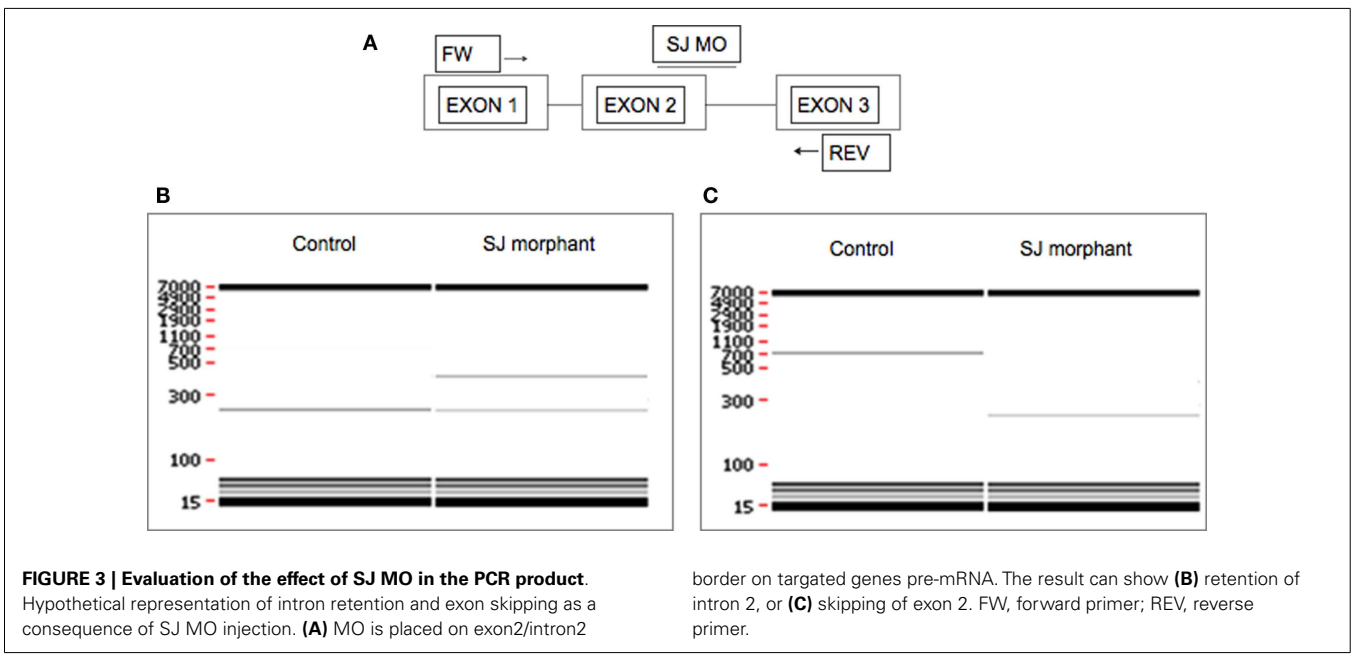

FIGURE 3 | Evaluation of the effect of SJ MO in the PCR product. Hypothetical representation of intron retention and exon skipping as a consequence of SJ MO injection. **(A)** MO is placed on exon2/intron2 border on targated genes pre-mRNA. The result can show **(B)** retention of intron 2, or **(C)** skipping of exon 2. FW, forward primer; REV, reverse primer.

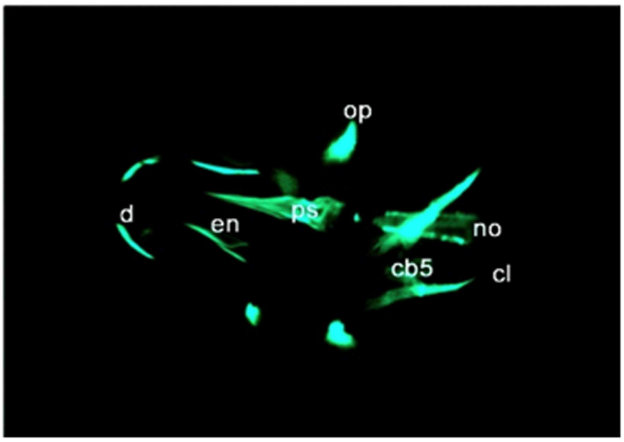

FIGURE 4 | Calcein staining of 6 dpf embryos showing staining of the ceratobranchials 5 (cb5), cleithrum (cl), dentary (d), entopterygoid (en), opercular bone (op), and parasphenoid (ps), in the skull, and anterior tip of the notochord (no).

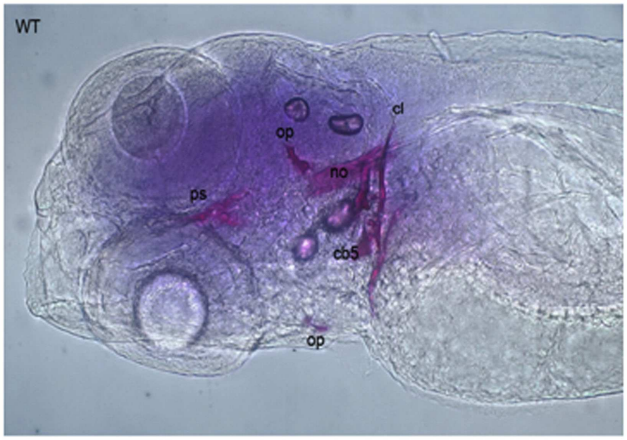

FIGURE 5 | Alizarin Red staining of a 5 dpf embryo showing cleithrum (cl), opercular bone (op), parasphenoid (ps), and ceratobranchials 5 (cb) with a set of three teeth.

μCT IMAGING

Micro computerized tomography (μCT) is a powerful and practical tool for the skeletal analysis of diverse model organisms including ZF. It is used to understand developmental processes of three-dimensional embryos, embryo phenotyping, and quantitative modeling of development (**Figure 6**) (Metscher, 2009). The method is analogous to that used for the 3D imaging of human structures, on a smaller scale. It is dependent upon the interaction of large atoms with X-ray beams and requires the use of contrast agents, still in development, for imaging anything other than bone. But the usefulness of μCT imaging for developmental biology has been limited by the low inherent contrast of embryonic tissues. Though it can be envisaged that μCT may also be useful to demonstrate ectopic calcification in, e.g., soft tissues, its limited sensitivity in embryos makes it a more useful technique in adult fish and hence the characterization of mutants instead of morphants. The ZF is the only well-developed vertebrate genetic model that is small enough to image the whole animal at cell resolutions using μCT (Cheng et al., 2011).

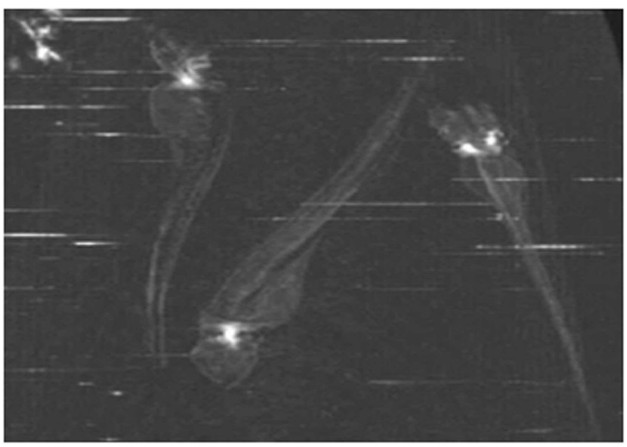

FIGURE 6 | MicroCT scanning of whole 4 dpf embryos. The white spot in the head region represents a signal for the mineralized otoliths.

MOLECULAR CHARACTERIZATION

IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) is a powerful and commonly used technique in ZF for determining both the presence and localization of antigens (e.g., endogenous protein) in cells of a tissue section, or whole mount embryos and larvae. Sample preparation, especially sectioning and fixation of tissue, is very critical to maintain cell morphology, tissue architecture and the antigenicity of target epitopes. Non-specific binding can cause high background staining which may mask the detection of the target antigen. It is also possible to label both whole mount specimens and sections with two different antibodies, known as the double-labeling technique. Double-labeling works particularly well with fluorescent conjugated secondary antibodies and these can be applied at the same time.

Immunohistochemical approaches to reveal the localization of some calcification-related proteins demonstrated tissue distribution and accumulation of Bgp and Matrix γ-carboxyglutamic acid (Gla) protein (Mgp) during the larval stage and in adult tissues of ZF by IHC shows that Bgp and Mgp proteins were located in all mineralized tissues during and after calcification including bone and calcified cartilage of branchial arches (Gavaia et al., 2006). Being a powerful method to identify specific proteins by using an antibody, the main obstacle is the availability of antibodies and the limited cross-reactivity of ZF protein with antibodies originating from other sources.

WHOLE MOUNT RNA *IN SITU* HYBRIDIZATION

Whole mount RNA *in situ* hybridization (ISH) is one of the oldest and most frequently used techniques used in the ZF research to investigate the site of expression of a particular gene in an intact

embryo (Jowett and Lettice, 1994). ISH allows specific nucleic acid sequences to be detected in morphologically preserved cells or embryos and is also used to identify novel genes involved in the same signaling pathways (Thisse and Thisse, 2008). Whole mount mRNA ISH can also be done to observe MO mediated gene expression changes. In this case, cDNA of a gene of interest is used as a template for the synthesis of an anti-sense mRNA probe, which is used to recognize and bind to the endogenous transcript through a color or fluorescence-based assay (Thisse and Thisse, 2008). Finally, the expression of the gene can be observed under a light or fluorescent microscope.

The major advantage for using the ISH technique in ZF compared to other animal models is that, because of its transparency and small size, the expression of a particular gene can be observed in the entire embryo. Though whole mount ISH is a quick and efficient method, the most significant caveat in ZF is the poor penetration of the RNA probes after 2 days of development; only superficial tissues (i.e., epithelial cells) are accessible to the probe at these later developmental stages (Thisse and Thisse, 2008). To overcome this, embryos can be treated with proteinase K with optimal concentration, incubation time and temperature for the embryonic stage to facilitate infiltration of the probes into the tissue.

ISH was performed to study the expression of the abcc6 gene (respectively the abcc6a and abcc6b isoform) during ZF development. While abcc6a was found to be expressed in Kupffer's vesicle, abcc6b expression was evident in the proximal tubules of the embryonic kidney (Li et al., 2010).

In general, ISH is a laborious technique, taking about 3 days to complete the whole protocol. Additionally, it is difficult to handle small embryos because of their fragility throughout the protocol. So, semi-high-throughput procedures can alternatively be used to facilitate these experiments by using multiwell plates (instead of using single tube) and robotics (e.g., IntavisS AG) (Thisse and Thisse, 2008; Bouzaffour et al., 2009). To detect the differential protein expression in morphant compared to control, ISH followed by image mapping software can be a useful tool. Alternatively, post-ISH embedding in plastic, followed by sectioning, yields high resolution images that allow detailed cellular localization of the transcripts (Verstraeten et al., 2012).

ASSESSMENT OF GENE EXPRESSION USING QUANTITATIVE REAL-TIME PCR AND MICROARRAYS

Real-time polymerase chain reaction, also called qPCR, is a laboratory technique based on PCR, which is used to detect and measure minute amounts of nucleic acids in a wide range of samples. The quantity can be either an absolute number of copies or a relative amount. The expression pattern of mineralization-related genes in morphants (i.e., osteocalcin, alkaline phosphatase, bone sialoprotein) can be assessed by performing quantitative RT-PCR. To reliably conduct qRT-PCR experiments it is highly recommended to follow the MIQE guidelines (Bustin et al., 2009). MIQE is a set of guidelines that describe the minimum information necessary for evaluating qPCR experiments. Among the requirements is the need to include at least three biological replicates (e.g., three independently injected clutches of embryos) to address the statistical significance of differences in qPCR results between morphants and controls. Furthermore, at least two reference genes should be included as internal controls for normalizing cellular mRNA data. Reference genes should be stably expressed, and their abundances should show strong correlation with the total amount of mRNA present in the samples. In ZF embryos tuba1, bactin1, and elfα are three stable reference genes which can be used for qPCR experiments comparing morphants and controls (McCurley and Callard, 2008).

Microarray technology can be used to monitor transcriptome wide expression of genes in ZF embryos. Microarray analysis has been employed to study the temporal activity of developmentally regulated genes during ZF embryogenesis (Mathavan et al., 2005) but also in numerous studies where gene expression was compared between morphants and controls (Jenny et al., 2012; Wei et al., 2012).

The principle of Next Generation Sequencing (NGS) has recently been applied to transcriptome profiling (Wang et al., 2009), which offers several advantages compared to microarrays or quantitative RT-PCR. Most importantly, RNA-Seq transcriptome profiling can be used to identify rare transcripts which are undetectable with microarrays. Moreover, RNA-Seq allows more precise quantification of different transcripts (Wetterbom et al., 2010).

PROTEOMICS

Physiological mineralization is governed by highly coordinated changes in the expression of a large number of proteins. Understanding these changes at the molecular level can provide important insights into physiological and disease mechanisms (Lucitt et al., 2008). To elucidate these regulatory genetic networks in a ZF disease model, quantitation of protein expression during growth and development is essential. In morphants, proteomics can be used to identify proteins that are differentially expressed compared to controls. It has been proposed that proteomic studies should complement genome-wide expression profiling (Love et al., 2004).

Many approaches can be used for quantitative protein studies including two dimensional poly acrylamide gel electrophoresis (2D PAGE), mass spectrometry (MS), liquid chromatography (LC) and western blotting. Proteomic approaches are however incrementally challenging in ZF because of unavailability of specific antibodies and the high abundance of yolk proteins in embryos (Akhtar et al., 2009; Lobner et al., 2012). Further, many protein identifications have low reproducibility if the sensitivity of detection is not carefully balanced against rates of false identification error (Lucitt et al., 2008). Therefore, rigorous statistical analysis is needed to obtain high quality profiles of proteins.

In the early embryo, the cells forming the embryo constitute only a minor volume compared to the large yolk sac. The major yolk protein is vitellogenin, a phospholipo-glycoprotein, which functions as a nutritional source for the development of the embryo (Denslow et al., 1999). Link et al. (2006) developed an effective protocol for protein analysis from deyolked embryos. By pipetting with a narrow tip, the yolk sac can be disrupted. A buffer of low osmolarity facilitates dissolving of the yolk. The deyolking efficiency can be further increased by two additional wash steps. By removing the yolk, recovery of cellular proteins remained

high with only a minor reduction of housekeeping gene (mek and tubulin) observed.

Mass spectrometry

Mass spectrometry is an important method used to characterize proteins by measuring the mass-to-charge ratio. The first step in MS is the ionization of proteins, for which two common methods can be used: electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). Mass analysis of proteins can be conducted using either time-of-flight (TOF) MS or Fourier transform ion cyclotron resonance (FT-ICR). Generally, a protein sample is a complex mixture of different proteins. The concentration of a protein can vary between samples and an overwhelming number of peptides can make it difficult to interpret the results. To overcome this problem, two methods, 2D gel electrophoresis and high performance liquid chromatography, can be used to fractionate the proteins or their peptide products after enzymatic digestion. In MS, two ways are mainly used to identify proteins, i.e., Peptide mass fingerprinting and Tandem MS. Observed fragment masses are matched with a database of predicted masses for given peptide sequences. Several methods also allow quantitation of proteins by MS, i.e., stable isotope labeling by amino acids in cell culture (SILAC), isotope coded affinity tagging (ICAT), iRRAC (isobaric tags for relative and absolute quantitation), or semi-quantitative MALDI analysis (in liner mode).

2D gel electrophoresis

After removal of the predominant yolk proteins, high resolution 2D gels in the acidic (pH 4–7) as well as in the basic range (pH 6–9) can be run, and proteins will be separated according to isoelectric point. Two biological replicates of each sample have to labeled with fluorophores (Cy3/Cy5), and an internal control from each sample labeled with different fluorophores (Cy2) can be used to normalize label differences. DIGE gels are then stained and imaged using emission wavelengths of the fluorophores. Link et al. (2006) established a protocol that is compatible with three color fluorescent labeling using the Ettan DIGE system, which significantly reduces inter-gel variability compared to one color stains with a detection limit less than 1 ng protein. Analysis of 2D gels also allowed to resolve protein isoforms. Finally, validation of protein expression changes can be confirmed by western blotting and real-time PCR studies.

Western blotting

Western blot is a widely accepted analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. The success of western blotting depends on the affinity and specificity of the antibodies and on the abundance of the target protein. If the yolk sac is not removed only 1 or 2 embryos (50–100 μg) can be loaded per lane on a gel to avoid overloading effects (Link et al., 2006). As previously stated, antibodies validated for ZF proteins are not always available which can hamper the use of this technique. Along with confirmation of TB MO efficiency, western blotting can also be used to detect differential expression of a targeted protein in morphant compared to control.

DISEASE SPECIFIC MECHANISMS

IDENTIFICATION OF PROGRAMED CELL DEATH (APOPTOSIS)

Apoptosis is a form of cell death in which a programed sequence of events leads to the elimination of cells without inflammation. Apoptosis plays a pathophysiological role in many mineralization-related disorders including calcific aortic valve disease (Côté et al., 2012), osteoarthritis (Sun et al., 2012), and PXE (Mungrue et al., 2011).

Several methods have been developed for visualizing apoptotic cells *in vitro* or in fixed tissues, but few tools are available for visualizing apoptotic cells in live animals. Methods exist for labeling apoptotic cells with fluorescent nucleic acid binding dyes, such as acridine orange, ethidium bromide, and propidium iodide (Lecoeur et al., 2002). A standardized technique to detect apoptotic cells in fixed tissue or fixed cells is terminal deoxynucleotidyl TUNEL, which is based on end labeling of DNA degradation products enzymatically or by a fluorescent probe (**Figure 7**) (Gavrieli et al., 1992). Another well-established method to detect apoptotic cells *in vitro* is based on loss-of membrane asymmetry during apoptosis (Fadok et al., 1992). During apoptosis, the normal asymmetric distribution of phospholipids in the cell membrane is lost, and phosphatidylserine (PS) is exposed on the outer leaflet of the lipid bilayer. The calcium-dependent protein Annexin V (A5) binds PS with high affinity and fluorescently labeled A5 probes have been widely used to detect apoptotic cells *in vitro* (Van Genderen et al., 2006).

Van Ham et al. (2010) recently introduced a new transgenic fluorescent marker allowing *in vivo* imaging of apoptotic cells to understand their dynamics. They fused secreted A5 (secA5) protein to yellow fluorescent protein (YFP) (secA5-YFP) and showed that this fusion product specifically labels apoptotic cells in living ZF; the fluorescent probe can characterize patterns of apoptosis in living ZF larvae and visualize cell death at single-cell resolution *in vivo*. Labeled cells exhibit several other characteristics of apoptotic cells, and the pattern of apoptotic cells observed by live imaging was similar to previous findings using TUNEL.

DETECTION OF OXIDATIVE STRESS

Oxidative stress is an imbalance between the systemic manifestations of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Oxidative stress is increasingly implicated as a possible pathogenic mechanism underlying a wide range of diseases including mineralization-related disorders such as atherosclerosis, cardiovascular disease, or diabetes (Stephens et al., 2009). Oxidative stress in fibroblasts (Pasquali-Ronchetti et al., 2006), and elevated oxidative stress markers in the circulation (Garcia-Fernandez et al., 2008) of patients with PXE reveal its possible role in the pathogenesis of this ectopic calcification disease.

To determine the level of ROS, fluorescent reporter molecules CMH2DCF and Dihydrorhodamine can be administered, both of which have been successfully applied to live embryos (Hermann et al., 2004; Craven et al., 2005). Dihydrorhodamine is an uncharged fluorescent ROS indicator which passively diffuses

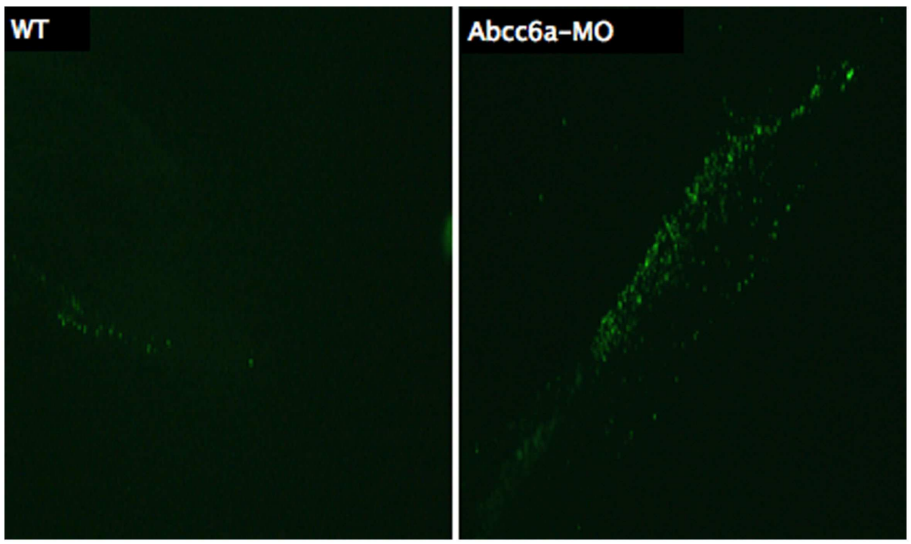

FIGURE 7 | Transferase dUTP nick end labeling staining of 5 dpf embryos. More fluorescent dots are observed in the tail region of Abcc6a-MO injected fish (right) demonstrating more apoptosis compared to the tail region of the wild-type fish (left).

across membranes where it is oxidized to cationic rhodamine 123 which localizes in the mitochondria and exhibits green fluorescence (Song et al., 2009).

ANALYSIS OF MITOCHONDRIAL MEMBRANE POTENTIALITY

Recent findings show that besides the production of ATP, mitochondria also contribute to several other cellular functions, including redox homeostasis, calcium homeostasis, and cell death (Scheffler, 2001). Mitochondrial mutations (inherited or somatic) are responsible for many developmental abnormalities (Zhang et al., 2012). Mitochondrial dysfunction also plays an important role in many mineralization diseases; i.e., in PXE and vascular calcification where decreased mitochondrial membrane potentiality (MMP) and reduced ion gradient has been reported. Measuring MMP allows assessment of mitochondrial function and integrity (Nicholls and Budd, 2000).

Different methods exist to measure MMP in ZF, including staining with membrane-potential-dependent dyes such as Rhodamine 123, tetramethylrhodamine ethyl ester (TMRE), fluorescent carbocyanine dye JC-1, or MitoTracker (Chazotte, 2010; Mitra and Lippincott-Schwartz, 2010). MitoTracker is a commercially available fluorescent dye, which labels mitochondria within live cells. Among available probes, MitoTracker Red is a red-fluorescent dye widely used for labeling mitochondria in live ZF embryos/cells and its accumulation depends upon membrane potential (**Figure 8**). Live ZF embryos are incubated in the dark with 25–500 nM MitoTracker Red CM-H2XRos working solution for 2 h at 28.5°C, and observed under a fluorescent microscope. MitoTracker can also stain the endoplasmic reticulum if embryos/cells are exposed to a higher MitoTracker solution for a prolonged period of time. However, high concentration and prolonged exposure can also block mitochondrial activity, so a low concentration with short time exposure is recommended to obtain specific staining (Ryu et al., 2011).

CHEMICAL SCREENING AND DRUG DISCOVERY

Due to easy diffusion of chemicals through the skin, ZF allows disease-driven drug target identification and *in vivo* validation, thus representing an interesting bioassay tool for small molecule testing and dissecting biological pathways (Pichler et al., 2003). In a MO injection based reverse genetic approach, chemical screening is very useful to find a chemical/drug which rescues morphant phenotypes (Taylor et al., 2010). Peterson et al. (2000) screened 1100 synthetic small molecules against ZF embryos arrayed in 96 well plates to identify molecules that specifically modulated developmental processes, and found that one tetrazole derivative affected otolith development. By adding and removing the chemical, they determined a critical stage for otolith development that occurs between 14 and 26 h after fertilization. This demonstrates that drug screening in ZF can provide more insights in physiological and developmental processes.

Pichler et al. (2003) proposed two broad strategies for drug development in ZF. First, large-scale random chemical screening against diseased or control ZF can be performed to observe biologically interesting phenotypic changes; secondly, functional understanding of disease pathways can allow to determine specific target genes directly associated with the disease followed by chemical screening against that gene. In combination with microarray, ZF promise to be a cost and efficient bioassay that can simultaneously uncover drug candidates, estimate toxicity, primary and secondary drug targets, and phenotypic outcomes (Pichler et al., 2004). As many ectopic mineralization disorders are currently still intractable, this model may prove efficient to further explore therapeutic options.

