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Globotriaosylceramide is correlated with oxidative stress and inflammation in Fabry patients treated with enzyme replacement therapy

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ABSTRACT

Fabry disease is an X-linked inborn error of glycosphingolipid catabolism due to deficient activity of α-galactosidase A that leads to accumulation of the enzyme substrates, mainly globotriaosylceramide (Gb3), in body fluids and lysosomes of many cell types. Some pathophysiology hypotheses are intimately linked to reactive species production and inflammation, but until this moment there is no *in vivo* study about it. Hence, the aim of this study was to investigate oxidative stress parameters, pro-inflammatory cytokines and Gb3 levels in Fabry patients under treatment with enzyme replacement therapy (ERT) and finally to establish a possible relation between them. We analyzed urine and blood samples of patients under ERT (n = 14) and healthy age-matched controls (n = 14). Patients presented decreased levels of antioxidant defenses, assessed by reduced glutathione (GSH), glutathione peroxidase (GPx) activity and increased superoxide dismutase/catalase (SOD/CAT) ratio in erythrocytes. Concerning to the damage to biomolecules (lipids and proteins), we found that plasma levels of malondialdehyde (MDA) and protein carbonyl groups and di-tyrosine (di-Tyr) in urine were increased in patients. The pro-inflammatory cytokines IL-6 and TNF-α were also increased in patients. Urinary Gb3 levels were positively correlated with the plasma levels of IL-6, carbonyl groups and MDA. IL-6 levels were directly correlated with di-Tyr and inversely correlated with GPx activity. This data suggest that pro-inflammatory and pro-oxidant states occur, are correlated and seem to be induced by Gb3 in Fabry patients.

1. Introduction

Fabry disease (FD) is an X-linked inborn error of glycosphingolipid catabolism due to deficient activity of α-galactosidase A (EC 3.2.1.22). This defect leads to a progressive accumulation of substrates, mainly globotriaosylceramide (Gb3), in body fluids and lysosomes of vascular endothelium and most other tissues. Clinical manifestations include pain and paresthesias in the extremities, angiokeratomas in skin and mucous membranes, hypohidrosis and corneal opacity [1]. Since 2001, enzyme replacement therapy (ERT) is available for FD. The treatment consists in intravenous infusion of recombinant human α-galactosidase A, every two weeks. Agalsidase beta and agalsidase alfa are the two recombinant forms of α-galactosidase A in use for FD [2].

Although it is known that Fabry patients usually die in adult life from renal, cardiac and/or cerebral complications of the vascular disease [1], the pathophysiology of the disease is largely unknown. There is some evidence in the literature demonstrating that oxidative stress

Abbreviations: Cr, creatinine; CAT, catalase; Di-Tyr, di-tyrosine; FD, Fabry disease; FU, fluorescence units; Gb3, globotriaosylceramide; GLA, α-galactosidase A; GPx, glutathione peroxidase; GSH, reduced glutathione; H_2O_2, hydrogen peroxide; HPLC, high-performance liquid chromatography; IL, interleukin; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MDA, malondialdehyde; SOD, superoxide dismutase; TNF, tumor necrosis factor.

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may be involved in FD pathophysiology. Moore et al. [3] found that ascorbate, a potent antioxidant, decreases hyperperfusion in Fabry patients under ERT treatment. In this context, Bodary et al. [4] demonstrated, in apolipoprotein-E (apo-E) deficient mice, that α-galactosidase A deficiency accelerates atherosclerosis and causes increased nitrotyrosine in the plaque. Moore et al. [5] also found excess dermal vascular nitrotyrosine in biopsies from Fabry patients. It is already known that reactive species are involved in a great number of chronic-inflammatory and vascular diseases [6,7]. Also, some studies have shown that lysosomes are quite vulnerable to oxidative stress and the lysosomal compartment is, at the same time, a place with many favorable conditions to pro-oxidant state and even Fenton reaction [8–10].

