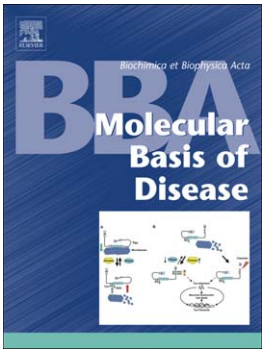

Parameters of oxidative stress are present in the circulation of pxe patients

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PARAMETERS OF OXIDATIVE STRESS ARE PRESENT IN THE CIRCULATION OF PXE PATIENTS.

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Key words: Pseudoxanthoma elasticum, oxidative stress, serum, plasma, redox balance

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ABSTRACT

Pseudoxanthoma elasticum (PXE) is an inherited disorder characterized by calcification of elastic fibres leading to dermatological and vascular alterations associated to premature aged features and to life threatening clinical manifestations. The severity of the disease is independent from the type of mutation in the ABCC6 gene, and it has been suggested that local and/or systemic factors may contribute to the occurrence of clinical phenotype. The redox balance in the circulation of 27 PXE patients and of 50 healthy subjects of comparable age was evaluated by measuring the advanced oxidation protein products (AOPP), the lipid peroxidation derivatives (LOOH), the circulating total antioxidant status (TAS), the thiol content and the extracellular superoxide dismutase activity (EC-SOD). Patients were diagnosed by clinical, ultrastructural and molecular findings. Compared to control subjects, PXE patients exhibited significantly lower antioxidant potential, namely circulating TAS and free thiol groups,and higher levels of parameters of oxidative damage, as LOOH and of AOPP, and. of circulating EC-SOD activity. Interestingly, the ratio between oxidant and antioxidant parameters was significantly altered in PXE patients and related to various score indices. This study demonstrates, for the first time, that several parameters of oxidative stress are present in the blood of PXE patients and that the redox balance is significantly altered compared to control subjects of comparable age. Therefore, in PXE patients the circulating impaired redox balance may contribute to the occurrence of several clinical manifestations in PXE patients, and/or to the severity of disease, thus opening new perspectives for their management.

Introduction

Pseudoxanthoma elasticum (PXE) is a genetic disorder characterised by calcification of elastic fibres in all soft connective tissues [1], with main alterations in the dermis and in the vessel compartment [2]. The disease is due to mutations in the ABCC6 gene that codes for the multidrug resistance protein-6 (MRP-6). The physiological role as well as the pathogenesis of clinical manifestations are still unknown [3,4]. The high heterogeneity of clinical manifestations, even in individuals of the same family with identical mutations, suggests that local and/or systemic factors might be involved.

Dermal fibroblasts, although expressing little or no MRP-6 [5,6], when isolated from PXE patients, exhibit a number of biochemical and behavioural alterations compared to age matched controls indicating that these cells bear permanent metabolic alterations affecting synthesis and deposition of several matrix molecules, thus altering connective tissue homeostasis and especially that of elastic fibers [7-11].

Recently, it has been demonstrated that in vitro PXE fibroblasts suffer from a condition of mild chronic oxidative stress, as they produce more malondialdehyde, a byproduct of lipid peroxidation, and have higher amounts of oxidised glutathione and of glutathione peroxidase activity, as well as higher superoxide dismutase activity [12] compared to controls.

In order to investigate whether the cellular impaired redox balance could reflect a more generalised condition in patients, the redox equilibrium in the blood of PXE patients was analysed and compared with controls of comparable age, by evaluating the amount of advanced protein oxidation products (AOPP) and of lipid hydroperoxide (LOOH) levels as indices of a general oxidative damage of plasma proteins, lipids and lipoproteins; the total antioxidant status (TAS) and the total thiol content representing the capacity of plasma factors to counteract oxidative stress; the activity of extracellular superoxide dismutase (EC-SOD), due to its important role in controlling damages induced by superoxide anion radicals [13,14].

Methods

Chemicals

All reagents were purchased from Sigma (St. Louis, MO) and were of analytical grade.

Plasma and dermal samples

Blood samples from 27 patients with Pseudoxanthoma elasticum (21 women and 6 men; 37 ±12 yr;) and from 50 healthy volunteers (40 women and 10 men; 41 ± 10 yr) were analysed. All subjects gave written informed consent and the investigation was approved by the Local Ethical Committee. Venous blood samples were drawn after 8 h fasting and collected into tubes containing EDTA or Gel-Clot Activator. Tubes were centrifuged at 3500 × *g* for 10 min at 4°C. Appropriate aliquots of plasma-EDTA and of serum were stored at -70°C and used, according to the method applied for each marker.

Diagnosis and clinical evaluations

PXE patients have been firstly clinically diagnosed on the basis of skin and eye alterations. Other clinical signs, such as intermittent claudication, cardiovascular complications and gastrointestinal bleeding were carefully evaluated when present. In order to uniformly describe these parameters, physicians employed an internationally standardized scoring system (Phenodex™) developed by the PXE International Consortium [4]. The "total score" used in the present study was the sum of all scores that, in each patient, characterized the alterations of the different organs/systems, and therefore a global index of the severity of clinical manifestation in each individual. Diagnosis was further confirmed by light and electron microscopy on skin samples and by the identification of mutations in the ABCC6 gene according to the procedures already described [3]. Disease onset in all patients occurred around puberty. Clinical data and genotypes for each patient are reported in Table 1.

Analytical Methods

Evaluation of Plasma Advanced Oxidation Protein Products (AOPP)

Plasma AOPPs were evaluated using a microassay adapted to Cobas Mira according to Matteucci et al. [15] and based on the original method of Witko-Sarsat et al. [16]. Briefly, 18 µL of plasma or chloramine-T (ch-T) standard solutions (400 – 6.25 µmol/L) were placed in each well of the Cobas Mira autoanalyser followed by addition of 200 µL of reaction mixture, consisting of 81% phosphate buffer solution (PBS), 15% acetic acid, and 4% 1.16 mM potassium iodide. Absorbance was read at 340 nm (the blank contained PBS instead of plasma). AOPP concentration was obtained on the basis of measured ch-T equivalents. Intra- and inter-assay variation coefficients were 1% and 5%, respectively.

