DR LOUKMAN OMARJEE (Orcid ID : 0000-0001-7766-396X)

NASTASSIA NAVASIOLAVA (Orcid ID : 0000-0002-6405-6498)

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Severe Early-Onset Manifestations of Pseudoxanthoma Elasticum Resulting from the Cumulative Effects of Several Deleterious Mutations in the *ENPP1*, *ABCC6* and *HBB* Genes: Transient Improvement in Ectopic Calcification with Sodium Thiosulfate

L. Omarjee[1,2,3,4,5]; Y. Nitschke[6]; S. Verschuere[7]; E. Bourrat[8]; M.-D. Vignon[9]; N. Navasiolava[1,2]; G. Leftheriotis[10]; G. Kauffenstein[1]; F. Rutsch[6], O. M. Vanakker[7]; L. Martin[1,2.]

1: MitoVasc Institute, UMR CNRS 6015 / INSERM 1083 49100 Angers, France

2: PXE Reference Centre (MAGEC Nord), University Hospital of Angers, Angers, France

3: Univ Rennes, CHU Rennes, INSERM CIC1414, Vascular Medicine Unit, Rennes, France

4: PXE Vascular Consultation Centre, CHU Rennes, 35000 Rennes, France

5: Vascular Medicine Unit, Redon Hospital, 8 Rue Etienne Gascon, 35600 Redon, France

6: Muenster University Children's Hospital, Muenster, Germany

7: Centre for Medical Genetics, Ghent University Hospital, Ghent, Belgium

8: Department of Paediatrics, APHP, Robert Debré Hospital, Paris, France

9: Department of Pathology, Saint-Louis Hospital, Paris, France

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10: Department of Physiology and Vascular Investigation, Nice University Hospital, France

Correspondence to:

Dr Loukman OMARJEE, MD-PhD
Vascular Physician and Clinical Immunologist
PXE Vascular Consultation Centre
Department of Vascular Medicine and Investigation
Pôle imagerie médicale et explorations fonctionnelles
Hôpital Pontchaillou – Rennes University Hospital
2 rue Henri Le Guilloux Rennes, F-35033. France.
Contact Phone Number: +33 (0) 2 99 28 43 21
Contact Mobile Number: +33 (0) 6 27 49 70 51
Contact Email: loukmano@yahoo.fr; Twitter: @LoukmanOmarjee

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ABSTRACT

Pseudoxanthoma Elasticum (PXE) is a rare disorder characterized by fragmentation and progressive calcification of elastic fibres in connective tissues. Overlap has been reported between the inherited PXE phenotype associated with *ENPP1*, *ABCC6* or *NT5E* mutations and acquired PXE clinical manifestations associated with haemoglobinopathies induced by *HBB* mutations. No treatment is available for PXE to date. A young boy presented with severe early-onset systemic calcifications occurring in the skin as elastosis perforans serpiginosa (EPS) and in the arteries causing mesenteric and limb ischemia. Molecular and functional analyses revealed deleterious *ABCC6, ENPP1* and *HBB* mutations. The diagnosis of severe PXE was retained and we coined the term of "PXE+ syndrome" to report the cumulative effects of the various mutations in this uncommon phenotype. Given severity, rapid progression and a potentially fatal prognosis, intravenous sodium thiosulfate (STS) was initiated at 25g 3-times/week for six months. Numerous side effects prompted dosage adjustment to 10g IV/day. Treatment efficacy was evaluated at 6

months. Asthenia, anorexia and pre/post prandial pain had completely subsided entailing weight gain. Abdominal EPS had diminished. Calcific stenosis of the celiac and mesenteric arteries was no longer detectable on arterial ultrasonography. Follow-up however revealed only transient efficacy of STS. Discontinuation of treatment to evaluate persistence of effects resulted in relapse of initial symptomatology after four months. STS efficacy is conceivably due to strong antioxidant properties and chelation of calcium to form soluble calcium thiosulfate complexes. This case is suggestive of PXE+ syndrome for which STS may represent potential treatment in severe cases.

