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Pseudoxanthoma Elasticum: Progress in Diagnostics and Research Towards Treatment Summary of the 2010 PXE International Research Meeting*

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Abstract

Pseudoxanthoma elasticum (PXE), a prototypic heritable disorder with ectopic mineralization, manifests with characteristic skin findings, ocular involvement and cardiovascular problems. The classic forms of PXE are due to loss-of-function mutations in the *ABCC6* gene, which encodes ABCC6, a putative transmembrane efflux transporter expressed primarily in the liver. While considerable progress has recently been made in understanding the molecular genetics and pathomechanisms of PXE, no effective or specific treatment is currently available for this disorder. PXE International, the premiere patient advocacy organization, organized a workshop in November 2010 to assess the current state of diagnostics and research to develop an agenda towards treatment of PXE. This overview summarizes the progress in PXE research, with emphasis on molecular therapies for this, currently intractable, disorder.

Keywords

Pseudoxanthoma elasticum; Heritable ectopic mineralization disorders; ABC transporters; Antimineralization factors; Molecular therapies for PXE

INTRODUCTION

Pseudoxanthoma elasticum (PXE) is the prototype of heritable multisystem diseases displaying ectopic mineralization of connective tissues, with clinical manifestations in the skin, the eyes and the cardiovascular system, associated with considerable morbidity and occasional mortality (for reviews, see Neldner, 1988; Uitto et al., 2010a). The precise prevalence of PXE is currently unknown, but it is estimated to be around 1 in 50,000, with a carrier frequency of ~1:150-300. PXE is inherited in an autosomal recessive manner and without ethnic or racial predilection. In the overwhelming majority of cases PXE is caused by mutations in the *ABCC6* gene which encodes a putative efflux transporter, ABCC6, expressed primarily in the liver, the kidneys, and the intestine, and at very low level, if at all, in tissues directly affected in PXE [Uitto et al., 2010a]. Based on different lines of evidence,

*This is a summary of presentations and discussions in the PXE Research Meeting organized by the PXE International, held November 29 & 30, 2010 in Bethesda, MD.

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including careful examination of *Abcc6* knockout mice, PXE is thought to be a metabolic disorder with the primary molecular defect in the liver [Jiang et al., 2009]. In spite of significant progress in molecular genetics of PXE over the past decade, there is currently no effective or specific treatment for PXE. Quite recently, however, new innovative approaches have been developed, some of which may potentially lead to treatment of PXE at the molecular level.

A cadre of prominent PXE researchers from around the world convened in Bethesda, MD, on November 29 and 30, 2010 to participate in a research meeting organized by the PXE International, the premier patient advocacy organization [Terry et al., 2007]. The presentations and ensuing discussions, providing state-of-the-art, up-to-date information on clinical diagnosis, management and research on PXE, with translational implications, are summarized below.

CLINICAL HETEROGENEITY AND DIAGNOSTIC FEATURES

PXE can pose a diagnostic challenge to practitioners for several reasons. First, although fully penetrant, clinical findings of PXE are rarely present at birth, and the skin findings usually become recognizable not until the second or third decade of life. In many cases, an accurate diagnosis is not made until after several years of delay when serious ocular or vascular complications develop. Secondly, there is a considerable both intra- and inter-familial heterogeneity, so that in some families the skin manifestations may be predominant with relatively little eye or cardiovascular involvement, while in other families involvement of the latter organ systems may have severe clinical consequences with limited skin findings [Neldner, 1988]. Reasons for this phenotypic heterogeneity are currently not clear, and attempts to establish genotype-phenotype correlations in different populations have yielded largely negative results [Pfundner et al., 2007]. There are recent suggestions, however, that certain mutations in *ABCC6* may be statistically associated with involvement of specific target organs, such as the p.R1268Q mutation being associated with early onset of angioid streaks [Sato et al., 2009; Li et al., 2011a], and the stop codon mutation p.R1141X possibly predisposing the individuals to cardiovascular involvement independent of hyperlipidemia in patients with PXE [Köblös et al., 2010; Pisciotta et al., 2010]. The precise role of these mutations in the development of complications in the eye and the cardiovascular system requires further evaluation of larger numbers of patients with defined clinical phenotypes. In addition, the roles of epigenetics, dietary factors, and life style variables need to be examined in larger cohorts of patients (see below). It is also clear from animal studies using the *Abcc6*^{-/-} mouse as a model system that genetic background can profoundly influence the degree of mineralization [Li and Uitto, 2010]. These issues can be further examined in humans by ascertainment of well documented populations and perhaps of the manifestations in monozygotic twin pairs with PXE.