Evaluation of serum lipid hydroperoxides (LOOH)

Lipid hydroperoxides were evaluated by the FOX2 method (Ferrous Oxidation automated by Arab and Steghens [17] adapted to Cobas Mira (wavelength 600 nm) for studying lipid peroxidation in serum samples. Xylenol orange (180 µL of a 167 µM solution) was added to 25 µL of sample. The first optical reading was recorded before the addition of 45 µL of 833 µM iron II D-gluconate. LOOH was calculated using a standard curve of tert-butylhydroperoxide. Intra- and inter-assay variation coefficients were 3% and 8%, respectively.

Total Antioxidant Status (TAS)

The total antioxidant capacity of serum 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]. The method was calibrated using the TROLOX standard included with the kit.

Determination of Plasma Sulfhydryl Groups (Total-thiol)

Plasma sulfhydryl (-SH) groups were determined by using Ellman's reagent 5,5'-dithiobis(2-nitrobenzoate)-DTNB adapted to Cobas Mira [19]. Plasma (10µL) was mixed with 200 µL of 0.1 M Tris buffer, containing 10 mM EDTA, pH 8.2. The absorbance at 405 nm, given by the plasma alone, was subtracted from that obtained from the same sample 10 min after addition of 8 µL of 10 mM DTNB. A blank containing only DTNB was also included, and -SH concentration was calculated by using a standard curve of glutathione. Intra- and inter-assay variation coefficients were 1.2% and 6%, respectively.

Separation of Extracellular Superoxide Dismutase (EC-SOD)

EC-SOD (EC 1.15.1.1) can be separated from SOD isoenzymes and other compounds having SOD-like activity applying the chromatography of samples on concanavalin A-substituted Sepharose (ConA-Sepharose; Amersham, NL). EDTA-plasma (200µL) [14] was applied to a 1mL ConA-Sepharose column equilibrated with 50 mM Na-HEPES (pH 7.0) - 0.25M NaCl. After 5 min, 3 mL of equilibration buffer were added, the eluted fluid was discarded and the column was washed with 10 mL of equilibration buffer. EC-SOD was finally eluted with 5 mL of 0.5 M α-methylmannoside added to 1 mL aliquots at 5 min intervals.

EC-SOD activity determination

Briefly, 160 µL of eluted plasma were processed using a system based on the oxidation of NAD(P)H [20], adapted to Cobas Mira. Briefly, plasma was combined with 160 µL of 100 mM triethanolamine/diethanolamine-HCl buffer, pH 7.4, 5 µL of a solution containing 100 mM EDTA and 50 mM $MnCl_2$, and 8 µL of 7.5 mM NAD(P)H. The reaction was started by addition of 20 µL of 10 mM mercaptoethanol. Superoxide was generated by molecular oxygen in the presence of EDTA, $MnCl_2$, and mercaptoethanol. NAD(P)H was oxidized by superoxide at a predictable rate and the absorbance at 340 nm decreased accordingly. The decrease in absorbance was inhibited by

the presence of endogenous EC-SOD. Samples were tested for NAD(P)H oxidase activity before addition of mercaptoethanol. EC-SOD activity was estimated from a standard curve constructed by measuring the activity of increasing known amounts of Cu/Zn-SOD (Sigma). One unit was equivalent to 21.5 ng Cu/Zn -SOD and resulted in a 50% inhibition of the rate of NAD(P)H oxidation compared with triethanolamine/diethanolamine buffer as a negative control. Intra- and inter-assay variation coefficients were 2.5% and 8%, respectively.

Statistical analysis

All determinations were performed in duplicate and data were expressed as mean ± SEM.

The Pearson's test was used to assess the significance of correlations between analytical values, age and clinical scores. Correlations were also evaluated by the Fisher test.

In order to verify if mean values of oxidative stress markers belonged to different populations, differences in the distribution of data were compared with the Student t test (critical value of $t=1.66$ with 75 d.f. and probability 0.95).

Finally, the influence of gender, age and clinical scores on analytical values was evaluated with the two and three way analysis of variance. Statistical significance was confirmed for $p<0.05$.

Results

Measurement of parameters of oxidative stress

Several parameters of oxidative stress, i.e. Advanced Oxidation Protein Products (AOPP), lipid peroxidation products (LOOH), Total Antioxidant Status (TAS), total Thiol content and the EC-SOD activity, were measured in the blood of PXE patients and of healthy subjects of comparable age.

Mean values indicate that all parameters were significantly modified in patients (Figure 1). In particular, AOPP and LOOH, as indices of oxidative damage of proteins and lipids, were significantly higher in PXE patients, whereas the antioxidant capacity of plasma, namely TAS and

total thiol content, were significantly lower in patients, compared to controls (Figure 1). Moreover, the EC-SOD activity was significantly higher in the plasma of PXE patients than in healthy individuals (Figure 1).

Although the great majority of patients were woman, the analysis of variance allowed to exclude the influence of gender in the evaluation of oxidative stress parameters between control and PXE patients (data not shown).

In order to assess if these parameters were differently distributed according to the age of subjects, values from each individual (controls and patients) were evaluated by linear regression analysis (Figure 1). Data indicate that age seems to influence total thiol content in control subjects ($p<0.001$) (figure 1D) and AOPP values in patients ($p<0.05$) (Figure 1a), whereas all other parameters did not appear to change with age in healthy individuals nor in PXE patients (Figure 1).

Looking at mean values of thiols and AOPP in each decade of life, we have observed that the total thiol content was significantly different in the third and fourth decades of life and became similar in healthy and diseased subjects after the fifth decade of life (Figure 2a). The opposite behaviour was noted for AOPP values that were similar in controls and PXE patients of younger age, whereas they became progressively more dissimilar after the third decade, being significantly different only in the sixth decade of life (Figure 2b).

