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Abstract: A number of beta-thalassemia (b-thal) patients in the course of the disease exhibit ectopic calcification affecting skin, eyes and the cardiovascular system. Clinical and histopathological features have been described similar to those in pseudoxanthoma elasticum (PXE), although different genes are affected in the two diseases. Cultured dermal fibroblasts from b-thal patients with and without PXE-like clinical manifestations have been compared for parameters of redox balance and for the expression of proteins, which have been already associated with the pathologic mineralization of soft connective tissues. Even though oxidative stress is a well-known condition of b-thal patients, our results indicate that the occurrence of mineralized elastin is associated with a more pronounced redox disequilibrium, as demonstrated by the intracellular increase of anion superoxide and of oxidized proteins and lipids. Moreover, fibroblasts from b-thal PXE-like patients are characterized by decreased availability of carboxylated matrix Gla protein (MGP), as well as by altered expression of proteins involved in the vitamin K-dependent carboxylation process. Results demonstrate that elastic fibre calcification is promoted when redox balance threshold levels are exceeded and the vitamin K-dependent carboxylation process is affected decreasing the activity of MGP, a well-known inhibitor of ectopic calcification. Furthermore, independently from the primary gene defect, these pathways are similarly involved in fibroblasts from PXE and from b-thal PXE-like patients as well as in other diseases leading to ectopic calcification, thus suggesting that can be used as markers of pathologic mineralization.

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*Highlights (for review)

Highlights

- Fibroblasts from β-thal patients with/without ectopic calcifications are studied
- Oxidative stress (OS) is higher in cells from patients with ectopic calcifications
- Matrix Gla Protein has a specific localization on mineralized elastic fibers
- Carboxylated MGP is reduced in cells from patients with ectopic calcifications
- High OS and low cMGP can be potential markers of ectopic calcification

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Ectopic calcification in β-thalassemia patients is associated with increased oxidative stress and lower MGP carboxylation.

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running head: ectopic calcification in beta-thalassemia

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Abstract

A number of beta-thalassemia (β-thal) patients in the course of the disease exhibit ectopic calcification affecting skin, eyes and the cardiovascular system. Clinical and histopathological features have been described similar to those in pseudoxanthoma elasticum (PXE), although different genes are affected in the two diseases. Cultured dermal fibroblasts from β-thal patients with and without PXE-like clinical manifestations have been compared for parameters of redox balance and for the expression of proteins, which have been already associated with the pathologic mineralization of soft connective tissues. Even though oxidative stress is a well-known condition of β-thal patients, our results indicate that the occurrence of mineralized elastin is associated with a more pronounced redox disequilibrium, as demonstrated by the intracellular increase of anion superoxide and of oxidized proteins and lipids. Moreover, fibroblasts from β-thal PXE-like patients are characterized by decreased availability of carboxylated matrix Gla protein (MGP), as well as by altered expression of proteins involved in the vitamin K-dependent carboxylation process. Results demonstrate that elastic fibre calcification is promoted when redox balance threshold levels are exceeded and the vitamin K-dependent carboxylation process is affected decreasing the activity of MGP, a well-known inhibitor of ectopic calcification. Furthermore, independently from the primary gene defect, these pathways are similarly involved in fibroblasts from PXE and from β-thal PXE-like patients as well as in other diseases leading to ectopic calcification, thus suggesting that can be used as markers of pathologic mineralization.

1. Introduction

Beta-thalassemias (β-thal) comprise a group of hereditary blood disorders characterized by abnormal synthesis of haemoglobin β-chains exerting into variable phenotypes ranging from life-threatening anaemia to clinically asymptomatic individuals, thus suggesting the existence of modifiers genes [1]. Regular transfusion therapy, although essential for adequate management of severely affected patients, is also responsible for a variety of iron overload-related manifestations such as endocrine complications, myocardiopathy and liver fibrosis, all contributing to increase morbidity and mortality. Several studies reported that approximately 16% of patients with major or intermediate β-thal display progressive skin, eye and vessel pathologies similar to those observed in pseudoxanthoma elasticum (PXE), a genetic disease in which soft connective tissues are specifically affected [2-6]. Consistently, ultrastructural studies on dermal biopsies clearly demonstrated that calcification of elastic fibres, deposition of abnormal matrix constituents in the extracellular space and abnormal collagen fibrillogenesis are analogous in these β-thal patients and in PXE [7]. Despite these similarities, β-thal is caused by mutations in the β-globin gene, whereas PXE is associated with mutations in the *ABCC6* gene encoding for the membrane transporter MRP6 [8-10]. Analysis of the *ABCC6* gene in a number of β-thal patients, either with or without PXE manifestations, did not reveal any mutation, thus excluding a digenic inheritance [11]. It was suggested that the clinical phenotype and the morphological features in PXE and in β-thal might be the result of abnormalities affecting similar metabolic pathways independently from the gene defect [12]. Recent investigation in a β-thal mouse model demonstrated that these animals are characterized by a significant NF-E2-dependent down-regulation of the *Abcc6* gene and protein, and it was suggested that a similar transcriptional regulation might occur also in humans [13], even though, in addition to lower *Abcc6* expression, a specific genetic background and/or additional factors may be necessary to have a pathologic calcification phenotype [13].

To further investigate on the mineralization of elastic fibres in β-thal and to look for markers that could be associated to the occurrence of ectopic calcification, parameters of oxidative stress and the expression of the calcification inhibitor Matrix Gla Protein (MGP) were analysed in dermal fibroblasts cultured from β-thal patients with and without PXE-like manifestations. We have previously demonstrated that these parameters are altered in PXE compared to control fibroblasts and we have therefore hypothesized that similar alterations may play a role in other ectopic mineralization diseases [14-16].

2. Materials and Methods

2.1. Patients and cells

In accordance with the guidelines of the Institutional Medical Ethical Committee and of the Helsinki Declaration of 1975 revised in 1983, dermal biopsies were obtained, after informed consent, from the axilla of 6 subjects affected by β-thal major (age 30 ± 6 yr) and from 6 subjects affected by β-thal major with PXE-like clinical manifestations (age 36 ± 8 yr).

All patients had started blood transfusion treatment within the first year of life, underwent splenectomy and, at present, undergo regular chelation therapy. Serum calcium and phosphate were within the normal range. β-thal PXE-like patients had angioid streaks as well as lax and redundant skin on the neck and on the axillae with coalescent papules. Consistently, dermal biopsies were positive for the von Kossa stain. All patients were negative for *ABCC6* mutations [11].

As additional reference controls, fibroblasts from 3 subjects not exhibiting any sign of connective or haematologic diseases (age 34 ± 6) and from 3 PXE patients (age 39 ± 7) were used. Diagnosis of pseudoxanthoma was performed on the basis of clinical symptoms and confirmed by ABCC6 mutations.

From each dermal bioptic sample, fibroblast cultures were established and grown as previously described [17].

2.2. Flow cytometry assays

Confluent fibroblasts, grown in DMEM with 10% fetal bovine serum (FBS), were incubated with appropriate fluorescent markers and analysed on an EPICS XL flow cytometer (Coulter, USA), using a WINMDI 2.8 program.

Intracellular levels of $O_2^{-\bullet}$ were estimated by incubating fibroblasts with 1 mM dihydroethidium probe (DH_2) (Molecular Probes, USA), whereas for H_2O_2 detection, trypsinized cells were stained with 2 mM H_2DCF-DA (Molecular Probes) [16].

2.3. Total Antioxidant Status (TAS)

The total antioxidant capacity of cell lysate was estimated using the commercial kit 'Total Antioxidant Status' (Randox, UK), adapted to the autoanalyser Cobas Mira (ABX, France), which measures at 600 nm the formation of the radical cation $ABTS^{\cdot +}$ using the Reagent ABTS® in the presence of H_2O_2 and peroxidase [18].

2.4. Advanced Oxidation Protein Products (AOPP)

AOPP were evaluated on cell lysates as a measure of the degree of oxidant mediated protein damage [19]. Confluent cells were cultured for 24 hours with complete DMEM supplemented with 0,5% bovine serum albumin. A microassay adapted to Cobas Mira [20] from the original method of Witko-Sarsat [21] was used.

2.5. Lipid hydroperoxides (LOOH)

LOOH have been evaluated as indicators of lipid peroxidation as they are intermediates of peroxidative reactions induced by hydroxyl radicals and singlet oxygen on unsaturated phospholipids, glycolipids and cholesterol [22]. LOOH were measured using the FOX2 method [22] adapted to Cobas Mira (wavelength 600 nm) [20]. LOOH was calculated using a standard curve of tert-butylhydroperoxide.

2.6. Malondialdehyde (MDA)

MDA was determined by HPLC on cell extracts incubated with 1ml of 0.5% thyobarbituric acid and 0.75 ml of 0.1N NaOH [24]. Subsequent treatments were carried out as described by Lepage [25]. Spectrophotometric reading was done at 490 and 600 nm wavelength.

2.7. Global DNA methylation

DNA was obtained from each cell line using the standard phenol–chloroform extraction method. Bisulfite modification was performed as previously described [26]. The method is based on the ability of sodium bisulfite to selectively deaminate cytosine but not 5-methylcytosine to uracil. Once this conversion has taken place, sequence differences between methylated and un-methylated cytosines can be exploited by direct sequencing and restriction digestion with MboI (Fermentas) [16].