What is already known about this topic?

- Generalized arterial calcification of infancy (GACI) may occur in association with *ABCC6* mutations and pseudoxanthoma elasticum (PXE) can be linked to *ENPP1* mutations.
- A PXE-like phenotype has also been reported in a subset of patients with inherited hemoglobinopathies, namely sickle cell disease or β-thalassemia related to beta-globin (*HBB*) gene mutations.
- To date there is still no cure for PXE.

What does this study contribute?

- We report a severe case of PXE resulting from the cumulative effects of several deleterious mutations in the *ENPP1*, *ABCC6* and *HBB* genes. We suggest to coin the term "PXE+ syndrome" to qualify such patients.
- Sodium thiosulfate therapy could represent a potential option in severe cases of PXE+ syndrome.

INTRODUCTION

Pseudoxanthoma Elasticum (PXE, OMIM #264800) is an autosomal recessive metabolic disorder characterized by the fragmentation and progressive calcification of elastic fibres in connective tissues such as the skin, vascular system and Bruch's membrane of the retina[1]. PXE is caused by mutations in the *ABCC6* gene which encodes a transmembrane ATP-binding cassette (ABC) transporter primarily expressed in the liver and kidney[2]. ABCC6 endogenous substrates are unknown but pyrophosphate (PPi) deficiency reliably accounts for remote calcification[3,4]. Typical PXE manifestations include yellowish papules on the neck and in large skin folds, loss of central vision and cardiovascular complications such as peripheral artery disease (PAD)[1]. Mutations in *ENPP1*, encoding ectonucleotide pyrophosphatase/phosphodiesterase 1 (NPP1), were found to cause generalized arterial calcification of infancy (GACI, OMIM #208000)[5].

In this disorder, calcification in the media of large- and medium-sized arteries is associated with intimal proliferation leading to arterial stenosis. Phenotype and genotype overlapping between GACI and PXE has been reported[6]. Recently, a phenotype resembling arterial calcification due to deficiency of CD73 (ACDC, OMIM #211800) commonly linked to ecto-5'-nucleotidase (*NT5E)* mutations was reported in a carrier of two *ABCC6* mutations[7]. A PXE-like phenotype has also been reported in a subset of patients with inherited hemoglobinopathies such as sickle cell disease (SCD, OMIM #603903)[8] or β-thalassemia (OMIM #613985)[9] related to beta-globin (*HBB*) gene mutations[9].

To date a cure for PXE has yet to be found. In the present case report, we illustrate improvement following sodium thiosulfate treatment (STS) in the condition of a young PXE boy with a complex genotype resulting in severe early-onset systemic calcifications.

CASE REPORT

An 11-year-old boy from sub-Saharan Africa was referred for management of symptomatic PAD, retinal angioid streaks and cutaneous lesions. On admission, symptomatology included asthenia, anorexia, weight loss of 10 kg, intermittent claudication and pre/postprandial abdominal pain presumably due to mesenteric angina. Examination revealed widespread yellowish papules in the large folds and papular lesions in the abdominal area suggestive of elastosis perforans serpiginosa (Figure 1; Panels 1&2). Histological analysis of skin biopsy showed short curled elastic fibres in the reticular dermis (Figure 2; Panels 1&2) and calcified elastic fibres on Von Kossa staining (Figure 2; Panel 3). Fundus examination revealed "peau d'orange" and peripheral angioid streaks with no haemorrhagic complications. Ankle-brachial index was <0.6 (N=0.9-1.3) confirming the diagnosis of PAD. Arterial ultrasonography revealed widespread medial calcific sclerosis extending from the abdominal aorta to the distal lower limb arteries with severe (>70%) stenosis in the celiac, superior mesenteric and superficial femoral arteries (Figure 5; Panels 1&2). Blood tests yielded no acute inflammation (hsCRP<1mg/l) and normal phosphorus/calcium levels. Non-iron deficient microcytic anemia was detected and haemoglobin electrophoresis revealed 40% haemoglobin S; 3.3% haemoglobin A2; and 56.7% haemoglobin A0 consistent with heterozygous sickle-cell disease, which was molecularly confirmed by one heterozygous mutation in the *HBB* gene p.(Glu7Val).