The analysis of variance confirmed that the different thiol content in control and PXE patients is partly related to the occurrence of the disease and partly to aging itself.

Parameters of oxidative stress and severity of disease

Although all patients included in the present study exhibited the first sign of the disease around puberty, the severity of the disease as well as the number of organs involved appeared to progress with time in patients at different degree, independently from the type of mutation.

Figure 3 describes the relationships between patients' age and the disease score measured in the different affected organs/system (i.e, skin, eyes, peripheral vessels, heart and gastrointestinal tract)

as well as the total disease score (i.e. the sum of all scores that, in each patient, characterized the alterations of the different organs/systems).

It appears that some organs show an age-depend progression of clinical manifestations. In particular, the eye score ($p<0.004$), the peripheral vessel score ($p<0,05$) and the total disease score ($p<0,01$) significantly correlate with age. By contrast, the skin score did not change with age as well as the cardiac and the gastrointestinal score, even though the number of patients with cardiac and gastric complications was probably to low for a significant correlation.

Furthermore, in order to assess if oxidative stress parameters were not only associated to the occurrence of PXE, but if they were also related to the severity of disease, all investigated parameters were correlated with the patient's total disease scores (Figure 4). Data indicate that there was a statistically inverse correlation between the total disease score and the thiol content (Figure 4d) ($p<0.02$). The thiol levels were inversely related also to the skin score ($p<0,01$) (not shown). By contrast, all other values did not significantly correlate with the overall severity of the disease, nor with the severity of clinical manifestations of each organ, at least in the cohort of patients we have examined (data not shown).

Analysis of variance indicated that changes in thiol levels were partly related to the age of patients and partly to the disease score, with the exception of the skin score that appeared to be independent from age (data not shown).

Oxidant / Antioxidant ratio in PXE patients

The ability of organisms to cope with oxidative damage depends on the efficiency of the antioxidant capabilities within tissues and/or blood to counteract oxidative damages. We have therefore evaluated the redox balance by correlating the oxidant/antioxidant ratio (OX/AntiOX ratio) by comparing parameters of oxidative damage (AOPP and LOOH) with those of antioxidant properties (TAS and total thiols). Data indicate that the mean OX/AntiOX ratio is $0,99 \pm 0,04$ in control subjects, whereas it is $1,32 \pm 0,08$ ($p<0.004$) in PXE patients (Figure 5).

Even though there is an age-dependent increase of the OX/AntiOX ratio in controls ($p<0,02$) as well as in patients ($p<0,03$), values are always higher in PXE, at all ages considered (Figure 5).

Difference between control and PXE patients can be even more pronounced if we consider the circulating EC-SOD as a parameter favouring oxidative damage in tissues. In these conditions, the ratio between parameters of oxidative damage (AOPP, LOOH and EC-SOD) and antioxidant parameters (TAS and total thiol) is $1,09 \pm 0,04$ vs $1,48 \pm 0,08$ ($p<0,001$).

Interestingly, in PXE patients the OX/AntiOX ratio appears to be significantly related not only to the total disease score ($p<0,02$) but also to the skin ($p<0,05$), the heart ($p<0.01$) (Figure 6). and the cardiovascular score ($p<0.05$, data not shown), being this last score calculated from all vascular complications, such as vessel occlusions, claudicatio, cardiac complications, gastrointestinal bleeding

Furthermore, looking at patients with identical ABCC6 mutations but with different disease score and/or number of organs involved there was a general correlation between the severity of the disease and the OX/AntiOX ratio (Table 2).

Discussion

Data from the present study demonstrate for the first time that different parameters of the redox status are significantly modified in the circulation of PXE patients compared to control subjects of comparable age and that the oxidant/antioxidant ratio (OX/AntiOX ratio) significantly increased in patients. These findings are consistent with recent evidence from our laboratory showing that PXE dermal fibroblasts in vitro are in a condition of mild chronic oxidative stress [12]. Therefore, a condition of permanent oxidative stress is actually present and not efficiently balanced in PXE patients at local and at general levels.

It is well known that oxidative stress is associated to age-related degenerative features in several tissues and organs [21]. All patients included in the present study showed first sign of clinical

manifestations at puberty and, for most of them, there was a progression of disease's severity with time, as clearly shown by the positive correlation between patient's age and total disease score.

Interestingly, as in the case of skin, it appeared that some organs did not show a significant correlation between age and progression of clinical manifestations.

In the normal population, vessel alterations are not clinically relevant until the fifth or the sixth decades of life [22]. By contrast, from the analysis of patients' clinical data it appears that 25% of patients (7/27) had vascular alterations before the fifth decade of life, and 13% (4/27) before the fourth decade of life. Moreover, claudication due to femoral artery damages is often present in PXE patients in the second-third and, occasionally, also in the first decade of life [2,3].

The majority of investigated parameters of the oxidative state did not change with age in the group of subjects we analyzed. This could be due to the relatively low number of old individuals and no one over the age of 67 years. Moreover, the heterogeneity among subjects may hide differences related to aging. Never the less, patients were always compared with controls of similar age and in some cases data were analyzed also within each decade of life.

Even though ROS are physiologically important mediators in biological signaling processes [23], they may also represent an important cause of structural damage. Harman has been the first to propose that the damaging effects of ROS may play a key role in the mechanism of aging [24], since ROS are continuously generated in living tissue and can potentially damage DNA, proteins and lipids, thus possibly influencing life span [25, 26]. Even though PXE patients are characterized by several features resembling premature aging syndromes (i.e. skin wrinkles and laxity, cardiovascular complications, visual impairment similar to macular degeneration), at present, we do not have any significant data on premature death in clinically and/or genetically diagnosed PXE patients. Therefore, the altered oxidant/ antioxidant balance in these patients could be eventually related only to the premature occurrence of clinical manifestations that can influence at least life's quality, if not life span expectancy.