An *in vitro* study from Shen et al. [11] demonstrates that Gb3 increases reactive oxygen species (ROS) generation and adhesion molecules expression in cultured Fabry endothelial cells. Also, the authors found that plasma from patients treated with ERT increased ROS generation in cultured endothelial cells when compared to plasma from controls.

Considering that oxidative stress is probably involved in Fabry disease but there are a few studies relating it directly, the aim of this study was to evaluate and correlate oxidative damage to biomolecules, antioxidant defenses, pro-inflammatory cytokines and Gb3 levels in patients with Fabry disease under treatment with ERT.

2. Material and methods

2.1. Subjects

The study was performed in 14 adult patients (11 male hemizygotes and three female heterozygotes) with the classic form of FD (median age 39.0 years; range 19–63) and 14 healthy controls matched by age and sex (median age 28.5 years; range 22–58). All patients were receiving ERT treatment (agalsidase alfa — Replagal® 0.2 mg/kg or agalsidase beta — Fabrazyme® 1.0 mg/kg) every two weeks by intravenous infusion, for about 24.5 months (median; range 2–77). At the moment of diagnosis, most of the patients presented the classic symptoms: angiokeratomas, hypohidrosis and pain in extremities. Diagnosis of male patients was done by detection of deficient α-galactosidase A activity in plasma and confirmed in leucocytes. For the heterozygotes, diagnosis was confirmed by molecular analysis of GLA gene. Table 1 shows age, gender, mutation, estimated glomerular filtration rate by Modification of Diet in Renal Disease equation (eGFR-MDRD) and chronic kidney disease (CKD) stage data from the patients.

Informed consent was obtained from the participants. The study was approved by The Ethics Committee of the Hospital de Clinicas de Porto Alegre (HCPA), RS, Brazil.

Table 1
Gender, age, mutation, estimated glomerular filtration rate and chronic kidney disease stage from FD patients.

	Gender[a]	Age (years)	Mutation[b]	eGFR-MDRD[c] (mL/min/1.73)	CKD stage[d]
1	M	40	I133N	79	2
2	M	19	570del16	154	1
3	M	39	L36F	133	1
4	M	34	G328R	15	5
5	M	21	30delG	150	1
6	M	54	30delG	61	2
7	M	39	30delG	25	4
8	M	51	30delG	7	5
9	M	32	30delG	105	1
10	M	23	30delG	177	1
11	M	35	30delG	111	1
12	F	63	30delG	57	3
13	F	53	30delG	72	2
14	F	44	570del16	77	2

[a] M = male and F = female.
[b] GLA mutation.
[c] Glomerular filtration rate (GFR) estimated by MDRD equation.
[d] Chronic kidney disease (CKD) stage according to the National Kidney Foundation.

2.2. Samples collection and preparation

Occasional urine and heparinized blood samples were obtained from patients immediately before the session of ERT. Samples were obtained from controls concomitantly. Urine samples were collected in sterile flask and mixed before being deposited on 10–10 cm virgin filter papers (Whatman 903), until saturation. The filter papers were dried at room temperature (for at least 4–6 h) and stored at 4 °C until Gb3 determination. The rest of urine collected was 1 mL aliquoted and frozen at −80 °C until analysis.

Whole blood was centrifuged at 1000 ×*g* for 10 min and plasma was removed by aspiration, aliquoted and frozen at −80 °C until biochemical determinations. Erythrocytes were washed three times with cold saline solution (0.153 mol/L sodium chloride) and lysates were prepared by addition of 1 mL of distilled water to 100 μL of washed erythrocytes and frozen at −80 °C until determination of GSH and antioxidant enzymes' activities. For these determinations, supernatant (after centrifugation at 13,500 ×*g* for 10 min) was diluted in order to contain approximately 0.5 mg/mL of protein.