Molecular analysis of the *ABCC6* gene (direct sequencing of the complete coding region and the intron-exon boundaries, coupled with MLPA analysis) revealed the heterozygous pathogenic nonsense variant p.(Arg1141*);c.3421C>T. Direct sequencing of the coding region of the other PXE-related genes (*ENPP1, GGCX)* demonstrated two previously unreported biallelic heterozygous *ENPP1* missense variants: p.(Glu866Lys);c.2596G>A and p.(Cys711Tyr);c.2132G>A. Segregation analysis of *ABCC6* and *ENPP1* variants in the mother confirmed she carried only the *ENPP1* p.(Cys711Tyr);c.2132G>A variant. The father was unwilling to participate in the segregation analysis. To determine the functional impact of the missense variants in *ENPP1*[10], we transfected a pcDNA3.1(+) expression vector harbouring the mutated full-length *ENPP1* coding sequence into the human embryonic kidney (HEK293) cells and measured NPP activity (Figure 3; Panel 1). Cys711Tyr completely abolished NPP activity, whereas Glu866Lys showed residual but significantly reduced NPP activity to about 60% of that associated with WT *ENPP1* when transfected into HEK293 cells (Figure 3; Panel 1, p<0.0001 vs.

WT ENPP1). Both mutant proteins were expressed similarly to the WT protein (Figure 1; Panel 2). We tested whether extracellular PPi generation was affected by the variants in *ENPP1*. PPi levels in the medium of HEK293 cells transfected with WT *ENPP1* increased rapidly over time (Figure 3; Panel 3). In the medium of HEK293 cells expressing the Cys711Tyr and Glu866Lys mutants, PPi levels were not different from the PPi levels in the medium of HEK293 cells transfected with the empty vector ($p>0.3$ vs. empty vector, $p<0.005$ vs. WT ENPP1, after 20 min) (Figure 3; Panel 3). Each mutant was individually expressed in Cercopithecus aethiops kidney (COS7) cells. WT *ENPP1* was found predominantly in the plasma membrane (Figure 4). The *ENPP1* mutant Cys711Tyr when expressed in COS7 cells demonstrated intracellular localization instead of WT-like cellular localization in the plasma membrane (Figure 4). The Glu866Lys mutant fully localized to the plasma membrane in the same manner as WT *ENPP1* (Figure 4).

Given the severity of the phenotype, its rapid progression and an extremely unfavourable, potentially fatal prognosis, intravenous (IV) STS was initiated at a dose of 25g 3-times/week for six months, resulting in impaired quality of life with numerous side effects including metabolic acidosis, vomiting and dizziness[7]. This prompted dosage adjustment to 10g IV/day[8]. Efficacy of STS was evaluated at six-month follow-up. Infiltration of the papular lesions on the abdomen had diminished (Figure 1; Panel3). Asthenia, anorexia and pre/post prandial pain had completely subsided and the patient had gained weight (+10kgs). On arterial ultrasonography, calcific stenosis of the celiac and mesenteric arteries had disappeared (Figure 5; Panels 3&4). Discontinuation of treatment to evaluate persistence of effects resulted in exacerbation of symptoms such as digestive angina, lower limb claudication and relapse of abdominal skin changes after four months. STS was resumed.

