Nonlinear microscopy for the visualization of calcification and assessment of connective tissue fibers in pseudoxanthoma elasticum

N Kiss^{1,2}, L Fésüs^{1,2}, F Szeri³, T Arányi³, M Van Gils⁴, O Vanakker⁴, L Martin⁵, R Szipőcs², NM Wikonkál¹ and M Medvecz¹

¹ Department of Dermatology, Venereology and Dermatocology, Semmelweis University, Budapest, Hungary
² Wigner RCP, Institute for Solid State Physics and Optics, Hungarian Academy of Sciences, Budapest, Hungary
³ Research Center for Natural Sciences, Institute of Enzymology, Hungarian Academy of Sciences, Budapest, Hungary
⁴ Center for Medical Genetics, Ghent University Hospital, Ghent, Belgium
⁵ PXE Reference Center, Angers University Hospital, Angers, France

1. Introduction

Pseudoxanthoma elasticum (PXE, OMIM#264800) is a rare genetic disorder caused by mutations of the *ABCC6* gene in which decreased plasma levels of inorganic pyrophosphate (PPI) lead to ectopic mineralization resulting in severe dermatological, ophthalmologic and cardiovascular complications [1]. Though PXE skin lesions can be identified clinically in most cases, skin biopsy and/or genetic analysis are required to confirm the diagnosis [2]. Components of the dermal connective tissue can be differentiated with nonlinear optical (NLO) techniques, as collagen emits a strong second harmonic generation (SHG) signal given its non-centrosymmetric structure, while elastin as an endogenous chromophore can be visualized by two-photon excitation fluorescence (TPF) [3] [4].

2. Materials and methods

Three female and two male PXE patients with a mean age of 53.8±13.1 years were included in this study. We carried out deep phenotyping, molecular genetic testing and plasma PPI measurement of five patients with PXE [5]. *Ex vivo* SHG and TPF imaging and conventional histopathological evaluation of their skin was performed and compared to five age- and gender-matched healthy controls. The generated TPF signal is mainly emitted by elastin in the dermis, while SHG displays the collagen structure. Unpaired, two-tailed Student's *t*-test, Mann Whitney's U-test and linear regression were used for statistical analysis using GraphPad Prism v6.0 software (**p*<0.05), as applicable.

3. Results

Phenodex scores can be found in Table 1. Pathological mutations were found in the *ABCC6* gene in all patients (Table 2.) Plasma PPI levels of PXE patients were significantly lower compared to controls (*p*=0.0005, Mean±SD: 0.232±0.063 μM vs 0.947±0.108 μM, respectively) (Fig. 1). Papillary dermis and deep layers of the dermis appeared to be unaffected in all patients. However, the mid-dermis of PXE patients exhibited prominent changes (Fig. 2.) We visualized and quantified the degradation and mineralization of elastin fibers and extensive mid-dermal calcium salt deposition as well as the alterations of collagen structure in the mid-dermis of patients with PXE utilizing TPF and SHG, respectively (Fig. 3 and 4.)

Fig. 1. PPI levels of our PXE patients compared to age- and gender-matched healthy controls. Blood samples were collected and platelet-free plasma was prepared. (a) Plasma PPI levels of all included patients were determined by first converting PPI to ATP, then measuring ATP content in a bioluminescent assay. PPI concentration of plasma was calculated based on calibration standards and corrected for initial plasma ATP concentration. (b) Plasma PPI levels in PXE vs control patients.

Table 1. Demographic data and Phenodex scores according to Legrand *et al.* of our patients with PXE.