Finally, adding to the clinical diagnostic difficulties relating to PXE is the observation that manifestations similar to those in PXE can be found in a number of unrelated, both acquired and heritable, clinical conditions [Neldner, 1988]. For example, PXE-like cutaneous changes, sometimes associated with angioid streaks, can be found in patients with β -thalassemia and sickle cell anemia, yet it has been demonstrated that these individuals do not harbor mutations in the *ABCC6* gene [Baccarain-Contrí et al., 2001; Hamlin et al., 2003]. Particularly intriguing observations, with potential pathomechanistic implications for PXE, have been recently made in families with PXE-like cutaneous findings, in association with vitamin K-dependent multiple coagulation factor deficiency. Some of these patients show lesions similar to those seen in PXE, and in many of these patients there is an excessive folding and sagging of the skin suggested to be similar to cutis laxa [Vanakker et al., 2007; Li et al., 2009a]. However, skin biopsy from these patients depicts characteristic

mineralization of elastic structures similar to PXE, a finding that is not present in patients with cutis laxa.

MOLECULAR GENETICS

Early studies in 1970s, primarily from the United Kingdom, suggested the presence of autosomal dominant and autosomal recessive forms of the disease. In support of this suggestion were demonstrations of clinical features suggestive of PXE in patients in two subsequent generations. It appears that many of these patients may have been misdiagnosed, for example due to extensive solar elastosis which can mimic PXE in elderly individuals, and in many of the cases the diagnosis was not confirmed by skin biopsy. Furthermore, subsequent to identification of mutations in the *ABCC6* gene, in many of the families depicting clinically confirmed diagnosis in two generations, the PXE phenotype was shown to result from pseudodominance, reflecting in some cases consanguinity in the family [Ringpfeil et al., 2000].

A question raised by genetics in the families also relates to the clinical presentation of obligate heterozygous carriers of a mutation in one allele of the *ABCC6* gene only. Several relatively large studies have failed to identify any clinical findings suggestive of PXE in heterozygous carriers [Miksch et al., 2005; Christen-Zäch et al., 2006; Pfendner et al., 2007]. However, few cases with relatively subtle clinical findings, particularly in the eyes, accompanied by mutations in one *ABCC6* allele, have been reported [Sherer et al., 2001; Martin et al., 2008]. Careful examination of these cases reveals the presence of only subtle findings, and in many cases the family relationships are not entirely clear. Possible explanations for the clinical findings in a putative heterozygous carrier of a mutation include the inability to identify the second *ABCC6* mutation based on PCR amplification of exons and flanking intronic sequences, a strategy that does not include sequencing of the entire introns or the 5' regulatory region of the gene [Pfendner et al., 2007, 2008; Vanakker et al., 2008]. Finally, a possible cause for this rare situation is suggested by a family in which the presence of heterozygous mutations both in the *ABCC6* and *GGCX* genes in affected individuals suggested digenic inheritance [Li et al., 2009b]. As a general rule, the overwhelming evidence suggests that virtually all families with PXE display autosomal recessive inheritance [Ringpfeil et al., 2000]. This situation allows appropriate counseling for the genetic risk of inheritance of an affected child either in the same or subsequent generations and allows presymptomatic testing of siblings with affected individuals in the family [Uitto et al., 2010; Pfendner et al., 2007; Li et al., 2010a].

MODEL SYSTEMS

Soon after the first demonstrations of mutations in the *ABCC6* gene in human patients with PXE, transgenic knockout mice were developed through targeted ablation of the *Abcc6* gene [Gorgels et al., 2005; Klement et al., 2005]. These *Abcc6*^{-/-} mice recapitulate many of the features of human PXE, including autosomal recessive inheritance with full penetrance and delayed onset of mineralization which becomes evident by histopathologic examination around 5-6 weeks of age. Similar to findings in humans, the PXE mice demonstrate deposition of mineral complexes in the skin, the retina and arterial blood vessels, in addition to widespread evidence of mineralization in other tissues as well. A characteristic feature of *Abcc6*^{-/-} mice is the early and progressive mineralization of the connective tissue capsule surrounding vibrissae [Klement et al., 2005]. Consequently, assessment of the degree of mineralization of vibrissae during the development and growth of the mice by histopathology coupled with computerized morphometric analysis, and by direct chemical assay of calcium and phosphate, serves as a biomarker that can be quantitated to follow the progression of mineralization in these mice [Jiang et al., 2007]. Thus, the *Abcc6* knockout

mice have provided a useful preclinical model system to explore the pathomechanisms of PXE and to test potential treatment modalities.