2.8. Superoxide dismutase (SOD) activities

SOD activities were determined at 37°C using the commercial kit ''Ransod'' (Randox) [20]. Cu-Zn-SOD activity was differentiated from Mn-SOD on the basis of its sensitivity to 3mM sodium cyanide. To analyse EC-SOD activity, the medium of cultured fibroblasts was concentrated 15-fold using the protein concentration spin columns (VIVASPIN 20, 10 000 WMCO, Sartorius, Germany) and samples processed as previously described [20, 27].

2.9. Glutathione-s-transferase (GST) and glutathione peroxidase (GPX) activities.

GST activity was measured according to Habig [28] using the substrate 1-chloro-2, 4-dinitrobenzene (CDNB) and monitoring absorbance changes at 340 nm [29].

GPX activity was determined at 37°C using the commercial kit 'Ransel' (Randox). One activity unit was defined as the oxidation of 1 μmol NADPH to NADP/min at 37°C [29].

2.10. Western Blot

For 1D-Western blot, protein concentration was measured according to Bradford [30], thereafter 30 μg proteins were loaded on 10% polyacrylamide gel under reducing conditions and then transferred to nitrocellulose according to standard protocols [16]. Primary antibodies against PDI and CALU were diluted in TBST 1:1000 and 1:100, respectively, whereas appropriate horseradish peroxidase-conjugated secondary antibodies (Abcam, UK) were used diluted 1:5000. Western blots were visualized using Super Signal West Pico (Pierce, USA). Densitometry of protein bands was performed using the ImageQuant TL v2005 software (GE Healthcare, USA).

For 2D-Western blot, confluent cells were trypsinised and then sonicated on ice in a Sonifier B-12 (Branson Sonic Power Company, USA) with 20 pulses of 2 s. The membrane-enriched fraction, obtained by centrifuging supernatant at 229 000 X g for 60 min (Beckman Instruments, USA), was stored frozen at -80°C until use. Proteins extracted from a pool of fibroblasts derived from twelve β-thal patients with (n=6) and without (n=6) PXE-like manifestations were used for MGP determination by 2D immunoblots as previously described [15]. Primary antibodies against uncarboxylated-matrix Gla protein (Glu-MGP) and carboxylated-matrix Gla protein (Gla-MGP) [31, 32] were used diluted 1:10000 in TBST plus 1% BSA. Anti-mouse IgG conjugated with horseradish peroxidase (GE HealthCare) and diluted 1:20000 were used as secondary antibodies. Immunoreaction was revealed by using the SuperSignal West Pico Chemoluminescent Substrate (Pierce) and normalized to β-actin.

2.11. Ultrastructure

Skin biopsies, cut into small fragments, were routinely fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, USA) in Tyrode's buffer, post-fixed in 1% osmium tetroxide (Fluka AG Chem, Swizerland), dehydrated in ethanol and then in propylene oxide and embedded in Spurr resin (Polysciences, USA). Ultrathin sections, obtained from the epidermis through the reticular dermis, were observed under a JEM-1200EXII transmission electron microscope (Jeol, Japan).

2.12. Immmuno-electron-microscopy

For MGP immune-localisation on mineralized elastic fibres, ultrathin sections from dermal biopsies of β-thal-PXE patients were processed as already described [15]. Sections were firstly incubated over night at 4°C with primary antibodies towards the non-carboxylated and the carboxylated forms of MGP [31, 32] and thereafter with secondary antibodies conjugated with 10nm gold particles (EY Laboratories, USA) for 3 hours at room temperature. Observations were made with a JEM-1200EXII transmission electron microscope (Jeol).

2.13. Statistical analyses

Results are expressed as means of data from each cell lines in duplicate experiments ± standard deviation (SD), unless otherwise specified. Statistical differences were determined using the Mann Whitney test (GraphPad Prism software 5.0) and significance was assigned as $p<0.05$.

3. Results

3.1. Parameters of oxidative stress in cultured fibroblasts

The amount of ROS was measured in the cellular monolayer by flow cytometry (Table I). Fibroblasts from β-thal PXE-like patients had a significantly higher content of anion superoxide compared to fibroblasts from β-thal patients ($p<0.05$), whereas changes were negligible for hydrogen peroxide.

To verify whether ROS levels in β-thal PXE-like cells were high enough to induce cellular damages, intermediate and end-products of lipid peroxidation, as well as oxidized proteins, were measured [22] (Table 1). Values of LOOH and MDA were higher in β-thal PXE-like fibroblasts compared to β-thal cells and a similar accumulation was observed also for the advanced oxidation protein products (AOPP) (Table I). By contrast, the total antioxidant status (TAS) was similar in all cell lines (Table I). Consequently, the oxidant/antioxidant ratio (OX/AntiOX ratio) between parameters related to oxidative damage (AOPP, LOOH and MDA) and to the antioxidant properties (TAS) of the cells was significantly altered in β-thal PXE-like cells in favour of a pro-oxidant status (Table I).

Evaluation of changes in total DNA methylation appeared not significant in all cell strains (data not shown), even if changes at specific promoters cannot be excluded.

Measurements of the activity of enzymes regulating the intracellular redox equilibrium revealed that mitochondrial Mn-SOD was similar in all cell strains (Figure 1), whereas cytoplasmic Cu-Zn-SOD, glutathione S-transferase (GST) and glutatione peroxidase (GPX) activities were significantly

higher in β-thal PXE-like compared to β-thal fibroblasts (Figure 1). To be noted, however, that the ratio between Cu-Zn-SOD/GPX was markedly up-regulated in β-thal PXE-like cells indicating that the increase was not of the same extent for the two parameters.

Moreover, EC-SOD activity measured in the culture medium was significantly higher in β-thal PXE-like compared to β-thal cultures (Figure 1).

3.2. Expression of proteins related to the vitamin K-dependent carboxylation

By 1D and 2D-WB, β-thal PXE-like fibroblasts had lower levels of PDI and higher expression of CALU in comparison with β-thal cells (Figure 2). In the case of CALU, antibodies stained two bands representing the glycosylated and the non-glycosylated protein, respectively. Densitometric analysis performed on both bands indicated that in β-thal PXE-like cells the glycosylated (i.e. active form) was more intense.

Since both PDI and CALU are involved in the carboxylation of MGP, un-carboxylated (Glu) and carboxylated (Gla) MGP were evaluated by 2D-WB using specific antibodies upon normalization towards the β-actin content (Figure 2). Glu-MGP was more abundant in β-thal PXE-like fibroblasts compared to β-thal, whereas the amount of Gla-MGP was higher in β-thal compared to β-thal PXE-like cells.

3.3. Immuno-electronmicroscopy of MGP on mineralized elastic fibres

In normal dermal biopsies, elongated elastic fibres were located between collagen bundles in close proximity to fibroblasts (Figure 3a). By contrast, in β-thal skin specimens, elastic fibres were characterized by irregular contours with sporadic small mineral precipitates in the amorphous core (Figure 3b), as those described at the ultrastructural level in PXE heterozygous healthy carriers [33]. In β-thal PXE-like patients the majority of elastic fibres exhibit abundant homogeneous tiny precipitates, as well as bulky deforming mineral aggregates (Figure 3c,d).

The localization of Glu- and Gla-MGP to calcified elastic fibres was evaluated by immuno-electronmicroscopy on ultrathin sections of dermal biopsies from β-thal PXE-like patients.

Antibodies recognizing Glu-MGP strongly reacted with polymorphous calcified areas and with the finely dispersed calcified core of elastic fibres (Figure 4 a,b). By contrast, antibodies towards Gla-MGP were localized at the interface between mineralized and normal elastin (Figure 4 c,d), similarly to observations made on PXE calcified elastic fibres [15].

4. Discussion

A number (approximately 16%) of β-thal patients suffer from clinical complications due to progressive ectopic calcification, leading to a PXE-like phenotype, in the absence of ABCC6 mutations [2-7]. We have already demonstrated that dermal fibroblasts can be regarded as a valuable model to investigate the occurrence of pathologic mineralization [34] and that in PXE, a disease which is considered a paradigm of ectopic calcification, oxidative stress and vitamin K-related proteins are involved in the pathologic calcific phenotype [16].

Therefore, in the present study we have investigated dermal fibroblasts from β-thal patients, with and without PXE-like manifestations, in order to look for parameters that could discriminate the two phenotypes and may be associated to ectopic calcification.

Altered redox balance, for instance, could significantly contribute to changes in fibroblast behaviour [35], to the severity of clinical manifestations in several disorders including PXE [14, 20] and to elastic tissue injury [36-37]. It has been actually demonstrated that, in β-thal patients, both iron overload and plasma membrane microparticles derived from the oxidative damage of red cell membranes could elicit inflammatory and oxidative reactions [38-39]. Consistently, the occurrence of an oxidative stress condition was associated with an abnormal morphology of elastic fibres in all β-thal patients. Since 16% of β-thal patients exhibit also elastic fibre calcification, we have compared fibroblasts from β-thal patients with and without ectopic mineralization in order to highlight difference in the redox-balance of these cells.