The antioxidant imbalance in PXE plasma is shown by the significantly lower level of thiols (namely glutathione, cysteine and protein-bound sulphydryl groups, albumin) detected in patients compared to controls. Thiols are recognized to play a fundamental antioxidant role by protecting cellular and extracellular functions against oxidative stress [27]. Their level normally decreases with age in humans as well as in mice [28] and this may contribute to the reduced capacity of plasma factors to counterbalance oxidative stress during senescence. Interestingly, thiol levels in young patients were generally as low as in normal old individuals. This finding and the inverse correlation between plasma thiol content and severity of clinical manifestations may highlight the importance of this parameter in the premature occurrence of clinical manifestations in PXE patients. Since the great majority of thiol groups are furnished by plasma proteins, the net decrease of the thiol content in PXE plasma is probably the result of abnormal oxidation of SH-containing proteins [29]. This finding is consistent with the high amount of AOPP in patients, similarly to what observed in the plasma of uremic patients [16] and in those with coronary artery disease [30], where the AOPP parameter has been used as indicator of oxidant-mediated protein damage. The present study shows that, in PXE, the permanent condition of oxidative stress affects also plasma lipids, as documented by the higher level of LOOH. Since lipoproteins account for the majority of plasma lipids, data suggest that oxidised lipoproteins could contribute to the precocity and severity of vessel alterations that are frequently and prematurely observed in PXE patients [2,3].

To further investigate the reduced capacity of PXE patients to cope with a generalized condition of oxidative stress, we have evaluated the activity of EC-SOD. Superoxide dismutases are enzymes that catalyse the rapid dismutation of superoxide radical to hydrogen peroxide and oxygen. The SOD3 isoform, also called EC-SOD, is produced by several cell types [31] and is the only antioxidant enzyme that removes superoxide radicals from the extracellular space of tissues [31]. Several reports have shown that high levels of circulating EC-SOD are associated with decreased tissue antioxidant levels and with increased risk of ischemic diseases indicating that the circulating form of EC-SOD is not efficient in protecting tissues [32]. PXE patients had always high levels of

circulating EC-SOD compared to controls. The high level of EC-SOD in PXE plasma could derive either from its release from tissues by proteolytic removal of its heparin-binding site [33] or by mechanisms involving binding of EC-SOD to sulphated glycosaminoglycans that are abundantly present in plasma and urine of PXE patients [11].

Even though vascular alterations are frequently observed in PXE patients, circulating EC-SOD did not appear to be significantly related to vascular nor to other clinical manifestations. Never the less the high levels of circulating EC-SOD may be regarded as a factor contributing to oxidative damage, further worsening the altered redox balance in PXE patients, as suggested by the striking divergence in the ratio between oxidant and antioxidant parameters measured in controls and in PXE patients.

The redox balance within tissues or in the circulation is due to a tightly regulated homeostasis between oxidant and antioxidant capabilities. By evaluating the OX/AntiOX ratio, i.e. ratio between measured values of oxidative damage (AOPP and LOOH) and of antioxidant properties (TAS and total thiols), it emerges that the OX/AntiOX ratio is related to the total disease score and to skin and cardiac complications. Moreover, in PXE patients with identical ABCC6 mutations, the total severity of clinical manifestations generally showed a positive correlation with levels of the OX/AntiOX ratio. This sustains the hypothesis that factors other than ABCC6 mutations may contribute to the heterogeneity of clinical manifestations and that OX/AntiOX ratio may play a relevant role.

In conclusion, present data demonstrate that abnormalities in the redox equilibrium are present in the circulation of PXE patients, affecting both plasma proteins and lipids, consistently with previous observations on in vitro cultured fibroblasts [12] and with very recent data from Zarbock and coworkers showing that the disease onset in PXE patients could be related to polymorphisms in genes encoding for catalase, superoxide dismutase 2 and glutathione peroxidase 1 [34]. Alterations in the plasma redox balance and have been repeatedly associated to age-related complications [35] and in particular to vascular damages [36]. Vascular complications are present and have been

repeatidly observed in PXE [2,3], and the plasma redox imbalance could contribute to these vascular alterations; actually, claudication and femoral calcification are often present in PXE patients already in the second and the third decades of life.

In the light of these results, it is conceivable to suggest that PXE manifestations are not the direct effect of the reduced/absent expression and function of MRP6, since connective tissue alterations, and in particular elastic fiber calcifications, are the consequence of an altered metabolism of mesenchymal cells, where MRP6 is actually poorly expressed, even in normal conditions [37]. Therefore, the pathologic behaviour of fibroblasts could be the consequence of a modified "environment", i.e. of an increased redox imbalance either general and/or local. The response of cells to oxidative stress might have epigenetic consequence on cell behaviour through altered DNA mathylation, abnormal regulatory mechanisms possibly by miRNA, accumulation of oxidised proteins, activation of an ER stress response.

However, it has to be mentioned that increased ROS production is present in several disorders where no calcification occurs, or where mineralization is only limited to the cardiovascular system [21, 38]. Therefore, oxidative stress per se, could not be the only responsible for calcification of elastic fibers.

In PXE, it has been recently observed that, as a consequence and/or in association with oxidative stress, are changes in the pathways regulating the carboxylation of Matrix Gla Protein (MGP) that, when efficiently carboxylated, acts as a potent inhibitor of ectopic calcification in soft connective tissues [39].

At present, studies are in progress in order to verify if the same molecular pathways are altered also in patients with PXE-like alterations, such as in haemolytic disorders and especially in beta-thalassemia where PXE-like structural and clinical manifestations have been described in several organs [40-42] and where increased markers of oxidative stress have been already demonstrated [43].

Since a globally preserved antioxidant ability is a fundamental prerequisite for a successful ageing, it could be suggested that, as already done in similar conditions of oxidative stress [44], antioxidant treatments could ameliorate the redox balance in PXE, thus opening new perspectives for the management of clinical complications.