One of the limitations of the mouse model is the long developmental lifespan and relatively slow onset of the disease. In addition, establishment and maintenance of mouse colonies is time consuming and costly. In search for complementary, and perhaps more expedient model systems to study heritable skin diseases in general, the zebrafish was examined as an alternate [Li et al., 2011b]. The zebrafish, a freshwater vertebrate, has nearly the same complement of genes as mammals, and assembly of the zebrafish genome database is essentially complete. The zebrafish embryos develop rapidly outside of the mother's body, so that the organ development, including skin, which is essentially complete at 5-6 days post fertilization, can be visualized by optical means.

Examination of the zebrafish genome database reveals the presence of two *ABCC6* orthologues, *abcc6a* and *abcc6b*, and the encoded proteins have a high degree of homology with human *ABCC6*, with conserved structural organization of the transmembrane domains, nucleotide binding folds, Walker motifs, and the ABC signature sequence, features of ABC family of proteins [Li et al., 2010b]. Morpholino-based antisense nucleotide knockdown of *abcc6a* expression revealed significant developmental abnormalities, including pericardial edema and curved tail with stunted growth, yet no evidence of mineralization at the time of demise of these mutant fish at 8 days post fertilization was noted. These results indicate that the *abcc6a* gene, an orthologue of human *ABCC6*, is essential for normal zebrafish development, but this knock-down model is not a suitable system to study the mineralization processes in PXE. However, this model system is useful for study of compounds modulating *abcc6* expression and for testing the pathogenicity of mutations in this gene through mRNA rescue.

Cellular model systems to study the function of *ABCC6* have been developed through expression of a full-length cDNA in insect cells (Sf9) or in mammalian kidney derived MDCKII cells. Formation of inside-out vesicles of the isolated membranes of the Sf9 cells allows testing of various substrates of *ABCC6*, and in fact, this system was first used to demonstrate that *ABCC6* can effectively transport small molecular weight compounds *in vitro*, including leukotriene-C4 and N-acetylcysteine S-glutathione [Iliás et al., 2002]. This model system has also been helpful in determining the lack of functionality of mutant proteins as an ATP-dependent transmembrane transporter. The MDCKII cells grown in polarized cultures have been shown to localize *ABCC6* expression to the basolateral surface of the plasma membrane, a feature critical for understanding the putative role of *ABCC6* as in efflux transporter [Sinkó et al., 2003].

Fibroblasts cultures established from the skin of patients with PXE have been studied to get insight into the pathomechanisms of this disease as well. Although these cells express relatively low levels of *ABCC6*, these cells, when compared to appropriate controls, demonstrate altered growth, migration and gene expression profiles, possibly relevant to PXE manifestations in the skin [Hendig et al., 2008; LeSaux et al., 2006]. Finally, an *in vitro* culture system consisting of human skin fibroblasts, or alternatively aortic smooth muscle cells, incubated in a mineralization inducing medium can be tested for the efficacy of anti-mineralization factors, such as fetuin-A or magnesium, in preventing the mineralization process [Jiang et al., 2010; LaRusso et al., 2009].

PUTATIVE PATHOMECHANISMS

Histopathologic evaluation of skin in patients with PXE reveals progressive mineralization of connective tissues, and the elastic structures in particular, as the characteristic feature. Accumulation of calcium/phosphate complexes in these lesions is apparently responsible for

clinical manifestations in the skin, as well as in the eyes and the cardiovascular system affected in PXE. The gene, *ABCC6*, harboring mutations in the majority of families with PXE is expressed primarily in the liver, the kidneys and the intestine, and at very low level, if at all, in tissues clinically affected with PXE [Belinsky et al., 1999; Scheffer et al., 2002]. This and subsequent observations in animal models of PXE have suggested that PXE is a metabolic disorder, postulating that absence of functional *ABCC6* primarily in the liver results in deficiency of a circulating factor(s) that are physiologically present to prevent aberrant mineralization under normal calcium and phosphate homeostatic conditions [Uitto et al., 2010; Jiang et al., 2009] (Fig 1). In this context, it is important to emphasize that no abnormalities in the serum calcium or phosphate content have been noted, and parathyroid hormone levels are normal [Neldner, 1998]. The nature of the molecules potentially transported by *ABCC6* from the liver to circulation is currently unknown. Previous studies, however, have identified a number of proteins that can act as powerful anti-mineralization factors in the circulation; these include fetuin-A and matrix Gla-protein (MGP) (Table I). The presence of many of these proteins has been demonstrated in association with mineral deposits in the mouse knockout model of PXE [Jiang et al., 2007]. Furthermore, it has been demonstrated both in patients with PXE and in *Abcc6*^{-/-} mice that the serum fetuin-A levels are reduced by about 20-30% from the normal, and less of the activated (γ -glutamyl carboxylated form) form of MGP is found in association with the mineral deposits [Hendig et al., 2006; Gheduzzi et al., 2007; Li et al., 2007; Jiang et al., 2010]. These observations suggest that restoration of the full anti-mineralization capacity of these molecules could be used to counteract the development of PXE phenotype. In fact, expression of fetuin-A in *Abcc6*^{-/-} mice under a liver-specific promoter has been shown to result in elevated serum fetuin-A levels, with subsequent reduction in the extent of calcium/phosphate deposits in the skin [Jiang et al., 2010].