SYSTEMS BIOLOGY APPROACHES

The multidisciplinary approaches of systems biology allow us to quantitatively study of the fundamental principles of a biological system, aiming at better understanding the connections

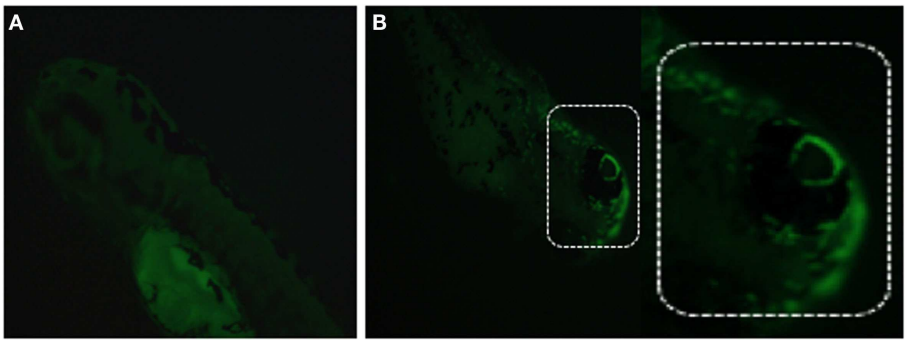

FIGURE 8 | Detection of mitochondrial membrane potentiality on 2 dpf embryos by MitoTracker Red CM-H2XRos staining. **(A)** Control fish showing no fluorescent staining, and **(B)** staining with 500 nM MitoTracker Red CM-H2XRos for 2 h showing fluorescent staining of mitochondria in the head region of a mitochondrial disease model.

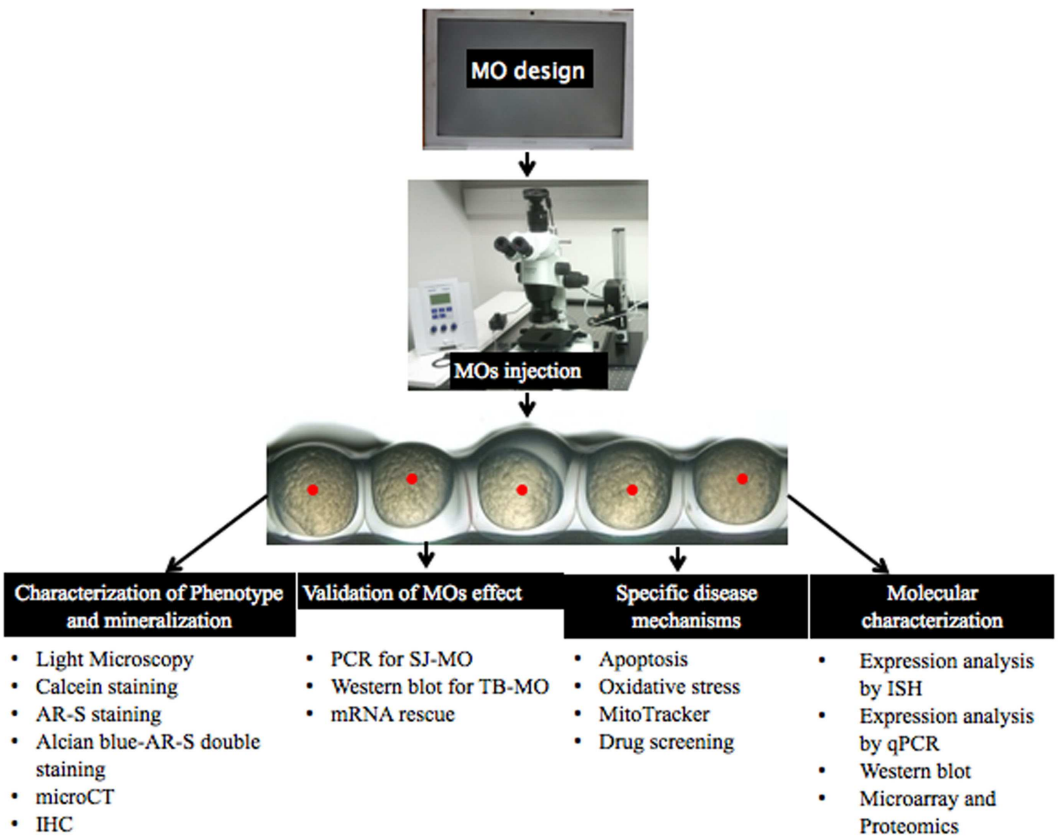

FIGURE 9 | Overview of MO injection and post-injection follow-up to study ectopic mineralization zebrafish models.

between gene, protein, and cellular networks. ZF is regarded as an ideal vertebrate model for studying genotype-phenotype relationships, pathway analysis, and systems biology. Deo and MacRae (2011) described the possibility of a systems biology approach in ZF for functional annotation of the genome, elucidation of multicellular processes *in vivo*, expression profiling and genome-wide association, phenotypic architecture, disease heterogeneity, and drug responses. Several systems biology approaches have already been developed to study ZF models; among them, the comparative study of embryonic development between morphants and controls can break new ground in understanding and uncovering disease mechanisms. However, the bottleneck of these approaches is the huge amount of data that needs to be analyzed.

IN TOTO IMAGING

Megason and Fraser (2007) recently described "Digital Fish project" to upload ZF development into computer for better understanding how genome computes the formation of an embryo from an egg. In their project they combine confocal/2-photon imaging with genetics, genomics, synthetic biology, and computational analysis to watch biological circuits function *in vivo* and use these data in cell-based modeling. They developed "in toto imaging" to track all cells in a developing tissue and record cell-based quantitative data by using fluorescent reporters. In this imaging process they used "segmentation marker" to track cells, captured 3D time-lapse movies on confocal and custom built 2-photon microscope, and finally used a software package called GoFigure to determine complete lineages and cell-based frameworks for use in modeling.

FLIP TRAPS

Flip traps is a novel gene trap technique to generate endogenously expressed functional fluorescent fusion protein, and they generate Cre conditional alleles. Fluorescent fusion protein is used to non-invasively quantify expression and localization of proteins *in vivo*. Flip Traps in combination with "in toto imaging" can digitize expression and phenotype with single-cell resolution for use in molecular and cellular modeling of developmental processes (Megason and Fraser, 2007).

COMPUTATIONAL APPROACH

Morelli et al. (2012) recently described the use of computational modeling (algorithm and simulation) to understand embryonic development. They investigated four developmental patterning strategies of the embryo: (1) gradients of signaling molecules released from localized source cell population, (2) balance between activator-inhibitor mechanism leading to formation of spatial patterns, such as stripes and spots in a two dimensional space, (3) synchronization of cellular oscillations controlling rhythmic and sequential subdivision of body axis into segments, and (4) mechanical deformation changing the pattern of a cellular population. It is shown that theoretical along with experimental/computational data can play an important role to disclose mechanisms of development. Following the same approach between control and morphant embryos will give an opportunity to understand underlying differential mechanisms. The Prerequisite for using this approach is that the level of description and model type are matched to quantitative, precise, and accurate data. This will beyond any doubt require a multidisciplinary team of specialists.

IN VIVO MODELING

Genetic and genomic features, conservation of intermolecular network, as well as physiologic and phenotypic features have made ZF an important model for systems biology studies. Deo and MacRae (2011) described homology and high-throughput phenotyping strategies which can be used for genetic or chemical screening on a scale compatible with *in vivo* validation for systems biology.

Advent and validation of MO and increasing efficiency of transgenesis have made it possible to study hundreds of genes with specific phenotypes. Generally, phenotypic investigation of these approaches has been limited by phenotyping throughput. Now, the possibility of automated, quantitative phenotypes can lead to more comprehensive screenings. Moreover, applying a known causal mutation background in ZF models can aid to uncover disease mechanism. This type of analysis is also very useful to understand genotype-phenotype correlations including penetrance, pleiotropy, and pathogenicity. ZF disease models combined with known/approved libraries of drugs may enable collection of datasets which can be highly informative not only for disease network architecture, but also for pharmacogenetics.

CONCLUSION

Ectopic mineralization disorders feature some important medical issues to be resolved because of their complex pathogenesis, uncertainties on the mechanisms that deposit mineral in tissues or how to remove it from the tissues, and the unavailability of specific drugs and treatments. Until now, many experiments have been done both on patients and mammalian model organisms but the pathophysiological mechanisms of many ectopic mineralization disorders are still incompletely understood. Because of the limitations to perform studies on human tissues – often due to the invasive procedures needed to obtain them – and the high cost, long breeding time and complexity to achieve knockdown of genes in mouse models, the ZF has come to attention as an alternative model organism. Several studies have shown that there are many similarities in the molecular pathways and mechanisms involved in (pathological) mineralization between ZF and mammalians, even if the phenotypic consequences are not identical for obvious reasons. Using MO injection based knockdown, ZF can be an important disease model to study mineralization disorders. This is because phenotypic and molecular genetic results can be obtained within hours, because of the possibility of close observation in the developing transparent embryos and because of the easy application of techniques in the post-injection period. The concern of off-target or aspecific findings can be addressed by applying one or more alternative knock-down approaches. By considering aforementioned advantages, ZF can be used as a novel model organism for ectopic mineralization disorders. After MO-based knockdown, the described (**Figure 9**) validation and post-injection follow-up can be applied to gain insights in the mechanisms and future therapeutics of mineralization disorders.

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Transcriptional regulation of the ABCC6 gene and the background of impaired function of missense disease-causing mutations

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The human ATP-binding cassette family C member 6 (*ABCC6*) gene encodes an ABC transporter protein expressed primarily in the liver and to a lesser extent in the kidneys and the intestines. We review here the mechanisms of this restricted tissue-specific expression and the role of hepatocyte nuclear factor 4α which is responsible for the expression pattern. Detailed analyses uncovered further regulators of the expression of the gene pointing to an intronic primate-specific regulator region, an activator of the expression of the gene by binding CCAAT/enhancer-binding protein beta, which interacts with other proteins acting in the proximal promoter. This regulatory network is affected by various environmental stimuli including oxidative stress and the extracellular signal-regulated protein kinases 1 and 2 pathway. We also review here the structural and functional consequences of disease-causing missense mutations of ABCC6. A significant clustering of the missense disease-causing mutations was found at the domain–domain interfaces. This clustering means that the domain contacts are much less permissive to amino acid replacements than the rest of the protein. We summarize the experimental methods resulting in the identification of mutants with preserved transport activity but failure in intracellular targeting. These mutants are candidates for functional rescue by chemical chaperons. The results of such research can provide the basis of future allele-specific therapy of ABCC6-mediated disorders like pseudoxanthoma elasticum or the generalized arterial calcification in infancy.

Keywords: ABCC6, HNF4α, 4-phenyl-butyrate, C/EBPβ, calcification, pseudoxanthoma elasticum, generalized arterial calcification in infancy

THE ABCC6 PROTEIN

ATP-binding cassette family C member 6 (ABCC6) encodes an ATP-dependent transporter primarily found in the plasma membrane of hepatocytes (Madon et al., 2000). *In vitro* studies supports that it functions as an organic anion efflux pump (Belinsky et al., 2002; Iliás et al., 2002) transporting (an) unidentified substrate(s) from the liver toward the circulation. The functional relationship between ABCC6 and ectopic calcification is not understood.

On the basis of sequence similarity forty-eight ABC proteins are annotated in the human genome and classified as members of seven subfamilies. The ABC proteins share the general structural features: they harbor two nucleotide-binding (ABC) domains and two transmembrane domains (TMDs), each with six membrane-spanning helices (the so called "core structure"). The ABCC-subfamily consists of 12 proteins; most of them are active transporters. Two additional domains are fused to this core structure N-terminally in some ABCC-type proteins ("long MRPs" like ABCC1, 2, 3, 6, 8, 9, and 10): a TMD with five membrane embedded helices and an intracellular approximately 80 amino acid long loop. The domain architecture of this type of proteins, including ABCC6, can be described like TMD0-L0-TMD1-ABC1-L1-TMD2-ABC2 [L0 and L1 are intracellular loop (ICL); **Figure 1B**]. ABCC6 consists of 1503 amino acids and *in vitro* studies demonstrated the transport of different organic anions, glutathione-conjugates like glutathione *S*-conjugated leukotriene C4 (LTC4), *N*-ethylmaleimide *S*-glutathione (NEM-GS), and *S*-(2,4-dinitrophenyl) glutathione (Iliás et al., 2002), while the rat ortholog transports an anionic cyclopentapeptide (Belinsky et al., 2002). It was found using an *in vitro* transport assays that three of the missense mutations described as causative variants in pseudoxanthoma elasticum (PXE) result in the loss of ATP-dependent transport of test substrates (Iliás et al., 2002). It has also been suggested that overexpression of ABCC6 is able to confer low levels of resistance to several commonly used natural product anticancer agents like etoposide, doxorubicin, daunorubicin, and actinomycin D (Belinsky et al., 2002). However, clinically relevant ABCC6-mediated drug resistance has never been found.

Since the first PXE-causing mutations were discovered (Bergen et al., 2000; Le Saux et al., 2000, 2001; Ringpfeil et al., 2000), the number of identified disease-causing variants has exceeded 350. By searching PubMed papers reporting

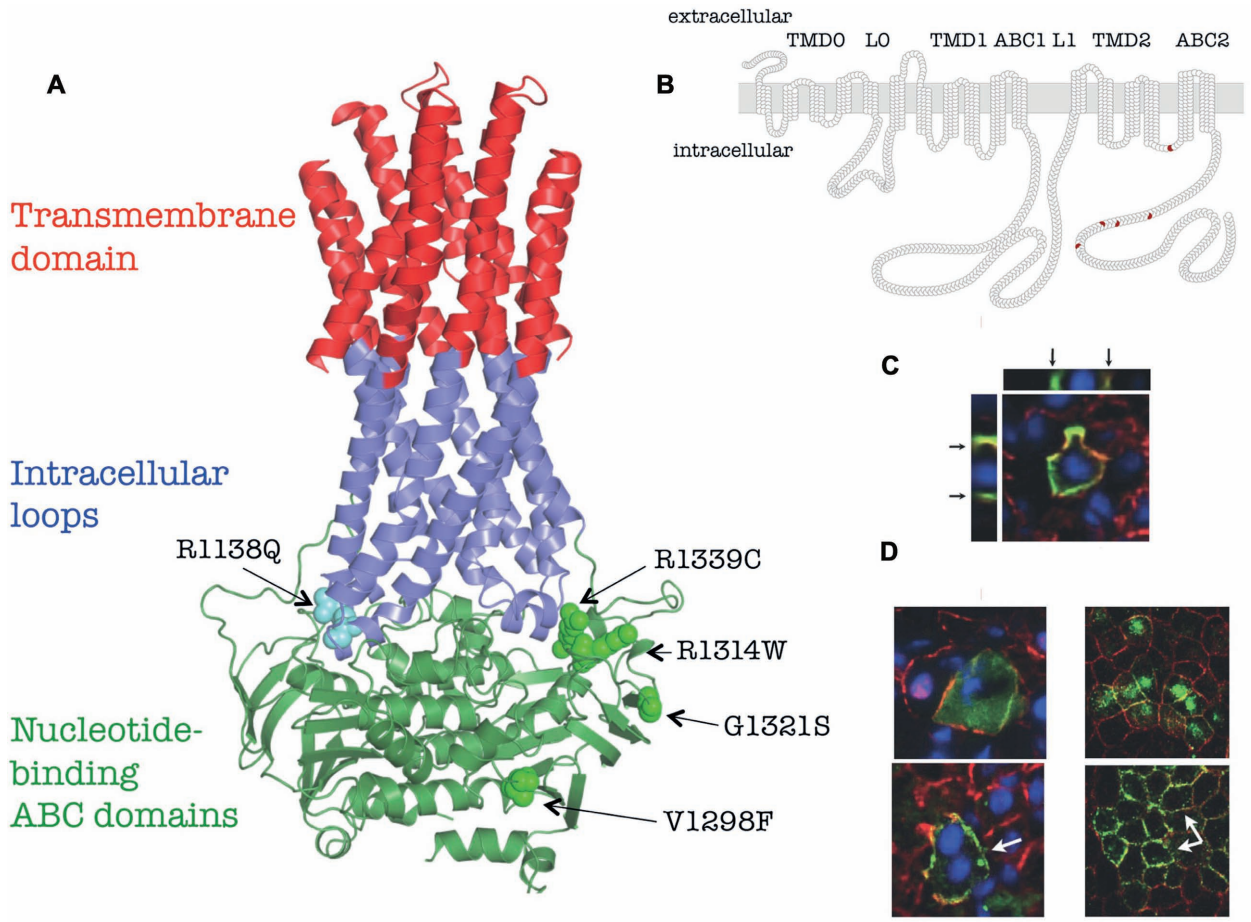

FIGURE 1 | Membrane topology, homology model and *in vivo* localization of human ABCC6 and its mutants (based on Le Saux et al., 2011). (A) Homology model of human ABCC6. The position of the missense mutants studied are indicated. **(B)** Membrane homology model of human ABCC6. The domain structure are indicated on the top, the position of missense mutants are shown in red. **(C)** Colocalization of the endogenous mouse Abcc6 (red) and human ABCC6 (green) *in vivo* in mouse liver after HTVI-delivery of ABCC6 cDNA by confocal microscopy. Arrows indicate the basolateral localization on Z-stack cross/sections. **(D)** Effect of 4-PBA on membrane targeting of Q1314W. *Left panel*: colocalization of the endogenous mouse Abcc6 (red) and human ABCC6 mutant (green) *in vivo* in mouse liver after HTVI-delivery of ABCC6 cDNA without 4-PBA treatment (*upper panel*) or with 4-PBA treatment (*lower panel*). *Right panel*: colocalization of the endogenous dog Na/K-ATPase (red) and human ABCC6 mutant Q1314W (green) *in vitro* in polarized MDCKII cells without 4-PBA treatment (*upper panel*) or with 4-PBA treatment (*lower panel*). White arrows point to the altered membrane localization.

ABCC6 mutations and polymorphisms an internet-based mutation/variation database of ABCC6 has been established. Upon creating the database approximately 300 published mutations in 51 publications by 23 corresponding authors were collected and 65 potential errors in the reported mutations or sequences were found. Only corrected (confirmed) mutations are included into the database (Váradi et al., 2011), which contains now nearly 400 mutation/variations of the gene (http://www.ncbi.nlm.nih.gov/lovd/home.php?select_db=ABCC6). The majority of the mutations are missense. Despite the large number of PXE-specific mutations that has been identified in ABCC6, no clear genotype–phenotype correlation has emerged.

No high-resolution three-dimensional structure of ABCC-type is available. However, a three-dimensional homology model of ABCC6 is already built and published (Fülöp et al., 2009), made possible by using the coordinates of high-resolution crystalline structures of other ABC proteins (Dawson and Locher, 2006; Aller et al., 2009). Newly recognized structural elements are the long "rigid" extensions of the transmembrane helices, called ICLs were also obvious in the homology models. Each half of the ABC proteins has two ICLs interacting with the ABC domains. The coupling helices contact with their "own" as well as with the "opposite" ABC domains, hence a special type of domain swapping can be recognized in the structure (see insert on **Figure 1A**). The homology models constructed can be interactively studied using the ABCC6 database.

The disease-causing mutations in the protein are distributed on an uneven fashion and performing statistical analysis significant clustering of the missense PXE mutations was found at the domain–domain interfaces: at the transmission interface that involves four ICLs and the two ABC domains as well as at the ABC–ABC contact surfaces. In the nucleotide-saturated model

the mutations affecting these regions are 2.75- and 3.53-fold more frequent than the average mutational rate along the protein sequence, respectively (Fülöp et al., 2009). At the predicted ICL–ABC interfaces in the nucleotide-free model the mutational rate is 4.25-fold more frequent than the average mutational rate along the protein sequence (the ABC domains are distant in this conformation; Váradi et al., 2011). The observed significant clustering means that the domain contacts are much less permissive to amino acid replacements than the rest of the protein. These results provide a "bridge" between genetic data and protein structure and can be viewed as novel proof of the importance of the studied domain–domain interactions in the ABCC6 transporter.

A systematic experimental study has been initiated to investigate the impact of missense disease-causing mutation in order to better understand the molecular failure triggered by different mutations. Amino acid substitutions in large plasma membrane proteins such as ABCC6 generally result in decreased activity, major conformation changes, low level of plasma membrane targeting or a combination thereof. Therefore, studying the consequences of naturally occurring disease-causing missense mutations can provide important insights into the relationship between protein structure and function, which may later assist in the development of therapeutic applications (Le Saux et al., 2011). We review here the major steps of this systematic experimental study.

The complex experimental repertoire consists of expression and biochemical characterization of the protein variants, investigation of their plasma membrane vs. intracellular localization in polarized cell cultures. Finally, investigation the *in vivo* stability and cellular location of mutated ABCC6 in fully differentiated hepatocytes by transiently expressing the human mutant proteins was performed in the liver of C57BL/6J mice. Five missense mutations, V1298F and G1321S in the C-proximal ABC domain and R1138Q, R1314W, and R1339C at the transmission interface were included into the study. The location of the mutated residues is illustrated on **Figure 1A**. An N-terminal truncated mutant, del_{1-277}ABCC6 (DABCC6) missing the TMD_0 and L_0 domains has also been constructed as a control (see **Figure 1B**). The results are summarized in **Table 1**. In detail: the wt ABCC6 is fully functional in the biochemical transport and enzymatic (MgATP-binding and catalytic intermedier formation) assays, it is integrated into the basolateral membrane of polarized Madin–Darby canine kidney type II (MDCKII) cells *in vitro* as well as that of the mouse hepatocytes *in vivo*. The DABCC6 appears to be an ideal negative control as it is inactive, and localized intracellularly both in MDCKII cells and in mouse liver cells.

The R1139C mutant did not result in native size protein when expressed in Sf9 cells indicating folding problems. Two of the variants (V1298F and G1321S) were found to be inactive, though they were capable of binding MgATP, their capacity to form a catalytic intermediate ("occluded nucleotide state") was impaired. R1138Q and R1314W were fully active in the transport assay. When overexpressed in MDCKII cells surprisingly, R1139C show a level of protein expression similar to the wild type, however, it was found intracellularly, indicating that the mutation triggered an altered conformation not compatible with plasma membrane targeting. The same was true for mutants G1321S, R1338Q, and R1314W, while the inactive V1298F was found mostly in the plasma membrane.

ATP-binding cassette transporters are traditionally studied in cultures of kidney-derived MDCKII cells, but these are not ideal for studying hepatic proteins, as they do not correspond to the physiology of the liver where ABCC6 is primarily present. To overcome these obstacles hydrodynamic tail vein injection (HTVI) method was utilized to study the human ABCC6 protein in the fully differentiated liver of a living mouse. This method delivered DNA to the liver very effectively (Liu et al., 1999; Zhang et al., 2004) and ensured the selective hepatic expression of the wt human protein in mouse liver with an adequate basolateral targeting (see **Figure 1C**).

The *in vivo* intracellular localization of the human variants was also studied. Only the inactive V1298F mutant showed plasma membrane localization at a similar high level as the wt. The R1339C and the G1321S proteins were found intracellularly (similar to the results obtained in the *in vitro* cell culture system). R1138Q and R1314W showed mostly intracellular appearance,

Table 1 | Function and intracellular localization of ABCC6 variants.

ABCC6 variant	Stability in Sf9	MgATP-binding	ATPase catalytic intermediate	Transport activity (% of WT)	Plasma membrane localization in MDCKII cells	Plasma membrane localization in mouse liver	Intracellular localization in mouse liver
WT	Stable	Yes	Yes	100	+++++	+++++	−
DABCC6	Stable	n.d.	n.d.	<10	−	−	+++++
R1138Q	Stable	Yes	Yes	~85	++++	++	+++
V1298F	Stable	Yes	No	<10	+++++	+++++	−
G1321S	Stable	Yes	No	<10	−	−	+++++
R1314W	Stable	Yes	Yes	~90	−	+	++++ (ER)
R1339C	Unstable	n.a.	n.a.	n.a.	−	−	+++++

n.d., not determined; n.a., not applicable; ER, mostly retained with the endoplasmic reticulum.
The symbol "+" represents semiquantitative estimate of the presence of immunohistochemical signal in the given compartment (+++++ is maximal abundance).
The symbol "−" represents absence of immunohistochemical signal in the given compartment.

however, a portion of the protein could reach the plasma membrane.

From the above the functional features and the pathogenic character of the mutants can be delineated. They clarify the biochemical and cellular effects of ABCC6 mutations that lead to dystrophic calcification in humans. Most probably the dramatically reduced transport activity of mutants V1298F and G1321S is the molecular background of the disease in patients with these variants (irrespective that in the case of the former one the correct plasma membrane targeting is preserved). In the case of R1338, R1314, and R1339 the aberrant folding resulted by the mutation prevents plasma membrane localization, which seems to be the prerequisite of the normal physiological function (irrespective that two of these mutants are active as organic anion transporter).

The successful completion of these experiments may provide valuable basic results and those in the field of translational medical research. They indicate the potential for therapeutic interventions to correct the defects of a mutant ABCC6 protein. Mutants with preserved transport activity but failure in intracellular targeting are candidates for functional rescue.