DISCUSSION

According to 2014 criteria[11], PXE diagnosis requires either two pathogenic *ABCC6* mutations or a combination of ocular findings (retinal angioid streaks > 1 Disc Diameter (DD) or “peau d'orange” in individuals aged <20) together with characteristic skin findings (pseudoxanthomatous papules/plaques on the neck or in flexural creases or diagnostic histopathological changes in lesional skin)[11]. In the present case report, although clinical diagnosis criteria for PXE were met, molecular analysis revealed a single heterozygous nonsense variant in the *ABCC6* gene in addition to two heterozygous missense variants in the *ENPP1* gene, and one heterozygous variant in the

HBB gene. We confirmed that both Cys711Tyr and Glu866Lys variants led to loss-of-function in the NPP1 protein, most likely due to impaired stability and/or conformation[5,10]. Moreover, our patient had SCT. A link between β-thalassemia or sickle cell disease (SCD) and PXE was first reported in 1964 and 1989, respectively[9]. The pathophysiology is still poorly understood[8,9,12,13]. Although histological changes similar to those found in PXE have been demonstrated in patients with β-thalassemia, studies have failed to reveal any abnormality in the *ABCC6* gene in these patients, strongly implying that it is an acquired rather than inherited form of PXE[14]. This was demonstrated in a mouse model of β-thalassemia where a liver-specific down-regulation of Abcc6 expression was observed[15]. The most widely accepted hypothesis revolves around oxidative damage to elastic tissue, while other theories evoking neutrophil elastase activity-related elastic degradation are less substantiated[9,16]. suggests that all genes producing PXE-like manifestations should be screened in patients with SCD, β-thalassemia and PXE findings[8]. We speculate that the severe early-onset PXE phenotype of our patient could result from the cumulative effects of several deleterious mutations in the *ENPP1*, *ABCC6* and *HBB* genes and as a result we would like to coin the term "PXE+ Syndrome" to better individualize this condition.

STS proved to be transiently effective in improving calcified cutaneous and vascular lesions in our PXE+ phenotype patient. STS therapy was adopted by virtue of its similarity to uremic and non-uremic calciphylaxis[7,13]. STS chelates divalent cations and possesses antioxidant and vasodilatory properties[7,13,14]. Its chelating action has been used in non-uremic patients with renal lithiasis to increase the solubility of deposits or calcium crystals, or to dissolve ectopic calcifications in blood vessels[13]. STS is also a reducing agent, thus enabling regeneration of the oxidized glutathione involved in cellular detoxification mechanisms. Moreover, chronic ischemia induces reactive oxygen species (ROS) and causes depletion of tetrahydrobiopterin, an essential cofactor in nitric oxide (NO) synthesis[17]. Consequently, STS restores endothelial function, NO production and enables vasodilation. It is believed to increase calcium phosphate solubility in soluble thiosulphate complexes[7]. This chelating effect of calcium appears to have longer action time of up to a month[14], as confirmed by cutaneous/vascular findings from our case report at 6-months post STS therapy. With respect to the multi-factorial positive effects observed in terms of calcium chelation, oxidative-stress and vasodilation, and despite the complexity of its treatment protocol and its numerous side-effects, STS could represent a viable therapeutic option in such severe cases of PXE.

Ethics Approval and Consent to Participate

All the procedures in this report have been approved by the ethics committee and were performed in accordance with the guidelines of the Helsinki Declaration of 2008.

Consent for Publication

Written consent to publish was obtained from the parents of this underage patient.

Availability of Data and Materials

Data sharing does not apply to this manuscript as no datasets were generated or analysed in the current investigation.

Conflict of Interest Disclosures

All authors declare that they have nothing to disclose.