An additional mechanism that has been suggested to contribute to the phenotypic presentation of PXE is based on the observations that skin fibroblasts cultured from patients with PXE display alterations in their biosynthetic expression profile as well in cell-cell and cell-matrix interactions, associated with changes in their proliferative capacity [Quaglino et al., 2000, 2005]. Specifically, such cultured fibroblasts have been shown to display enhanced synthesis of elastin and glycosaminoglycan/proteoglycan complexes, and they also show enhanced degradative potential because of elevated matrix metalloproteinase-2 activity. These phenotypic features of cultured cells have shown to be maintained through several passages, suggesting genetic or epigenetic alterations in the cell genome as a result of overall metabolic disturbance due to *ABCC6* mutations. The mechanisms by which such apparently permanent changes are elicited in the fibroblasts are currently unknown, and how such cell biological perturbations might contribute to the clinical findings in PXE remain to be studied further. Nevertheless, the cells in peripheral organs may serve as a target of the metabolic alterations that facilitate the mineralization of the adjacent connective tissues. In this context, it is important to note that ectopic mineralization is a complex process and that a number of modifiers involving soluble factors, cellular elements and the state and composition of the extracellular matrix can influence the degree of mineralization. Attesting to the complexity of this process are the demonstrations that development of knockout mice with targeted ablation of a single gene, such as those encoding matrix gla protein, fetuin-A, and others, can result in extensive mineralization primarily of vascular connective tissues [Jahner-Dechent et al., 1997; Luo et al., 1997]. Recent observations on patients with arterial calcification due to deficiency of CD73 (ACDC) have provided additional evidence, perhaps directly relevant to pathomechanism of PXE, of the complexity of ectopic mineralization. Specifically, these patients harbor mutations in the *NT5E* gene which encodes CD73, a 5'-exonucleotidase. As a result, these patients are deficient in adenosine, associated with extensive calcification of the lower extremity arteries as well as hand and foot joints capsules [St. Hilaire et al., 2011]. Since the vascular pathology in ACDC has distinct

similarities with PXE, it has been suggested that adenosine may be a ligand for ABCC6, and its absence in peripheral tissues would explain the mineralization in PXE [Markello et al., 2011]. This hypothesis is currently being tested in the different model systems available for PXE.

Another factor potentially modifying the phenotype of PXE in patients may relate to tissue damage by free radical oxygen. This suggestion is based on the observations that patients with β -thalassemia and sickle cell anemia, conditions associated with oxidative stress, develop PXE-like cutaneous findings, yet no gene defect in the *ABCC6* gene has been found [Baccarani-Contrì et al., 2001; Hamlin et al., 2003]. Part of the explanation for this observation may come from recent demonstrations that a mouse model of β -thalassemia shows a liver-specific downregulation of *Abcc6* expression [Martin et al., 2011]. Thus, generalized oxidative imbalance could also lead to clinical manifestations in the classic form of PXE. This hypothesis was initially supported by observations that cultured fibroblasts derived from the skin of patients with PXE demonstrate changes in oxidative stress markers *in vitro*, and parameters of oxidative stress were also detected in the circulation of PXE [Garcia-Fernandez et al., 2008; Boraldi et al., 2009]. Subsequently, genetic variations in antioxidant genes have been suggested to be a risk factor for early disease onset in PXE and specifically, single-nucleotide polymorphisms in the genes encoding catalase, superoxide dismutase 2 and glutathione peroxidase 1 showed a correlation between the polymorphisms in these genes and the age of onset of PXE [Zarbock et al., 2007]. These genetic variants were shown to affect the activities of the corresponding antioxidant enzymes, suggesting a pathomechanistic role in modulating the PXE phenotype. In this context, it should be noted that single nucleotide polymorphism in association with phenotypic features of PXE have also been reported in other genes, including the genes for vascular endothelial growth factor and matrix metalloproteinase-2 [Zarbock et al., 2009, 2010], but their pathomechanistic significance has not been established.