In β-thal PXE-like fibroblasts, compared to β-thal cells, there is an increased amount of ROS, in particular $O_2^{-\bullet}$. This redox disequilibrium can be due to higher production of ROS and/or to the inability of cells to counteract oxidative stress. However, a general failure of antioxidant mechanisms in β-thal PXE-like is unlikely, as increased Cu-Zn-SOD, GPX and GST activities are actually elevated in response to sublethal levels of oxidant pressure. GPX catalyses the reduction of lipid hydroperoxides and, at low concentration, of H_2O_2 and SOD reduces the ability of superoxide to form strong oxidants and nitrating agents. However, these two enzymes must increase in a well balanced way, as low SOD relative to GPX might lead to an accumulation of $O_2^{-\bullet}$. By contrast, high levels of SOD relative to GPX could lead to increased production of H_2O_2 intermediates that may be responsible for the additional production of noxius ROS [40]. In β-thal PXE-like cells we have found that the SOD/GPX ratio was significantly increased, thus contributing to altered cellular functions. Moreover, GST and GPX enhancement does not seem to be high enough to counteract oxidative stress and, in line with the above described data, AOPP, LOOH and MDA are significantly increased in cultured fibroblasts from β-thal PXE-like patients compared to β-thal fibroblasts, thus leaving unaffected the total antioxidant power measured in these cells.

An additional target and also source of ROS is the endoplasmic reticulum (ER). In dermal biopsies of β-thal PXE-like patients, fibroblasts exhibit cisternae of the ER filled with electrondense material [7]. Interestingly, the vitamin K-dependent γ-carboxylation of Matrix Gla Protein (MGP), one of the most efficient inhibitors of calcium precipitation, takes place in the ER [41, 42].

In this study, we have demonstrated *in vitro* and *ex vivo* that low amount of carboxylated MGP is present in β-thal PXE-like fibroblast's cultures and on mineralized areas of dermal elastic fibres. In figure 5a data from the present study are compared with those obtained in PXE compared to control fibroblasts [15]. In both cases, the occurrence of ectopic calcifications is associated to a marked reduction of carboxylated MGP. Additionally, in β-thal PXE-like compared to β-thal cells, CALU and PDI are up- and down-regulated, respectively. PDI plays a fundamental role in protein folding

and trafficking and, to be functional, it must be present in the reduced form [43]. Moreover, PDI, providing electrons to the VKOR system, interacts with part of the VKORC1 pool forming, on membranes of the endoplasmic reticulum, the active warfarin-sensitive Vitamin K>O reducing complex of the vitamin K cycle [44]. If the level of PDI is reduced and/or the protein is oxidised by ROS, as it could happen in the high oxidative environment of β-thal PXE-like fibroblasts, the recycling of vitamin K is impaired and protein γ-carboxylation may be reduced [45].

Furthermore, in β-thal PXE-like fibroblasts we have also found a significant up-regulation of calumenin, an ER chaperone with inhibitory activity on both γ-carboxylase and VKORC1 [46] and consequently on MGP carboxylation [47].

Dermal fibroblasts from β-thal PXE-like patients exhibit functional alterations similar to those of fibroblasts isolated from patients with inherited PXE (higher oxidative stress and reduced carboxylation of the calcification inhibitor MGP) suggesting that these altered parameters could be taken as potential markers for the occurrence of ectopic calcifications. As shown in figure 5b ectopic calcification occurs at higher levels of redox disequilibrium. Looking in details at the activity of several antioxidant enzymes, there are some dissimilarities in fibroblasts from PXE and from β-thal PXE-like patients, consistently with the involvement of different genes, although leading to analogous mineralization of elastic fibres and to similarly altered parameters (i.e. oxidative stress and low MGP carboxylation).

Furthermore, data support the hypothesis that serum factor(s) as well as mesenchymal cells (e.g. dermal fibroblasts) are involved in the occurrence of ectopic calcification. Consistently, in both PXE and β-thal PXE-like patients fibroblasts grown in culture in a controlled environment exhibit and maintain pathologic characteristics. Since it has been shown that dermal fibroblasts do not express ABCC6/MRP6, at least at significant levels, their phenotype could be the result of epigenetic regulation and/or of a favourable genetic background rendering these cells more susceptible to calcification inducers/stimuli.

Evidence in the animal models already disclosed the importance of *Abcc6* deregulation in the pathogenesis of PXE [48, 49] and possibly of PXE-like manifestations [13]. Actually, the occurrence of oxidative stress and/or of insufficient MGP carboxylation cannot be directly related to different levels of *ABCC6* expression in β-thal patients, since this parameter can be evaluated only in tissues/cells highly expressing the gene (i.e. liver cells). Nevertheless, present data support the hypothesis that, at least in human dermal fibroblasts, starting from mutations in different genes (*HBB* and *ABCC6*), elastic fibre mineralization is associated with common pathways. Slightly lower *ABCC6* expression, either due to NF-E2-mediated transcriptional regulatory mechanisms [13], as it could be in β-thal patients, or due to the presence of only one functional allele [33], as in PXE carriers, may affect elastin metabolism and morphology, without causing severe calcification.

Additional factors are necessary to induce mesenchymal cells to alter their behaviour and to favour the mineralization of the elastic component. Within this context, altered redox balance and low MGP carboxylation could be good candidates and may be regarded as markers of ectopic calcification contributing to the severity of clinical manifestations in β-thal, as already demonstrated in PXE [14, 20].

6. Conclusion

Studies on dermal fibroblasts cultured from β-thal patients with and without PXE-like manifestations demonstrate that the occurrence of a chronic oxidative stress condition is associated to an altered, possibly functionally damaging, morphology of elastic fibres. However, in order to have ectopic calcification a threshold levels must be exceeded. Furthermore, loss or insufficient availability of carboxylated MGP is associated to pathologic mineralization, consistently with the importance of this calcification inhibitor, as demonstrated in other different pathologic conditions as PXE [15], GGCX mutation associated disorders [50], vascular calcification [41-42] and chronic kidney disease [47].

In the light of these results, high oxidative stress and low carboxylated MGP can be considered potential markers of ectopic calcification in β-thal patients, as well as in other ectopic calcification disorders.

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Legend to figures

Figure 1. Manganese- (Mn-SOD), Copper-zinc- (Cu-Zn-SOD) and extracellular (EC-SOD) superoxide dismutase activities, glutathione-S-trasferase (GST) and glutathione peroxidase (GPX) activities as well as the ratio Cu-Zn-SOD/GPX have been measured in fibroblasts from β-thalassemia (β-thal) patients with and without PXE-like (PXE-L) manifestations. Data are expressed as mean values ± SD of three experiments performed in duplicate using all cell lines. $*p<0.05$; $**p<0.01$

Figure 2. Representative immunoblots of protein disulfide isomerase (PDI), calumenin (CALU), uncarboxylated- (Glu-) and carboxylated- (Gla-) matrix Gla protein (MGP) on proteins isolated from fibroblasts from β-thalassemia (β-thal) patients with and without PXE-like (PXE-L) manifestations. Histograms represent mean values expressed as percentage of densitometric values ± SD with β-thal values set at 100.

$*p<0.05$ β-thal PXE-L vs β-thal values obtained in each cell strain analysed in duplicate in two experiments.

# $p<0.05$ β-thal PXE-L vs β-thal values obtained in duplicate from a pool of fibroblasts, thus indicating experimental variability

Figure 3. Transmission electron microscopy of dermal elastic fibres (E) in normal subjects (a) and in β-thalassemia (β-thal) patients with (c,d) and without (b) PXE-like manifestations. Mineralization (arrows) is limited to tiny small deposits in β-thal patients (b), whereas it dramatically affects and deforms elastic fibres in β-thal PXE-like patients (c-d).

Bar= 1 μm

Figure 4. Immuno-electron-microscopy shows the different localization of uncarboxylated- (a,b) and carboxylated- (c,d) matrix Gla protein on mineralized elastic fibres in the dermis of β-thalassemia patients with PXE-like manifestations. Epitopes are visible as electrondense colloidal gold particles.

Bar= 0.5 μm

Figure 5. Levels of carboxylated- (Gla-) matrix Gla protein (MGP) evaluated by 2D-Western blot are reported for fibroblasts from controls and PXE patients as well as from β-thalassemia (β-thal) patients with and without PXE-like manifestations (a). Parameters of redox balance (glutathione-S-trasferase,GST; glutathione peroxidase, GPX; extracellular (EC-SOD), copper-zinc- (Cu-Zn-SOD) and manganese- (Mn-SOD) superoxide dismutase activities, as well as the oxidant/antioxidant ratio, AOPP+LOOH+MDA/TAS) in fibroblasts from patients affected by PXE, β-thal and β-thal PXE-like diseases are compared with those in fibroblasts from healthy subjects set at 1 (b).

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Ectopic calcification in β-thalassemia patients is associated with increased oxidative stress and lower MGP carboxylation.