Several studies have shown that sodium 4-phenylbutyrate (4-PBA) can act as a chemical chaperone (e.g., Rubenstein and Zeitlin, 2000; van der Velden et al., 2010) for misfolded proteins in the endoplasmic reticulum (ER). Therefore, it was studied whether pre-treating mice with 4-PBA before HTVI would restore normal cellular trafficking of those mutants that retained transport activity. R1138Q and R1314W were tested, along with R1339C and the WT protein as non-functional and functional controls respectively. It is expected that those mutant proteins retained in the ER are to be rescued by 4-PBA. We have found that R1314 shows ER retain, as it colocalized with an ER-marker in mouse hepatocytes, while R1338Q and R1339C was not found in this location.

Oral treatment of the animals with 4-PBA before HTVI resulted in no effect on the intracellular localization of R1338Q and R1339C. However, treatment of mice with 4-PBA improved the cellular localization of R1314W (**Figure 1D**, left panels), which was confirmed in MDCKII cells (**Figure 1D**, right panels). This result suggests that the incorrect intracellular trafficking of this otherwise functional mutant is due to ER retention, implicating protein misfolding, and that correct intracellular trafficking of an otherwise functional mutant can be restored. As 4-PBA is an FDA-approved drug, these results may facilitate further clinical research for allele-specific therapy of PXE.

TRANSCRIPTIONAL REGULATION OF THE ABCC6 GENE

However, we should also keep in mind that PXE is not a single disease but a group of similar phenotypes, which is constituted by PXE, PXE-like diseases and generalized arterial calcification of infancy (GACI; Aessopos et al., 1992, 2008; Vanakker et al., 2007; Nitschke et al., 2012) based on various, mostly unknown pathomechanisms. Therefore, another therapeutical approach for at least some PXE(-like) diseases could be targeted to the gene instead of the misfolded protein. In order to explore this idea we have to go back to the transcriptional regulation of *ABCC6*.

Although some studies have reported ubiquitous expression of ABCC6 (Beck et al., 2003, 2005), there is a consensus in the literature that the gene is primarily expressed in the liver and to a much lower extent in the kidneys (Kool et al., 1999; Madon et al., 2000; Beck et al., 2005; Maher et al., 2005, 2006). There is also intestinal expression of the gene based on the data published by (Kool et al., 1999) and findings in human cell lines (Ratajewski et al., 2012). This expression pattern is similar in rodents and human even if the gene is in a dynamically evolving genomic region (Eichler et al., 2007; Symmons et al., 2008). This fast evolution is characterized by numerous recent segmental duplications but also some primate-specific insertions/deletions with uncovered regulatory role, in spite of the relatively high conservation level of the coding region and some other regulatory sequences (Eichler et al., 2007; Symmons et al., 2008; Ratajewski et al., 2012). Why the gene is expressed essentially in the liver and to a lower level in very few other tissues, while in the organs suffering from the loss-of-function ABCC6 mutations ABCC6 is normally not expressed? To formulate this question in a different way: what is responsible for this intriguing tissue-specificity?

To answer this question a battery of molecular biological tools and assays were used, e.g., luciferase reporter gene assay, gel shift assay (EMSA), transcription factor microarray, DNase I hypersensitivity assay (DHA), and chromatin immunoprecipitation (ChIP). Bioinformatic studies revealed the existence of two evolutionarily well-conserved sequence blocks: one in a distal 5′ region, near to the neighboring NOMO3 gene, and one in the proximal promoter of the *ABCC6* gene (Arányi et al., 2005). Similar data were reported in human cell lines (Arányi et al., 2005) and mouse tissues (Douet et al., 2007) showing that in the proximal promoter, the presence of silencing epigenetic factors, namely DNA methylation, correlates inversely with the expression level of the gene, suggesting that the regulation of tissue-specific expression is determined by this region.

An important number of luciferase reporter gene assays were performed to find the regulatory elements in the promoter and later for the identification of transcription factors binding those elements (Arányi et al., 2005; Douet et al., 2006, 2007; Jiang et al., 2006; Ratajewski et al., 2006, 2008, 2009, 2012; de Boussac et al., 2010; Martin et al., 2011). In these experiments the firefly luciferase gene is under the transcriptional control of a target sequence in a plasmid vector. After transient transfection of the plasmid the transcriptional regulatory capacity of the target sequence is estimated by the luminometric luciferase enzymatic activity measurement. The use of this technique revealed a DNA methylation-dependent activatory (−332/+72 relative to the translation start site) and a repressor region (−718/−332; Arányi et al., 2005; Jiang et al., 2006). The −332/+72 fragment turned out to be composed of a −145/+72 core promoter (characterized by a ubiquitous low activity in the assay in all cell types tested and not sensitive to DNA methylation (Arányi et al., 2005; Ratajewski et al., 2006, 2012; de Boussac et al., 2010) and two other fragments located between −209/−145 and −233/−209 both sequences active in a cell type-specific manner (Ratajewski et al., 2009, 2012; de Boussac et al., 2010). Furthermore, it has also been shown that the activity of the primate-specific −233/−209 element depends on the activity of the −209/−145 sequence (Ratajewski et al., 2009, 2012).

Another set of luciferase reporter gene assays were carried out based on the results obtained by DHA. This classical method

reveals accessible regions to partial DNase I digestion in intact nuclei (Lu and Richardson, 2004). The accessible regions frequently represent active transcriptional regulatory elements. Surprisingly, the data suggested that not only the proximal promoter but also the first intron of the human *ABCC6* gene might have an important tissue- or cell type-specific regulatory role in the control of the expression of the gene (Ratajewski et al., 2012). Later on, with the help of the luciferase assays the presence of an important primate-specific sequence in the first intron of the gene (+629/+688) was found, which multiplies by several fold the transcriptional activity of the proximal promoter alone (Ratajewski et al., 2012). This intronic sequence and the proteins binding to turned out to depend on the presence of the proximal promoter and more specifically on the −233/−209 element, with which it might interact directly. Interestingly both sequences are primate-specific. These two elements also interact with the protein binding the sequence −209/−145, which is an evolutionarily highly conserved fragment (Ratajewski et al., 2012).

In another series of experiments, the different proteins recognizing the response elements were identified. Either luciferase assays with mutated target regulatory sequences or luciferase plasmids co-expressed with candidate transcription factors were used (Douet et al., 2006; Jiang et al., 2006; Ratajewski et al., 2006, 2008, 2009, 2012; de Boussac et al., 2010). Another approach was the EMSA, when the *in vitro* binding of a protein from a nuclear extract or its recombinant form is tested. In these cases in general short, labeled oligonucleotides are used as probe and in case of binding a protein, upon acrylamide gel electrophoresis a delay in the migration can be observed relative to that of the free probe (Douet et al., 2006, 2007; Jiang et al., 2006; Ratajewski et al., 2008, 2009). The transcription factor array is based on a similar principle [the hybridization of DNA and specific transcription factors (Jiang et al., 2006; Martin et al., 2011). Finally, ChIP can immunoprecipitate a target protein reversibly linked to its target sequence in the natural chromatin context after fragmentation of the chromatin. The immunoprecipitated fraction can then be tested by qPCR to determine the binding of the target protein to the sequences of interest (Douet et al., 2006, 2007; Ratajewski et al., 2006, 2008, 2012; de Boussac et al., 2010; Martin et al., 2011)]. These techniques led different groups to demonstrate the crucial regulatory role of hepatocyte nuclear factor 4 (HNF4) both in the human (de Boussac et al., 2010) and mouse (Douet et al., 2006) *ABCC6* gene. HNF4 binds to an evolutionarily highly conserved degenerate site between −209/−145 (de Boussac et al., 2010; Ratajewski et al., 2012). These data demonstrate the role of this transcription factor, which is a master regulator of metabolic genes in the liver. Interestingly, ABCC6 is expressed only in tissues where HNF4 is also present, suggesting that this transcription factor determines the tissue-specific expression of ABCC6. Our data also showed that in spite of the very important role of HNF4, it does not confer very high transcriptional activity to the gene. Further experiments suggested the binding of several other transcription factors to the proximal promoter [PLAG family members (Ratajewski et al., 2008, 2009), RXR (Ratajewski et al., 2006), SP1 and TGF-β (Jiang et al., 2006), and SP1 and NF-E2 in mouse (Douet et al., 2006, 2007; Martin et al., 2011)], however, the potential role of these different proteins is still unclear. The important role of CCAAT/enhancer-binding protein beta (C/EBPβ) in activating the transcription of the gene by binding the primate-specific sequence in the first intron was also shown (Ratajewski et al., 2012). Our data also suggest the binding of C/EBPα (unpublished).

In the previous sections we have summarized the regulatory elements in the ABCC6 gene and the transcription factors binding them. Altogether this network governs the tissue-specific transcriptional regulation of the expression of the gene. However, there is a third layer of investigations, which focused on the regulation of the *ABCC6* gene expression by environmental factors. These experiments showed that the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) cascade negatively regulates the expression of the gene (de Boussac et al., 2010). It was shown that this inhibition converges on HNF4, which due to the activation of ERK1/2 looses its activator potential in two phases. Similarly, it was shown that oxidative stress inhibits the expression of the gene in human cell lines. This effect is probably also at least partially acting via the ERK pathway (de Boussac et al., 2010). The role of oxidative stress in the downregulation of the mouse *Abcc6* gene expression was shown in a beta-thalassemia model mouse. In this model the role of NF-E2 transcription factor was described (Martin et al., 2011). Finally, the oxidative stress factors or other agents present in the serum of PXE patients were shown to downregulate the expression of the human gene in cell culture systems. The detailed molecular mechanism behind this observation remains to be deciphered (Le Saux et al., 2006). Altogether these findings described in these sections above are summarized on (**Figure 2**).

What are the future questions in the field? Can we use the obtained information either in the better understanding or in the cure of the disease? The major, still remaining question in the PXE field is the identification of the molecule the ABCC6 protein is transporting. Although, the better understanding of the transcriptional regulation of the gene will not directly provide an answer to this question however, it might give some hints. For example, since the gene is downregulated by reactive oxygen species (ROS) it probably does not play a role in the transport of molecules participating in the ROS pathways. Similarly, as HNF4 and C/EBP transcription factors regulate the expression of metabolic genes, this might suggest that ABCC6 transports a common metabolite.

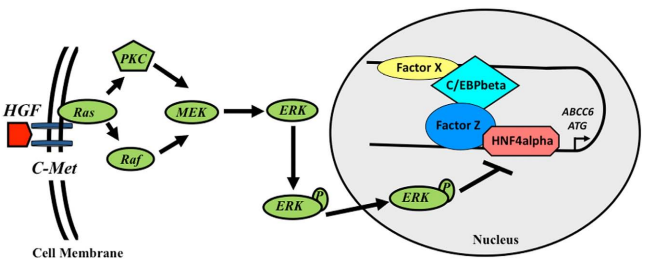

FIGURE 2 | Schematic model of the transcriptional regulation of the human ABCC6 gene. Growth factors inhibit the expression of ABCC6 via the activation of ERK1/2 cascade, which reduces HNF4α binding to the promoter. In the intron, a transcriptional activator sequence binds C/EBPβ and another hypothetical protein (Factor X). These proteins interact with a complex formed by HNF4α and another factor (Y) binding the promoter, activating the transcription of the gene.

Concerning the use of the knowledge obtained from these studies in the clinics, the situation is encouraging as in various clinical conditions [carriers of PXE mutations (Köblös et al., 2010; Vanakker et al., 2011) and secondary PXE (Aessopos et al., 1992, 2008)] the increase of the expression of the ABCC6 gene could lead to an improvement of the symptoms. Therefore, we consider that continuing the research to find the molecular mechanisms governing the regulation of the ABCC6 expression will lead to both a better understanding of the pathomechanism of the disease and an improvement of PXE related clinical phenotypes.

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New insights into the pathogenesis of pseudoxanthoma elasticum and related soft tissue calcification disorders by identifying genetic interactions and modifiers

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Screening of the adenosine triphosphate binding cassette transporter protein subfamily C member 6 gene (*ABCC6*) in pseudoxanthoma elasticum (PXE) revealed a mutation detection rate of approximately 87%. Although 25% of the unidentified disease alleles underlie deletions/insertions, there remain several PXE patients with no clear genotype. The recent identification of PXE-related diseases and the high intra-familiar and inter-individual clinical variability of PXE led to the assumption that secondary genetic co-factors exist. Here, we summarize current knowledge of the genetics underlying PXE and PXE-related disorders based on human and animal studies. Furthermore, we discuss the role of genetic interactions and modifier genes in PXE and PXE-related diseases characterized by soft tissue calcification.

Keywords: PXE, rare disease, mineralization, calcification, genetic interaction, genetic modifier

INTRODUCTION

Mutations in *ABCC6*, a gene encoding for the ABC transporter protein 6 of subfamily C, formerly known as multidrug resistance-associated protein 6 (MRP6), are the cause of pseudoxanthoma elasticum (PXE; Kool et al., 1999; Bergen et al., 2000; Le Saux et al., 2000; Ringpfeil et al., 2000; Miksch et al., 2005; Schulz et al., 2006). PXE is an autosomal recessive disorder characterized by soft tissue calcification primarily in the skin, Bruch's membrane in the retina and the vessel walls (Neldner and Struk, 2002). To date, more than 300 – mostly unique – PXE-associated *ABCC6* mutations have been identified {http://www.ncbi.nlm.nih.gov/lovd/home.php?select_db=ABCC6}. Despite large epidemiological studies including more than 500 well-characterized PXE patients, no genotype–phenotype correlations have been discovered so far. Moreover, there is a great clinical variability between PXE patients, even within families with more than one person affected. This heterogeneity between patients raised the question of whether factors, e.g., environmental or genetic background, contribute to PXE manifestation, disease progression, and severity. The assumption is supported by the description of PXE-related diseases (Vanakker et al., 2007) as well as clinical overlaps with other rare monogenic syndromes and common cardiovascular disorders (Trip et al., 2002). A recent publication in this special issue on soft tissue mineralization focused on the clinical phenotype of PXE and its parallels to other cardiovascular diseases (Lefthériotis et al., 2013). Here, we summarize the role of *ABCC6* mutations as cause of PXE, and discuss the current knowledge of genetic co-factors (modifiers) and genetic interactions for PXE and related disorders.

MUTATIONAL ANALYSIS OF THE *ABCC6* GENE

Recently, the PXE candidate gene, *ABCC6* (MIM #603234), was identified and mutations in this gene were found to cause PXE (exemplarily: Le Saux et al., 2000). The *ABCC6* gene contains 31 exons and encodes a 165 kDa transmembrane transporter of 1503 amino acids. The physiological function of ABCC6 is still unknown. To date, more than 300 causative *ABCC6* mutations have been identified. These include missense, nonsense, and splice site mutations, as well as deletions and insertions. According to current studies, PXE seems to be inherited in an exclusively autosomal recessive mode (Plomp et al., 2004; Miksch et al., 2005; Pfendner et al., 2007). Hence, PXE patients are homozygous or compound heterozygous carriers of *ABCC6* mutations. Most mutations observed in *ABCC6* are unique and the majority of them are located within cytoplasmic domains of ABCC6. The most frequent mutation found in PXE patients is a nonsense mutation in exon 24, p.R1141X (c.3421C>T, rs72653706), which is found in approximately 25% of the European patient population. A larger deletion of exons 23–29 (c.2996_4208del) represents the most common mutation (25%) in American PXE patients. The mutation detection rate lies between 80 and 90%. Consequently, in up to 20% of patients clinically diagnosed with PXE, only one or no *ABCC6* mutations were detected. As a consequence of methodological limitations, small *ABCC6* deletions/insertions in homozygous state could be missed by direct sequencing approaches. Costrop et al. (2010) uncovered about 25% of the missing alleles as deletions of various sizes by performing multiplex ligation-dependent probe amplification (MLPA). These findings emphasize the importance of screening

for deletion/insertions in the molecular diagnostics of PXE. Nevertheless, there still remain PXE patients with an incomplete *ABCC6* genotype. Consequently, other genetic causes or environmental factors may be involved in PXE manifestation.

GENETIC INTERACTIONS

The recent identification of inherited disorders related to PXE and characterized by soft tissue calcification suggests multiple genetic factors. Vanakker et al. (2007) described a novel PXE-like syndrome (MIM #610842), which is inherited in an autosomal recessive mode, but caused by mutations in the *GGCX* gene, encoding γ-glutamate carboxylase. Patients present with generalized redundant skin folds, a mild retinopathy and a coagulation defect of vitamin K-dependent clotting factors (Vanakker et al., 2007). Similar cases had already been reported in the early 1990s (Le Corvaisier-Pieto et al., 1996). Histological examination of a skin biopsy reveals fragmentation and calcification of mid-dermal elastic fibers identical to PXE. However, ultrastructural analysis by electron microscopy showed differences in elastic fiber mineralization: in PXE-like syndrome, the calcification occurred in the periphery of the elastic fiber rather than in the fiber core. Li et al. (2009a,b) described two different families with combined features of PXE and a vitamin K-dependent coagulation deficiency. In both studies mutational screening of *ABCC6*, *GGCX,* and *VKORC1* revealed two putative pathogenic mutations in *GGCX* in patients presenting with distinct skin lesions and a coagulation factor deficiency. Interestingly, the authors observed similar skin lesions in the mother and her twin sister with no evidence of a coagulation disorder (Li et al., 2009a). Mother and maternal aunt were identified as compound heterozygous carriers of the recurrent *ABCC6* nonsense mutation p.R1141X and a missense mutation in *GGCX* (p.S300F). The authors suggested that the skin phenotype is because of digenic inheritance.

Generalized arterial calcification of infancy (GACI, MIM #208000) is another rare autosomal recessive trait characterized by soft tissue calcification (Rutsch et al., 2003). Mutations in *ENPP1*, encoding for ectonucleotide pyrophosphatase 1, are the primary cause of GACI. Patients present with extensive hydroxyapatite deposition in large and medium-sized arteries, leading to arterial stenosis and visceral ischemia. A recent study reported on a family with two brothers (Le Boulanger et al., 2010). One was suffering from PXE, while the other died at 15 months of age from a condition similar to GACI. Molecular analysis of *ABCC6* and *ENPP1* in this family revealed two pathogenic *ABCC6* mutations in heterozygous state as the primary cause of disease. Pathogenic ENPP1 mutations were not identified except for two common ENPP1 sequence variations. It remains speculative whether these lead to a more severe disease progression. Following on from these results, Nitschke et al. (2012) screened *ABCC6* and reanalyzed *ENPP1* for pathogenic mutations in a larger cohort of patients with clinically proven GACI ($n = 92$). The authors found 30 patients who did not carry any *ENPP1* mutations. Pathogenic *ABCC6* mutations in homo- or compound-heterozygosity were found as the primary cause for GACI in 15 patients (Nitschke et al., 2012). The fact that mutations in *ENPP1* and *ABCC6* manifest in overlapping clinical phenotypes of GACI and PXE suggests similar metabolic pathways are involved in the pathogenesis.

The significance of genetic interactions is further supported by experimental studies with $Abcc6^{-/-}$;$Ggcx^{+/+}$ mice in which the onset of ectopic calcification was delayed, whereas $Abcc6^{-/-}$;$Ggcx^{-/+}$ mice presented with accelerated mineralization (Li and Uitto, 2010).

GENETIC MODIFIERS OF PXE

The variability in the outcome and progression of PXE may include variations in several of the functional pathways involved in the pathophysiology of the disease in addition to the loss of ABCC6 function. Detection of such "modifier genes" may uncover major pathways involved in the pathogenesis and provide targets for therapeutic intervention. Studies with $Abcc6^{-/-}$ mice support the hypothesis of the contribution of other genetic factors in PXE-related calcification since these mice present with different phenotypes (Gorgels et al., 2005; Klement et al., 2005).

DEFINITION OF MODIFIER GENES

The "genetic co-factor" concept is not new, and was introduced by Haldane as early as 1941 (Haldane, 1941). A primary reason for searching for genetic factors modifying disease is to provide information on the disease course and to improve therapies (if available). Several definitions of so-called "modifier genes" exist in the literature. We define a modifier gene as a gene that if "mutated," is insufficient on its own to cause disease, but when coupled with another genetic mutation, produces or enhances its pathogenesis. "Modifier genes" have been detected for cystic fibrosis, another monogenic disorder caused by an ABCC transporter defect (*ABCC7* gene) with variable disease onset and progression (Cutting, 2005; Drumm et al., 2005).

SELECTION OF CANDIDATE GENES

In PXE several metabolic pathways seem to be affected, e.g., the regulation of biological calcification, extracellular matrix (ECM) remodeling and lipid transport and biosynthesis. Sequence variations in genes regulating these pathways may be involved in the development and clinical course of PXE.

Regulation of biological calcification

Ectopic calcification is the result of a complex interplay between stimulating and inhibitory proteins and metabolites. One candidate gene for PXE susceptibility is secreted phosphoprotein 1 (*SPP1*, formerly known as osteopontin). SPP1 fulfils important functions in the regulation of biological calcification and was also found to be a constitutive component of elastic fibers in order to prevent them from calcification (Baccarani-Contri et al., 1994). A study by Aherrahrou et al. described a dramatic up-regulation of SPP1 expression in mice suffering from dystrophic cardiac calcification (DCC, Aherrahrou et al., 2004). The *Abcc6* gene was identified as the potential candidate gene for DCC in mice (Korff et al., 2006). *SPP1* is a predominantly transcriptional regulated gene and the *SPP1* promoter is highly conserved among the human, murine, and porcine genes (Hijiya et al., 1994). Several polymorphisms in *SPP1* were shown to affect SPP1 expression (Giacopelli et al., 2004; D'Alfonso et al., 2005; Hummelshoj et al., 2006). We found that the c.−1748G, the c.155_156insG and the c.244_245insTG alleles appear to be significantly more common in PXE patients (Hendig et al., 2007). The polymorphism

c.−155_156insG generates a RUNX2 (runt-related transcription factor 2) binding site closed to a second RUNX2 binding site in the *SPP1* promoter. RUNX2 is an essential transcriptional regulator of osteoblast differentiation and bone formation. Conclusively, we interpreted three *SPP1* promoter polymorphisms and the haplotype combining these disease-associated alleles as a putative genetic risk pattern for PXE.

Fetuin-A, was found to be a major systemic inhibitor of calcification (Jahnen-Dechent et al., 2001). Carriers of fetuin-A genotype 2 have been shown to have the lowest serum fetuin-A concentration (Stenvinkel et al., 2005). Even though we did not observe an association between PXE manifestation and fetuin-A genotype, it could be speculated that fetuin-A genotype 2 is an additional disease-promoting risk factor (Hendig et al., 2006).

Matrix gla protein (MGP) is a calcification inhibitor acting locally. MGP was also found in the circulatory system, where it is part of the so-called calciprotein particles, together with fetuin-A and hydroxyapatite. The importance of MGP in preventing pathological calcification is supported by Mgp-deficient mice, which develop severe arterial calcification (Munroe et al., 1999). Analysis of the *MGP* promoter polymorphism frequencies revealed one *MGP* haplotype to be a potential protective genetic co-factor in PXE (Hendig et al., 2008).

ECM-remodeling and oxidative stress

Several studies reported an increased ECM remodeling in skin of PXE patients. In this context increased expression of proteases, a mild oxidative stress, and an altered proteoglycan metabolism was detected. The latter resulted in increased accumulation of proteoglycans in the ECM, and in changes in the structure and composition of urinary glycosaminoglycans (Longas et al., 1986; Maccari et al., 2003; Kornet et al., 2004; Volpi and Maccari, 2005).

Many of the alterations observed in PXE could be explained by oxidative stress, for instance ECM remodeling. We found a correlation between genotype and age of disease onset for polymorphisms in the genes catalase (*CAT*, c.262C>T), superoxide dismutase 2 (*SOD2*, c.47C>T), and glutathione peroxidase *1* (*GPX1*, c.593C>T), encoding essential antioxidant enzymes (Zarbock et al., 2007). Furthermore, the age of disease onset was inversely correlated with the number of mutated alleles, indicating a cumulative effect on the time of PXE onset.

Elevated production of MMP2 (matrix metallopeptidase 2) in PXE fibroblasts and increased levels of MMP2 and MMP9 in serum from PXE patients were shown (Quaglino et al., 2005; Diekmann et al., 2009). Increased MMP expression may at least partly result from genetic variation. We have previously shown that variations in *MMP2* are a genetic co-factor for PXE (Zarbock et al., 2010).

We propose that xylosyltransferase I (*XYLT*, XT-I), as the initial and most important enzyme in proteoglycan biosynthesis, and XT-II, as a highly homologous protein, might contribute to the increased ECM synthesis rate in PXE. As most XT-I is secreted into the ECM, XT activity was proposed as a diagnostic marker for the determination of enhanced proteoglycan biosynthesis and tissue destruction (Götting et al., 2007). Moreover, elevated XT activity was found in sera from PXE patients, reflecting the higher proteoglycan biosynthesis rate (Götting et al., 2005). We further showed that three sequence variants in the *XYLT2* gene result in a severe disease course of PXE (Schön et al., 2006).