Utilization of the *Abcc6*^{-/-} mouse model of PXE has confirmed the presence of chronic oxidative stress as reflected by reduced total antioxidant capacity and the presence of enhanced protein oxidation and lipid peroxidation markers [Li et al., 2008]. However, an antioxidant diet containing vitamins C and E, selenium and N-acetylcysteine, while counteracting the oxidative stress, did not modify the process of aberrant mineralization of connective tissues in these mice. Thus, these animal studies do not support the possibility that ingestion of antioxidants may be directly beneficial in counteracting the disease process in PXE, but carefully controlled clinical trials on patients with PXE could potentially provide an unequivocal answer. At the same time, it should be noted that the progression of the phenotype in patients with PXE often parallels the development of chronic, age-associated diseases, such as arteriosclerosis and age-associated macular degeneration, resulting in a combination of clinical manifestations. Thus, treatments suggested to be beneficial in ameliorating age-associated complications in general, such as antioxidants, could also be beneficial for patients with PXE.

An interesting observation, with potential pathomechanistic implications, is offered by patients with mutations in the *GGCX* gene, with the development of skin findings reminiscent of PXE [Vanakker et al., 2007; Li et al., 2009a]. The *GGCX* gene encodes a vitamin K dependent enzyme, γ -glutamyl carboxylase, which catalyzes γ -carboxylation of glutamyl residues in a number of proteins (Gla proteins), including several coagulation factors and a number of extracellular matrix proteins, such as MGP (Table 1) [Berkner, 2008]. The carboxylation reaction is required for activation of these proteins, and as a result of deficient γ -glutamyl carboxylation of clotting factors, these patients demonstrate vitamin K-dependent coagulation factor deficiency manifesting with a bleeding disorder. The action of γ -glutamyl carboxylase is dependent on reduced vitamin K as a cofactor [Berkner, 2008].

Based on the similarity of cutaneous findings in PXE and patients with *GGCX* mutations in both alleles, it has been suggested that the *ABCC6* deficiency in PXE patients may result in reduced concentrations of vitamin K or its derivatives in serum causing reduced activation of anti-mineralization proteins, such as MGP [Borst et al., 2008; Vanakker et al., 2010]. In fact, the ratio of active (carboxylated) compared to inactive (undercarboxylated) forms of MGP have been shown to be reduced in the tissues of patients with PXE and in PXE mouse model [Gheduzzi et al., 2007; Li et al., 2007], suggesting a critical role for vitamin K in pathogenesis of PXE. While this hypothesis is currently being tested in different model systems of PXE, it was first reported at this conference that feeding of *Abcc6*^{-/-} mice with high doses of vitamin K1 or K2 or systemic injection of vitamin K3-glutathione conjugate into these mice had no effect on the level of tissue mineralization [Jiang et al., 2011; Brampton et al., 2010]. The results in the mouse model therefore suggest that supplementation of the diet of PXE patients with vitamin K may not be effective and will require further study.

The key question to advance the translational investigation on PXE relates to the nature of the substrate molecules potentially transported by *ABCC6* from the liver to the circulation (Table II). Once the identity of such molecules has been deciphered, then more specific approaches to counteract the mineralization processes of this metabolic disorder can be developed. It should be noted that these postulates are based on the assumption that liver is the primary site of molecular pathology in PXE, and that the lack of expression of *ABCC6* in other tissues, such as the kidneys, does not play a pathogenic role in PXE. The primary role of liver vs. kidneys in the pathogenesis of PXE is currently being examined through experiments that aim at developing a transgenic *Abcc6*^{-/-} mouse in which *Abcc6* expression is restored selectively either in the liver or in the kidneys. Furthermore, microsurgical experiments allowing liver or kidney transplantation from wild-type mice to their *Abcc6*^{-/-} counterparts would help in deciphering the role of these organ systems. Finally, it should be noted that anecdotally, development of late-onset acquired PXE following liver transplantation has been noted in three patients [Bercovitch et al., 2011]. Identification of additional patients with liver or kidney transplantation from PXE donors with documented *ABCC6* mutations would provide further insight into the specific role of these organ systems in the pathogenesis of PXE.

TRANSLATIONAL RESEARCH ON PXE

Over the past few years, different approaches have been developed to counteract the manifestations of PXE at the preclinical level, primarily using the *Abcc6*^{-/-} mouse as a platform. At the same time, new pharmacological approaches have been adopted from the treatment of other diseases, such as those affecting the eyes. For example, recent reports on the use of intra-ocular injection of anti-angiogenic agents targeting vascular endothelial growth factor in age-associated macular degeneration have suggested that significant improvements in the visual acuity also in patients with PXE could be achieved [Finger et al., 2011]. Currently, clinical trials organized by National Eye Institute, National Institutes of Health, are ongoing to compare different anti-angiogenic compounds, with significant price differences, for their clinical efficacy, while new anti-angiogenic agents are being developed. In fact these anti-angiogenic agents appear to be so effective and they have largely replaced previously used laser photocoagulation and photodynamic therapy to counteract neovascularization of the retina in PXE patients.