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Abstract

A number of beta-thalassemia (β-thal) patients in the course of the disease exhibit ectopic calcification affecting skin, eyes and the cardiovascular system. Clinical and histopathological features have been described similar to those in pseudoxanthoma elasticum (PXE), although different genes are affected in the two diseases. Cultured dermal fibroblasts from β-thal patients with and without PXE-like clinical manifestations have been compared for parameters of redox balance and for the expression of proteins, which have been already associated with the pathologic mineralization of soft connective tissues. Even though oxidative stress is a well-known condition of β-thal patients, our results indicate that the occurrence of mineralized elastin is associated with a more pronounced redox disequilibrium, as demonstrated by the intracellular increase of anion superoxide and of oxidized proteins and lipids. Moreover, fibroblasts from β-thal PXE-like patients are characterized by decreased availability of carboxylated matrix Gla protein (MGP), as well as by altered expression of proteins involved in the vitamin K-dependent carboxylation process. Results demonstrate that elastic fibre calcification is promoted when redox balance threshold levels are exceeded and the vitamin K-dependent carboxylation process is affected decreasing the activity of MGP, a well-known inhibitor of ectopic calcification. Furthermore, independently from the primary gene defect, these pathways are similarly involved in fibroblasts from PXE and from β-thal PXE-like patients as well as in other diseases leading to ectopic calcification, thus suggesting that can be used as markers of pathologic mineralization.

1. Introduction

Beta-thalassemias (β-thal) comprise a group of hereditary blood disorders characterized by abnormal synthesis of haemoglobin β-chains exerting into variable phenotypes ranging from life-threatening anaemia to clinically asymptomatic individuals, thus suggesting the existence of modifiers genes [1]. Regular transfusion therapy, although essential for adequate management of severely affected patients, is also responsible for a variety of iron overload-related manifestations such as endocrine complications, myocardiopathy and liver fibrosis, all contributing to increase morbidity and mortality. Several studies reported that approximately 16% of patients with major or intermediate β-thal display progressive skin, eye and vessel pathologies similar to those observed in pseudoxanthoma elasticum (PXE), a genetic disease in which soft connective tissues are specifically affected [2-6]. Consistently, ultrastructural studies on dermal biopsies clearly demonstrated that calcification of elastic fibres, deposition of abnormal matrix constituents in the extracellular space and abnormal collagen fibrillogenesis are analogous in these β-thal patients and in PXE [7]. Despite these similarities, β-thal is caused by mutations in the β-globin gene, whereas PXE is associated with mutations in the *ABCC6* gene encoding for the membrane transporter MRP6 [8-10]. Analysis of the *ABCC6* gene in a number of β-thal patients, either with or without PXE manifestations, did not reveal any mutation, thus excluding a digenic inheritance [11]. It was suggested that the clinical phenotype and the morphological features in PXE and in β-thal might be the result of abnormalities affecting similar metabolic pathways independently from the gene defect [12]. Recent investigation in a β-thal mouse model demonstrated that these animals are characterized by a significant NF-E2-dependent down-regulation of the *Abcc6* gene and protein, and it was suggested that a similar transcriptional regulation might occur also in humans [13], even though, in addition to lower *Abcc6* expression, a specific genetic background and/or additional factors may be necessary to have a pathologic calcification phenotype [13].

To further investigate on the mineralization of elastic fibres in β-thal and to look for markers that could be associated to the occurrence of ectopic calcification, parameters of oxidative stress and the expression of the calcification inhibitor Matrix Gla Protein (MGP) were analysed in dermal fibroblasts cultured from β-thal patients with and without PXE-like manifestations. We have previously demonstrated that these parameters are altered in PXE compared to control fibroblasts and we have therefore hypothesized that similar alterations may play a role in other ectopic mineralization diseases [14-16].

2. Materials and Methods

2.1. Patients and cells

In accordance with the guidelines of the Institutional Medical Ethical Committee and of the Helsinki Declaration of 1975 revised in 1983, dermal biopsies were obtained, after informed consent, from the axilla of 6 subjects affected by β-thal major (age 30 ± 6 yr) and from 6 subjects affected by β-thal major with PXE-like clinical manifestations (age 36 ± 8 yr).

All patients had started blood transfusion treatment within the first year of life, underwent splenectomy and, at present, undergo regular chelation therapy. Serum calcium and phosphate were within the normal range. β-thal PXE-like patients had angioid streaks as well as lax and redundant skin on the neck and on the axillae with coalescent papules. Consistently, dermal biopsies were positive for the von Kossa stain. All patients were negative for *ABCC6* mutations [11].

As additional reference controls, fibroblasts from 3 subjects not exhibiting any sign of connective or haematologic diseases (age 34 ± 6) and from 3 PXE patients (age 39 ± 7) were used. Diagnosis of pseudoxanthoma was performed on the basis of clinical symptoms and confirmed by ABCC6 mutations.

From each dermal bioptic sample, fibroblast cultures were established and grown as previously described [17].

2.2. Flow cytometry assays

Confluent fibroblasts, grown in DMEM with 10% fetal bovine serum (FBS), were incubated with appropriate fluorescent markers and analysed on an EPICS XL flow cytometer (Coulter, USA), using a WINMDI 2.8 program.

Intracellular levels of $O_2^{-\bullet}$ were estimated by incubating fibroblasts with 1 mM dihydroethidium probe (DH_2) (Molecular Probes, USA), whereas for H_2O_2 detection, trypsinized cells were stained with 2 mM H_2DCF-DA (Molecular Probes) [16].

2.3. Total Antioxidant Status (TAS)

The total antioxidant capacity of cell lysate was estimated using the commercial kit 'Total Antioxidant Status' (Randox, UK), adapted to the autoanalyser Cobas Mira (ABX, France), which measures at 600 nm the formation of the radical cation $ABTS^{\cdot+}$ using the Reagent ABTS® in the presence of H_2O_2 and peroxidase [18].

2.4. Advanced Oxidation Protein Products (AOPP)

AOPP were evaluated on cell lysates as a measure of the degree of oxidant mediated protein damage [19]. Confluent cells were cultured for 24 hours with complete DMEM supplemented with 0,5% bovine serum albumin. A microassay adapted to Cobas Mira [20] from the original method of Witko-Sarsat [21] was used.

2.5. Lipid hydroperoxides (LOOH)

LOOH have been evaluated as indicators of lipid peroxidation as they are intermediates of peroxidative reactions induced by hydroxyl radicals and singlet oxygen on unsaturated phospholipids, glycolipids and cholesterol [22]. LOOH were measured using the FOX2 method [22] adapted to Cobas Mira (wavelength 600 nm) [20]. LOOH was calculated using a standard curve of tert-butylhydroperoxide.

2.6. Malondialdehyde (MDA)

MDA was determined by HPLC on cell extracts incubated with 1ml of 0.5% thyobarbituric acid and 0.75 ml of 0.1N NaOH [24]. Subsequent treatments were carried out as described by Lepage [25]. Spectrophotometric reading was done at 490 and 600 nm wavelength.

2.7. Global DNA methylation

DNA was obtained from each cell line using the standard phenol–chloroform extraction method. Bisulfite modification was performed as previously described [26]. The method is based on the ability of sodium bisulfite to selectively deaminate cytosine but not 5-methylcytosine to uracil. Once this conversion has taken place, sequence differences between methylated and un-methylated cytosines can be exploited by direct sequencing and restriction digestion with MboI (Fermentas) [16].

2.8. Superoxide dismutase (SOD) activities

SOD activities were determined at 37°C using the commercial kit ''Ransod'' (Randox) [20]. Cu-Zn-SOD activity was differentiated from Mn-SOD on the basis of its sensitivity to 3mM sodium cyanide. To analyse EC-SOD activity, the medium of cultured fibroblasts was concentrated 15-fold using the protein concentration spin columns (VIVASPIN 20, 10 000 WMCO, Sartorius, Germany) and samples processed as previously described [20, 27].

2.9. Glutathione-s-transferase (GST) and glutathione peroxidase (GPX) activities.

GST activity was measured according to Habig [28] using the substrate 1-chloro-2, 4-dinitrobenzene (CDNB) and monitoring absorbance changes at 340 nm [29].

GPX activity was determined at 37°C using the commercial kit 'Ransel' (Randox). One activity unit was defined as the oxidation of 1 μmol NADPH to NADP/min at 37°C [29].

2.10. Western Blot

For 1D-Western blot, protein concentration was measured according to Bradford [30], thereafter 30 μg proteins were loaded on 10% polyacrylamide gel under reducing conditions and then transferred to nitrocellulose according to standard protocols [16]. Primary antibodies against PDI and CALU were diluted in TBST 1:1000 and 1:100, respectively, whereas appropriate horseradish peroxidase-conjugated secondary antibodies (Abcam, UK) were used diluted 1:5000. Western blots were visualized using Super Signal West Pico (Pierce, USA). Densitometry of protein bands was performed using the ImageQuant TL v2005 software (GE Healthcare, USA).