Angiogenesis

Choroidal neovascularization (CNV) in PXE-associated retinopathy is believed to be mediated by the action of vascular endothelial growth factor (VEGF). Intravitreal anti-VEGF therapy with ranibizumab or bevacizumab is beneficial for the treatment of CNV secondary to angioid streaks associated with PXE (Gliem et al., 2013). It seems justified to assume that polymorphisms leading to altered VEGF expression may modify the severity of PXE retinopathy. Five *VEGFA* sequence variants showed significant association with severe retinopathy in PXE (Zarbock et al., 2009). We identified the most significant association for the variant c.-460C>T with an Odds Ratio of 3.8 (95% confidence interval 2.0–7.3, $P_{\text{corrected}}$ 0.0003). *VEGFA* gene polymorphisms might prove useful as a prognostic marker for development of PXE-associated retinopathy leading to earlier therapeutic intervention in order to prevent loss of central vision.

Lipid biosynthesis and metabolism

About 50% in the general population present with dyslipidemia which is a high risk factor for coronary artery disease (CAD). Familial hypercholesterolemia is caused by genetic variations in different genes, including low density lipoprotein receptor (*LDLR*) and the apolipoprotein B (*APOB*). Heterozygous carriers are found with a frequency of 1 in 500. Dyslipidemia is likewise observed in PXE patients and case reports suggested an impact of genetic risk factors of lipid metabolism (*LDLR* mutations), for severe complications such as stroke in PXE (Pisciotta et al., 2010).

GENETIC MODIFIERS IN Abcc6 DEFICIENT MOUSE STRAINS

Genetic examination of nine different mice strains predisposed to more or less severe soft tissue calcification reveal a common *Abcc6* sequence variant in exon 14 as the primary trigger in four of them (c.1866G>A, p.R621C, rs32756904). All these mice strains are prone to soft tissue calcification, but present with immense phenotypic heterogeneity. However, no modifier genes have been identified so far, even though not the correct candidate genes (Ssp1 instead of Spp1) were evaluated in a recent study (Berndt et al., 2012). Whole genome screening and careful evaluation of putative candidate genes will shed light on the role of genetic factors contributing to soft tissue calcification caused by Abcc6 deficiency. It will be of high interest to gain a deeper insight into the genetic background of these mice strains.

ABCC6 VARIANTS AS GENETIC MODIFIER OF OTHER RARE AND COMMON DISEASES

The occurrence of a mild skin phenotype was previously reported in heterozygous carriers of *ABCC6* mutations, although the findings could not be replicated in another study (Martin et al., 2007, 2008; Plomp et al., 2009). By searching for biomarkers in biological fluids of PXE patients, we and others observed that unaffected first-degree relatives mostly display results, e.g., for serum fetuin-A, desmosine, that were different from normal and intermediate between that of the PXE patients and the controls (Annovazzi et al., 2004; Hendig et al., 2006). Most of these individuals had

been identified as heterozygous carriers of only one *ABCC6* mutation. Taken together, these results underline the assumption that *ABCC6* mutations on a single allele might determine a mild PXE phenotype. This hypothesis might have great impact considering *ABCC6* as genetic modifier of other rare monogenic disorders (GACI), or common disease (stroke, myocardial infarction, and CAD). Wang et al. (2001) reported an association of the frequent *ABCC6* p.R1268Q variant (c.3803G>A, rs2238472) with plasma triglyceride and low HDL (high-density lipoprotein) cholesterol. Peloso et al. (2010) replicated these data by identification of an *ABCC6* sequence variant (rs150468; c.3736-334A>C) as a susceptibility allele for low HDL cholesterol. Both studies led to the suggestion that ABCC6 may be an important determinant of plasma lipoproteins.

Few studies investigated the correlation of *ABCC6* mutations with cardiovascular disease and identified the frequent mutation p.R1141X as a strong genetic risk factor for CAD (Trip et al., 2002; Wegman et al., 2005; Köblös et al., 2010). However, a large Danish Study including more than 13,600 cases, presenting with ischemic vascular disease, could not replicate this findings (Hornstrup et al., 2011). In conclusion, the impact of *ABCC6* variants as a genetic risk factor for common cardiovascular diseases remains to be elucidated.

LIMITATIONS AND PERSPECTIVE

Monogenic diseases such as PXE and GACI represent simple models that teach us many things about the genetic basis of more complex disorders, e.g., arterial calcification (Antonarakis and Beckmann, 2006). Considerable variety in the clinical expression of a monogenic disease might be explained by the effect of other genetic factors that modify the expression of the disease phenotype. Many arguments support the concept of modifier genes and genetic interactions. We suggest that genetic interactions, as one example between *ABCC6* and *ENPP1*, point toward new disease entities which seem to be phenotypically similar to PXE or GACI at first sight but differ in yet unknown features. Genetic modifiers are discussed as concomitant factors contributing to the course of the disease. The search for modifier genes is difficult but worth being performed in view of better knowledge of biological pathways affected by the disease. Moreover, numerous studies have identified such genes in mice. The availability of various mouse strains carrying the same *Abcc6* sequence variation associated with more or less severe soft tissue calcification provides an excellent starting point for functional studies and the investigation of interactions between *ABCC6* and specific modifier genes (Berndt et al., 2012). The recent identification of modifier genes for PXE underscores the importance of the analysis of gene–gene environment interactions in understanding the development of complex phenotypes such as PXE. As genetic modifiers are known to alter the course and expression of disease, their gene products become interesting targets for therapeutic intervention. Discovery of genetic modifiers for example affecting the success of anti-neovascular therapy could be, in the future, a key issue to obtain a personalization of therapy and to avoid unnecessary costs in PXE. Nevertheless, replication studies analyzing the new association of modifier genes in other PXE patient cohorts are essential to determine whether these modifier genes are indeed a genetic risk factor for PXE and related disorders. Moreover, the importance of consistent phenotype measures and complementary study designs cannot be overemphasized. Here, family studies and sibling analysis may help to estimate the contribution of genetic and non-genetic factors.

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Generalized arterial calcification of infancy and pseudoxanthoma elasticum: two sides of the same coin

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Generalized arterial calcification of infancy (GACI) is associated with biallelic mutations in *ENPP1* in the majority of cases, whereas mutations in *ABCC6* (ATP-binding cassette subfamily C number 6) are known to cause pseudoxanthoma elasticum (PXE). However, *ABCC6* mutations account for a significant subset of GACI cases, and *ENPP1* mutations can also be associated with PXE lesions. Based on the considerable overlap of GACI and PXE, both entities appear to reflect two ends of a clinical spectrum of ectopic calcification rather than two distinct disorders. *ABCC6* and *ENPP1* mutations might lead to alterations of the same physiological pathways.

Keywords: *ENPP1*, *ABCC6*, GACI, PXE, arterial calcification

GACI AND *ENPP1* MUTATIONS

Generalized arterial calcification of infancy (GACI; OMIM208000) is a rare autosomal recessive disease, which is characterized by severe calcification of the internal elastic lamina in large- and medium-sized arteries associated with intimal proliferation leading to arterial stenoses and heart failure within the first months of life. Although survival to adulthood has been reported, GACI is often lethal in the first 6 months of life. In the past, few patients survived the neonatal period (Moran, 1975; Morton, 1978), whereas more recently, patients treated with bisphosphonates have experienced a more favorable outcome (Rutsch et al., 2008; Ramjan et al., 2009). Some patients may also develop hypophosphatemic rickets with hyperphosphaturia, a finding associated with improved survival beyond infancy in patients with GACI (Rutsch et al., 2008; Levy-Litan et al., 2010; Lorenz-Depiereux et al., 2010). The disease has been found to be caused by inactivating mutations in *ENPP1* (MIM 173335; Rutsch et al., 2003). Mutations in *ENPP1* have been identified as the underlying defect in about 75% of the cases of GACI (Rutsch et al., 2008, 2011). *ENPP1* encodes the ectonucleotide pyrophosphatase/phosphodiesterase 1 (NPP1), a cell surface protein that catalyzes the hydrolysis of ATP to AMP and extracellular inorganic pyrophosphate (PP_i; Rutsch et al., 2001; Goding et al., 2003). PP_i potently inhibits hydroxyapatite crystal deposition and growth and regulates chondrogenesis, collagen I expression and synthesis, and other cell differentiation processes (Bollen et al., 2000; Goding et al., 2003).

PXE AND *ABCC6* MUTATIONS

Pseudoxanthoma elasticum (PXE; OMIM 264800) is a hereditary, autosomal recessive, multisystemic disease characterized by ectopic mineralization and fragmentation of elastic fibers of soft connective tissues such as the skin, the retina, and the arterial blood vessels. The clinical manifestations of classic PXE center on the skin, the eyes, and the cardiovascular system. The primary cutaneous lesions are small, yellowish papules on the neck and in large flexural areas, and these lesions progressively coalesce to form larger plaques, and skin folding occasionally develops. The eyes are frequently involved by calcification of Bruch's membrane leading to angioid streaks, and bleeding from the choroidal vessels can result in loss of visual acuity and, occasionally, in central blindness. The cardiovascular manifestations derive from mineralization of arterial blood vessels, and include gastrointestinal bleeding, intermittent claudication, hypertension, and sometimes early myocardial infarcts. Additionally, PXE can manifest with gastrointestinal hemorrhage and abnormal tissue mineralization in different organs, including the liver, kidneys, spleen, breast, and testes (Li et al., 2009; Plomp et al., 2010). Although dermatological signs are common, the main burden of PXE results from the complications in the visual and cardiovascular systems (Hu et al., 2003). Cutaneous and eye involvement usually occurs in adolescence, but may appear earlier in childhood. Cardiovascular complications usually develop later, in mid-adulthood (Naouri et al., 2009). The prevalence of PXE is estimated to 1/25,000 to 1/75,000 in the general population (Chassaing et al., 2005; Li et al., 2009). Mutations in the *ABCC6* (ATP-binding cassette subfamily C number 6) gene are demonstrated in about 66–97% of patients who are genotyped (Bergen et al., 2000; Le Saux et al., 2000, 2001; Miksch et al., 2005; Chassaing et al., 2007; Vanakker et al., 2008). The ABCC6-transported substrate or substrates, which modulate arterial calcification and other phenotypic changes of PXE, are not known, and hepatic abnormalities that have effects on calcification-regulating plasma proteins such as fetuin have been suggested to at least partially mediate the pathogenesis of PXE (Hendig et al., 2006).

Generalized arterial calcification of infancy and PXE have been considered to be two distinct entities in the past and have been

primarily linked to mutations in *ENPP1* and *ABCC6*, respectively. But recent findings indicate that GACI and PXE might be more closely related than previously thought.

THE FIRST CASE OF GACI AND PXE IN ONE FAMILY

Recently, we reported on a family with two brothers born to unrelated parents. The elder developed uncomplicated PXE in adolescence. Interestingly, the younger brother died after his second myocardial infarction at 15 months of age. Autopsy demonstrated calcifications of the endocardium, with extensive calcifications of the coronary arteries and of medium-sized arteries and the aorta, leading to the diagnosis of GACI. We performed molecular genetic analyses in the family. Unfortunately, no DNA of the deceased younger brother with GACI was available. The elder brother had two heterozygous missense mutations of *ABCC6*. Each mutation was inherited from one of his heterozygous asymptomatic parents. However, no *ENPP1* mutations were found in the three living family members (Le Boulanger et al., 2010).

This case was the first one suggesting a correlation between PXE and GACI. We hypothesized that GACI could be independent of *ENPP1*, but related to *ABCC6* mutations and that on the other hand PXE could be related to *ENPP1* mutations.

PATIENTS WITH GACI CARRY MUTATIONS IN *ABCC6*

Based on this case of GACI and PXE in one family with *ABCC6* mutations, we sequenced the *ABCC6* gene in 30 patients with a typical GACI phenotype but without disease-causing *ENPP1* mutations. In 14 of these patients, we detected pathogenic mutations in *ABCC6* (biallelic mutations in eight patients, monoallelic mutations in six patients). This study showed that biallelic mutations in the *ABCC6* gene account for a substantial number of typical GACI cases (Nitschke et al., 2012). The fact that even monoallelic mutations in *ABCC6* were associated with the severe phenotype of GACI cannot fully be explained on the basis of autosomal recessive inheritance. However, mutations of other disease-associated genes have not been ruled out so far.

PATIENTS WITH GACI AND PXE CARRY MUTATIONS IN *ENPP1*

Three of our GACI patients, who showed extensive calcifications of the large- and medium-sized arteries, arterial stenoses, and periarticular calcifications in infancy, carried biallelic *ENPP1* mutations. These patients developed clinical features of PXE in childhood between 5 and 8 years of age. The patients showed angioid streaks and typical pseudoxanthomatous skin lesions (Nitschke et al., 2012). Most recently, one additional 2-year-old patient with a relatively mild form of GACI developed PXE with pseudoxanthomatous lesions of the neck, inguinal folds, and lower abdomen. The patient was also found to harbor a homozygous missense mutation in *ENPP1* (Li et al., 2012).

GENOCOPY AND PHENOCOPY IN GACI AND PXE

GACI and PXE have been considered to be two distinct entities in the past and have been primarily linked to *ENPP1* and *ABCC6*, respectively. But based on the overlap of genotype and phenotype of GACI and PXE, both entities appear to reflect two ends of a clinical spectrum of ectopic calcification and other organ pathologies, rather than two distinct disorders (**Figure 1**). It was shown, that biallelic mutations in *ABCC6* account for a significant number of typical GACI cases, which involve widespread arterial calcifications, arterial stenoses, periarticular calcifications, and hypophosphatemic rickets. *ABCC6* mutations can be associated with a much more severe phenotype, including death in infancy from myocardial infarction, than was previously known. We conclude that the phenotypic spectrum of diseases associated with *ABCC6* mutations is much broader than was previously assumed. In fact, the infantile phenotype of patients carrying *ABCC6* mutations can be indistinguishable from the phenotype associated with *ENPP1* mutations. The fact that the same *ABCC6* mutations can cause the severe GACI phenotype associated with death in early infancy and the

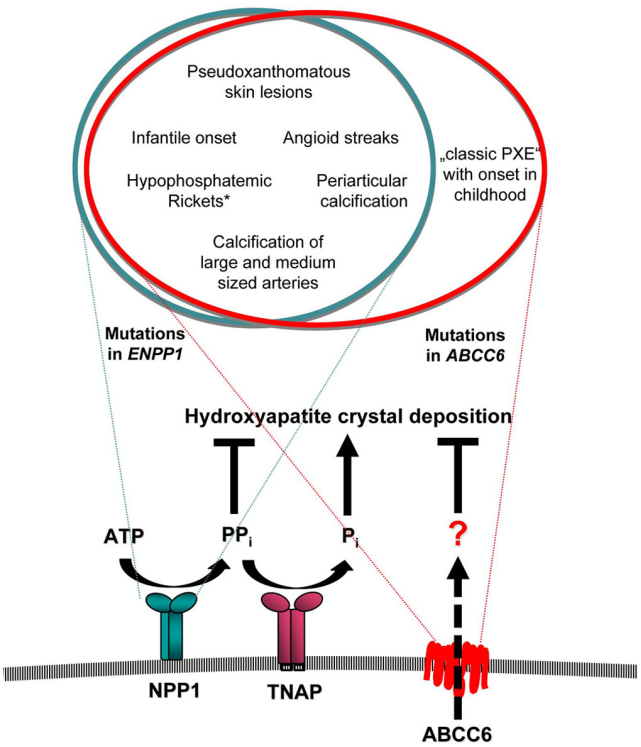

FIGURE 1 | Overlap of clinical manifestations associated with mutations in *ENPP1* and *ABCC6*. NPP1 and ABCC6 serve as a minor component in the function of a network of factors that exert balanced effects to promote and suppress arterial calcification. The transmembrane ectoenzyme NPP1 generates AMP and PP_i from ATP. PP_i is hydrolyzed by the tissue non-specific alkaline phosphatase (TNAP) to generate P_i, which is a component of hydroxyapatite crystal deposition and plays a role in the regulation of osteoblast differentiation. PP_i suppresses hydroxyapatite deposition and inhibits ectopic chondrogenesis (and modulates artery calcification by other effects). The role of ABCC6 has to be defined. Mutations of either *ABCC6* or *ENPP1* can cause the severe phenotype of GACI, which frequently leads to death within the first year of life. While mutations in *ENPP1* can also cause typical pseudoxanthomatous skin lesions and angioid streaks of the retina in children with GACI, who survived the critical period of infancy, the later onset of "classic PXE" phenotype without GACI was only observed in patients with mutations in *ABCC6*. *Hypophosphatemic rickets has been observed frequently in patients with *ENPP1* mutations, but was observed only in one proband carrying a mutation in *ABCC6* on one allele.

relatively mild phenotype of PXE warrants further explanation. Because of the difficulty with charting a clear pattern of inheritance to phenotype, it is likely that mutations in other disease-associated genes may play a role here.

Up to date, four patients who presented with GACI and carried biallelic *ENPP1* mutations developed the clinical manifestation of PXE in childhood. Symptoms included angioid streaks and histologically proven calcifications of elastic skin fibers. Thus, given the poor prognosis of severe GACI, affected patients might die of the cardiovascular complications of the disease before they develop typical signs of PXE. This might be the reason that no previous case of GACI has been described in the PXE literature. Also, many PXE characteristics, including angioid streaks of the retina and peau d'orange skin lesions might frequently be overlooked in the clinical examinations of GACI patients. Hence, the true number of patients carrying *ENPP1* mutations and showing PXE lesions might be higher. In summary, these findings show that mutations in the different genes *ENPP1* and *ABCC6* can lead to similar pathophysiological consequences and that GACI and PXE do not simply represent two distinct disorders. They rather represent a spectrum of different peculiarities of ectopic calcification. It can therefore be hypothesized that the pathophysiology of *ENPP1* and *ABCC6* related disorders is based on common downstream mechanisms.

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An update on the ocular phenotype in patients with pseudoxanthoma elasticum

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Pseudoxanthoma elasticum (PXE) is an inherited multi-system disorder characterized by ectopic mineralization and fragmentation of elastic fibers in the skin, the elastic laminae of blood vessels and Bruch's membrane in the eye. Biallelic mutations in the ATP-binding cassette (ABC) transporter gene *ABCC6* on chromosome 16 are responsible for the disease. The pathophysiology is incompletely understood. However, there is consent that a metabolic alteration leads to dysfunction in extracellular calcium homeostasis and subsequent calcification of connective tissues rich in elastic fibers. This review summarizes and aims at explaining the variety of phenotypic ocular findings in patients with PXE. Specialized imaging techniques including white light fundus photography, blue light autofluorescence, near-infrared confocal reflectance imaging, high resolution optical coherence tomography, fluorescein and indocyanine green (ICG) angiography have revealed characteristic lesions at the ocular fundus of PXE patients. These include the classic signs of angioid streaks, peau d'orange, comet lesions, and choroidal neovascularizations (CNVs), but also the more recently recognized features such as chorioretinal atrophy, subretinal fluid independent from CNV, pattern dystrophy-like changes, debris accumulation under the retinal pigment epithelium, reticular drusen and a decreased fluorescence on late phase ICG angiography.

Keywords: pseudoxanthoma elasticum, angioid streaks, retina, Bruch's membrane, choroidal neovascularization

Pseudoxanthoma elasticum (PXE; OMIM# 264800) is a rare autosomal recessive multi-system disease affecting mainly the cardiovascular system, the skin, and the eyes (Finger et al., 2009b; Li et al., 2009b; Plomp et al., 2010; Uitto et al., 2011). It is due to mutations in the *ABCC6* gene (Bergen et al., 2000; Plomp et al., 2008). The prevalence has been estimated to range between 1:25,000 and 1:100,000 (Chassaing et al., 2005).

The pathophysiology of PXE remains to be elucidated. However, it is clear that the various disease manifestations are a consequence of slow but progressive calcification of connective tissue rich in elastic fibers. *ABCC6* encodes a transmembrane transporter protein that is primarily expressed in the liver and kidneys, and to a lower extent, if at all, in the tissues that define the PXE phenotype (Matsuzaki et al., 2005). Therefore it has been hypothesized that one or several factor(s) that inhibit calcification of elastic fibers is/are secreted from liver cells into the blood using ABCC6 as a shuttle. This hypothesis has recently been supported by experimental evidence in a PXE animal model, the *Abcc6*$^{-/-}$ mouse (Jiang et al., 2008, 2010).

The ocular phenotype is defined by pathology of the ocular fundus, i.e., changes of the complex consisting of the choroid, Bruch's membrane (BM), retinal pigment epithelium (RPE), and the retina. The initial event appears to be the calcification of BM, which is rich in elastic fibers. BM is situated between the RPE, the cellular monolayer directly adjacent to the photoreceptors, and the choriocapillaris. The latter itself is the inner part of the choroid, the heavily perfused vascular layer at the back of the eye. RPE and choroid – both essential for normal functioning of the retina – may become compromised morphologically and functionally subsequent to Bruch membrane calcification. Moreover, neovascular membranes may sprout from the choroidal vessels through breaks in the calcified BM into the subretinal space, leading to subretinal hemorrhage and scar formation. Eventually, permanent vision loss will ensue.

A recent study assessed the impact of PXE on vision and health related quality of life (Finger et al., 2011b). Accordingly, visual impairment seems to have a larger impact than cardiovascular disease on reported quality of life in PXE patients. Therefore, a thorough understanding of the PXE-associated ocular disease is required for adequate patient counseling, screening, and treatment.

Herein, we describe the morphological and functional ocular changes observed in patients with PXE.

HISTORICAL NOTES AND TERMINOLOGY

Angioid streaks, one of the most striking ocular findings in patients with PXE, were first reported in a case presentation by Robert Doyne from the Oxford Eye Hospital in 1889 as *irregular jagged lines* (Doyne, 1889). The presented patient had a history of blunt trauma to both eyes, and the streaks were interpreted as "*rupture to the pigment layer of the retina*." In 1891, the German ophthalmologist Otto Plange from Münster independently reported a patient he observed during his residency in Bochum (Plange, 1891). In his extensive description of angioid streaks, he interpreted them as intraretinal pigmented deposits following hemorrhage. Due to their "*appearance of an obliterated system of blood-vessels*," Knapp subsequently coined the term *angioid streaks* (Knapp, 1892). In their descriptions, Plange and Knapp also mentioned that "*streaks seemed to fade within a mottled area*" (Plange, 1891), possibly representing what we now call peau d'orange. It took more than two further decades before Kofler, based on meticulous clinical observations, eventually suggested that angioid streaks are due to breaks in BM (Kofler, 1917). His observations were later supported by histological studies (Böck, 1938; Hagedoorn, 1939; Verhoeff, 1948; McWilliam, 1951; Jensen, 1977; Dreyer and Green, 1978) and were more recently confirmed by *in vivo* imaging (Charbel Issa et al., 2009). In 1941, Scholz provided an excellent thorough review of the early literature on angioid streaks, analyzing a total of 188 reported cases (Scholz, 1941).

The term *peau d'orange* was suggested by Smith and colleagues in 1964 to describe the mottled fundus appearance (Smith et al., 1964). Gass (2003) suggested the term "comet tail lesions" for the characteristic spot-like chorioretinal lesions with a tail pointing toward the posterior pole.

As pointed out by Hagedoorn (Hagedoorn, 1939), Hallopeau and Laffitte were the first to report a possible relation between PXE and retinal disease (Hallopeau and Laffitte, 1903). However, it took until 1929 for the Swedish ophthalmologist Ester Grönblad together with the dermatologist James Strandberg to recognize the syndromic association between the characteristic ocular and skin phenotypes (Grönblad, 1929; Strandberg, 1929). Although the term PXE was originally introduced in 1896 to specifically describe the dermal pathology, it is generally accepted to use the term synonymously with Grönblad–Strandberg syndrome.

FUNDUSCOPIC FINDINGS

Pseudoxanthoma elasticum retinopathy (**Figure 1**) is characterized by a mottled aspect of the temporal retinal midperiphery, called peau d'orange, and angioid streaks, representing ruptures in BM. The latter may lead to subretinal choroidal neovascularization (CNV) with a risk for spontaneous hemorrhage and scar formation with subsequent loss of vision. In addition, comets and comet tail lesions in the midperiphery with a variable degree of RPE atrophy represent a unique sign of the condition (Gass, 2003). The clinical heterogeneity, inherent to PXE, is also reflected in the retinal phenotype: although invariably present, the retinopathy remains rather limited in some patients, making an early diagnosis more difficult, whereas in others the phenotype is severe.

Retinal involvement generally increases with age. Due to the high phenotypic variability (Plomp et al., 2009) even within families and the large number of mutations and polymorphisms in the *ABCC6* gene, no clear genotype–phenotype correlations have been established. As well, no clear correlations between severity of the ocular phenotype and that of other PXE-related manifestations, such as cardiovascular disease, have so far been identified. A minor retinal phenotype, limited to comet lesions, was observed in 8 of 25 carriers of ABCC6 mutations in one study (De Zaeytijd et al., 2010).