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Reprint Permission

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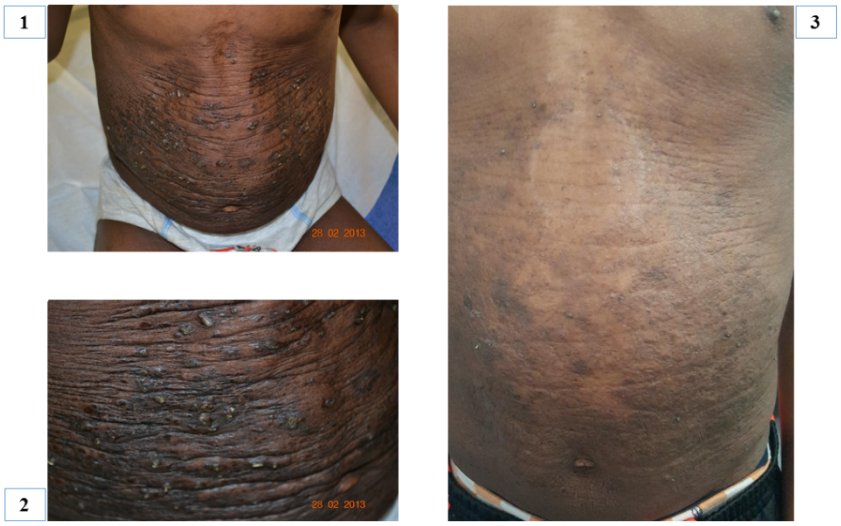

Figure 1: Skin involvement with yellowish papules, perforating and acneiform lesions in the abdominal area before sodium thiosulfate (STS) therapy (Panel 1&2). Radical improvement in papular lesions of the anterior abdomen area, less infiltrated than in prior perforating lesions and less acneiform after 6 months of STS therapy (Panel 3).

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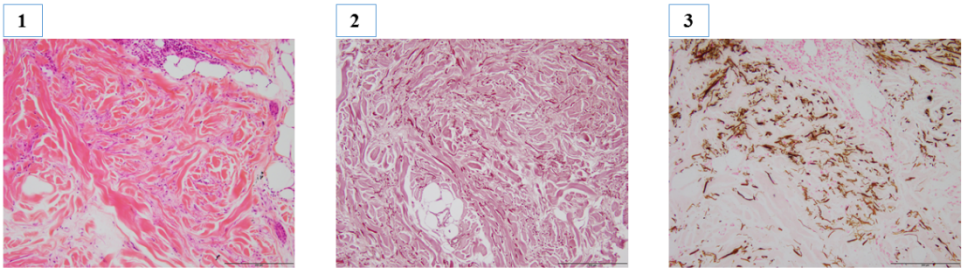

Figure 2: Collagen fibers in the reticular dermis (Hematoxylin-eosin-saffron (HES) stain x200) (Panel 1); Short curled elastic fibers in the reticular dermis (Orcein stain x200) (Panel 2). Calcium salts deposited on the abnormal elastic fibers (von Kossa stain x200) (Panel 3).

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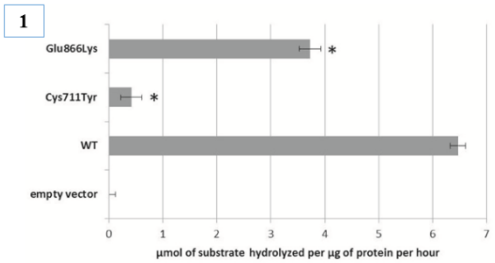

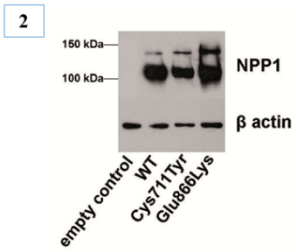

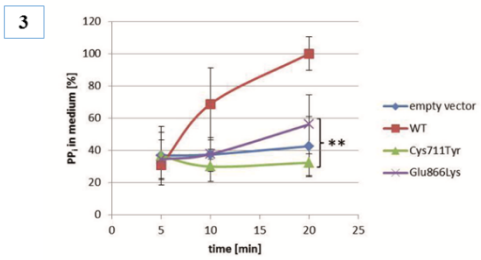

Figure 3: Biochemical characterization of ENPP1 enzyme activity (Panel 1), expression (Panel 2) and extracellular PPi generation (Panel 3). Expression of NPP activity in HEK 293 cells transfected with empty vector, WT ENPP1, and the mutants Cys711Tyr and Glu866Lys. Data represent the mean ± standard error of four independent experiments; *p<0.0001 vs. WT (Panel 1). Western-Blot of ENPP1 and β-actin. ENPP1 monomers are known to migrate as doublets at 118 kDa and 128 kDa on SDS PAGE (Panel 2). Extracellular PPi generation in medium of COS7 cells transfected with empty vector, WT ENPP1, and the mutants Cys711Tyr and Glu866Lys. Data represent the mean ± standard error of four independent experiments; *p<0.005 vs. WT (Panel 3).