For 2D-Western blot, confluent cells were trypsinised and then sonicated on ice in a Sonifier B-12 (Branson Sonic Power Company, USA) with 20 pulses of 2 s. The membrane-enriched fraction, obtained by centrifuging supernatant at 229 000 X g for 60 min (Beckman Instruments, USA), was stored frozen at -80°C until use. Proteins extracted from a pool of fibroblasts derived from twelve β-thal patients with (n=6) and without (n=6) PXE-like manifestations were used for MGP determination by 2D immunoblots as previously described [15]. Primary antibodies against uncarboxylated-matrix Gla protein (Glu-MGP) and carboxylated-matrix Gla protein (Gla-MGP) [31, 32] were used diluted 1:10000 in TBST plus 1% BSA. Anti-mouse IgG conjugated with horseradish peroxidase (GE HealthCare) and diluted 1:20000 were used as secondary antibodies. Immunoreaction was revealed by using the SuperSignal West Pico Chemoluminescent Substrate (Pierce) and normalized to β-actin.

2.11. Ultrastructure

Skin biopsies, cut into small fragments, were routinely fixed in 2.5% glutaraldehyde (Electron Microscopy Sciences, USA) in Tyrode's buffer, post-fixed in 1% osmium tetroxide (Fluka AG Chem, Swizerland), dehydrated in ethanol and then in propylene oxide and embedded in Spurr resin (Polysciences, USA). Ultrathin sections, obtained from the epidermis through the reticular dermis, were observed under a JEM-1200EXII transmission electron microscope (Jeol, Japan).

2.12. Immmuno-electron-microscopy

For MGP immune-localisation on mineralized elastic fibres, ultrathin sections from dermal biopsies of β-thal-PXE patients were processed as already described [15]. Sections were firstly incubated over night at 4°C with primary antibodies towards the non-carboxylated and the carboxylated forms of MGP [31, 32] and thereafter with secondary antibodies conjugated with 10nm gold particles (EY Laboratories, USA) for 3 hours at room temperature. Observations were made with a JEM-1200EXII transmission electron microscope (Jeol).

2.13. Statistical analyses

Results are expressed as means of data from each cell lines in duplicate experiments ± standard deviation (SD), unless otherwise specified. Statistical differences were determined using the Mann Whitney test (GraphPad Prism software 5.0) and significance was assigned as $p<0.05$.

3. Results

3.1. Parameters of oxidative stress in cultured fibroblasts

The amount of ROS was measured in the cellular monolayer by flow cytometry (Table I). Fibroblasts from β-thal PXE-like patients had a significantly higher content of anion superoxide compared to fibroblasts from β-thal patients ($p<0.05$), whereas changes were negligible for hydrogen peroxide.

To verify whether ROS levels in β-thal PXE-like cells were high enough to induce cellular damages, intermediate and end-products of lipid peroxidation, as well as oxidized proteins, were measured [22] (Table 1). Values of LOOH and MDA were higher in β-thal PXE-like fibroblasts compared to β-thal cells and a similar accumulation was observed also for the advanced oxidation protein products (AOPP) (Table I). By contrast, the total antioxidant status (TAS) was similar in all cell lines (Table I). Consequently, the oxidant/antioxidant ratio (OX/AntiOX ratio) between parameters related to oxidative damage (AOPP, LOOH and MDA) and to the antioxidant properties (TAS) of the cells was significantly altered in β-thal PXE-like cells in favour of a pro-oxidant status (Table I).

Evaluation of changes in total DNA methylation appeared not significant in all cell strains (data not shown), even if changes at specific promoters cannot be excluded.

Measurements of the activity of enzymes regulating the intracellular redox equilibrium revealed that mitochondrial Mn-SOD was similar in all cell strains (Figure 1), whereas cytoplasmic Cu-Zn-SOD, glutathione S-transferase (GST) and glutatione peroxidase (GPX) activities were significantly

higher in β-thal PXE-like compared to β-thal fibroblasts (Figure 1). To be noted, however, that the ratio between Cu-Zn-SOD/GPX was markedly up-regulated in β-thal PXE-like cells indicating that the increase was not of the same extent for the two parameters.

Moreover, EC-SOD activity measured in the culture medium was significantly higher in β-thal PXE-like compared to β-thal cultures (Figure 1).

3.2. Expression of proteins related to the vitamin K-dependent carboxylation

By 1D and 2D-WB, β-thal PXE-like fibroblasts had lower levels of PDI and higher expression of CALU in comparison with β-thal cells (Figure 2). In the case of CALU, antibodies stained two bands representing the glycosylated and the non-glycosylated protein, respectively. Densitometric analysis performed on both bands indicated that in β-thal PXE-like cells the glycosylated (i.e. active form) was more intense.

Since both PDI and CALU are involved in the carboxylation of MGP, un-carboxylated (Glu) and carboxylated (Gla) MGP were evaluated by 2D-WB using specific antibodies upon normalization towards the β-actin content (Figure 2). Glu-MGP was more abundant in β-thal PXE-like fibroblasts compared to β-thal, whereas the amount of Gla-MGP was higher in β-thal compared to β-thal PXE-like cells.

3.3. Immuno-electronmicroscopy of MGP on mineralized elastic fibres

In normal dermal biopsies, elongated elastic fibres were located between collagen bundles in close proximity to fibroblasts (Figure 3a). By contrast, in β-thal skin specimens, elastic fibres were characterized by irregular contours with sporadic small mineral precipitates in the amorphous core (Figure 3b), as those described at the ultrastructural level in PXE heterozygous healthy carriers [33]. In β-thal PXE-like patients the majority of elastic fibres exhibit abundant homogeneous tiny precipitates, as well as bulky deforming mineral aggregates (Figure 3c,d).

The localization of Glu- and Gla-MGP to calcified elastic fibres was evaluated by immuno-electronmicroscopy on ultrathin sections of dermal biopsies from β-thal PXE-like patients.

Antibodies recognizing Glu-MGP strongly reacted with polymorphous calcified areas and with the finely dispersed calcified core of elastic fibres (Figure 4 a,b). By contrast, antibodies towards Gla-MGP were localized at the interface between mineralized and normal elastin (Figure 4 c,d), similarly to observations made on PXE calcified elastic fibres [15].

4. Discussion

A number (approximately 16%) of β-thal patients suffer from clinical complications due to progressive ectopic calcification, leading to a PXE-like phenotype, in the absence of ABCC6 mutations [2-7]. We have already demonstrated that dermal fibroblasts can be regarded as a valuable model to investigate the occurrence of pathologic mineralization [34] and that in PXE, a disease which is considered a paradigm of ectopic calcification, oxidative stress and vitamin K-related proteins are involved in the pathologic calcific phenotype [16].

Therefore, in the present study we have investigated dermal fibroblasts from β-thal patients, with and without PXE-like manifestations, in order to look for parameters that could discriminate the two phenotypes and may be associated to ectopic calcification.

Altered redox balance, for instance, could significantly contribute to changes in fibroblast behaviour [35], to the severity of clinical manifestations in several disorders including PXE [14, 20] and to elastic tissue injury [36-37]. It has been actually demonstrated that, in β-thal patients, both iron overload and plasma membrane microparticles derived from the oxidative damage of red cell membranes could elicit inflammatory and oxidative reactions [38-39]. Consistently, the occurrence of an oxidative stress condition was associated with an abnormal morphology of elastic fibres in all β-thal patients. Since 16% of β-thal patients exhibit also elastic fibre calcification, we have compared fibroblasts from β-thal patients with and without ectopic mineralization in order to highlight difference in the redox-balance of these cells.

In β-thal PXE-like fibroblasts, compared to β-thal cells, there is an increased amount of ROS, in particular $O_2^{-\bullet}$. This redox disequilibrium can be due to higher production of ROS and/or to the inability of cells to counteract oxidative stress. However, a general failure of antioxidant mechanisms in β-thal PXE-like is unlikely, as increased Cu-Zn-SOD, GPX and GST activities are actually elevated in response to sublethal levels of oxidant pressure. GPX catalyses the reduction of lipid hydroperoxides and, at low concentration, of H_2O_2 and SOD reduces the ability of superoxide to form strong oxidants and nitrating agents. However, these two enzymes must increase in a well balanced way, as low SOD relative to GPX might lead to an accumulation of $O_2^{-\bullet}$. By contrast, high levels of SOD relative to GPX could lead to increased production of H_2O_2 intermediates that may be responsible for the additional production of noxius ROS [40]. In β-thal PXE-like cells we have found that the SOD/GPX ratio was significantly increased, thus contributing to altered cellular functions. Moreover, GST and GPX enhancement does not seem to be high enough to counteract oxidative stress and, in line with the above described data, AOPP, LOOH and MDA are significantly increased in cultured fibroblasts from β-thal PXE-like patients compared to β-thal fibroblasts, thus leaving unaffected the total antioxidant power measured in these cells.

An additional target and also source of ROS is the endoplasmic reticulum (ER). In dermal biopsies of β-thal PXE-like patients, fibroblasts exhibit cisternae of the ER filled with electrondense material [7]. Interestingly, the vitamin K-dependent γ-carboxylation of Matrix Gla Protein (MGP), one of the most efficient inhibitors of calcium precipitation, takes place in the ER [41, 42].