Calcification in BM predisposes to breaks within this membrane most likely through the physiological tensions exerted on the eye by the extraocular muscles, with a point of convergence at the optic nerve head. Moreover, even minor trauma to the eye that otherwise would not have a major effect on ocular morphology and function, may cause retinal hemorrhage independent from presence of a CNV (**Figure 1L**) and/or development and growth of angioid streaks (Britten, 1966; Hagedoorn, 1975). Therefore, activities with potential trauma to the eye should be avoided, or adequate protection should be worn. Calcification of BM may also impede the exchange of nutrients, growth factors, and waste products between RPE and choroid, eventually leading to functional and structural compromise of the RPE, the choriocapillaris and the retina.

PEAU D'ORANGE

Peau d'orange appears to be the earliest funduscopically visible alteration in patients with PXE, preceding the development of angioid streaks (Gills and Paton, 1965; Krill et al., 1973; Naouri et al., 2009). Peau d'orange is characterized by small dark spots, within an area of a slightly whitish or opaque fundus reflex (**Figures 1A–C,M, 2A,** and **6K**). This pattern may be observed at the posterior pole very early in the disease and more peripheral in later disease stages. In the latter case, the slightly whitish or opaque fundus reflex may have become more uniform posteriorly to the area of peau d'orange. Peripheral to the peau d'orange region, the fundus reflex is usually darker (**Figure 1M**). The difference between the central and peripheral fundus reflex is more obvious in dark pigmented patients in whom the higher overall fundus pigmentation contrasts better with the more posteriorly located whitish areas. It may remain unnoticed in less pigmented individuals as well as in very late disease stages (Charbel Issa et al., 2010a).

Bruch's membrane is believed to be the primarily affected anatomic structure of the ocular fundus in patients with PXE. Anatomical differences in thickness and integrity between central and peripheral BM (Chong et al., 2005) may account for a higher vulnerability and thus earlier calcification at the posterior pole, with a subsequent centrifugal disease spread.

Based on clinical observations, it has been hypothesized that peau d'orange represents a visible transition zone of BM calcification (Charbel Issa et al., 2010a). Replacement of peau d'orange by a more uniform area of whitish fundus reflex posteriorly may occur once calcification has become continuous. A centrifugal spread of calcification, with the leading edge representing the transition zone of calcifying BM, appears to be the rational for the observation that peau d'orange is seen increasingly more peripheral with age. Currently, there is little

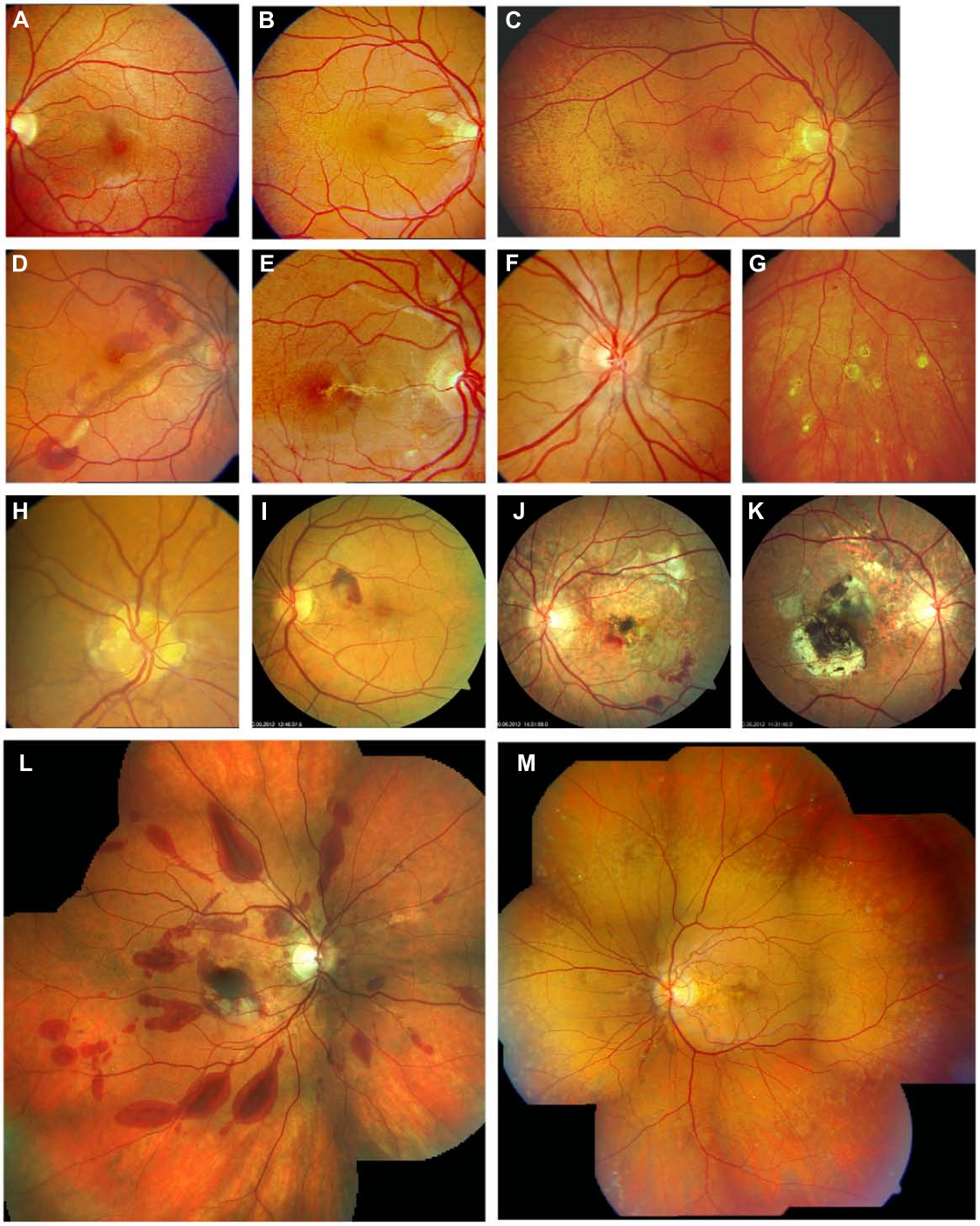

FIGURE 1 | Clinical features of pseudoxanthoma elasticum on funduscopic examination. Peau d'orange is characterized by small dark spots on a whitish or opaque background **(A–C)** and likely represents the transition between calcified and non-calcified Bruch's membrane. Peau d'orange appears to begin at the posterior pole **(A)**, spreading peripherally over time **(B,C,M)**. Peau d'orange is most pronounced temporaly but may be visible circular within the retinal periphery **(M)**. Angioid streaks are reddish or brownish irregular lines that often form a peripapillary ring from where they radiate into the periphery **(D–F,H,M)**. Small roundish chorioretinal atrophies are frequently found in the retinal periphery eccentric to peau d'orange, but may also occur closer to the optic nerve head. They often present with a tail pointing toward the optic nerve head, leading to the descriptive term of comet tail lesions **(G)**. Not a pathognomonic but frequent finding are optic disc drusen **(H)**. Angioid streaks may be complicated by the development of choroidal neovascularizations **(D,I)** leading to subsequent atrophy and scarring **(J,K)**. Progressive chorioretinal atrophy may also enlarge without presence of active neovascularizations **(J)**. The resistance of Bruch's membrane to ocular trauma is reduced leading to extensive bleeding after minor traumata **(L)**. PXE-associated fundus features are summarized in **(M)** with peau d'orange encompassing the circular periphery, comet tail lesions within the periphery, Angioid streaks not exceeding peau d'orange and central pattern dystrophy-like changes.

published longitudinal data to confirm such cross sectional clinical observations.

Peau d'orange often is assumed to be most pronounced and widespread in the temporal midperiphery. However, there is good evidence that this characteristic fundus finding simply is the most prominent manifestation of a transition zone that in actual fact is present circumferentially, though not symmetrically (Charbel Issa et al., 2010a; **Figures 1M** and **5**).

ANGIOID STREAKS

Angioid streaks, which are breaks in BM, are the most obvious and most consistently observed funduscopic finding in patients with in PXE (**Figures 1D–F,H,M**, and also visible in most other figures).

Angioid streaks present ophthalmoscopically as irregular and jagged lines that radiate from a concentric peripapillary ring toward the equator of the eye. Visibility and colour of angioid streaks may depend upon the degree of secondary alterations, such as loss of the choriocapillaris, depigmentation or loss of RPE cells, or fibrosis. In some patients, they may be limited to a few almost imperceptible lines whereas in others they present as a complicated interlacing network or grid. The streaks are most prominent at the posterior pole of the eye and typically taper and fade toward the equator of the eye, often dividing into smaller branches. Occasionally they continue for a short distance as irregular, white, depigmented lines. Pigmented wing-like hyperpigmentations may be found along angioid streaks.

Alterations in the RPE adjacent to streaks commonly occur. Loss of pigment imparts a "feathered" appearance to the streak (De Zaeytijd et al., 2010). Angioid streaks enlarge in length and width over time (Mansour et al., 1993), and usually do not cross areas of peau d'orange (Charbel Issa et al., 2010a). The latter finding supports the notion that calcification of BM predisposing to angioid streak formation is present centrally from peau d'orange. In addition, stress lines on the posterior pole of the eye converge on the optic nerve head, which is both the anchor point for the optic nerve, and the hinge point at which, despite the flexibility of the optic nerve itself, a certain degree of mechanical tilting of the eyeball occurs, relative to the optic nerve, in a direction dependent on the gaze. The combination of these stress lines in a calcified BM probably leads to angioid streak formation.

Angioid streaks are usually obvious on funduscopy. Confocal near-infrared (NIR) reflectance imaging was found to be superior to other imaging modalities (Charbel Issa et al., 2009; De Zaeytijd et al., 2010) to document angioid streaks (Charbel Issa et al., 2009). In areas of widespread chorioretinal atrophy, angioid streaks are usually not visible anymore. However, breaks in BM may still be detected in such cases using spectral domain optical coherence tomography (SD-OCT; Charbel Issa et al., 2009).

Although other ocular diseases such as high myopia and ocular trauma may also lead to breaks in BM, the pattern of break formation is different in those diseases (Pruett et al., 1987). While myopic lacquer cracks are short and usually found in a reticular distribution within a posterior staphyloma, traumatic tears are characteristically curved parallel to the optic disk margin and are usually located temporal to the disk in only one eye.

Angioid streaks are not necessarily associated with a noticeable decrease in retinal function, and visual acuity may remain normal even in presence of a streak crossing the fovea. Angioid streaks may occur with or without overt damage to the RPE (Charbel Issa et al., 2009). Probably, the overlying RPE first needs to be compromised before loss of function occurs. However, high resolution structure–function correlations, e.g., with microperimetry, has only recently become available (Charbel Issa et al., 2010b) and has not yet been performed in patients with PXE. Definite vision loss occurs when angioid streaks are complicated by the development of a CNV or atrophy of the RPE.

PERIPHERAL COMET AND COMET TAIL LESIONS

Comet lesions with or without comet tail (**Figures 1G, 3D,** and **4J**) are observed as solitary, subretinal, nodular, white bodies with a tapering white tail extending posteriorly of the comet body pointing toward the optic disk. The body may have some pigmentation at its margin. Sometimes a spray of comets and comet tails can be observed, creating an aspect of "comet rain." Comets and comet tails are found in the (mid)periphery of the fundus and are the only PXE-related finding that may occur peripheral to peau d'orange (Gass, 2003; Charbel Issa et al., 2010a). They have been suggested to be the only pathognomonic characteristic of PXE (Gass, 2003) and may also occur in heterozygous carriers of *ABCC6* mutations (De Zaeytijd et al., 2010). Especially in young patients, in whom angioid streaks are often not yet present, they might thus be of significant diagnostic value.

CHOROIDAL NEOVASCULARIZATION

Choroidal neovascularization of the macular region is a frequent complication in patients with PXE and commonly leads to pronounced vision loss (**Figures 1D,I** and **3F**). Often, CNV in PXE are classic membranes (Nakagawa et al., 2013), i.e., they are located between the RPE and the photoreceptor layer. Classic CNV usually occurs in association with angioid streaks and may develop from occult CNV which is situated underneath the RPE (Nakagawa et al., 2013). The predisposition for the posterior pole is consequent upon the higher frequency of angioid streaks in that area. CNV leads to subretinal hemorrhage and exudation, and eventually formation of a fibrovascular scar (**Figures 1J,K**). Occasionally, eccentric CNV may remain unnoticed due to its lower impact on visual function. Before the development of a scar, CNV is the only ocular PXE complication that is currently eligible for treatment. Intravitreal inhibitors of vascular endothelial growth factor (VEGF) are currently most effective in the attempt to prevent or limit fibrovascular scar formation with consequent visual loss (Gliem et al., 2013). Older treatment options such as photodynamic therapy with verteporfin or argon laser photocoagulation have been largely abandoned. Overall, classic CNV appears to have a worse prognosis with regards to visual function compared to occult CNV (Nakagawa et al., 2013).

Recently, polypoidal choroidal vasculopathy (PCV) was also described to occur in patients with angioid streaks (Baillif-Gostoli et al., 2010; Nakagawa et al., 2013). PCV may occur as an initial vascular change or secondary to CNVs. In contrast to classic CNV, those polyps appear not to be associated with angioid streaks.

PATTERN DYSTROPHY-LIKE CHANGES

Pattern dystrophy-like changes (**Figures 4F** and **7**) are frequently observed in patients with PXE varying between 10% (Finger et al., 2009a) and almost 70% of cases (Agarwal et al., 2005). Based on a classification suggested by Agarwal and Gass, findings can be categorized due to their similarity to pattern dystrophies, into vitelliform, butterfly, and reticular dystrophy, or fundus flavimaculatus or pulverulentus (von Winning and Oosterhuis, 1974; Shiraki et al., 2001; Agarwal et al., 2005; Sawa et al., 2006; Finger et al., 2009a). It has been postulated that the presence of a pattern dystrophy is a prognostic sign for CNV development although further longitudinal data are needed for confirmation (Finger et al., 2009a).

CHORIORETINAL ATROPHY

Chorioretinal atrophy may develop secondary to CNVs, usually surrounding a fibrovascular scar. Atrophy can also occur in the absence of CNV (**Figures 7A–D**) – a process which, however, has not been well characterized yet. It may initially be observed as patches of atrophy mostly between or along the major vascular arcades, frequently originating within areas of pattern dystrophy. During the subseqent course of the disease, such lesions may grow and – if initially multifocal – become confluent.

OPTIC NERVE HEAD DRUSEN

Optic nerve head (ONH) drusen (**Figure 1H**) seem to be more common in PXE patients than in the general population. The reported prevalence ranges from 6–8% (Meislik et al., 1979; Finger et al., 2009a) to just over 20% (Pierro et al., 1994) compared to ∼0.3% in the general population. To date, it remains unclear why PXE patients are at an increased risk to develop ONH drusen though it may be assumed that a common process of abnormal mineralization might be involved, e.g., through direct calcification or increased rigidity of the lamina cribrosa. Similar to ONH drusen not associated with PXE, ocular ultrasound or fundus autofluorescence (AF) may be required to detect them and may be useful for documentation and follow up (Finger et al., 2009a; De Zaeytijd et al., 2010).

ADDED VALUE OF RETINAL IMAGING

OPTICAL COHERENCE TOMOGRAPHY

Spectral domain OCT, which allows for quasi histologic assessment of the posterior ocular fundus *in vivo*, has been used to study PXE-related fundus features (**Figure 2**). On OCT, the calcification of BM may appear as increased reflectivity, and the transition zone from calcified to un-calcified areas correlates well with peau d'orange (Charbel Issa et al., 2009; **Figures 2A–E**). Angioid streaks have consistently been found to be associated with breaks in BM (**Figures 2F–H**, arrows). There may be differences with regards to the width of the gap, presence or absence of fibrovascular tissue extending through the breaks (**Figures 2I,J**), and alterations or preservations of the overlying RPE layer (Charbel Issa et al., 2009). SD-OCT imaging provided the first direct evidence that breaks in BM are indeed the underlying pathology of angioid streaks (Charbel Issa et al., 2009). Disruption and undulation (inward and outward deformation) of BM on OCT images are much more frequent in eyes of (older) PXE patients than in eyes of AMD patients (Ellabban et al., 2012a), facilitating somewhat the differentiation between those two causes for CNV and chorioretinal atrophy.

Comet tail lesions, the peripheral chorioretinal atrophic spots, may be difficult to record using OCT due to their preferably peripheral localization. The few available scans show hyporeflective spaces involving the outer neurosensory retina with a slightly hyperreflective inner lining and focal debris-like deposits just above the RPE level (Charbel Issa et al., 2009).

Pattern dystrophy-like fundus changes are associated with material deposited below the neurosensory retina, and this may be located either within the RPE layer or just below or above the RPE (Charbel Issa et al., 2009; Zweifel et al., 2011).

Spectral domain OCT has also revealed presence of subretinal fluid in the absence of CNV (**Figures 2N,Q**) in a subset of patients (Zweifel et al., 2011), which may remain undetected by funduscopy. Such fluid accumulation may appear similar to that observed in chronic serous chorioretinopathy. It is probably due to either abnormalities of the RPE pump function, increased hydrophobicity of BM, or a combination thereof. It does not respond to intravitreally applied VEGF inhibitors (Zweifel et al., 2011 and unpublished own observations) or systemic acetazolamide (unpublished own observation). Longstanding cases might develop a vitelliform lesion characterized by deposition of yellowish hyperautofluorescent material at the bottom of the lesion (**Figures 2O–Q**).

In daily practice, SD-OCT is a very efficient means to identify neovascular leakage (**Figure 2K**) and monitor treatment efficacy of intravitreal VEGF inhibition. This is of particular importance because it may be difficult in patients with PXE to distinguish between low-grade leakage, which would mean CNV activity, and staining on fluorescein angiography. However, at late stages, atrophic lesions may occur with or without intraretinal cysts (**Figures 2L,M**), which sometimes makes it difficult to distinguish between atrophic retinal cystic alterations and true leakage on OCT, too.

So far there is only little known about pathologic alterations of the choroid in PXE. Histopathologic works reported atrophic changes and disruptions of the choroid in areas of angioid streaks as well as calcification of choroidal vessels (McWilliam, 1951; Dreyer and Green, 1978). Recently, one study measured choroidal thickness in eyes affected by PXE using enhanced depth imaging-OCT and found a reduced choroidal thickness in the subset of eyes that presented with CNV (Ellabban et al., 2012b).

FLUORESCEIN ANGIOGRAPHY

Fluorescein angiography highlights several features of PXE (**Figure 3**) and, despite the importance of novel techniques such as SD-OCT, still remains the gold standard for detecting and documenting leakage from a CNV in patients with angioid streaks.

Several authors have presented and discussed the fluorescein angiographic findings of angioid streaks (Smith et al., 1964; Patnaik and Malik, 1971; Federman et al., 1975). In the absence of other signs of CNV, however, fluorescein angiography does not usually add clinically relevant information and therefore may be refrained from in asymptomatic patients.

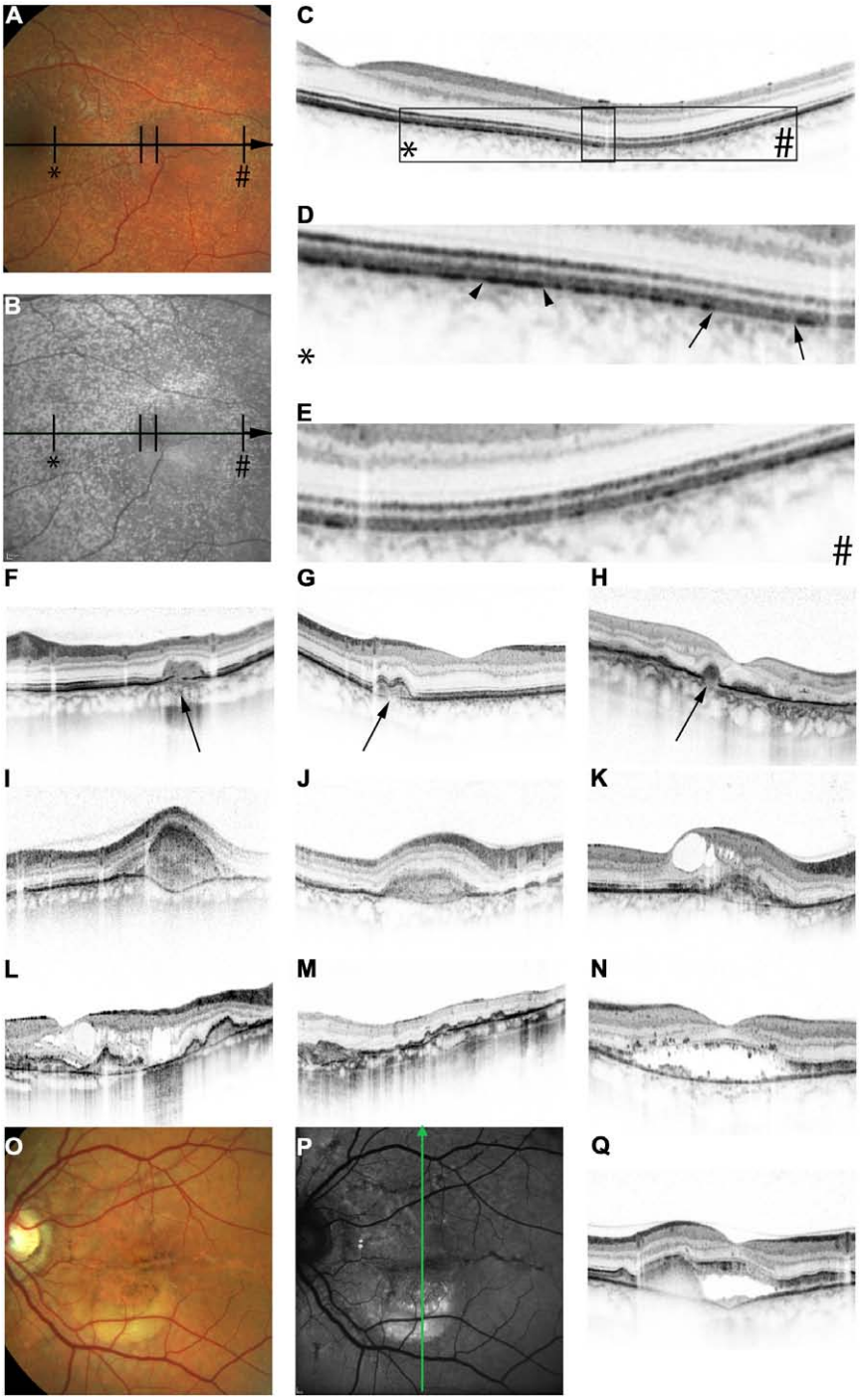

FIGURE 2 | Features of pseudoxanthoma elasticum on optical coherence tomography. Calcification of Bruch's membrane may be seen on OCT images. This is best illustrated within areas of peau d'orange, the transition zone between calcified and un-calcified Bruch membrane **(A–E)**. The horizontal arrow in **(A)** and **(B)** indicates the placement of the OCT scan in **(C)**. 2.5x magnifications of characteristic details in **(C)** are shown in **(D,E)**. Area * corresponds to **(D)** and area # to **(E)**. Areas of increased reflectivity within the outer zone of RPE-Bruch's membrane complex (arrow heads in **D**) correlate to the whitish opaque fundus reflex on color images **(A)** and the increased signal on near-infrared reflectance images **(B)**. Areas of lower reflectivity (**E**, arrows in **D**) correlate to the normal fundus reflex. Angioid streaks correlate to breaks within the thickened and hyperreflective Bruch's membrane (**F–H**, arrows). Fibrovascular tissue may grow through such breaks **(I,J)**. A typical complication of angioid streaks is the development of choroidal neovascularizations leading to retinal exsudation **(K)**. Eventually, atrophy of the retinal pigment epithelium is associated with atrophic changes in the photoreceptor layer with **(L)** or without **(M)** cystoid retinal lesions. In some patients there may be persistent subretinal fluid independent of choroidal neovascularizations **(N,Q)**. If longstanding, a vitelliform lesion may present with deposition of yellowish hyperautofluorescent material at the bottom of the lesion **(O–Q)**. The green arrow in **(P)** indicates the placement of the OCT scan in **(Q)**.