As indicated above, the key challenge for development of effective and specific treatment modalities for PXE resides in identification of the transport substrate(s) for *ABCC6*. At the same time, it is recognized that clinical manifestations in PXE are primarily, if not exclusively, related to ectopic mineralization of peripheral tissues. Thus, anti-mineralization

approaches might provide a treatment for PXE. In this context, recent studies have been exploring the role of the mineral content of the diet in modifying the severity of the disease in PXE. These studies were originally based on early retrospective surveys which suggested that individuals with a history of high intake of dairy products, rich in calcium and phosphate, during adolescence developed more severe disease later in life [Neldner, 1988; Renie et al., 1984]. More recent, genetically controlled studies utilizing *Abcc6*^{-/-} mice as a model system have specifically shown that magnesium, but not calcium, content of the diet can influence the extent of ectopic mineralization in peripheral tissues [LaRusso et al., 2008, 2009; Li et al., 2009c; Gorgels et al., 2010]. Specifically, magnesium carbonate, when added to the mouse diet in amounts that increase the magnesium content by 5-fold over the standard diet, was able to completely prevent the mineralization noted in *Abcc6*^{-/-} mice. Conversely, an experimental diet with low magnesium was shown to accelerate the mineralization process [Li and Uitto, 2010]. In similar studies, increased content of calcium or phosphate in the mouse diet did not show significant changes in the degree of mineralization. While the mechanisms by which magnesium may prevent calcium/phosphate deposition in tissues are currently not clear, there was a marked increase in the urinary output of calcium with concomitant reduction in phosphate [LaRusso et al., 2009; Li et al., 2009c]. It is possible, therefore, that magnesium replaces calcium in the calcium/phosphate complexes, and since magnesium phosphate is more soluble than calcium phosphate in the corresponding concentrations, this may result in reduced or absent mineralization of tissues. In this context, it should be noted that the calcium content of the bones measured by chemical assays and bone density determined by micro computerized tomography scan did not show any long-term adverse effects on the bone as a result of extended increased magnesium intake [Li et al., 2009c]. Thus, these findings support the notion that changes in the diet, and specifically changes in dietary magnesium, might be helpful for treatment of patients with PXE. In this context, it should be noted that preliminary studies on oral phosphate binders have suggested improvement in the degree of skin manifestations of patients with PXE [Sherer et al., 2005]. More recently, a randomized, investigator-blinded, placebo-controlled clinical trial testing the efficacy of sevelamer hydrochloride, an agent that is widely used for hemodialysis with proven safety and efficacy in binding phosphate, has been concluded [Yoo et al., 2011]. This study enrolled 40 patients with PXE, randomly assigned to receive sevelamer or a placebo for one year. Skin examination and ophthalmic evaluation showed a significant improvement in patients administered the phosphate binder. Somewhat surprisingly, however, the patients assigned to the placebo group also showed clinical improvement essentially to the same degree as those receiving the sevelamer hydrochloride. The latter observation can be retrospectively explained by the fact that, unbeknownst to the investigators at the time of the trial, the magnesium content in placebo was 2.5 times higher than in the capsules containing the sevelamer hydrochloride (400 mg. vs. 180 mg, per capsule, respectively), again emphasizing the potential role of magnesium in modifying the degree of tissue mineralization. Thus, the verification of the data on the efficacy of phosphate binders in counteracting clinical manifestations of PXE requires further clinical studies with large number of patients with PXE in comparison to an appropriate placebo control group. Furthermore, a clinical trial exploring the effects of supplementary dietary magnesium on the progression of cutaneous and ocular signs in PXE is currently being developed (Mark Lebwohl, personal communication in this meeting).

There are a number of hurdles/obstacles that may hamper efficient design of clinical trials for PXE. A major impediment is the lack of knowledge of the precise pathomechanisms leading from the mutations in the *ABCC6* gene to the mineralization of the peripheral connective tissues. Specifically, the role of systemic vs. local factors in promoting mineralization, particularly towards identification of the *ABCC6* ligands of transport, requires more focused research. Some of the difficulties in establishing clinical trials specifically for PXE involve phenotypic heterogeneity and slow and unpredictable

progression of the disease that makes documentation of the effectiveness of the test compounds in humans difficult and extends the time period required for determination of an unequivocal improvement in the end points. This uncertainty reflects the fact that currently there are no established and validated surrogate biomarkers for PXE which would allow earlier determination of the efficacy of the treatment modalities.