In this study, we have demonstrated *in vitro* and *ex vivo* that low amount of carboxylated MGP is present in β-thal PXE-like fibroblast's cultures and on mineralized areas of dermal elastic fibres. In figure 5a data from the present study are compared with those obtained in PXE compared to control fibroblasts [15]. In both cases, the occurrence of ectopic calcifications is associated to a marked reduction of carboxylated MGP. Additionally, in β-thal PXE-like compared to β-thal cells, CALU and PDI are up- and down-regulated, respectively. PDI plays a fundamental role in protein folding

and trafficking and, to be functional, it must be present in the reduced form [43]. Moreover, PDI, providing electrons to the VKOR system, interacts with part of the VKORC1 pool forming, on membranes of the endoplasmic reticulum, the active warfarin-sensitive Vitamin K>O reducing complex of the vitamin K cycle [44]. If the level of PDI is reduced and/or the protein is oxidised by ROS, as it could happen in the high oxidative environment of β-thal PXE-like fibroblasts, the recycling of vitamin K is impaired and protein γ-carboxylation may be reduced [45].

Furthermore, in β-thal PXE-like fibroblasts we have also found a significant up-regulation of calumenin, an ER chaperone with inhibitory activity on both γ-carboxylase and VKORC1 [46] and consequently on MGP carboxylation [47].

Dermal fibroblasts from β-thal PXE-like patients exhibit functional alterations similar to those of fibroblasts isolated from patients with inherited PXE (higher oxidative stress and reduced carboxylation of the calcification inhibitor MGP) suggesting that these altered parameters could be taken as potential markers for the occurrence of ectopic calcifications. As shown in figure 5b ectopic calcification occurs at higher levels of redox disequilibrium. Looking in details at the activity of several antioxidant enzymes, there are some dissimilarities in fibroblasts from PXE and from β-thal PXE-like patients, consistently with the involvement of different genes, although leading to analogous mineralization of elastic fibres and to similarly altered parameters (i.e. oxidative stress and low MGP carboxylation).

Furthermore, data support the hypothesis that serum factor(s) as well as mesenchymal cells (e.g. dermal fibroblasts) are involved in the occurrence of ectopic calcification. Consistently, in both PXE and β-thal PXE-like patients fibroblasts grown in culture in a controlled environment exhibit and maintain pathologic characteristics. Since it has been shown that dermal fibroblasts do not express ABCC6/MRP6, at least at significant levels, their phenotype could be the result of epigenetic regulation and/or of a favourable genetic background rendering these cells more susceptible to calcification inducers/stimuli.

Evidence in the animal models already disclosed the importance of *Abcc6* deregulation in the pathogenesis of PXE [48, 49] and possibly of PXE-like manifestations [13]. Actually, the occurrence of oxidative stress and/or of insufficient MGP carboxylation cannot be directly related to different levels of *ABCC6* expression in β-thal patients, since this parameter can be evaluated only in tissues/cells highly expressing the gene (i.e. liver cells). Nevertheless, present data support the hypothesis that, at least in human dermal fibroblasts, starting from mutations in different genes (*HBB* and *ABCC6*), elastic fibre mineralization is associated with common pathways. Slightly lower *ABCC6* expression, either due to NF-E2-mediated transcriptional regulatory mechanisms [13], as it could be in β-thal patients, or due to the presence of only one functional allele [33], as in PXE carriers, may affect elastin metabolism and morphology, without causing severe calcification.
Additional factors are necessary to induce mesenchymal cells to alter their behaviour and to favour the mineralization of the elastic component. Within this context, altered redox balance and low MGP carboxylation could be good candidates and may be regarded as markers of ectopic calcification contributing to the severity of clinical manifestations in β-thal, as already demonstrated in PXE [14, 20].

6. Conclusion

Studies on dermal fibroblasts cultured from β-thal patients with and without PXE-like manifestations demonstrate that the occurrence of a chronic oxidative stress condition is associated to an altered, possibly functionally damaging, morphology of elastic fibres. However, in order to have ectopic calcification a threshold levels must be exceeded. Furthermore, loss or insufficient availability of carboxylated MGP is associated to pathologic mineralization, consistently with the importance of this calcification inhibitor, as demonstrated in other different pathologic conditions as PXE [15], GGCX mutation associated disorders [50], vascular calcification [41-42] and chronic kidney disease [47].

In the light of these results, high oxidative stress and low carboxylated MGP can be considered potential markers of ectopic calcification in β-thal patients, as well as in other ectopic calcification disorders.

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8. References

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Legend to figures

Figure 1. Manganese- (Mn-SOD), Copper-zinc- (Cu-Zn-SOD) and extracellular (EC-SOD) superoxide dismutase activities, glutathione-S-trasferase (GST) and glutathione peroxidase (GPX) activities as well as the ratio Cu-Zn-SOD/GPX have been measured in fibroblasts from β-thalassemia (β-thal) patients with and without PXE-like (PXE-L) manifestations. Data are expressed as mean values ± SD of three experiments performed in duplicate using all cell lines. *$p<0.05$; **$p<0.01$

Figure 2. Representative immunoblots of protein disulfide isomerase (PDI), calumenin (CALU), uncarboxylated- (Glu-) and carboxylated- (Gla-) matrix Gla protein (MGP) on proteins isolated from fibroblasts from β-thalassemia (β-thal) patients with and without PXE-like (PXE-L) manifestations. Histograms represent mean values expressed as percentage of densitometric values ± SD with β-thal values set at 100.

*$p<0.05$ β-thal PXE-L vs β-thal values obtained in each cell strain analysed in duplicate in two experiments.

# $p<0.05$ β-thal PXE-L vs β-thal values obtained in duplicate from a pool of fibroblasts, thus indicating experimental variability

Figure 3. Transmission electron microscopy of dermal elastic fibres (E) in normal subjects (a) and in β-thalassemia (β-thal) patients with (c,d) and without (b) PXE-like manifestations. Mineralization (arrows) is limited to tiny small deposits in β-thal patients (b), whereas it dramatically affects and deforms elastic fibres in β-thal PXE-like patients (c-d).

Bar= 1 μm

Figure 4. Immuno-electron-microscopy shows the different localization of uncarboxylated- (a,b) and carboxylated- (c,d) matrix Gla protein on mineralized elastic fibres in the dermis of β-thalassemia patients with PXE-like manifestations. Epitopes are visible as electrondense colloidal gold particles.

Bar= 0.5 μm

Figure 5. Levels of carboxylated- (Gla-) matrix Gla protein (MGP) evaluated by 2D-Western blot are reported for fibroblasts from controls and PXE patients as well as from β-thalassemia (β-thal) patients with and without PXE-like manifestations (a). Parameters of redox balance (glutathione-S-trasferase,GST; glutathione peroxidase, GPX; extracellular (EC-SOD), copper-zinc- (Cu-Zn-SOD) and manganese- (Mn-SOD) superoxide dismutase activities, as well as the oxidant/antioxidant ratio, AOPP+LOOH+MDA/TAS) in fibroblasts from patients affected by PXE, β-thal and β-thal PXE-like diseases are compared with those in fibroblasts from healthy subjects set at 1 (b).

Figure 1

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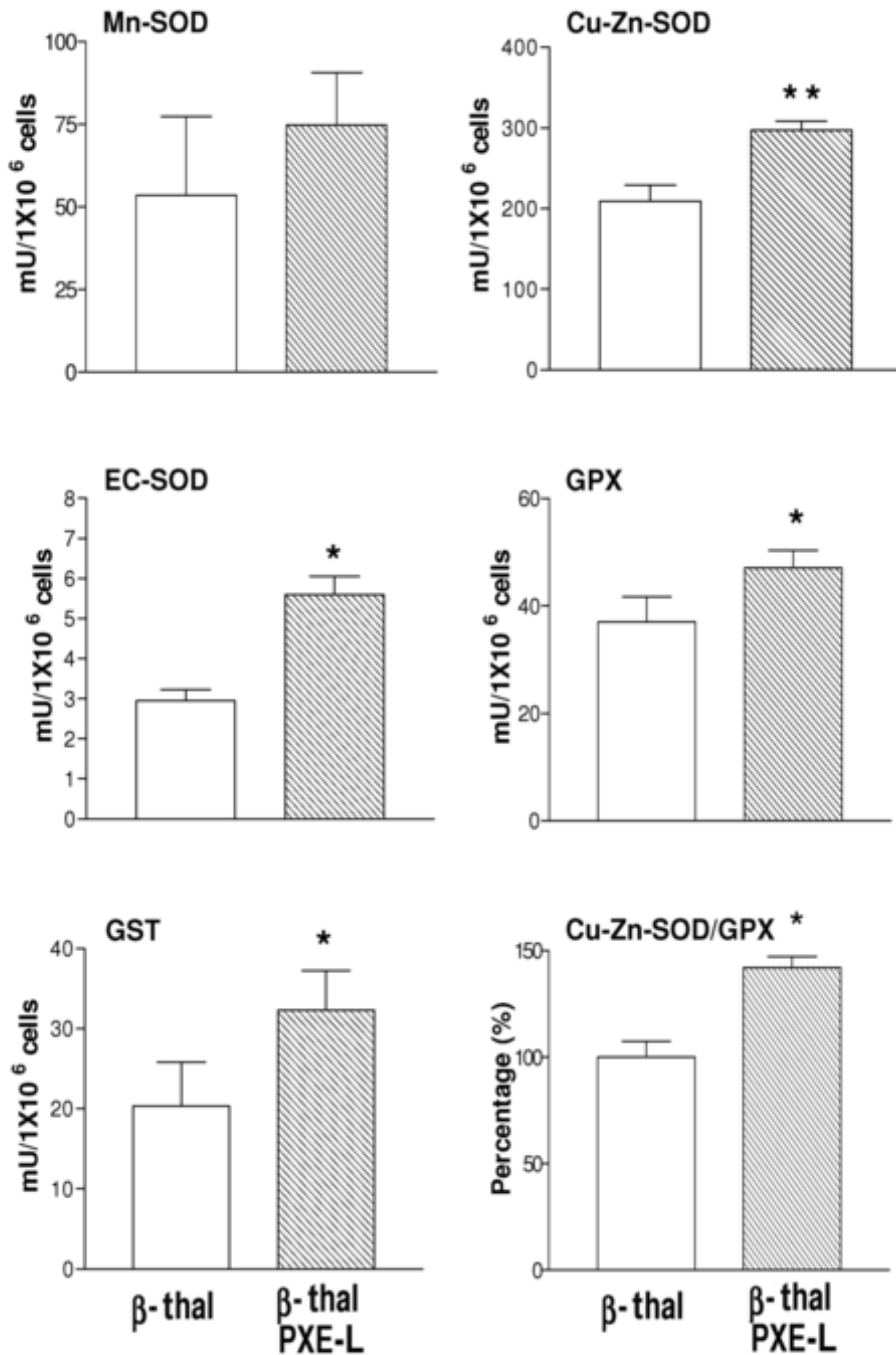