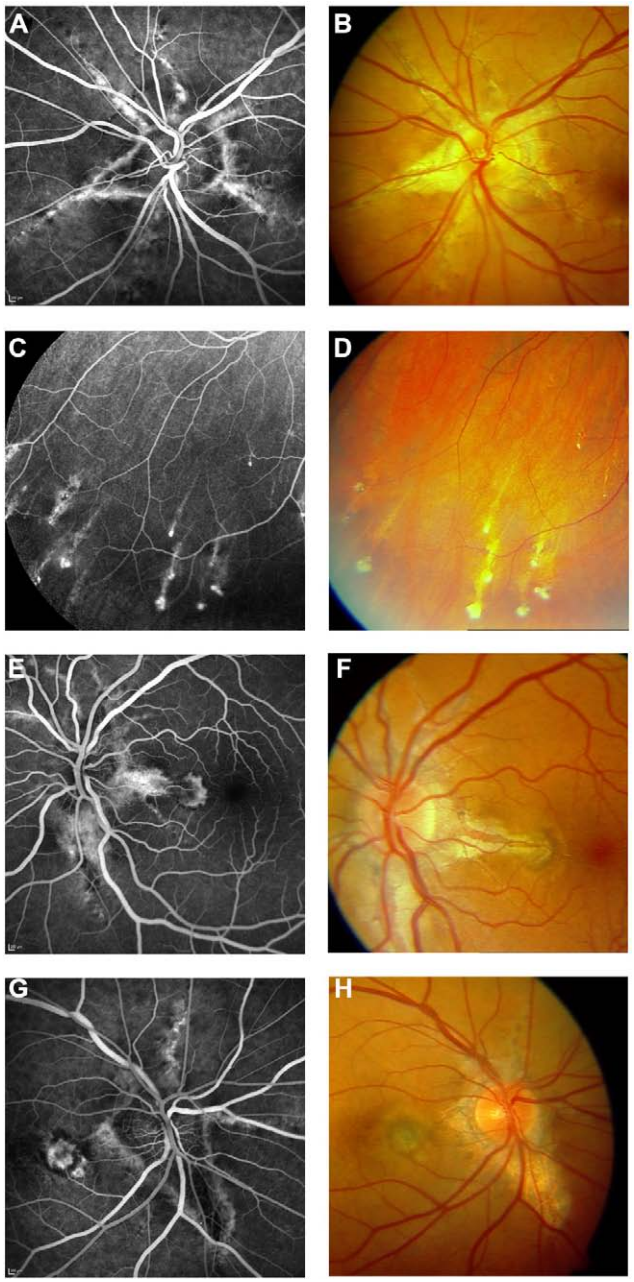

FIGURE 3 | Features of pseudoxanthoma elasticum on fluorescein angiography. Angioid streaks typically show a variable staining on fluorescein angiography **(A,B)**. Comet tail lesions appear as hyperfluorescent spots with their tail toward the optic disk **(C,D)**. Choroidal neovascularizations are mostly classic membranes. Sometimes, their detection may be difficult due to adjacent staining of angioid streaks **(E–H)**.

INDOCYANINE GREEN ANGIOGRAPHY

Indocyanine green angiography uses NIR light for excitation of the chromophore, which is superior to fluorescein angiography in detecting abnormalities under the RPE. ICG differs from fluorescein amongst others in terms of its much more extensive blood-protein binding, and its limited vascular leakage. ICG angiography is capable to outline angioid streaks much better than fluorescein angiography in the majority of cases (Lafaut et al., 1998). Angioid streaks are usually not visible in the early phase, but can be delineated with high sensitivity in the angiographic late phase (**Figure 4**). In the latter, angioid streaks usually are hyperfluorescent within the area of reduced late phase ICG-fluorescence (see below). Outside this area, they may also appear hypofluorescent or invisible in some cases.

A highly characteristic PXE-related fundus feature on ICG angiography is a reduced late phase fluorescence centered at the posterior pole (Charbel Issa et al., 2010a), while more eccentric areas exhibit a normal late phase fluorescence. In between these two areas, there is a spotted transition zone, which is most prominent on the temporal side of the fundus in most cases (**Figures 4** and **5**). There are no related obvious alterations on fundus photography, early phase ICG angiography, or fundus AF. Notably, the spotted pattern of peau d'orange is located more eccentric. It was hypothesized that the reduced late phase fluorescence may be due to BM calcification or a dysfunctional RPE (Charbel Issa et al., 2010a).

Indocyanine green is less well suited to detect leakage from CNV compared to fluorescein angiography. Due to its invasive nature it is therefore not recommended to use routinely for monitoring patients but may be used to confirm suspected PCV or occult CNV and to further investigate ocular pathophysiology in PXE.

FUNDUS AUTOFLUORESCENCE

Fundus AF imaging with most commonly used blue or green excitation light allows evaluation of the integrity and health of the RPE *in vivo*. Many fundus alterations commonly found in PXE may present with characteristic fundus AF abnormalities. Angioid streaks can show areas of increased as well as areas of decreased fundus AF. The latter usually suggests more severe damage of the RPE with cell loss. Wing-like focal spots of increased AF alongside angioid streaks, consisting of pigmentations visible on fundus photography, constitute the parastreak phenomenon (Finger et al., 2009a; De Zaeytijd et al., 2010). Patterns of AF in the macular area in PXE patients are often similar to those observed in patients with pattern dystrophies (**Figure 7**). Comets – and to a lesser extent their tails – typically show a hyperautofluorescent signal. Not all comets can be detected individually on AF imaging (Finger et al., 2009a; De Zaeytijd et al., 2010). Whether this hyperautofluorescence is caused by lipofuscin, or the presence of calcification, or a combination thereof is as yet unclear.

Peau d'orange is usually not highlighted on fundus AF images even when visibility was marked on color photography, irrespective of the severity of changes (**Figures 6A-I**; Finger et al., 2009a; De Zaeytijd et al., 2010). This seems logical when accepting that the appearance of peau d'orange originates from alterations in Bruch membrane, which is located underneath the RPE (with melanin as strong absorber of short wavelength visible light) and therefore is less effectively imaged using blue light.

Fundus AF illustrates RPE atrophy as areas of hypoautofluorescence, which are often more extensive than the areas of atrophy seen on funduscopy (**Figures 7A–D**). Thus, fundus AF

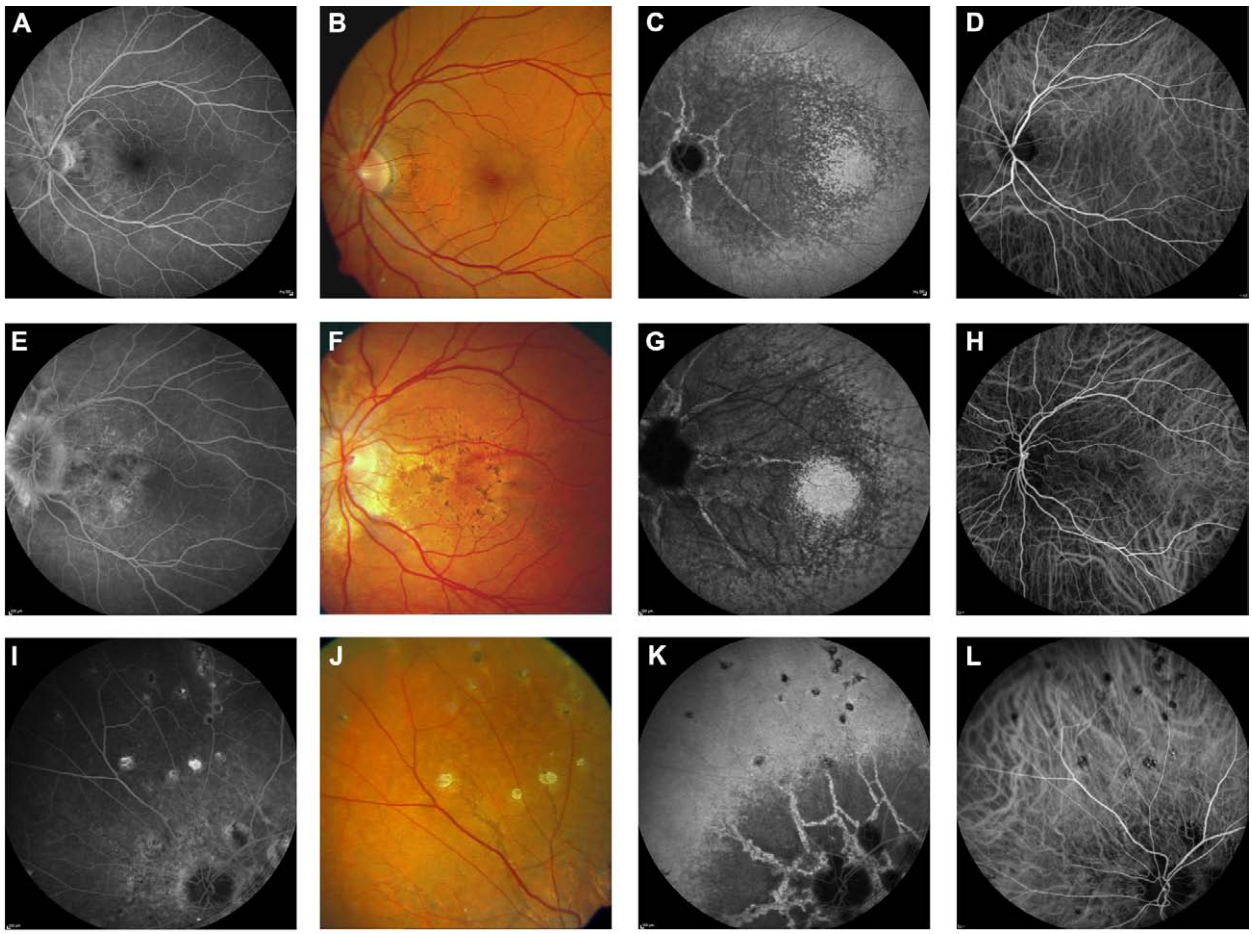

FIGURE 4 | Comparison of clinical features on late phase fluorescein angiography, early and late phase ICG angiography, and funduscopy. Late phase fluorescein angiography shows variable staining of angioid streaks **(A,E,I)** which corresponds well with findings on funduscopy **(B,F,J)**. A characteristic finding on late phase ICG angiography is a centrally reduced fluorescence with a spotted transition zone to normal peripheral fluorescence **(C,G,K)**. Angioid streaks are well visible within the dark non-fluorescent area. Note that there is no correlate on color images **(B,F,J)** or early ICG angiography frames **(D,H,L)**. Comet tail lesions **(J)** usually are hyperfluorescent on late phase fluorescein angiograms **(I)** and hypofluorescent on ICG late phase angiogram **(K)**.

is useful as a non-invasive tool to monitor progression of RPE changes, including chorioretinal atrophy.

CONFOCAL REFLECTANCE IMAGING

Confocal NIR reflectance imaging is highly sensitive in detecting peau d'orange and angioid streaks (Charbel Issa et al., 2009; De Zaeytijd et al., 2010). The low absorption rate of 790 nm light by melanin within the RPE, in combination with the high contrast of a confocal imaging system, leads to the superior illustration of such structural alterations underneath the RPE cell layer.

With NIR reflectance imaging, angioid streaks appear as uniform, well-demarcated dark fissures against a lighter background, even when they remain unnoticed on color imaging (**Figure 6**).

Near-infrared reflectance imaging always revealed a diffuse, speckled pattern of peau d'orange, extending beyond the area considered affected on white light digital fundus images. In the absence of extensive macular atrophy or scarring, the peau d'orange area may cover the entire posterior pole and the midperiphery up to the equator (**Figures 5B,C**; Charbel Issa et al., 2009; De Zaeytijd et al., 2010).

Near-infrared reflectance imaging is superior to visualize comets, which went undetected on color images. Comets appear as small white hyperintensities, suggesting high reflectivity for light of NIR wavelengths.

Recently, confocal NIR reflectance imaging has been shown to be highly sensitive for detecting reticular drusen in patients with age-related macular degeneration. Calcification of Bruch membrane due to PXE also seems to predispose to the development of reticular drusen (**Figures 6H,I**). However, those own preliminary observations have not yet been investigated systematically.

FUNCTIONAL ALTERATIONS

VISUAL ACUITY AND VISUAL FIELD

There are very little data available on the natural history of visual acuity loss in PXE, and the same is true for visual field testing.

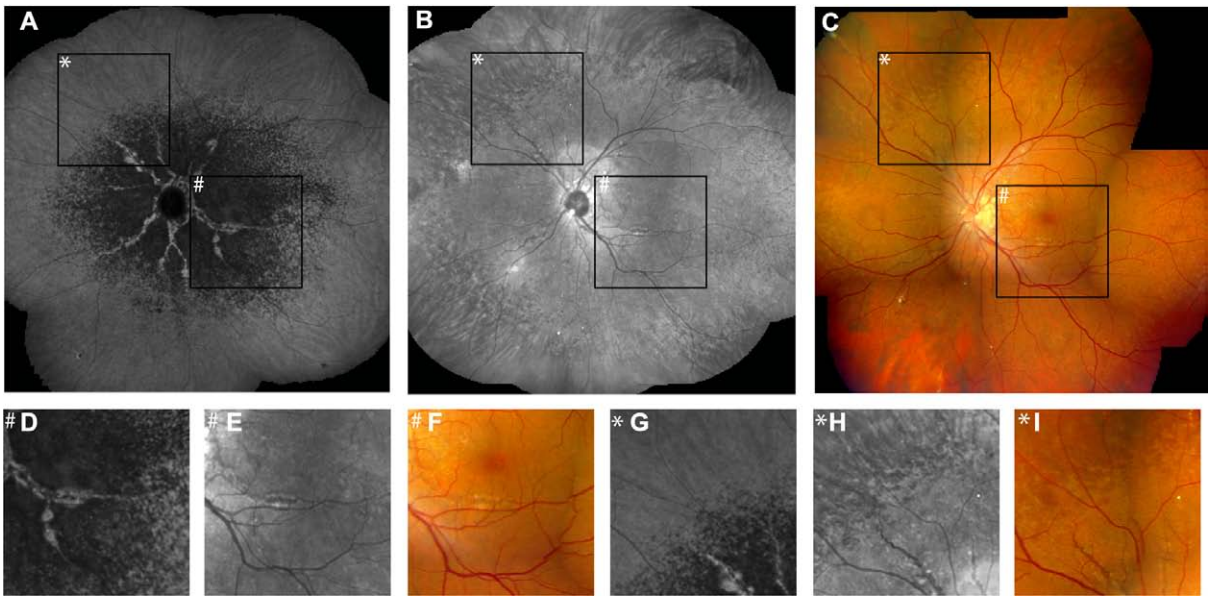

FIGURE 5 | Comparison of findings on late phase ICG angiography, NIR reflectance imaging and funduscopy. The transition zone from reduced to normal fluorescence on ICG angiography **(A)** is located more centrally relative to peau d'orange **(B,C)**. Note that there is no correlate of this phenomenon on IR reflection imaging or funduscopy **(D–F)**. Non-invasive near-infrared (NIR) reflectance imaging **(B)** often shows peau d'orange and angioid streaks with greater detail and contrast compared to fundus photography **(C)**. Angioid streaks do not cross peau d'orange **(G–I)**. **(D–I)** Represent a ×3.3 magnification of characteristic details of corresponding areas within **(A–C)**. **(D–F)** Correspond to area #, **(G–I)** correspond to area *.

Visual acuity frequently drops to 20/200 or less around the fourth to fifth decade of life and it is not uncommon that only hand movements may be seen (Gliem et al., 2013). Dark adaptation and scotopic perimetry over peau d'orange has not shown significant functional alterations (Holz et al., 1994).

ELECTROPHYSIOLOGY

Although electrophysiological techniques provide important additional information regarding the underlying causes of visual failure, it has not yet been extensively used to investigate the PXE retinopathy.

Early electrophysiological studies in 15 PXE patients (Francois and De Rouck, 1981) found mild reduced responses on electroretinography (ERG) and electro-oculography (EOG) testing in about 50% of eyes, mostly in those with advanced pathology. Audo et al. (2007) have extended the spectrum of ERG findings in PXE patients suggesting that general retinal dysfunction may occur, which may explain difficulties with night vision, which are frequently reported by PXE patients.

HISTOLOGY

In the eye, the first histologically detectable alteration appears to be an abnormal calcification and thickening of the elastic and subsequently of the collagenous layers of BM. Fragmentation and clustering of calcified elastic and collagenous fibres shows similarities to histopathological findings in skin specimen. These changes within BM secondarily may lead to alterations of the adjacent choriocapillaris as well as the overlying RPE and the neurosensory retina.

Particularly at the posterior pole, BM is thicker than normal and reveals basophilic staining with hematoxylin which is related to calcium deposition. Several authors noted a patchy transition zone towards the rather normal appearing peripheral BM anterior to the equator (Böck, 1938; Hagedoorn, 1939; Klien, 1947; Verhoeff, 1948; Jensen, 1977). This transition zone may be the histopathological correlate for peau d'orange, although no direct evidence has been provided for this interpretation. In late disease stages, the severely altered BM may undergo atrophy (Hagedoorn, 1939).

The histopathological correlate for angioid streaks– breaks in the calcified BM – has been documented in donor eyes of patients with PXE (Böck, 1938; Hagedoorn, 1939; Verhoeff, 1948; Gass, 1967; Jensen, 1977; Dreyer and Green, 1978; Mansour, 1998), Paget's disease (Gass and Clarkson, 1973), sickle cell disease (Jampol et al., 1987), and of patients without defined underlying disease (Klien, 1947; McWilliam, 1951; Domke and Tost, 1964; Gass, 1967; Dreyer and Green, 1978). Small breaks may remain without morphological changes in the overlying layers of the RPE and retina or of the underlying choriocapillaris. With increasing width of the angioid streaks, RPE cells may become irregular and loose melanin granules. Larger defects in Bruch membrane are often associated with ingrowth of fibrous tissue, RPE cell atrophy and thinning of the choriocapillaris.

Breaks in BM of PXE patients are a predilection site for the ingrowth of fibrovascular tissue from the choroid. Fibrovascular proliferation may grow underneath the RPE, leaving the detached RPE layer relatively or partially (Böck, 1938; Gass, 1967) intact. Histopathological observations of RPE detachments in patients with angioid streaks have also been described with exudation-like

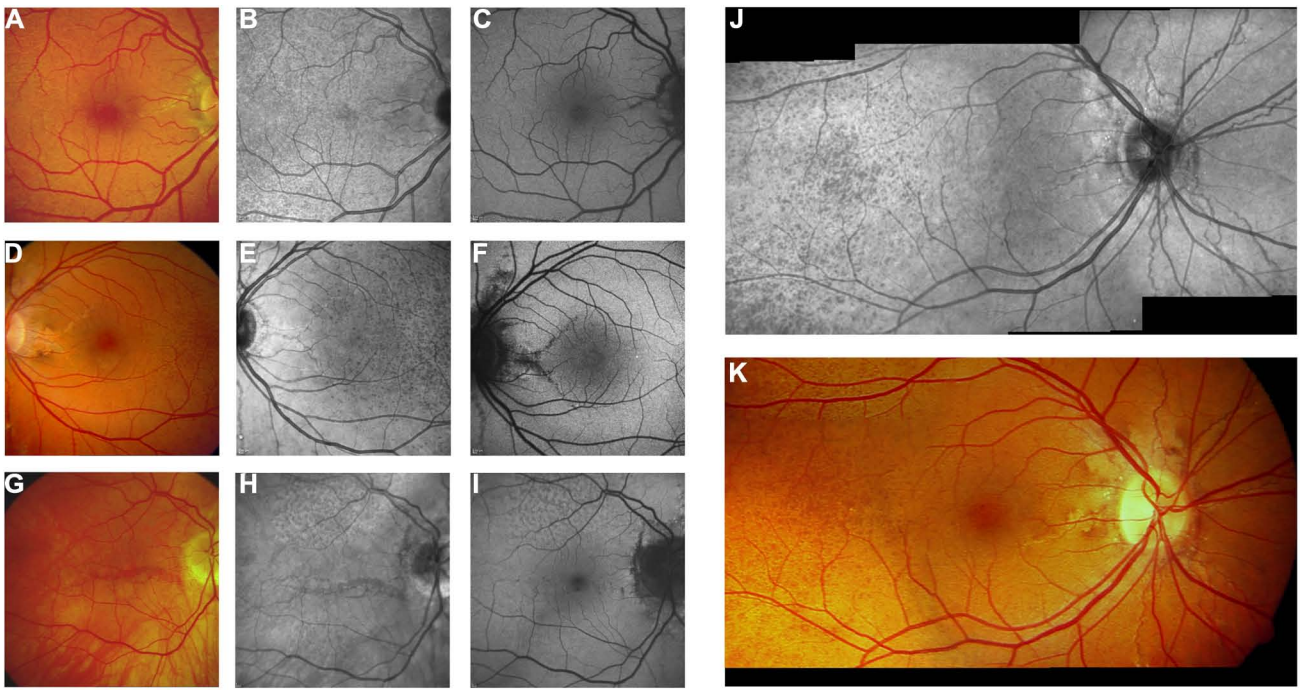

FIGURE 6 | Near-infrared reflectance imaging and 488 nm fundus autofluorescence in pseudoxanthoma elasticum. Angioid streaks and peau d'orange are best and most reliably visible on NIR reflectance imaging **(B,E,J)** correlating well with findings on funduscopy **(A,D,K)**. Peau d'orange is usually not discernible on 488 nm fundus autofluorescence images **(C,F)**. Angioid streaks may present with a reduced autofluorescence **(C,F)** but may as well remain undetected on autofluorescence imaging **(H,I)**. Note the reticular drusen on NIR reflectance and 488 nm autofluorescence which are sometimes associated with pseudoxanthoma elasticum **(H,I)**.

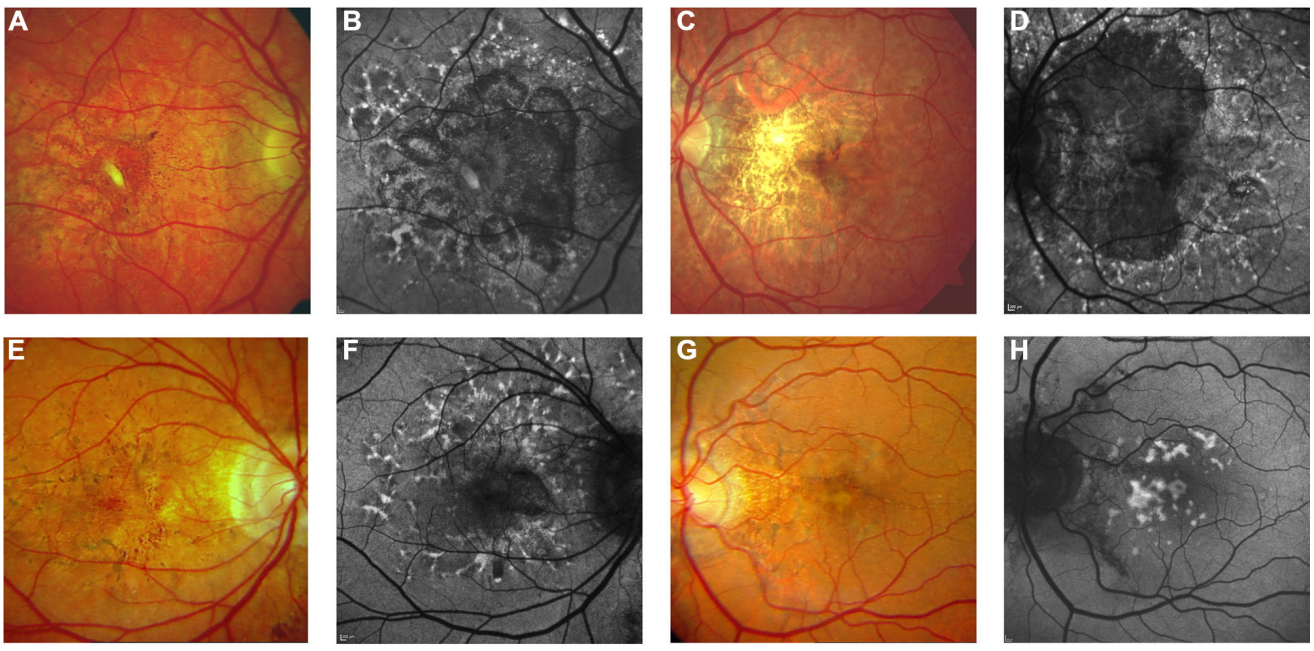

FIGURE 7 | Pattern dystrophy-like changes and atrophy in pseudoxanthoma elasticum on 488 nm fundus autofluorescence imaging. Atrophic lesions and pattern dystrophy-like changes are typical features of advanced pseudoxanthoma elasticum. Compared to funduscopic images **(A,C,E,G)** these lesions are best visible on 488 nm fundus autofluorescence images **(B,D,F,H)**. Pattern dystrophy-like lesions encompass different patterns of increased autofluorescence. Depending on the stage of pattern dystrophy changes, atrophy of the retinal pigment epithelium with reduced autofluorescence may be present **(B,D,F)**.

fluid or amorphous material within the sub-RPE space (Böck, 1938; Hagedoorn, 1939; Klien, 1947; Jampol et al., 1987). Frequently, there is very active CNV-proliferation through breaks in BM with subretinal exudation, haemorrhage and subsequent fibrosis, leading to degeneration of the RPE and retina.

Progressive and diffuse loss of the choriocapillaris paralleling degenerative changes in BM has been noted (Gass, 1967). However, apart from the focal changes along angioid streaks, little attention has been paid to such histopathological changes of the choroid and choriocapillaris. Also, little is known about the histopathological correlate of several clinically described fundus features in patients with PXE. This includes the pattern dystrophy-like changes, subretinal fluid in the absence of CNV, peripheral comet lesion, or reticular drusen. Thus, considering PXE as a well-defined model disease, better knowledge of the PXE-associated ocular pathology may shed light on the pathophysiology of other diseases, such as inherited pattern dystrophy of the retina or age-related macular degeneration.