2.3. Biochemical determinations

2.3.1. Malondialdehyde (MDA) plasmatic levels

MDA, an end product of lipoperoxidation, was measured by HPLC following the method described by Esterbauer and Cheeseman [12] with slight modifications. Six hundred microliters of 28% trichloroacetic acid and 1.4 mL of distilled water were added to a 100 μL aliquot of plasma. After centrifugation, supernatant was removed and MDA was separated by HPLC using an amino-phase column with 30 mM acetonitrile, Tris buffer pH 7.4 (1:9, v/v). The effluent was monitored at 267 nm, the wavelength of maximum absorption of the enolate anion form of free MDA. The calibration was done with a standard solution of MDA. Results were expressed as log of mM of MDA.

2.3.2. Urine di-tyrosine (di-Tyr) levels

In order to determine in urine the levels of protein oxidation, the intensity of fluorescence of di-Tyr was measured according to the method described by Kirschbaum [13] using a SpectraMax M2e (Molecular Devices, USA) microplate reader at wavelengths of 315 and 410 nm (excitation and emission, respectively). Results were expressed as log of fluorescence units per mg urine creatinine (log FU/mg Cr).

2.3.3. Carbonyl groups determination in plasma

The formation of carbonyl groups, a parameter of oxidative damage to proteins, was measured based on the reaction of these groups with dinitrophenylhidrazine (DNPH), as previously described by Levine et al. [14]. The absorption of the product of reaction was measured in a spectrophotometer at 370 nm. Results were expressed as nmol carbonyl/mg protein.

2.3.4. Total plasmatic level of sulfhydryl (SH) groups

The plasmatic concentration of SH groups was determined as described by Aksenov and Markesbery [15]. The method is based on the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by SH groups into a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm. The SH content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB/mg protein.

2.3.5. Reduced glutathione (GSH) content in erythrocytes

In order to measure the levels of GSH, the main intracellular antioxidant, lysates of erythrocytes were processed as described by Browne and Armstrong [16] and the fluorescence measured (λexcitation = 350 nm, λemission = 420 nm) was compared to a calibration curve prepared with GSH solutions. Results were expressed as nmol/mg protein.

2.3.6. Erythrocyte glutathione peroxidase (GPx) activity

Erythrocyte GPx activity was measured by using a commercially available kit (RANSEL®; Randox Lab). GPx catalyses the oxidation of glutathione (GSH) to GSSG (oxidized glutathione). In the presence of glutathione reductase (GR) and NADPH, the oxidized GSSG is converted to the reduced form with a concomitant oxidation of NADPH to NADP. The decrease in absorbance after 1 and 2 min at 340 nm was measured and results were expressed in U/mg protein.

2.3.7. Erythrocyte SOD/CAT ratio

Erythrocyte CAT activity was evaluated by observing the rate of decrease in hydrogen peroxide (H_2O_2) absorbance in a spectrophotometer at 240 nm according to the method described by Aebi [17]. SOD activity was measured using the RANSOD® kit (Randox Lab, Antrim, United Kingdom). The method is based on the formation of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and superoxide radical, which is produced by the incubation with the xanthine–xanthine oxidase reaction system. The absorbance of the product is measured spectrophotometrically at 505 nm. One unit of SOD corresponds to a 50% inhibition of red formazan formation. The specific activity of SOD and CAT were expressed as U/mg protein. Since both enzymes work in sequence to reduce the superoxide anion to water, the ratio SOD/CAT was applied in order to verify the existence of an impairment that could lead to a pro-oxidant status. Results were expressed as log of SOD/CAT ratio.

2.3.8. Pro-inflammatory cytokines (IL-6 and TNF-α)

Plasma IL-6 and TNF-α levels were measured by the MILLIPLEX™ MAP Human Cytokine/Chemokine Kit (Millipore Corp., Billerica, Massachusetts, USA). Results were expressed in pg/mL.