Figure 2
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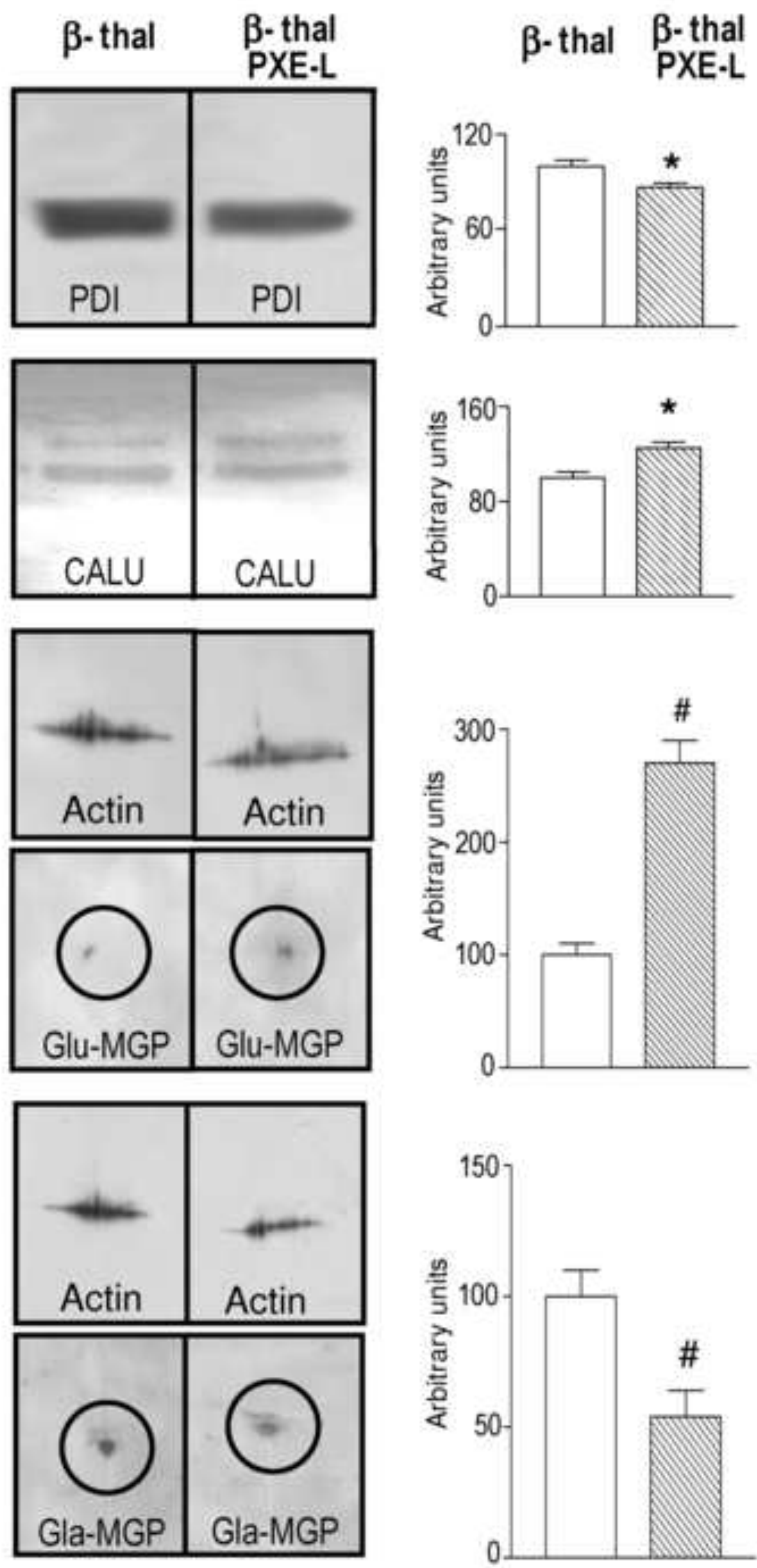

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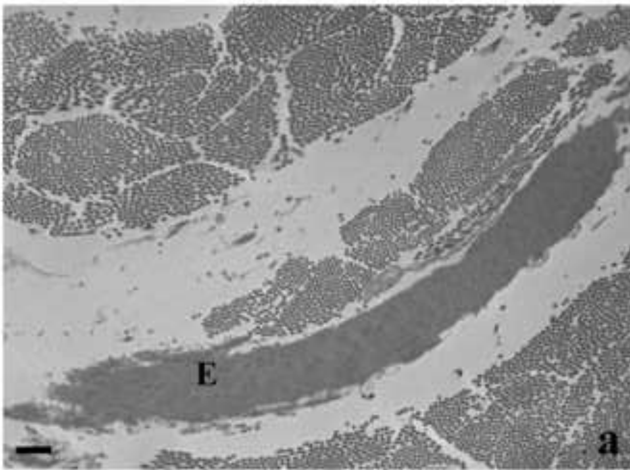