DIFFERENTIAL DIAGNOSIS

The term *PXE-like syndrome* has been used to describe vascular, dermal, and ocular alterations characteristic of PXE that occur secondary to other diseases or due to genetic mutations different from those in *ABCC6*. These include hemoglobinopathies, such as beta-thalassemia or sickle cell disease (Aessopos et al., 2002), Paget's disease of the bone (Gass and Clarkson, 1973), congenital dyserythropoietic anemia, *ENPP1* mutations causing generalized arterial calcification of infancy (GACI) syndrome (Kalal et al., 2012), and mutations in *GGCX* (Li et al., 2009a). The ocular phenotype described in such patients includes peau d'orange and angioid streaks.

Angioid streaks, the most obvious feature of PXE-related fundus abnormalities, are almost always observed in patients with PXE. The second strongest association appears to be with hemoglobinopathies, including beta-thalassemia and sickle cell disease. Paget's disease of the bone has been reported to be associated with angioid streaks in 1.4–14% (Scholz, 1941) and peau d'orange in 0–22% of cases (Clarkson and Altman, 1982). It has been reported that angioid streaks may also occur in the absence of any of the PXE-like systemic or ocular alterations (Clarkson and Altman, 1982), including two members of a family with Ehlers–Danlos syndrome (Green et al., 1966). However, up to date phenotyping in such patients would be needed to support such associations.

SUMMARY AND FUTURE DIRECTIONS

Pseudoxanthoma elasticum may be regarded as a model disease in which calcification of BM leads to a number of secondary effects at the ocular fundus. Of those, the development of CNV is the most vision threatening complication. However, novel therapies using intravitreally applied VEGF inhibitors appear successful in preserving vision over several years (Myung et al., 2010; Finger et al., 2011a; Gliem et al., 2013). Thus, other ocular disease manifestations such as atrophy and/or incompetence of the RPE may be additional future challenges for ophthalmologists caring for PXE patients. In this respect, a better understanding of the pathophysiology leading to several of the classic funduscopic findings is required. For instance, little is currently known on precursors of RPE atrophy and its rate of progression, or the origin of reduced ERG responses in some patients. Finally, it remains to be studied if the ocular phenotype may provide biomarkers that would allow assessing effects of future therapies aiming at systemically reducing soft tissue calcification.

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The vascular phenotype in pseudoxanthoma elasticum and related disorders: contribution of a genetic disease to the understanding of vascular calcification

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Vascular calcification is a complex and dynamic process occurring in various physiological conditions such as aging and exercise or in acquired metabolic disorders like diabetes or chronic renal insufficiency. Arterial calcifications are also observed in several genetic diseases revealing the important role of unbalanced or defective anti- or pro-calcifying factors. Pseudoxanthoma elasticum (PXE) is an inherited disease (OMIM 264800) characterized by elastic fiber fragmentation and calcification in various soft conjunctive tissues including the skin, eyes, and arterial media. The PXE disease results from mutations in the ABCC6 gene, encoding an ATP-binding cassette transporter primarily expressed in the liver, kidneys suggesting that it is a prototypic metabolic soft-tissue calcifying disease of genetic origin. The clinical expression of the PXE arterial disease is characterized by an increased risk for coronary (myocardial infarction), cerebral (aneurysm and stroke), and lower limb peripheral artery disease. However, the structural and functional changes in the arterial wall induced by PXE are still unexplained. The use of a recombinant mouse model inactivated for the Abcc6 gene is an important tool for the understanding of the PXE pathophysiology although the vascular impact in this model remains limited to date. Overlapping of the PXE phenotype with other inherited calcifying diseases could bring important informations to our comprehension of the PXE disease.

Keywords: pseudoxanthoma elasticum, calcium, vessels, cardiovascular diseases, elasticity, ankle-brachial index

ARTERIAL CALCIFICATION IS AN INDEPENDENT RISK FACTOR OF CARDIOVASCULAR DISEASES

Arterial calcification is gaining an increasing interest as an independent marker for cardiovascular (CV) diseases in acquired metabolic diseases, such as type II diabetes (Becker et al., 2008) and chronic renal insufficiency (Goodman et al., 2000). Vascular calcification increases physiologically with age and studies from Egyptian mummies have revealed that arterial calcification is not a feature of modern life style due to the absence of risk factors such as smoking, high fat cholesterol diets encountered in these ancient civilizations (Allam et al., 2011).

Calcification of the intimal layers can complicate atherosclerotic plaques favoring the risk of rupture whereas deposit within the medial layer contributes to stiffen arterial wall leading to hypertension, cardiac hypertrophy, and heart failure (Demer and Tintut, 2008). Similarly to the bone, ectopic calcium sediment within the arterial wall is a dynamic and tightly regulated biological process involving a large number of cytokines and cellular pathways (Giachelli, 2004; Persy and D'Haese, 2009). Contrary to the bones, the artery is a tubular organ that should remain soft and flexible but resilient to the high distending blood pressure. The elastic properties of the arterial wall plays a key role in damping the cyclic pressure changes produced by the beating heart (O'Rourke et al., 2002). This dampening effect predominates within the large elastic vessels such as aorta and progressively decreases downstream as the vessel wall becomes more muscular in the medium and small-sized arteries allowing a continuous flow and to protect the thin walled capillaries against high pressures. Therefore, any changes in arterial wall elasticity, i.e., the recoiling force, and distensibility, (the capacity to be distended), either physiologically with aging (so called arteriosclerosis) or in response to abnormal metabolic conditions such as type II diabetes or chronic renal insufficiency, contribute to stiffen the arterial wall. Stiffening of the arterial wall will reduce the dampening effect leading to the increase in systemic arterial pressure, mainly pulse pressure, and ultimately damage the small capillaries in end-organs such as brain, kidneys, or heart (Laurent et al., 2009).

Our understanding of the direct or indirect contribution of calcification in the vascular system remains limited due to the multifactorial mechanisms. Although both elastic lamina and medial calcification could share similar genetic determinants, whether or not calcification precedes or follows the disruption and/or the degeneration of the elastic fibers is often difficult to establish (Wang et al., 2009). The resulting elasto-calcinosis refers to a timely

and well-balanced interaction between several local and remote factors (see review Atkinson, 2008). The mechanisms underlying calcification of the elastic fibers are multifactorial including physico-chemical conditions, inflammation and oxydative stress, metabolic dysfunction, and unbalanced promoters/inhibitors of calcification. This process can occur focally in the intimal layer and may complicate atheromatous plaques whereas it occurs more diffusely in the media.

The role of genetics in the calcification process is likely to take an important place since than >40% of the variance of aortic and coronary calcification phenotype could be under the control of genes. The roles of genes have been deciphered in various monogenic diseases but also in the general population (O'Donnell et al., 2002; Assimes et al., 2008; Rampersaud et al., 2008) and have been recently reviewed by Rutsch et al. (2011).

The pathophysiology of calcification in metabolic diseases is of a particular concern and the present review will focus mainly on pseudoxanthoma elasticum (PXE), an inherited disease displaying specific and unusual characteristics that belongs to a larger group of genetically and metabolically determined calcifying vascular diseases.

PSEUDOXANTHOMA ELASTICUM: AN ENIGMATIC CALCIFYING GENETIC DISEASE

Pseudoxanthoma elasticum is an inherited autosomal recessive multisystem disorder affecting connective tissues. Its phenotypic expression is characterized by the fragmentation and mineralization of elastic fibers in the skin termed elastorrhexis, the Bruch's membrane of the retina and the vasculature (Neldner, 1988; Hu et al., 2003; Uitto et al., 2011). Its prevalence is estimated from 1/25,000 to 1/50,000 and the causative mutations have been identified in the *ABCC6* gene encoding a trans-membrane ATP-binding cassette transporter, subfamily C, member 6 (ABCC6/MRP6; Le Saux et al., 2000) that is primarily expressed in the liver and the kidney, but with much lower expression in other affected tissues such as skin, eyes, or arteries (Kool et al., 1999). The biological function of the ABCC6 transporter and its substrates remains totally unknown to date. The phenotype seems to result from an unknown defect originating from the liver and the kidney leading to the extracellular calcium sediment but probably intracellular (Martin et al., 2012). Several studies have demonstrated that normal tissues exposed to the serum from PXE patients or mice knockout for Abcc6 are able to calcify (Le Saux et al., 2006; Jiang et al., 2007, 2010). Therefore, PXE is considered as a prototypical metabolic calcifying disease of genetic origin (Jiang and Uitto, 2006). PXE is also characterized by its delayed onset and a variability in its phenotypic expression suggesting that a large number of co-factors contribute to its phenotype. The classical risk factors involved in arteriosclerosis, such as tobacco, hypertension, dyslipidemia, could be greatly suspected to interfere with the severity of the vascular expression of the disease, although most of the vascular complications in PXE occur later during the life (>40 years) than the skin and eyes lesions, with an unexplained female preponderance (Uitto et al., 2010). The 2/3 female–1/3 male ratio in PXE leads to an unusual and unexplained prevalence of arterial disease in female compared to the general population. A PXE-like phenotype has also been reported in other genetic diseases such as beta-thalassemia and sickle cell anemia (Fabbri et al., 2009), cutis laxa (Vanakker et al., 2007), generalized arterial calcification in infancy (Kalal et al., 2012), a defect in gamma-glutamyl carboxylase (Vanakker et al., 2007), familial idiopathic basal ganglia (Wang et al., 2012) and more rarely induced by pharmacological substances such as seen with D penicillamine (Ratnavel and Norris, 1994). The fact that PXE phenotype could overlap with other genetic diseases suggests that these diseases share a common pathophysiology (Nitschke et al., 2012). The main phenotypical differences reported in the literature between the genetic calcifying diseases are summarized in **Table 1**.

A number of candidate substrates for ABCC6 have been hypothesized. The observation that PXE patients exhibit a low plasma vitamin K level and that anti-vitamin K drugs accelerate calcification in normal and in Abcc6$^{-/-}$ mice (Li et al., 2012) raised the hypothesis for a role of vitamin K in the calcification process. Vitamin K is a key factor for the activation of tissue calcification inhibitor factors such as matrix Gla proteins (MGPs). This hypothesis has not been confirmed due to no changes in the calcification process occurring in PXE animal models supplemented with vitamin K (Brampton et al., 2011; Fülöp et al., 2011; Gorgels et al., 2011). The implication of adenosine as a calcifying factor in PXE has also been hypothesized (Markello et al., 2011), as well as oxidative stress (Pasquali-Ronchetti et al., 2006; Zarbock et al., 2007), although the role of oxidative stress has not been evidenced by endothelial dysfunction at present in PXE.

THE VASCULAR LESIONS AND HISTOLOGICAL FINDINGS IN PXE

The elementary arterial lesions observed in PXE are characterized by mineralization of the elastic fibers of the medial layer, predominantly within the medium and small-sized musculo-elastic arteries. Abnormal elastic fibers are thought to be produced by the PXE skin fibroblasts (Quaglino et al., 2000), but could also occur with normal fibroblasts in the presence of PXE serum (Le Saux et al., 2006) or in presence of elastin degradation products (Simionescu et al., 2005). These findings suggest that PXE is a disorder of the mechanisms controlling the production of matrix constituents and that elastic fiber mineralization is caused by factors abnormally produced and entrapped within the fiber during elastin fibrogenesis (Baccarani-Contri et al., 1994). These finding called for the "elastosis hypothesis" as the primary mechanism in PXE and that calcification is secondary. A primary role for smooth muscle cells and fibroblasts in PXE is suspected as they are a source of many regulatory proteins involved in the calcification process such as alkaline phosphatase and MGP (Shanahan et al., 1999; Simionescu et al., 2005). Finally, an abnormal balance between elevated proteolysis activity with increased P-selectin (Gotting et al., 2008), matrix metallo-proteinase (MMP) 2 and 9 (Diekmann et al., 2009), suggests an abnormal remodeling of the extra cellular matrix (ECM) in PXE. Despite unknown mechanism sequence for elastosis and calcification, the nature and affected sites of calcification lead to different functional expression.

The macroscopic distribution of the calcification along the arterial tree can be mapped using standard X-ray, although 3D

Table 1 | Comparative characteristics of the arterial phenotype in PXE and other related disorders.

	Beta-thalassemia/ Sickle cell disease	PXE-like + cutis laxa	PXE	GACI	ACDC (or CALJA)
Elementary arterial phenotype					
Intima-media thickness	Increased	Unknown	Increased		
Endothelial dysfunction	Defective	Unknown	Unknown	Unknown	Unknown
Stenosis	Yes (26.7% mostly cerebral)	Yes (PAD 50%)	Yes (mostly coronary and cerebral)	Yes (mostly coronary, cerebral, and renal)	
Dilatation/aneurysm	Sporadic	Yes (mostly cerebral)	Infrequent		Popliteal
Calcification	Present	Present	Present	Present	Present
Arterial malformations			gastrointestinal, eyes (neovascularization) and carotids (?)		
Coagulation defects	Present (50%)	Present	Secondary?	Present	Present
Defective gene	HBB/HBF	GGCX	ABCC6	ENPP1	NT5E

For each disorder, bibliographical references for vascular phenotype are indicated. GACI, generalized arterial calcification of infancy (Rutsch et al., 2001; Kalal et al., 2012); ACDC, arterial calcification or CALJA, calcification of joints and arteries due to deficiency of CD73 (Markello et al., 2011); HBB/HBF, hemoglobinopathies Beta and S (Aessopos et al., 1998; Fabbri et al., 2009; Musallam et al., 2011); GGCX, gamma-glutamyl carboxylase (Vanakker et al., 2007, 2011; Li et al., 2009a); ABCC6, adenosine triphosphate-binding cassette (ABC) gene subfamily C (Boutouyrie et al., 2001; Germain et al., 2003; Kornet et al., 2004; Leftheriotis et al., 2011; Kupetsky-Rincon et al., 2012); ENPP1, ectonucleotide pyrophosphatase/phosphodiesterase (Mackenzie et al., 2012; Nitschke et al., 2012); NT5E, Ecto-5′-nucleotidase (Markello et al., 2011; St. Hilaire et al., 2011).

reconstruction using helicoidal X-rays scans provides a more precise quantification and site identification (see **Figure 1**). In our experience, calcification accumulates mostly within the distal superficial femoral and below-knee arteries (tibialis and pedalis), a distribution mostly observed in the arteriosclerotic process associated with aging. Skin lesions are extensively documented in PXE, as skin biopsy are routinely done for diagnosis. However, histologic findings of the PXE-related vascular lesion are sparse and obtained from rare available autopsy samples. Ultrastructural analysis from the ascending aorta, iliac arteries, and vena cava from 2 males with PXE (36 and 80 years old), revealed that veins and arteries were similarly damaged (Gheduzzi et al., 2003). The alterations were not distributed homogeneously along the vessels with spotty alterations of elastic fibers, and aggregates of thin strands of amorphous elastin. The von Kossa staining revealed calcium sediment within the medial layers of the arterial wall of medium (e.g., carotids)- or small (radial)-sized arteries. In carotids, calcification was found extracellularly around elastin fibers although slight increase in intracellular calcium is also observed. Elastic fibers appears fragmented and proteoglycans accumulated preferentially within the media rather than intima compared to controls (Kornet et al., 2004). Qualitative and quantitative alteration in proteoglycans metabolism have been reported with increased heparin sulfate and decreased chondroitin sulfate in patient's urine (Maccari et al., 2003).

FUNCTIONAL CHANGES IN PXE ARTERY

The functional impacts of the arterial lesions, represented by elasto-calcinosis and proteoglycans accumulation, have been examined in a limited number of studies. Most of our knowledge is derived from non-invasive structural and dynamic observations using ultrasound techniques in living subjects and have provided important data for the understanding of the arterial lesions in PXE and their clinical expression. Three functional studies involving a small number (≈25) of PXE patients have focused on the carotid artery, an easily accessible large-sized musculo-elastic artery (Boutouyrie et al., 2001; Germain et al., 2003; Kornet et al., 2004). One of these studies has reported changes in the radial artery, a medium-sized muscular artery and the aorta, the main large-sized elastic artery of the body (Germain et al., 2003).

INCREASED INTIMA MEDIA THICKNESS

An increased carotid intima media thickness (IMT) has been reported in two human studies (Germain et al., 2003; Kornet et al., 2004) and another more recently involving mice (Kupetsky-Rincon et al., 2012). Compared to age and gender-matched patients, these changes were more marked in older patients than in younger ones under the age of 40. An increased carotid IMT is independently associated with a higher risk for CV events which could represent a relevant argument to explain an accelerated arteriosclerosis in PXE with higher than normal CV incidents (Eigenbrodt et al., 2008). This increase in IMT was not associated with an enlargement of the lumen size and was responsible for a 19% increase in the arterial wall mass. By contrast, the radial arteries exhibited a smaller lumen with a thicker IMT, suggesting an inward remodeling (Germain et al., 2003).

The larger IMT seems to result from the higher amount of proteoglycans without proliferative change in the vascular smooth muscle cells (VSMC). Proliferation of the VSMC toward the lumen leads to arterial stenosis and occlusion, and is observed in response to an abnormal mechano-transductive signaling in elastin deficiency diseases, such as Williams–Beuren syndrome (Li et al., 1998). But this may not be the sole mechanism since a higher IMT with a progressive loss of VSMC has also been reported in Progeria (Gerhard-Herman et al., 2011). More complex

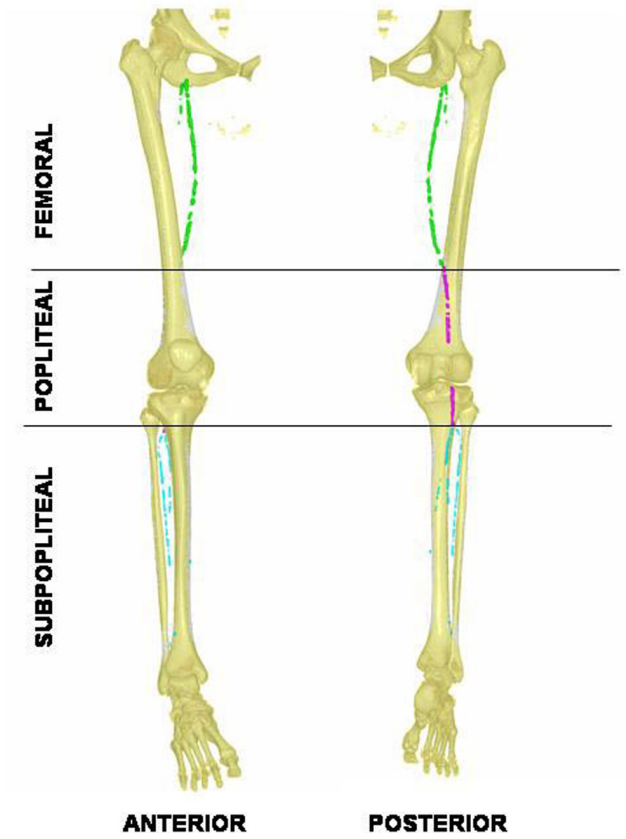

FIGURE 1 | Anterior and posterior 3D-views of lower limb arterial calcification in pseudoxanthoma elasticum revealed with helicoidal X-ray tomodensitometry. Calcification in the femoral artery is tagged in green, the popliteal in purple, and the visible distal arteries are tagged in blue. Note the absence of calcifications within the middle popliteal segment.

changes have also been reported with multilayered aspects in PXE coronary (Miwa et al., 2004) or mammary arteries (Sarraj et al., 1999).

Although these conclusions remain to be confirmed in larger cohorts, the arterial remodeling in PXE peripheral arteries exhibits differences from other arterial remodeling such as aging, hypertension, and atherosclerosis. This remodeling is characterized by intima-media thickening predominantly in the large and medium-sized musculo-elastic arteries (Boutouyrie et al., 1992). Aging is also defined by an outward remodeling whereas hypertension and atherosclerosis are rather characterized by an inward remodeling. This remodeling is variable along the arterial tree (Bortolotto et al., 1999; Bjarnegard and Länne, 2010) and with gender, women exhibiting a higher wall/lumen ratio than men (Green et al., 2010). The use of imaging techniques with higher spatial resolution will be very helpful to confirm and discriminate the relative changes of thickness between the intimal and medial layers, and between the different vascular beds.

Arterial wall stiffness and elasticity in PXE arteries: It is expected that both fragmentation of elastic fibers and calcification will affect the arterial elasticity of the PXE arteries.

Compared to age and sex-matched controls, the distensibility was found either unchanged (Germain et al., 2003) or higher (Kornet et al., 2004) in the large-sized artery, such as carotid, and in medium-sized arteries such as radial (Germain et al., 2003). Furthermore, in the older female patients, the elastic modulus was found unchanged in small-sized but was higher in the medium-sized artery. A lower or unchanged compressibility of the small-sized arterial wall was also reported in the ankle arteries of PXE patients (Leftheriotis et al., 2011).

Therefore, it is likely that the complex rearrangement of the extracellular matrix in PXE arterial wall resulting from the combination of elastin fragmentation associated to proteoglycans replacement, focal accumulation of calcium and activated MMP (Zarbock et al., 2010) in the media could mask a predicted arterial stiffness.

CLINICAL EXPRESSION OF THE VASCULAR PXE PHENOTYPE

The keystone clinical manifestations in PXE are represented by visual impairment due to the loss of central vision, large skin folds of esthetic concern, and CV complications (Neldner, 1988; Uitto et al., 2011). The vascular lesion in PXE takes a major place in the complications and the clinical outcome. Although, it seems that the lifespan of these patients is relatively preserved despite no clear reports on this point, the vascular impact of the disease is crucial and will be detailed in the following paragraphs.

ARTERIAL HYPERTENSION

Since the earliest reports, the presence of an arterial hypertension in PXE has been controversial with a highly variable prevalence reported in the literature ranging from 8% (Neldner, 1988) and up to 25% of the patients (Gotting et al., 2005). Even for the highest range, this reported prevalence remains within the overall limits of hypertension estimated to be 26.4% of the world's population (Kearney et al., 2005). In PXE, a higher than normal prevalence was likely explained on the basis of a renal artery stenosis or increased arterial stiffness resulting from the elasto-calcinosis (Goodman et al., 1963). A higher serum xylosyltransferase (XT-1), a fibrosis marker of the extracellular matrix was found in hypertensive PXE patients (Gotting et al., 2005), though this is not supported by functional data (cf paragraph above). The association between ABCC6 and angiotensin polymorphisms (T174M and M235T) was not demonstrated in hypertensive PXE compared to normals (Gotting et al., 2005). Therefore, the higher prevalence of arterial hypertension in PXE remains to be demonstrated.

ANEURYSMS AND DISSECTIONS

An abnormally dilated and/or ruptured arterial wall is the most life-threatening complication of the PXE disease. Contrary to other genetic diseases where the connective tissues are affected, as in Marfan's or Elhers–Danlos diseases, reports of aneurysm in PXE patients are sparse and almost anecdotic.

In the cerebral vasculature, the prevalence of intracranial aneurysms is difficult to estimate. A Belgian PXE cohort ($n = 100$) based on self-reported data concluded that it was an unrelated association (van den Berg et al., 2000) while the association

between intracranial malformations, including aneurysms, and PXE may not be fortuitous (Vasseur et al., 2011).

Aneurysms in the other vascular beds such as aorta or lower limbs are very rarely reported. ABCC6 mutations were found in a minority of non-PXE patients (5/133) with abdominal aortic aneurysms (Schulz et al., 2005), but this was not statistically significantly different from healthy controls and could not be considered as a genetic risk factor for aortic aneurysms. Aorto-coronary aneurysm has also been reported (Heno et al., 1998) but seems not specific to PXE as it has also been reported in other PXE-like syndromes, such as beta-thalassemia (Farmakis et al., 2004). Common genetic factors underlie medial calcification, such as ABCC6 and aneurysm development, suggesting that although medial disruption and calcification may occur in parallel, medial disruption does not strictly occur as a result of vascular calcification (Wang et al., 2009). The possibility for a higher prevalence for arterial dissection, such as the spontaneous disruption of the internal layers of an artery, common in the carotids of PXE, is still under discussion (Brandt et al., 2005), but remains anecdotic at present. Although several missense mutations (H623Q, R3190W, and R1268Q) were found in the patients with carotid dissection, these mutations were not disease-causing as they were also detected in healthy subjects (Morcher et al., 2003).

ISCHEMIC STROKE

Beside the risk of stroke due to cerebral hemorrhage with ruptured intracranial aneurysms, the risk of ischemic stroke (IS) is another feared complication in PXE but remains difficult to establish. IS was reported in 15% of the PXE patients from a cohort of 38 patients compared to the general population (0.3–0.5%; Vanakker et al., 2008). In a cohort of 100 patients, IS was reported in seven patients with one patient having recurrent IS leading to a relative risk of 3.6 (95% confidence interval 3.3–4.0) of ISs in patients under 65 years (van den Berg et al., 2000). Focal cerebral ischemia in PXE was predominantly caused by small-vessel occlusive disease. Atherosclerotic plaques could co-exist with PXE lesions, but results from our cohort (unpublished data) showed that carotid plaques were absent in 55/93 (59.1%), unilateral in 17/93 (18.3%), and bilateral in 21/93 (22.6%) compared to age and gender-matched controls ($p = 0.987$) suggesting that it is not a primary mechanism for stroke in PXE. Transient cerebral ischemic attack could result from intermittent hemodynamic cerebral insufficiency due to intracranial arterial malformation (Vasseur et al., 2011).