2.3.9. Urinary Gb3 concentration

A 5-cm diameter filter paper disc was punched from each sample and processed as described by C. Auray-Blais [18] with some modifications. $C_{17:0}$–Gb3 was used as internal standard (IS) and added to the samples before injection into the LC-MS/MS (liquid chromatography–tandem mass spectrometry) system. For LC, it was used an Alliance 2695 system with stepwise gradient elution with mobile phases A (ammonium acetate 2 mM + 0.1% formic acid in water) and B (ammonium acetate 2 mM + 0.1% formic acid in methanol). A Discovery® C8, 5 μm column (577508-U) was used for separation, at 45 °C and the total analysis run time was 4.5 min. MS/MS was carried out using a Quattro micro tandem quadrupole instrument (Waters Micromass, Manchester, UK) with electrospray ionization operated in positive ion mode. The multiple reaction monitoring mode was used for the measurement of IS and total Gb3 isoforms. Gb3 levels were expressed as μg/mL and after divided by creatinine (Cr) levels (mg/mL), finally resulting in μg Gb3/mg Cr. Results were expressed as log of μg Gb3/mg Cr.

2.3.10. Urinary creatinine (Cr)

Cr was determined by picric acid method — Creatinine K kit of Labtest® (Labtest Diagnóstica, Lagoa Santa, MG, Brazil). Urinary creatinine reacts with picric acid under alkaline conditions producing an orange color whose absorbance was determined in a spectrophotometer at 492 nm. Results were expressed as mg Cr/mL.

2.3.11. Protein determination

Plasma and erythrocyte protein concentrations were determined, respectively, by Biuret method — using the commercial kit of Labtest® (Labtest Diagnóstica, Lagoa Santa, MG, Brazil) — and by the method of Lowry [19].

2.4. Statistical analysis

All results were expressed as mean ± standard error of the mean (SEM). Normal distribution was tested by the Shapiro–Wilk test. Logarithmic (log) transformation was done in data not normally distributed in order to transform them in parametric. Unpaired Student's *t* test was used for all comparisons between the two groups, using the first sample collected of each patient. Correlations between Gb3 levels and the other parameters were performed by Pearsons's correlation test.

Differences were considered significant when $p<0.05$. Analyses were performed by using the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL, USA — SPSS version 19.0) software, and graphics were constructed in GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA — version 5.0) software.

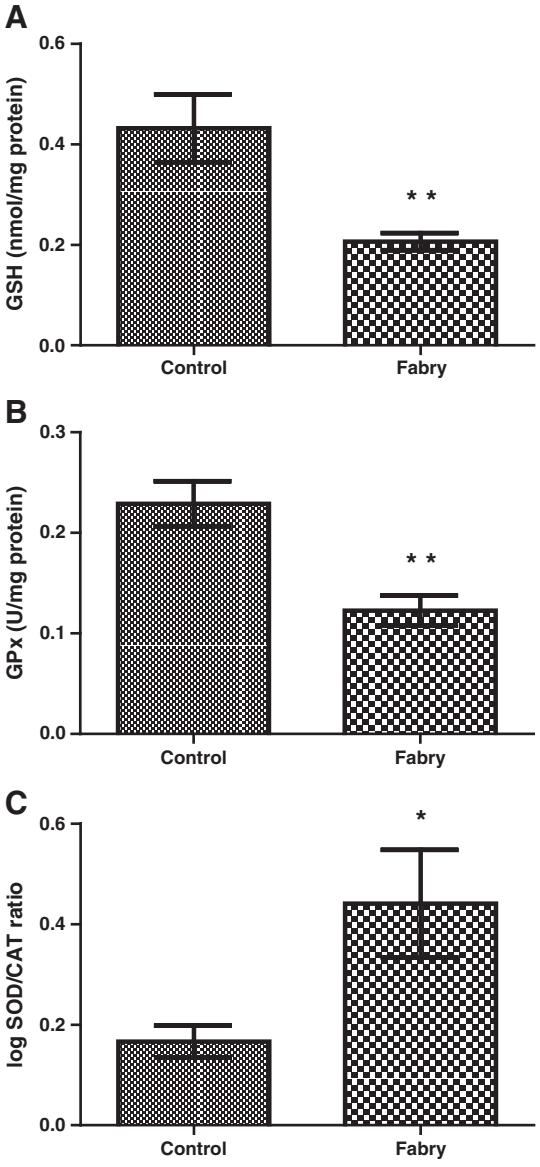

Fig. 1. Antioxidant defenses [GSH (A), GPx (B) and SOD/CAT ratio (C)] in Fabry patients under ERT ($n = 11$) and controls ($n = 12$–14). Data represent mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$ (Student's *t* test for unpaired samples) compared to the control group.