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Figure legend

Figure 1. Power regression lines show the distributions of different markers of oxidative stress AOPP (advanced oxidation protein products, A, a), LOOH (lipid peroxidation derivatives, B, b), TAS (total antioxidant status, C, c), thiol content (D, d) and EC-SOD (extracellular superoxide dismutase activity, E, e) measured in the blood of controls (A,B,C,D,E,F) and PXE patients (a, b, c, d, e, f) in relation to the age of subjects. There was no significant correlation between measured values and age of individuals, except for the total thiol content that significantly ($p<0.001$) decreased with age in controls (D) and the Advanced Oxidation Protein Products (AOPP) that were significantly ($p<0.05$) increased with age in patients (a)..

Values expressed as mean values ± SE are reported in the upper part of the figure for control subjects and for PXE patients. * $p<0,05$, ** $p<0,01$, *** $p<0,001$

Figure 2. The total thiol content (a) and the AOPP level (b) were compared in control and PXE patients according to the decade of life of individuals. The thiol content was always lower in patients up to the fifth decade of life, whereas .After AOPP levels were always higher in patients after the third decade of life. *$p<0.05$

Figure 3. Power regression lines show the distributions of disease scores according to the age of patients. The score indices of clinical manifestations occurring in the skin, in the eyes, in peripheral vessels, in the heart and in the gastrointestinal tract were evaluated for each patient. Moreover, the total score was taken as the sum of all scores that, in each patient, characterized the alterations of the different organs/systems (see also material and methods). The eye ($p<0,004$), the vascular ($p<0,05$) and the total scores ($p<0,01$) appeared to significantly progress with the age of patients.

Figure 4. Correlation between the total score, representing the severity of clinical complications as well as the number of organs involved, and AOPP (advanced oxidation protein products, a), LOOH (lipid hydroperoxides, b), TAS (total antioxidant status, c), total thiol content (d) and EC-SOD (extracellular superoxide dismutase, e). A power regression line is shown in each panel and parameters appeared to be significantly ($p<0.02$) related to the total disease score only in the case of total thiol content (d).

Figure 5. Correlation between the oxidant/antioxidant ratio (OX/AntiOX ratio) and the age of control subjects (a, $p<0,02$)) and of PXE patients (b, $p<0,03$). The increase of the OX/AntiOX ratio is more evident in patients at all ages examined (C). Mean values ± SE are reported in panels a and b ($p<0,004$).

* $p<0.05$

Figure 6. Power regression lines showing the distribution of disease scores according to the OX/AntiOX ratio. The score indices of clinical manifestations occurring in the skin, in the eyes, in peripheral vessels, in the heart and in the gastrointestinal tract were evaluated. Moreover, the total score was taken as the sum of all scores that, in each patient, characterized the alterations of the different organs/systems (see also material and methods). The skin ($p<0,05$), the heart ($p<0,01$) and the total scores ($p<0,01$) appeared to be significantly related to the OX/AntiOX ratio.

Table 1. Clinical data of patients

Patients' GENDER /AGE	Clinical scores	Mutations	
		Allele 1	Allele 2
M / 10	S2E2	c.3413G>A (p.R1138Q)	c.3413G>A (p.R1138Q)
F / 16	S1	c.1171A>G (p.R391G)	c.1552C>T (p.R518X)
F / 18	S3E2V2	c.1484T>A (p.L495H)	c.1484T>A (p.L495H)
F / 21	S2E2	c.2420G>A (p.R807Q)	ND
F / 21	S2E2	c.184T>C (p.Y62H)	c.2996_4208del (p.A999_S1403del)
F / 24	S2E2	c.1799G>A (p.R600H)	c.2420G>A (p.R807Q)
F / 27	S3E2	c.184T>C (p.Y62H)	c.2996_4208del (p.A999_S1403del)
F / 30	S2E2G1	c.2996_4208del (p.A999_S1403del)	c.4198G>A (p.E1400K)
F / 30	S2E3	c.2996_4208del (p.A999_S1403del)	c.4198G>A (p.E1400K)
M / 30	S2E1	c.3421C>T (p.R1141X)	c.3735G>A
F / 32	S2	c.3421C>T (p.R1141X)	c.3735G>A
F / 33	S3E2	c.1987G>A (p.G663S)	ND
F /33	S3E3	c.1609_1609delG (p.V537fsX26)	c.1763_1769del ins56
F / 36	S3E2V3	c.3421C>T (p.R1141X)	ND
F / 36	S3E3V2G1	c.3421C>T (p.R1141X)	c.3421C>T (p.R1141X)
M / 39	S1E2V2	c.1552C>T (p.R518X)	c.2996_4208del (p.A999_S1403del)
M / 42	S1E3V2G1	c.1552C>T (p.R518X)	c.2996_4208del (p.A999_S1403del)
F / 43	S3E3	c.1552C>T (p.R518X)	c.1552C>T (p.R518X)
F / 44	S3E2	c.3341G>A (p.R1114H)	c.3542G>A (p.G1181D)
F / 45	S3E3V2C1G1	c.3421C>T (p.R1141X)	c.3421C>T (p.R1141X)
F / 48	S2E2V2	c.1553G>A (p.R518Q)	ND
M / 51	S1E3	c.3662G>A (p.R1221H)	ND
F / 52	S3E3V2	c.3088C>T (p.R1030X)	c.3088C>T (p.R1030X)
M / 54	S1E2G1	c.1799G>A (p.R600H)	c.3941G>A (p.R1314Q)
F / 56	S3E3V2	c.3662G>A (p.R1221H)	ND
F / 60	S2E3V2C1G1	c.951C>A (p.S317R)	c.3421C>T (p.R1141X)
F / 62	S2E3	c.1552C>T (p.R518X)	c.3421C>T (p.R1141X)

Scores describe the severity of clinical manifestations.
In particular, for **skin**, **S1** denoted the presence of papules on the neck and/or other flexural sites, **S2** the presence of coalescent papules (or plaques) and **S3** lax, redundant skin.
For **eyes**, **E1** denoted peau d'orange, **E2** angioid streaks, **E3** retinal haemorrhages and disciform scarring.
For the **vascular system**, **V1** was given to weak or absent pulses, **V2** to intermittent claudication and **V3** to vascular occlusion or other symptoms requiring surgery.
For **cardiac symptoms**, **C1** denoted electrocardiographic changes of ischemia and/or angina, and **C2** myocardial infarction.
For the **gastrointestinal system**, **G1** was given to any gastrointestinal bleeding for which no cause other than PXE could be found.