CARDIAC DISEASES

Cardiac diseases in PXE are mainly represented by myocardial infarction, angina pectoris, and valvular malfunction (Neldner, 1988; Vanakker et al., 2008). Data from the largest cohorts (Neldner, 1988; Vanakker et al., 2008) have reported symptoms of myocardial origin ranging from 13 to 15% for angina pectoris but lower for infarction (1–5%) of the patients occurring at age <55 years and sometimes causing death. In the coronary arterial bed, the association with a heterozygous R1141X mutation in ABCC6 and ischemic vascular events including stroke, was not demonstrated in the general population ($n = 66831$ participants; Hornstrup et al., 2011), although a strong association was reported only with coronary artery disease (Koblos et al., 2010). Additionally, Abcc6 deficiency was found to increase infarct size and apoptosis in a mouse cardiac ischemia–reperfusion model, although there were no differences in cardiac calcification following ischemia/reperfusion (Mungrue et al., 2012). Abnormal coronary wall suggests that specific structural factors are likely present in these vascular beds (Miwa et al., 2004). Interestingly, the transferability of the PXE phenotype, i.e., calcification, to the arterial graft is still questioned (Sarraj et al., 1999; Iliopoulos et al., 2002; Song et al., 2004).

PERIPHERAL ARTERIAL DISEASE

Contrary to the other arterial beds, an early and severe peripheral arterial disease (PAD) described as a slowly worsening lower limb claudication is consistently and extensively reported in PXE patients without obvious CV risk factors. PAD is detected clinically by absence of ankle pulse, and the presence/absence of symptoms of a lower limb claudication such as a calf pain that limits or interrupts a walk. Additionally, the severity of PAD can be objectively determined by measuring the ankle-brachial systolic pressure index (ABI) which corresponds to the ratio of the systolic ankle and brachial pressures that normally ranges from 0.9 to 1.39, and the treadmill walking distance with the help of transcutaneous PO2 (Abraham et al., 2003). The prevalence of lower limb claudication, the symptomatic expression of PAD, is very high in PXE (53% in the Belgian cohort Vanakker et al., 2008 and 42% in ours) (Leftheriotis et al., 2011), a proportion clearly higher than the 9% men and 5% women with PAD reported in the general population. By contrast, the treadmill test, an objective evaluation of the arterial claudication, showed that only 56% of PXE with an ABI <0.90 were symptomatic during the test. The discrepancy between a high proportion of PAD detected by ABI with relatively less symptoms of intermittent claudication suggests that the PAD is well compensated by an efficient collateral circulation in PXE. This tolerance to ischemia was further demonstrated in our cohort by relatively well-preserved tissue oxygenation in PXE patients during walking. Interestingly, a relatively high incidence of subclinical peripheral artery disease (41%) was also reported in the carrier population ($n = 21$) suggesting that PAD could represent a frequent clinical manifestation, even in heterozygous patients. In addition to the presence/absence of ankle artery pulse, the ABI is a validated diagnostic tool for the detection of PAD and estimation of its severity, mainly in the asymptomatic patient (Diehm et al., 2009). PAD is also an independent marker of atherosclerosis associated with higher rates of CV diseases in the general population (Golomb et al., 2006). Calcifications in the tunica media of PXE patients are expected to increase arterial stiffness and thus decrease arterial wall compressibility (Kim et al., 2012). Although calcification predominates in the small-sized ankle arteries, the preserved arterial compressibility in these arteries remains unexplained.

HEMORRHAGE

Hemorrhages are a frequent ophthalmologic complication in PXE due to the rupture of proliferative choroidal neovascularization

secondary to angioid streak. To a lesser extent, gastrointestinal hemorrhage have also been reported and represent a life-threatening condition in PXE. The mechanism of hemorrhage remains unknown, but suggests sub-mucosal arterial malformations fragilized by the medial calcification with risk of rupture likely to the Bruch's membrane in the eyes. Neovessels and/or malformation could also result from defective regulatory pathways and vascular endothelial growth factor (VEGF) gene polymorphisms such as the c.-460T and the c.674C alleles that are independent risk factors for development of severe retinopathy (Zarbock et al., 2009). The genetic deficiency gamma-glutamyl carboxylase (GGCX), which is a PXE-like syndrome associated with an abnormal production of coagulation factors, are more prone to severe uncontrol bleeding (Li et al., 2009a).

WHAT DOES PXE MODELS TEACH US ABOUT CARDIOVASCULAR DISEASES?

Similar to other genetic diseases, the use of genetically engineered organisms such as mouse or zebrafish (*Danio rerio*) is helpful in our understanding of the PXE pathophysiology. Abcc6$^{-/-}$ mice exhibit a low-to-mild level of medial arterial calcifications (Gorgels et al., 2005; Klement et al., 2005), although most calcification processes develop markedly in specific organs such as the vibrissae, and represents an interesting marker of peripheral calcification (Le Corre et al., 2012). Other mouse strains such as C3H/HeOuJ are spontaneously prone to soft connective tissues calcification, due to the defective function of Abcc6, that can be easily followed and quantified using X-rays (Le Corre et al., 2012). Finally, the zebrafish model exhibits severe abnormal development that were fully rescued by co-injection of mouse Abcc6 mRNA (Li et al., 2010). Although the mechanism of the arterial lesions in PXE remains unexplained, the fact that exteriorized tissue lesions develop remotely from the predominant sites of ABCC6 demonstrates that modifying factors are at play and limits our conclusions from these mouse models. There is now a constellation of data arguing for the concomitant role of modifying genes (Li et al., 2007; Hendig et al., 2008), polymorphisms (Schon et al., 2006) and mutations, regulatory pathways (Martin et al., 2011) as well as nutritional and environmental factors (Zarbock et al., 2007, 2009, 2010; Pisciotta et al., 2009) considerably widening the phenotypic expression and severity of the disease. Furthermore, the selective involvement of anatomical sites such as skin, eyes, arteries is usually explained by the presence of elastic fibers (with the exception of the lungs), but recent data suggests that collagen fibers could also be involved (Gorgels et al., 2012).

THERAPEUTIC ISSUES IN THE PXE ARTERIAL DISEASE

In absence of validated and specific therapeutic targets in PXE, the treatment of arteriopathy remains limited. Vitamin K was not proven as an efficient treatment, although no data are presently available in human but the lack of a consistent result in proof-of-concept studies conducted in mice are not encouraging (Gorgels et al., 2011; Jiang et al., 2012). Despite disappointing initial results from a pilot study conducted in humans (Yoo et al., 2012), attempt to reduce calcification using phosphate binder (LaRusso et al., 2009) or magnesium supplementation has recently gained renewed interest with conclusive results in Abcc6$^{-/-}$ mice (LaRusso et al., 2009; Li et al., 2009b; Kupetsky-Rincon et al., 2012). Treatment of acute ischemic complications is sporadically reported in the literature without obvious difference compared to the general population, although reports of limb amputation in the literature have never been made to our knowledge.

Finally, the careful management of CV risk factors remains an important point since atheroma could mask pre-existing lesions. Factors that could aggravate arterial calcification should be avoided in these patients such as anti-vitamin K which is an oral anticoagulant drug known to favor calcification (Price et al., 1998) that can now be advantageously replaced by anti-Xa oral therapy after the hemorrhage risk/benefit balance has been properly evaluated. The question of preventive anti-platelet therapy for IS is unsolved at present. In the general population, anti-platelet treatment such as aspirin is advised for secondary prevention of IS, but the higher prevalence of upper gastrointestinal hemorrhages in PXE remains a firm contraindication for the use of aspirin as well as for anticoagulants. A major challenge for all clinical trials attempted in PXE is the need for an objective and reproducible quantification of the soft-tissue calcification. In this way, quantification of the arterial calcification could be an interesting tool complementary to skin biopsy or ultrasound imaging and ophthalmologic follow-up since the severity of cutaneous manifestations and angioid streak of PXE are likely predictive of CV involvement (Utani et al., 2010).

In conclusion, although the arterial remodeling observed in PXE shares some of the features of arteriosclerosis and other calcifying vascular diseases of metabolic origin, it is not readily comparable to atherosclerosis. This raises challenging questions on the central role of the hepato-renal axis in the soft-tissue calcifying processes and PXE represents a prototypical systemic metabolic disease of genetic origin. The severity of the disease is progressive and highly variable in which CV symptoms and PAD seems a constant finding in these patients. Furthermore, the higher than expected compressibility of the arterial wall may represent a useful marker as well as quantification of the arterial calcium load. Data from larger cohorts are awaited with more detailed phenotypic descriptions. From a clinical point of view, the absence of efficient therapy, the follow-up of these patients first requires a tight control and management of the usual CV risk factors in addition to the limitation of pro-calcifying factors. The place of other anti-calcifying drugs, such as denosumab remains unknown at present. Finally a comparative analysis of the overlapping phenotypes such as PXE, generalized arterial calcification of infancy (GACI), and other PXE-like diseases is likely to add valuable information on the elusive mechanism of calcifying genetic diseases.

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Disease advocacy organizations catalyze translational research

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Disease advocacy organizations have long played an important role in the continuum from basic science to therapy development in rare disease research. PXE International has led the field in innovative ways, venturing into specific activities that have traditionally been conducted by scientists. As lay founders, we have engaged in gene discovery, gene patenting, diagnostic test development, epidemiological studies, clinical trials, and therapy research and development. This article will describe the steps that we took, and the ways in which we have scaled these efforts for the larger community.

Keywords: rare diseases, advocacy, ABCC6, PXE, open access

ONE DISEASE

This perspective is that of individuals, families, and communities engaging in the scientific process to accelerate and improve health. We are ordinary parents, like hundreds working to better the lives of their children. Our original focus on our children's disease has become agnostic to disease, and expanded to include broad systemic change in the clinical and translational research enterprise.

Our quest began in 1993, when we noticed some small lesions on the sides of our 7-year-old daughter Elizabeth's neck. After a year of the diagnostic odyssey, we took her, out of plan and out of pocket, to a dermatologist, Lionel Bercovitch, MD, who recognized pseudoxanthoma elasticum (PXE) immediately. Looking at Elizabeth's 5-year-old brother, Ian's neck, and said, "He has it too." Then he examined Elizabeth's eyes. He was the perfect diagnostician for this condition; he was also trained as an ophthalmologist. Before this experience, we had no idea that a skin disease could be systemic. We had no frame of reference for all of this foreign information: "systemic, genetic, recessive, papules, angioid streaks. . ."

Our response, besides showering our children with gifts that Christmas 1994, in the pre-internet age, was to photocopy every article we could find on the disease: a stack of about 400 articles. We could not understand them and so turned to medical dictionaries and reference material.

By the middle of January, we understood several important things: (1) no one knew how this disease progressed, there were conflicting conclusions in the papers we read, (2) there was no comprehensive plan to study the disease, nor was there a plan emerging, (3) no one even knew how many people had the disease, and (4) there was no treatment, the gene had not even been discovered yet!

In the midst of this morass, two researchers from two different prominent biomedical research institutions appeared. After the first took blood from all of us, the second wanted the same. We told him to go get some from the first one. The chuckled and we learned the astounding fact that that scientists competed: they did not collaborate.

Within a few months of the start of our informal education in PXE, genetics, dermatology, ophthalmology, cardiology, biomedical research, and therapy development, we devised a plan largely influenced by Patrick's background in building engineering. **Figure 1** shows the "wiring diagram" plan for advancing research to lead to interventions (Terry and Boyd, 2001).

We first enlisted Dr. Bercovitch, asking him to be medical director and board member of a foundation we named PXE International. Then we asked the nearest lab engaged in the search for the PXE gene, if we could wash test tubes to speed up their research. They generously allowed us to come into the lab in the evenings and eventually gave us keys. They did not want us washing test tubes, they wanted us to "score gels." And so we scored gels night after night. Patrick often stayed until the wee hours of the morning. We had a wonderful neighbor who would watch our kids in the evenings while they slept.

Simultaneous with this we started to build a cohort of well-characterized individuals affected by PXE. We contacted dermatologists and ophthalmologists around the world and started adding people to our registry. We held meetings in Boston, New York, California, Paris, Gent, Modena, Amsterdam, and Cape Town. We used the nascent internet and created listservs.

We also contacted all of the researchers who had written numerous papers on PXE. We asked if we could meet with them, and to a person, they were generous and open with their time. Ken Neldner (Neldner, 1988), Mark Lebwohl (Lebwohl et al., 1994), Jouni Uitto (Christiano et al., 1992; Christiano and Uitto, 1994), Anne De Paepe (Godfrey et al., 1995), Ivonne Ronchetti (Contri et al., 1996), Charles Boyd (Lebwohl et al., 1994), Arthur Bergen (van Soest et al., 1997), Michael Pope (Pope, 1975), and Dennis Viljoen (Viljoen, 1988), all met with us in their labs, gave us tours and educated us.

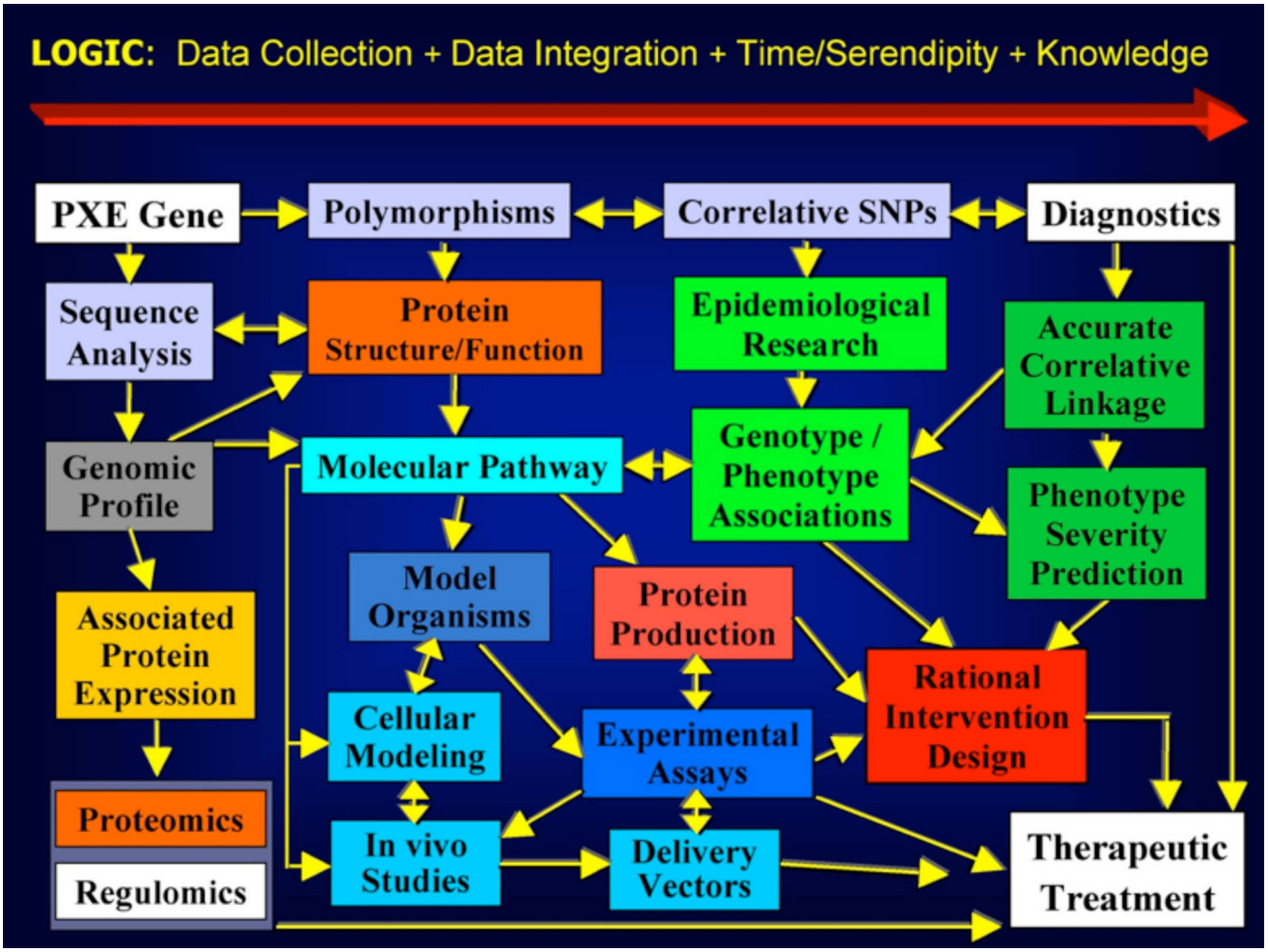

FIGURE 1 | PXE International's 1995 Strategic Plan (published in Terry and Boyd, 2001).

We recommended to the researchers that they all work together to find the gene. People told us "you can't herd cats, so stop trying." We retorted, "yes you can, you just need to move the food." We set to work building the first ever lay-owned blood and tissue bank (Terry et al., 2007; Terry, 2008). We collected blood samples by sending kits to affected individuals all over the world. With epidemiologists, we created a survey instrument and administered it, including collecting pedigrees. We then gave researchers access to the de-identified samples and data if they agreed to play by our then novel data sharing rules.

We had some sophisticated help in those days. Having met Francis Collins, then director of the National Human Genome Research Institute (NHGRI) at the National Institutes of Health, at the 10th anniversary of the Alliance of Genetic Support Groups (now known as Genetic Alliance), we asked for advice and he shared NHGRI's technology transfer wizard with us. Claire Driscoll helped us craft state of the art consents, protocols, material transfer agreements, and then joined our board upon which she still serves. The work she did formed the underpinning of our later cross-disease efforts.

We were not able to get all research groups to share data in those early days, but we did get a few to combine forces resulting in back-to-back papers in Nature Genetics (Bergen et al., 2000; Le Saux et al., 2000) when the gene was discovered through our wet bench work and that of several other groups (Ringpfeil et al., 2000). We were also able to encourage focus on the discovery of the gene associated with PXE, despite the attractiveness of several unknown genes in the locus. This is evidence of the contributions communities can make even in basic research related to a disease.

Our work moved from scoring gels to entering and analyzing the data, meeting with the various teams around the world search for the gene, and materially participating in the discovery. With a group of about five scientists we discovered the: one of the known ones-ABCC6. This discovery taught us a very important lesson. It was thought that the gene would code for a protein involved in a structural aspect of elastin, since degredated elastin fibers are common in all of the organs affected by PXE. Instead, ABCC6 codes for a membrane transport protein, in the same family as cystic fibrosis. The big learning for us, which we encounter over and over in biology, is that we do not know which discoveries are

going to benefit one disease or another. We often quote: "a rising tide lifts all boats." This experience was critical to our thinking about this disease and others on a system level. It later informed a policy position Genetic Alliance took about not earmarking federal funds for specific diseases (Terry, 2010).

We are co-inventors and patent holders of ABCC6 with the other scientists. We have assigned our rights to PXE International and as such are stewards of the gene, making sure there is open access to it for research and therapeutic development.

ALL DISEASES

Throughout these years, we frequently met with individuals affected by PXE and their families around the world. We built a robust website at pxe.org and created volumes of information on the disease to help mitigate the diagnostic odyssey and lack of information.

From the beginning we had excellent mentors in disease advocacy organizations. We also had hundreds of requests to help other organizations set up registries, biobanks, and research enterprises. I moved my work to a dynamic umbrella organization called Genetic Alliance, and was joined by remarkable colleagues who also sought the most effective systems-level solutions to accelerate translational research and services for all. Together we created a collaborative network that has led to the development of many tools, resources, and even legislation (**Table 1**).

CULTURE CHANGE

Changes taking place in society in the areas of information technology and networks, if parlayed for improving health, will be an essential catalyst for the transformation of biomedical research. The current biomedical research system was modeled after an industrial age culture of scarcity, win–lose, linear progress, and competition. We live in an age where raw materials are abundant: information is being produced today at rates we cannot manage. A newborn has more information shared about her in the first days of life than the US Library of Congress contains. Networks, concurrent processes, win–win engagements, and ever increasing transparency and openness are now available to transform the research enterprise.

These changes have initiated a wonderful rebirth in systems surrounding non-profits such as Genetic Alliance. It is critical for us to work with other organizations in a boundary-less way. This requires that we ask "how is it true of me?" when we encounter an obstacle that appears to be external. This is our practice both as individuals in Genetic Alliance and PXE International and for the organizations themselves. It is our belief that each of us is responsible because we not only represent the whole: we are the whole. It is also critical for us to be ever vigilant of the downfall of all systems: that they begin to exist to largely to protect the system rather than to serve the mission (Meadows, 2008). Just as we each look in the mirror every day and ask, "Am I the best person for

Table 1 | Resources and tools to accelerate research and services.

Need	Tool or resource	Year	Reference
Cross-disease, common platform, biobank, and registry	Genetic Alliance Registry and BioBank (www.biobank.org)	2003	Landy et al. (2012)
Toolbox/manual for maintaining an advocacy organization	WikiAdvocacy (www.wikiadvocacy.org)	2004	Weiss (2004)
Disease information provided by the experts (disease advocacy organizations) and filtered from federal sources to be at the right literacy level	Disease InfoSearch (www.diseaseinfosearch.org)	2006, revised in 2013	Landy et al. (2012)
The power of family history	Does It Run in the Family? (www.familyhealthhistory.org)	2006	O'Leary et al. (2011)
Protection against discrimination based on genetics	Coalition for Genetic Fairness and passage of the Genetic Information Nondiscrimination Act of 2008 (www.ginahelp.org)	2008	Dressler and Terry (2009); Terry (2009)
Clarity about the reliability of health information on the internet	Trust It or Trash It (www.trustortrash.org)	2009	NA
Information about newborn screening in all 50 states	Baby's First Test (www.babysfirsttest.org) as a result of the Newborn Screening Saves Lives Act of 2008	2010	NA
Drug development seen as a network, rather than a pipeline	Navigating the Ecosystem of Translational Science (www.geneticalliance.org/nets)	2011	Baxter et al. (2013)
Cross-disease registries for all that allow the individual to set their sharing and data access settings	Registries for All (www.reg4all.org)	2013	Terry et al. (2013)
Clinical trials need to find the individual, not the other way around	TrialsFinder (www.trialsfinder.org)	2013	NA
Resources for the public to understand genetic technologies	Genes In Life (www.genesinlife.org)	2013	NA

this job?," it is critical we ask if Genetic Alliance or PXE International are the best organization for their respective missions.

THE FUTURE

Advocacy organizations of the future will not look like today's organizations that were built on models such as Alcoholics Anonymous. Today's young parents do not join one group, one organization. They join many affinity groups and are adept at managing them. They create custom solutions that meet their family's needs. They use multiple ways of interacting, without compartmentalizing their lives. They do not experience the same level of isolation based on their children's diagnosis as we did in 1994. Parents today do not identify with one aspect of life to the detriment of others.

It's never wise to predict the future. I do believe, however, that we need to be bold in our vision of the future. I think that if we do not risk it all, and lead to the highest place we can envision, we will not succeed in our lofty, and essential, goals. I believe that we need to work together, without regard for the histories of our organizations, or body geography of the diseases for which we seek to find therapies.

Tools to help us achieve grand challenges have emerged. Data sharing in the information age is transformative – it will break down barriers and accelerate translational and clinical science. Giving individuals and communities the tools to decide with whom to share their data and samples, and how much to share is essential. When we understand that our fear of sharing information is hugely detrimental to accelerating solutions we will free up a great deal of energy. The old system will not work. Advocacy organizations, academic institutions, companies, and legislators still cling to it since it is familiar and safe. It is hard for us to see how unsafe it is to remain in the old models, and that it will impede our efforts. We have ample examples in other industries: music, travel, and publishing. Consumers have effected that change. As consumers in the cottage industry we call healthcare we are disconnected from our needs, and cannot feel them in the same way as we feel the need for music or air travel options and accessibility. This is remarkable because so much is at stake. Special interests, uncoordinated systems, lack of evidence, a "non-learning" healthcare system and fear keep us from achieving better health for all.

The advocacy organizations of the future will be flexible and dynamic. Their boards of directors will not focus on sustaining the organization; they will focus on maximizing the advance to the goals. They will be cross-disease, and be constellated around biological pathways, phenotypes, and biomarkers. They will come into being to address a very critical problem and dissolve or move on once that problem is addressed. The advocacy organization of the future will be an integral part of the research enterprise and not so novel in its work that it would be worthy of this sort of paper.

It is time to align incentives to serve the millions around the globe who suffer. It is time to risk what we think is unthinkable, share information and be bold. There is no time to hesitate – our loved ones cannot wait.

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