MOLECULAR THERAPIES FOR PXE

Analogous to other heritable diseases, different molecular strategies could be envisioned for PXE, and many of these strategies could be adopted from the technological advances made in other fields (Table 2). In fact, the “diseasome” approach – taking advantage of cross-cutting mechanisms of diseases, such as those caused by mutations in other members of the ABC-family of genes – would be expected to benefit the PXE research. For example, one could envision molecular strategies that are based on the consequences of specific mutations identified in the *ABCC6* gene in patients with PXE. Among such strategies, development of drugs that facilitate read-through of premature termination codon-causing mutations in *ABCC6*, so as to allow synthesis of the full-length protein which should be structurally normal and functional, would potentially benefit many patients with PXE [Rowe and Clancy, 2009]. Such compounds, that have shown to be efficient in *in vitro* situations and are being tested in a limited number of patients with heritable diseases, include aminoglycosides and other small molecular weight compounds promoting efficient translation. Another mutation-specific approach would involve assisted trafficking of mistargeted and misfolded proteins by utilizing chaperones, modifiers of protein conformation, and transporter activators, approaches that have been developed for cystic fibrosis, a heritable disease caused by mutations in another ABC transporter gene, *CFTR*. It has been demonstrated that some missense mutations in the *ABCC6* gene result in retention of the protein in internal cellular compartments [Iliás et al., 2002], and treatment with modifiers of protein conformation, such as sodium 4-phenylbutyrate, a FDA approved drug, would potentially allow targeting of the protein to the plasma membrane [Prulioère-Escabasse et al., 2007]. In this meeting, it was reported that selected mutant *ABCC6* proteins harboring missense mutations, such as p.R1314W, showed initially a problem in intracellular trafficking leading to incorrect localization, as determined *in vitro* in the Sf9 and MDCKII cell systems and in an *in vivo* liver-targeted system in mouse [Pomozi et al., 2010]. However, exposure to sodium 4-phenylbutyrate restored the plasma membrane targeting with restoration of substantial transport activity. These data suggest, therefore, that intracellular trafficking of functionally active but misdirected *ABCC6* mutants, such as p.R1314W, can be pharmacologically corrected by sodium 4-phenylbutyrate. In case of some mutations, the protein is appropriately targeted to the plasma membrane, but does not function efficiently as a transporter molecule. The paradigm of the development of such treatment approaches is also provided by cystic fibrosis, in which different small molecular weight proteins have been proposed to activate the cystic fibrosis transmembrane conductance regulator gene [Accurso et al., 2010]. Furthermore, examination of the consequences of mutations in other heritable diseases, such as epidermolysis bullosa, have suggested that some splice-site mutations are “leaky” allowing a low level of baseline expression of the functional, fully spliced protein [Uitto et al., 2010]. If such is the case with *ABCC6* mutations as well, one could envision that up-regulation of the corresponding allele by cytokines, transcription factors, and small molecular weight compounds that can be screened in large-scale fluorescence-based assay systems, would be beneficial. Towards this aim, better understanding of the regulatory factors and the promoter region polymorphisms in *ABCC6* would be of help in upregulating the expression of this gene [Jiang et al., 2006; Ratajowski et al., 2009; Váradi et al., 2010]. In this context, it is not clear as to how much normal *ABCC6* expression is required to overcome the mineralization phenotype in PXE. It is clear that 50% of expression compared to normal level is sufficient, since heterozygous

carriers by and large do not show any clinical manifestations. Development of new animal models that would allow titration of the level of *Abcc6* expression, such as inducible on-off model systems, would be helpful in this regard.

In addition to mutation based molecular approaches, gene therapy approaches targeting a transgene to the liver could potentially be beneficial. This could include expression of wild-type *Abcc6* restoring the function of this transmembrane transporter. Several problems inherent to the gene therapy approaches in case of PXE revolve around the ability to target the expression to the liver with subsequent insertion of the protein to the baso-lateral surface in topographically correct orientation. Furthermore, issues relating to the sustained and durable expression of the transgene, the type of delivery of the gene for permanent expression (integrating retroviral vectors), and potential development of antibodies to the newly introduced protein in patients with premature termination codon mutations pose formidable challenges. In addition to expression of the *ABCC6* gene, one could envision over-expression of anti-mineralization factors, such as fetuin-A and MGP. While the anti-mineralization protein, fetuin-A, is expressed primarily in the liver, one could express this gene in extrahepatic tissues, perhaps with easier targeting that would allow expedient secretion of this protein to the circulation.