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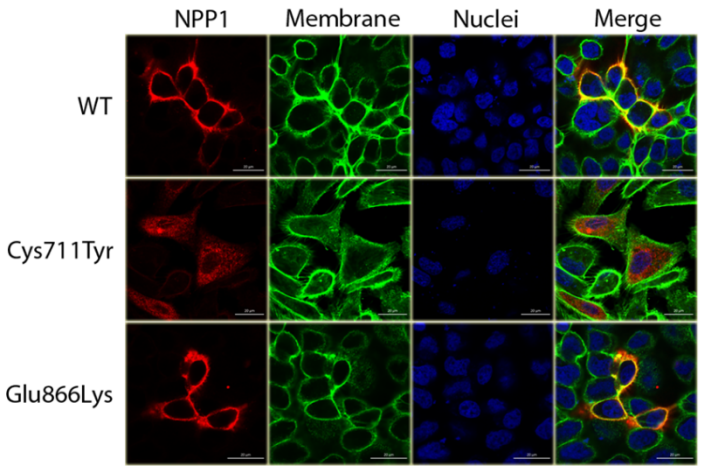

Figure 4: Cellular localization of WT ENPP1 and the mutants Cys711Tyr and Glu866Lys. COS7 cells expressing WT ENPP1 or the mutants Cys711Tyr and Glu866Lys were analyzed by double immunofluorescence. First column: staining with the 3E8 antibody to ENPP1 (red). Second column: staining with phalloidin for plasma membrane (green). Third column: DAPI staining for nuclei (blue). Fourth column: merged images. Co-localization of ENPP1 and plasma membrane was confirmed by yellow staining.

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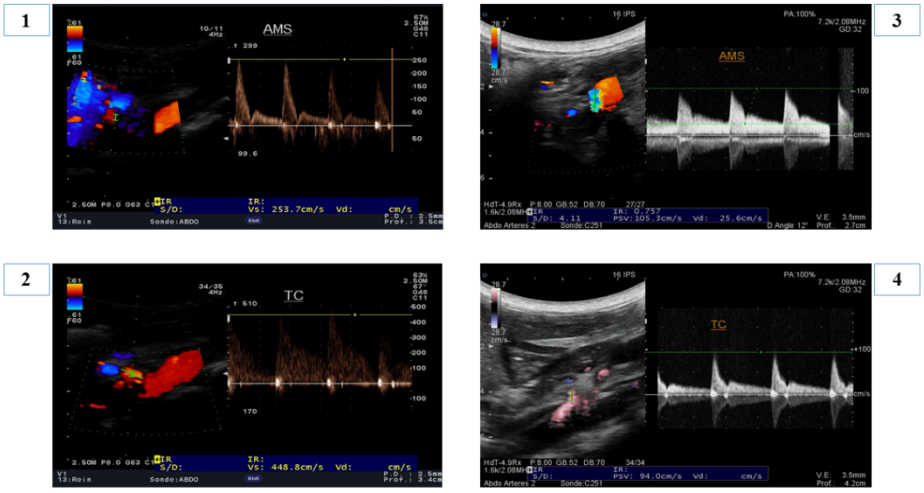

Figure 5: Hemodynamically significant severe arterial stenosis >70% due to ectopic vascular calcifications in superior mesenteric (Panel 1) and celiac arteries (Panel 2) before STS therapy. Ectopic vascular calcification reversal entailing radical improvement in hemodynamically arterial stenosis in superior mesenteric (Panel 3) and celiac arteries (Panel 4) after 6 months of STS therapy.

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