Pt. No.	Sex	Age (y)	Skin	Eye	Gastrointestinal	Vascular	Cardiac	Renal
1	F	52	S2	E3	G0	V1	C0	R0
2	F	52	S2	E3	G0	V1	C1	R0
3	M	34	S2	E2	G0	V0	C0	R0
4	M	68	S3	E2	G0	V0	C0	R0
5	F	63	S3	E3	G0	V1	C0	R0

5. References

- [1] Favre G, Laurain A, Arányi T, Szeri F, Fulop K, Le Saux O, et al. The *ABCC6* Transporter: A New Player in Biomineralization. International journal of molecular sciences 2017;18(9).
- [2] Hosen MJ, Lamoën A, De Paeppe A, Vanakker OM. Histopathology of pseudoxanthoma elasticum and related disorders: histological hallmarks and diagnostic clues. Scientifica 2012;2012:598262.
- [3] Kiss N, Haluszka D, Lorincz K, Gyongyosi N, Bozsanyi S, Barvolgyi A, et al. Quantitative Analysis on *Ex Vivo* Nonlinear Microscopy Images of Basal Cell Carcinoma Samples in Comparison to Healthy Skin. Pathol Oncol Res 2019;25(3):1015-1021
- [4] Kiss N, Haluszka D, Lorincz K, Kuroli E, Harsing J, Mayer B, et al. *Ex vivo* nonlinear microscopy imaging of Ehlers-Danlos syndrome-affected skin. Arch Dermatol Res 2018;310(5):463-73.
- [5] Jansen RS, Duijst S, Mahakena S, Sommer D, Szeri F, Varadi A, et al. *ABCC6*-mediated ATP secretion by the liver is the main source of the mineralization inhibitor inorganic pyrophosphate in the systemic circulation-brief report. Arterioscler Thromb Vasc Biol 2014;34(9)

Figure 2. Representative histology images of the mid-dermis of healthy and PXE-affected skin, stained with hematoxylin and eosin (H&E), Weigert's elastic (WE), von Kossa (VK) and Van Gieson's (VG) stainings. Scale bars display 200 μm.

Figure 3. TPF and SHG images of PXE-affected and healthy skin. A 525/50 nm band pass filter was used to separate TPF signal and a 405/20nm filter for the SHG signal. 420x420 μm² field of views were captured and assembled into (a, b) mosaic images of skin samples with 300x300 μm² high zoom images of the dermis (α,β,γ,δ). (a) Control skin, (b) skin of Patient 3. Representative high zoom inserts are marked with of the mid-dermis (c-j). (c,d) Patient 1, (e,f) Patient 2, (g,h) Patient 4, (i,j) Patient 5. Scale bars display 500 μm for mosaic images and 100 μm for high zoom images.

Figure 4. Extent of calcification and amount of elastin fibers in TPF images of the dermis of PXE patients and healthy. Number (a) and length (b) of elastin fibers in PXE vs control patients were measured following manual labeling and free-hand selection in every field of view (FOV). Relative calcification surface area (c) in each PXE patient was measured after manual outlining of the calcified areas using ImageJ software (NIH, USA). Right panels show representative TPF FOVs of PXE for each parameter. Size of the images: 420 x 420 μm².

Figure 5. Results of CT-FIRE analysis of SHG images of collagen in the dermis of PXE-affected and control skin. (a,b) Collagen fiber length (a) and width (b) were compared using Student's *t*-test after customized CT-FIRE analysis of raw SHG images. (c) Representative images of CT-FIRE analysis of Patient 1 and a healthy control. The size of the images is 420 x 420 μm².

Table 2. Molecular genetic data of the *ABCC6* gene in our PXE patients.

PXE patient No.	Allele 1	Allele 2
1	c.3421C>T ^a	c.1132C>T ^a
2	c.1552C>T ^a	c.3662G>A ^b
3	c.105delA ^a	c.3421C>T ^a
4	c.1944-1G>C ^d	exon 24-27del ^c
5	c.3421C>T ^a	c.1484T>A ^b

4. Discussion

The occurrence of severe complications among PXE patients highlights the importance of early diagnosis, so that prompt multidisciplinary care can be provided. Based on our data, NLO enables high-detail imaging and quantification of the specific features of PXE-affected skin. *In vivo* NLO may be a promising method in the assessment of PXE in the future, facilitating early diagnosis and monitoring of the disease.