Another molecular approach would involve cell-based therapies that have been advanced for a number of heritable disorders [Uitto et al., 2010]. This approach could include transfer of allogeneic stem cells derived from bone marrow or from umbilical cord, although delivery of stem cells to the liver requires special surgical considerations. One could also envision allogeneic hepatocyte transplantation as a way to correct the deficiency of *ABCC6* in patients with PXE. It is apparent, however, that in order to externally introduce hepatocytes to the liver, a regenerative process elicited either by partial hepatectomy or by chemical means is required. Finally, utilization of induced Pluripotent Stem (iPS) cells would provide an autologous source of cells, for example from skin fibroblasts, that would then be allowed to differentiate into hepatoblasts for delivery of such cells to the hepatic system [Inamura et al., 2011]. Bone marrow transfer has recently been shown to provide a source of stem cells that enter the circulation and then home to the tissues in need of repair [Chino et al., 2008]. The utility of this approach has been assisted by identification of a number of factors that facilitate the homing of bone marrow derived stem cells to damaged tissue. However, in the case of PXE, a major question is whether such bone marrow derived cells, upon bone marrow transplantation, would home to the liver since there is no gross pathology that would require tissue repair apparent in PXE. In this context, identification of homing factors and their ligands on the surface of the stem cells directing their trafficking to the liver might facilitate the application of this approach.

CONCLUSIONS

PXE is a complex heritable disorder at the genome/environment interface. The penetrance of the disease in individuals with mutations in both *ABCC6* alleles appears to be complete, but the severity of the phenotype and the age of onset of the disease are likely modulated by the genetic background, epigenetic factors, diet, and lifestyle variables [Neldner, 1988; Uitto et al., 2010]. Significant progress has been made in understanding the molecular genetics of PXE, and several promising avenues for treatment at the pre-clinical stage are currently being explored, primarily using mouse model systems as platforms. Promising organ-specific approaches are already in clinical use, as exemplified by the use of VEGF antagonists to counteract neovascularization in the eye, with encouraging clinical outcomes. Preliminary clinical studies have also attested to the potential efficacy of phosphate binders in treatment of PXE, and clinical trials are currently being planned to prevent mineralization by supplementation of the diet with high levels of magnesium, an approach that has been

shown to be effective in counteracting the mineralization in mouse models of PXE. However, development of global, pathophysiology related approaches are dependent on further research, including the identification of molecules transported by ABCC6 and establishment of the liver as the site of molecular pathology critical for the development of PXE phenotypes. Even before global molecular strategies can be brought to the clinical arena, however, a combination of empiric therapies might improve the quality of life for patients with PXE.

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Figure 1. Schematic illustration of the proposed “metabolic hypothesis” of PXE

Under physiologic conditions, the ABCC6 protein is expressed at high levels on the basolateral surface of the liver where it facilitates the transport of currently uncharacterized molecule(s) from hepatocytes to the circulation. These molecules are postulated to serve physiologically as potent anti-mineralization factors which under normal Ca/P homeostasis prevent precipitation of metastable calcium/phosphate complexes in the peripheral tissues. In the absence of ABCC6 transporter activity in the liver in PXE, the concentration of the anti-mineralization factors in circulation and consequently in peripheral tissues is reduced, allowing mineralization of connective tissues to ensue. The presence of mineral deposits in the eye, blood vessels, kidney and skin (left panels) is demonstrated by histopathological staining (Alizarin Red) of tissues from *Abcc6*^{-/-} mice which recapitulate features of PXE in humans. (Reproduced from Uitto et al., 2010).

Table I

Anti-Mineralization Factor with a Potential Role in the Pathogenesis and/or Treatment of Pseudoxanthoma Elasticum

-
- Fetuin-A
 - Matrix Gla protein
 - Osteocalcin
 - Transmembrane Gla proteins (TMG1-4)
 - Periostin
 - Gla-rich protein
 - Ankylosis protein
-

* Those proteins marked with an asterisk are known to undergo vitamin K-dependent γ -glutamyl carboxylation [Berkner, 2008]

Table II**Outstanding Research Questions on Pseudoxanthoma Elasticum**

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- What is the nature of molecule(s) transported by ABCC6, a transmembrane efflux transporter expressed primarily in the liver, kidneys and the intestine?
 - Is liver the site of molecular pathology in PXE?
 - What is the role of genetic modifier genes, epigenetics, and environmental factors in modulating the disease phenotype?
 - Can manipulation of diet (*e.g.*, increased magnesium content) lessen the degree of tissue mineralization, with clinical improvement?
 - Do phosphate binders counteract the mineralization processes?
 - Does vitamin K deficiency play a role in PXE?
 - Could adenosine deficiency contribute to the vascular mineralization in PXE?
 - Is development of mutation-specific, ABCC6 targeted therapies feasible?
 - Does development of DNA-, protein-, and cell-based therapies provide novel avenues of treatment for PXE?
-