Table 2 : **Oxidant /AntiOxidant Ratio in PXE patients with identical mutations and different severity of disease**

ABCC6 mutations Allele 1	ABCC6 mutations Allele 2	Age	Gender	Total disease score	N. affected organs	OX/AntiOX Ratio
c.184T>C (p.Y62H)	c.2996_4208del (p.A999_S1403del)	21	F	4	2	**0,80**
c.184T>C (p.Y62H)	c.2996_4208del (p.A999_S1403del)	27	F	5	2	**1,19**
c.2996_4208del (P.A999_S1403del)	c.4198G>A (p.E1400K)	30	F	5	2	**1,10**
c.2996_4208del (P.A999_S1403del)	c.4198G>A (p.E1400K)	30	F	5	3	**1,09**
c.3421C>T (p.R1141X)	c.3735G>A	32	F	2	1	**0,91**
c.3421C>T (p.R1141X)	c.3735G>A	30	M	3	2	**1,42**
c.1552C>T (p.R518X)	c.2996_4208del (p.A999_S1403del)	39	M	5	3	**1,29**
c.1552C>T (p.R518X)	c.2996_4208del (p.A999_S1403del)	42	M	7	4	**1,42**
c.3421C>T (p.R1141X)	c.3421C>T (p.R1141X)	36	F	9	4	**1,62**
c.3421C>T (p.R1141X)	c.3421C>T (p.R1141X)	45	F	10	5	**1,90**