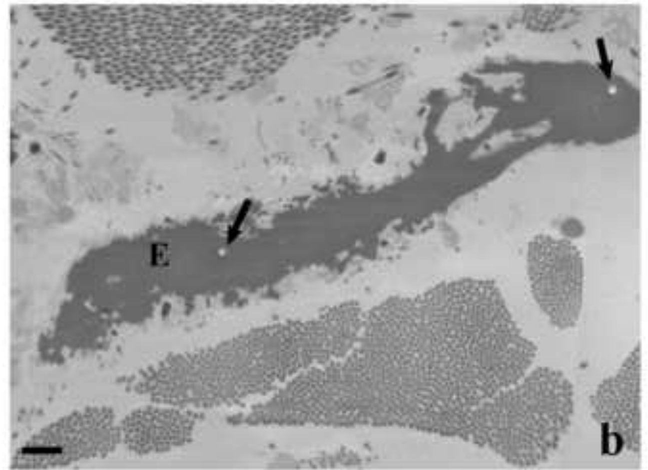

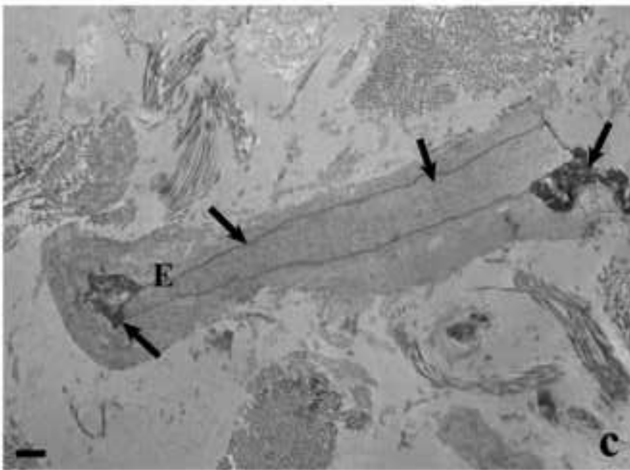

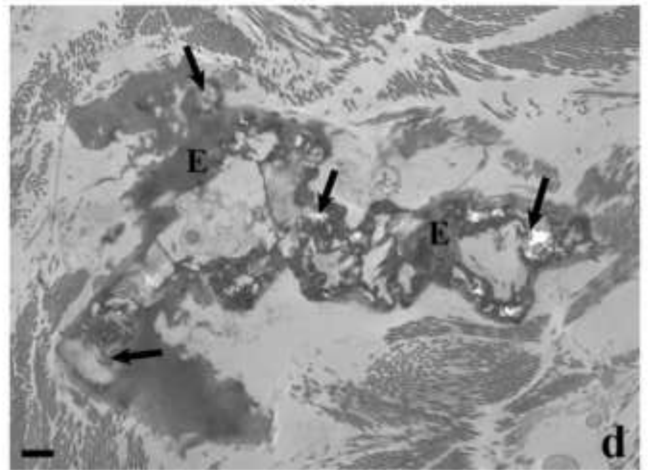

Figure 4
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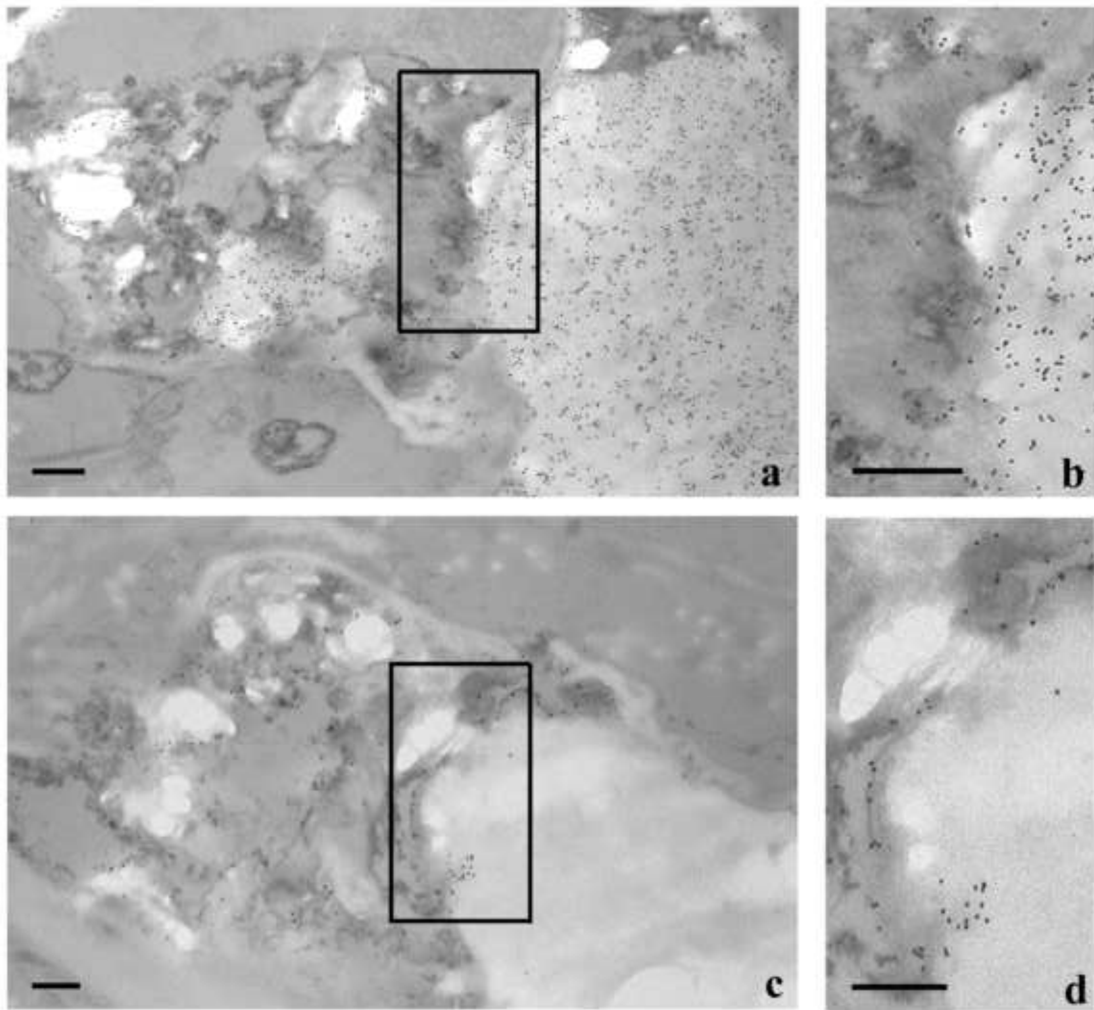

Figure 5
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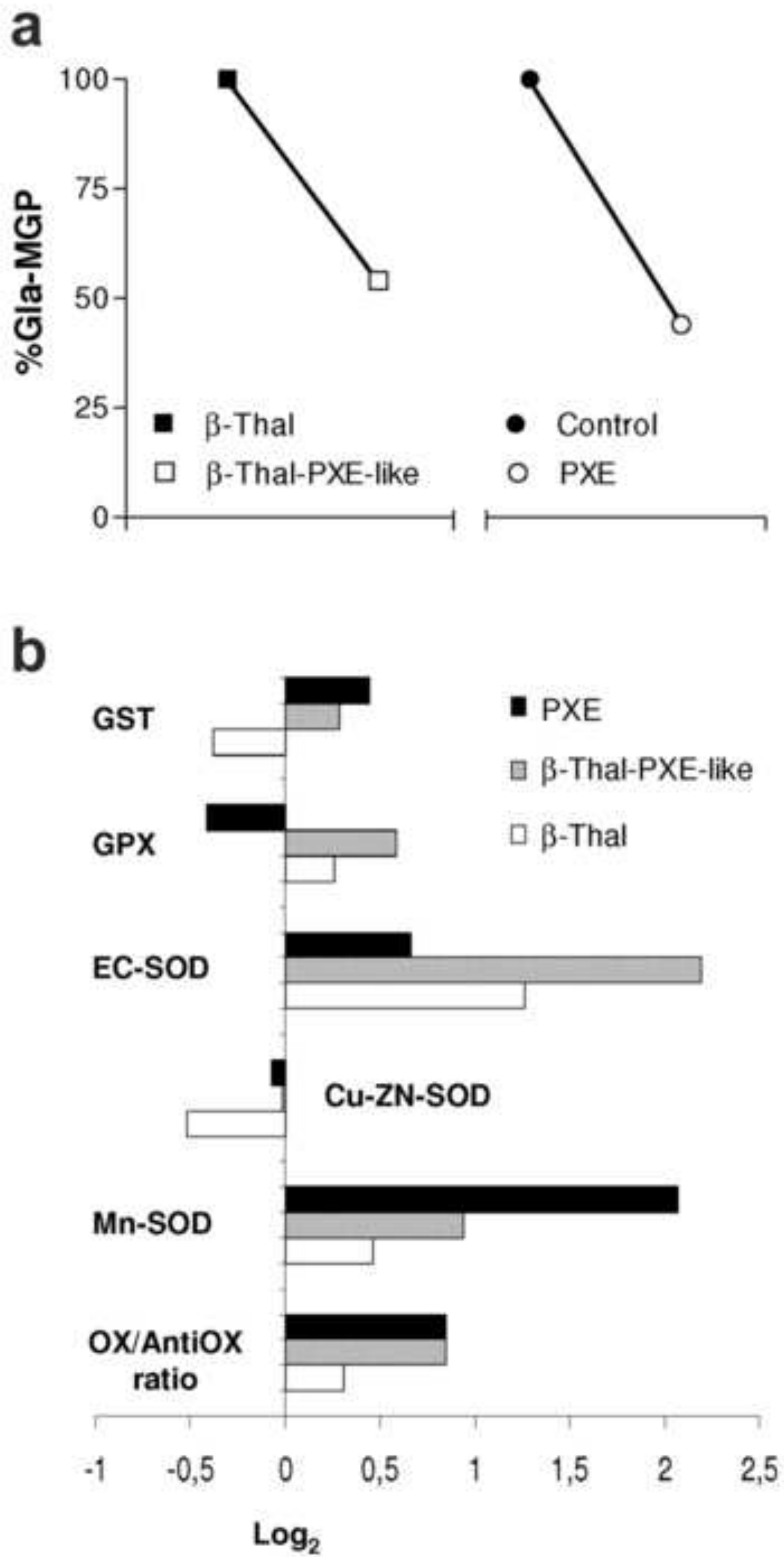

Table 1

Table I: Parameters of redox balance in fibroblasts from patients with β-thalassemia (β-thal) and with β-thal and PXE-like manifestations (β-thal PXE-like).

	beta-thal[1]	beta-thal PXE-like[1]	P value
O_2•- (arbitrary units)	100±3.1	116±10	<0.05
H_2O_2 (arbitrary units)	100±3.8	107±19	n.s
AOPP (nmol/ $1x10^6$ cells)	16.77±4.7	22.78±4.9	<0.05
LOOH (nmol/$1x10^6$ cells)	0.585±0.08	0.654±0.02	<0.05
MDA (pmol/mg protein)	5.30±0.6	7.44±2.7	<0.05
TAS (µmol/ $1x10^6$ cells)	0.075±0.002	0.070±0.009	n.s
OX/AntiOX ratio[2] (arbitrary unit)	100±20	145±24	<0.05

1) Data are expressed as mean values ± SD of experiments performed two times in duplicate with all cell lines.

2) The oxidant/antioxidant ratio (OX/AntiOX ratio) is the ratio between parameters of oxidative damage (AOPP, LOOH and MDA) and antioxidant properties (TAS).