Fig 1

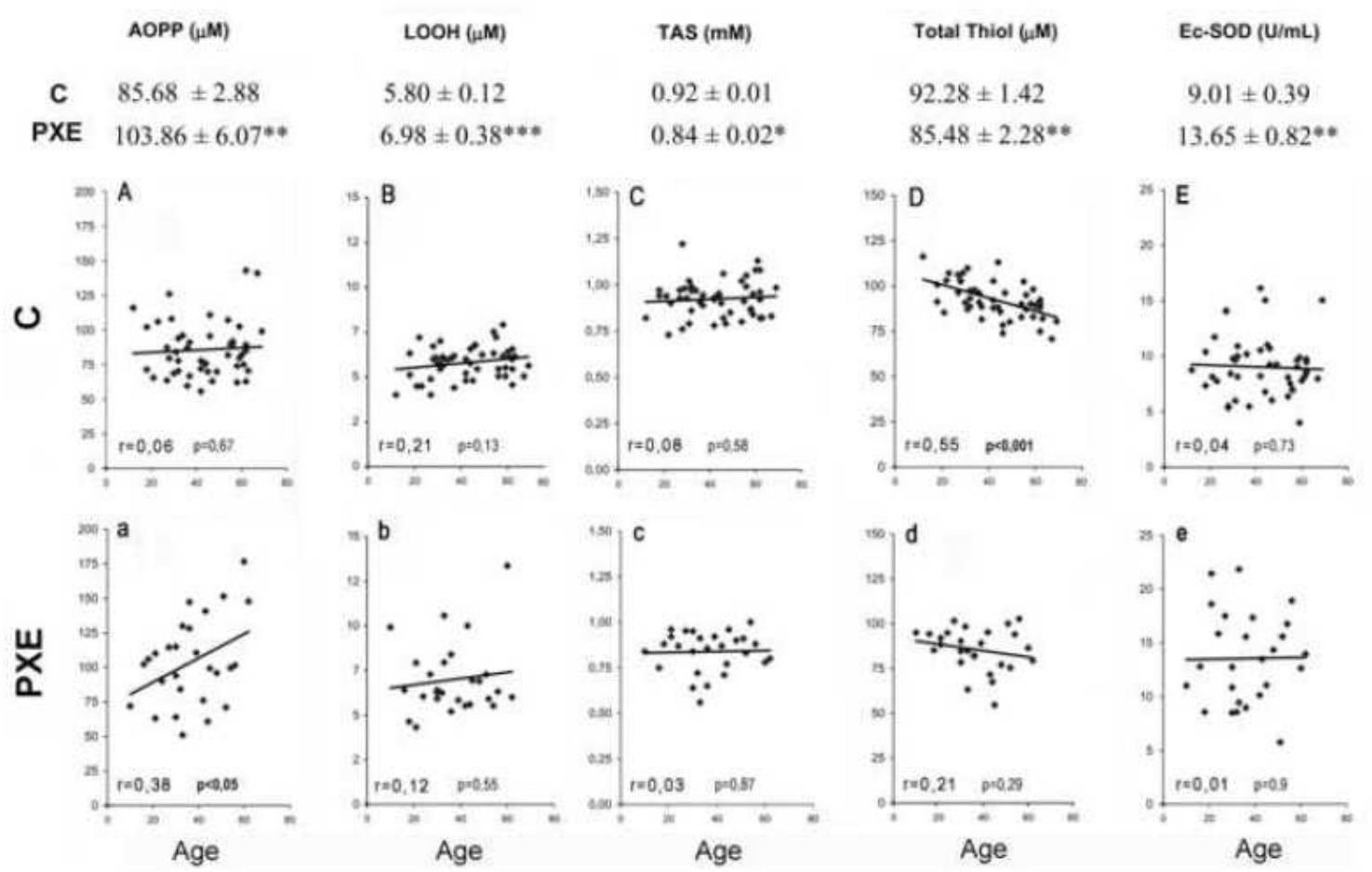

Fig 2

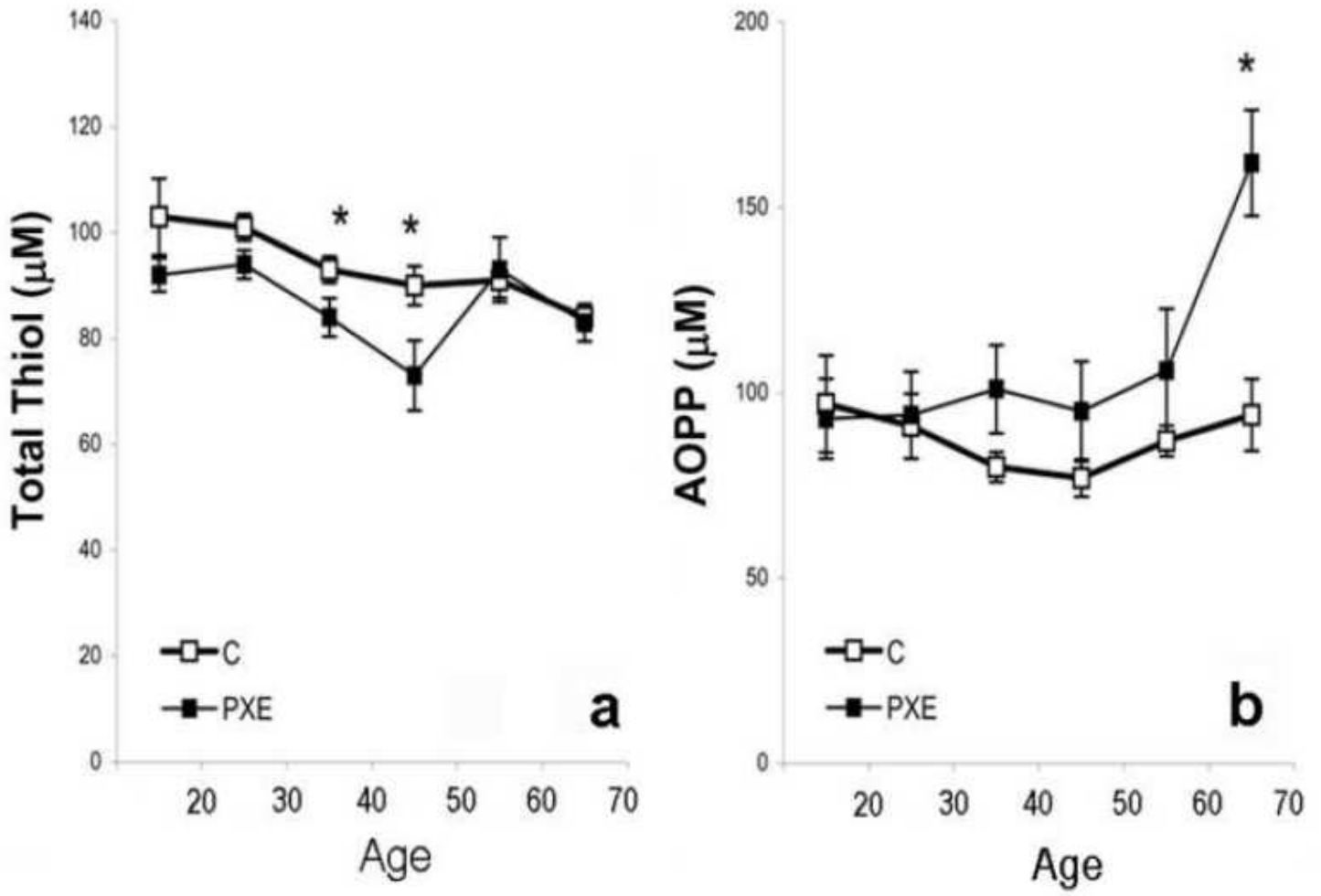

Fig 3

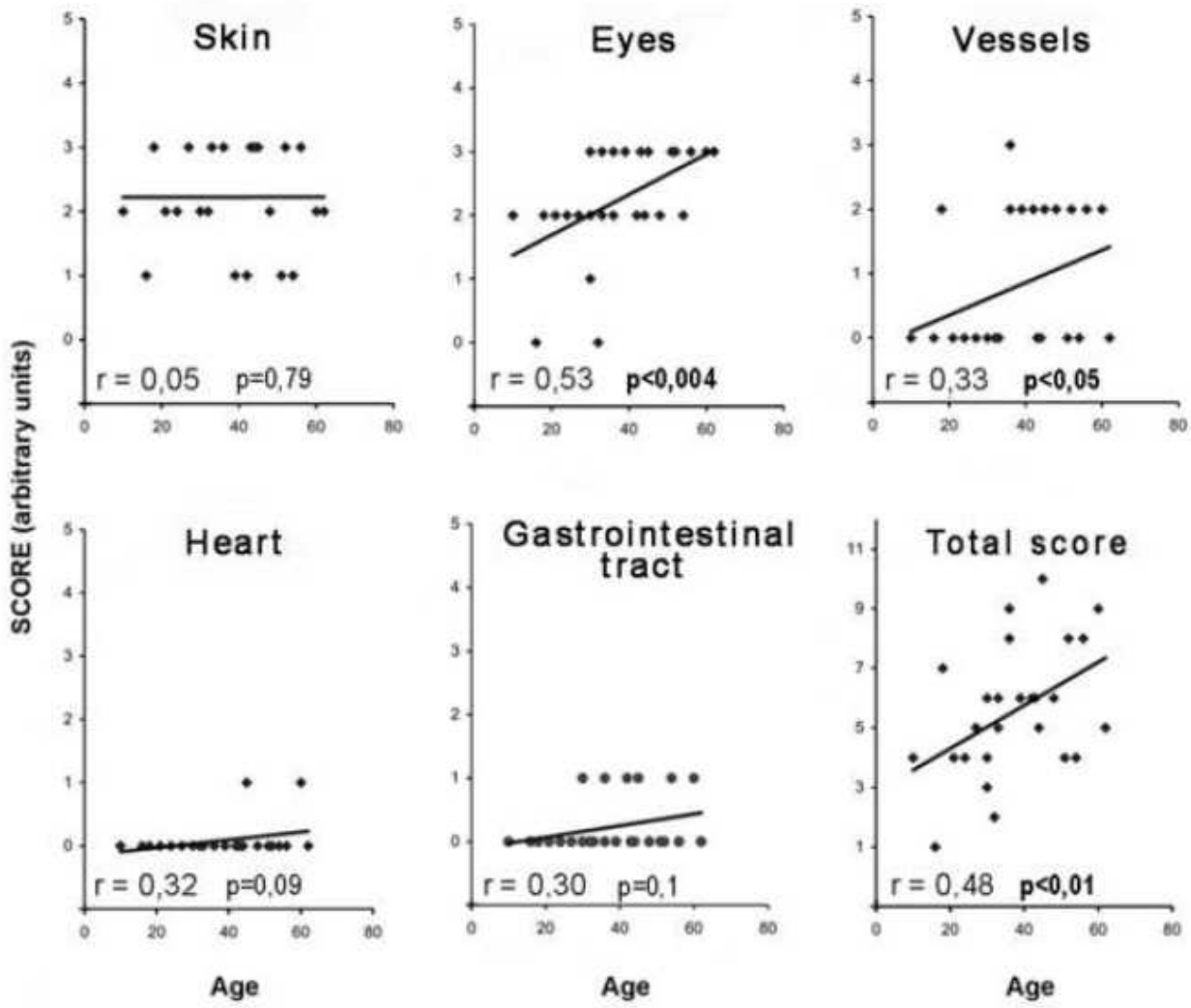

Fig 4

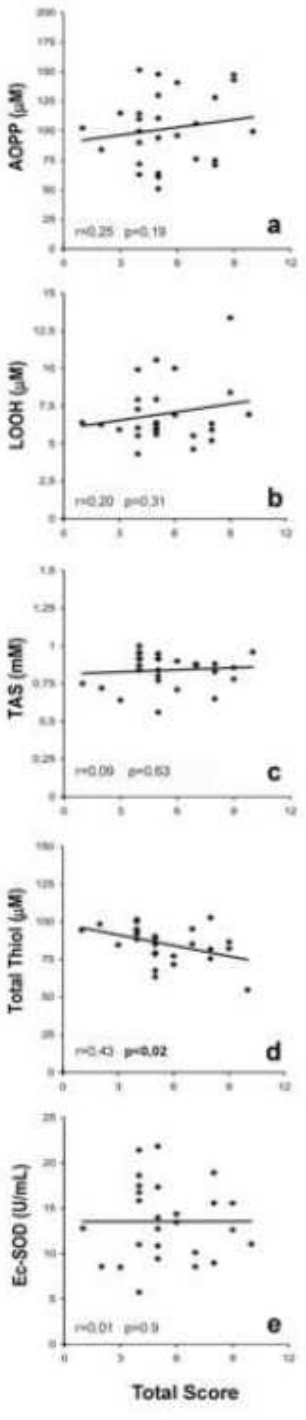

Fig 5

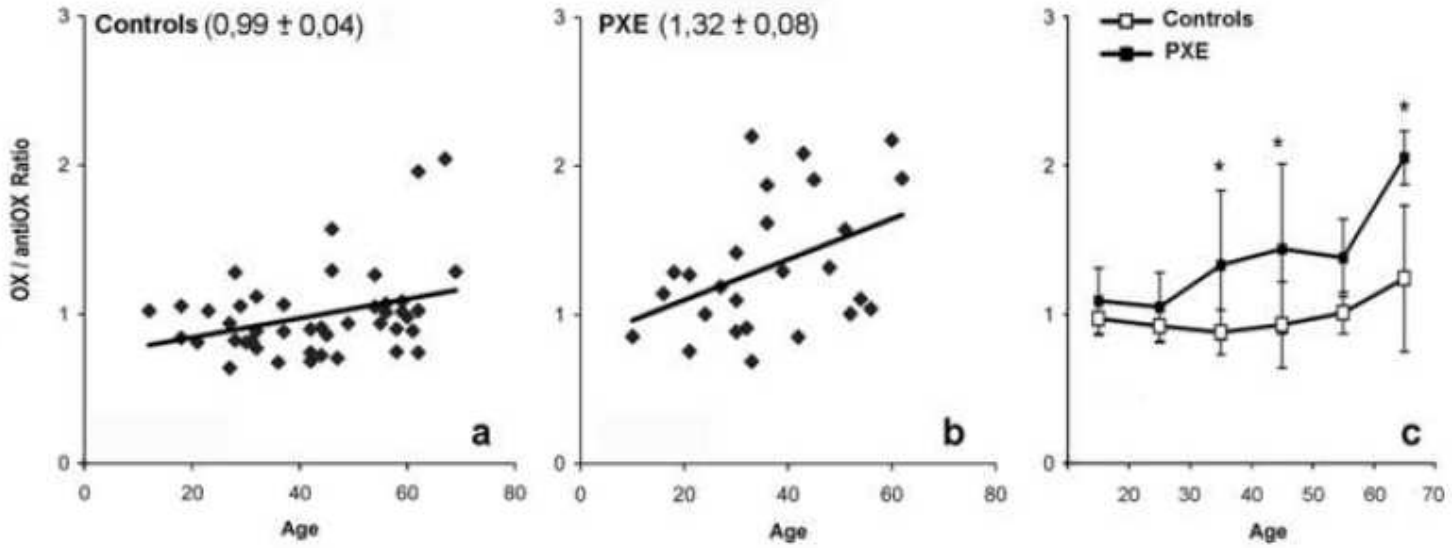

Fig 6