3. Results

3.1. Antioxidant defenses

The concentration of erythrocyte GSH, the main non-enzymatic antioxidant in cells, was significantly reduced in patients when compared to the control group (Fig. 1A) [$t(12.376) = 3.240$, $p<0.01$. Erythrocyte GPx activity (Fig. 1B) was also reduced in patients [$t(23) = 3.717$, $p<0.01$] and directly correlated with GSH content ($r = 0.290$, $p<0.05$). Moreover, SOD/CAT ratio was increased in patients [$t(12.191) = 1.800$, $p<0.05$], indicating a pro-oxidant status (Fig. 1C) since the product of the reaction catalyzed by SOD, hydrogen peroxide (H_2O_2), cannot be reduced to water in the same velocity by CAT and then becomes available to oxidize biomolecules.

3.2. Oxidative damage to biomolecules

To determine the oxidative damage to lipids and proteins we measured MDA and carbonyl groups in plasma and di-Tyr levels in urine. Results showed that Fabry patients under treatment with ERT have increased levels of oxidative damage to lipids (MDA plasma levels, Fig. 2A) [$t(11.403) = 5.069$, $p<0.01$] and proteins (di-Tyr in urine, Fig. 2B; carbonyl groups plasma levels, Fig. 2C) [$t(13.987) = 2.160$, $p<0.05$; $t(25) = 3.986$, $p<0.01$, respectively] when compared to controls. No statistical difference was found in plasma sulfhydryl groups (Fig. 2D) [$t(22) = 0.155$, $p>0.05$] between patients and controls.

3.3. Pro-inflammatory cytokines

Pro-inflammatory cytokines assayed in this study, TNF-α (Fig. 3A) and IL-6 (Fig. 3B) were increased in Fabry patients when compared to controls [$t(21) = 3.600$, $p<0.01$; $t(16) = 3.000$, $p<0.01$, respectively].

3.4. Gb3 levels

To determine Gb3 levels we measured urinary Gb3 concentration. Results showed that Fabry patients receiving ERT presented significant higher levels of urinary Gb3 when compared to controls [$t(19) = 8.138$, $p<0.01$] (Fig. 4).

3.5. Correlations between oxidative stress, pro-inflammatory cytokines and Gb3 levels

Gb3 levels were positively correlated with the plasma levels of IL-6, MDA and carbonyl (Fig. 5A, B and C; $r = 0.971$, $p<0.01$; $r = 0.622$, $p<0.05$; $r = 0.745$, $p<0.05$, respectively). No significant correlations were found between Gb3 levels and the other parameters. IL-6 levels were directly correlated with urinary levels of di-Tyr (Fig. 6A; $r = 0.642$, $p<0.05$) and inversely correlated with erythrocyte GPx activity (Fig. 6B; $r = -0.653$, $p<0.05$).

4. Discussion

Lysosome storage disorders (LSDs) are well known monogenic disorders caused by mutations that lead to an impairment of the activity of lysosome enzymes and consequently, a progressive accumulation of substrates. Although the primary cause of LSDs is established, the mechanisms of how the intra-lysosomal accumulation leads to cell and tissue dysfunction remain not completely clear (including in Fabry disease) [8,10,20,21].

Vitner et al. published in 2010 a minireview focusing on the pathogenic cascades activated in LSDs [22]. These cascades include autophagy, altered lipid trafficking, endoplasmic reticulum (ER) stress, autoimmune response, altered calcium homeostasis, inflammation and oxidative stress. Lysosomes are quite vulnerable to oxidative stress as a natural consequence of the physiological conditions underlying the organelle [8–10]. Probably, the accumulation of substrates

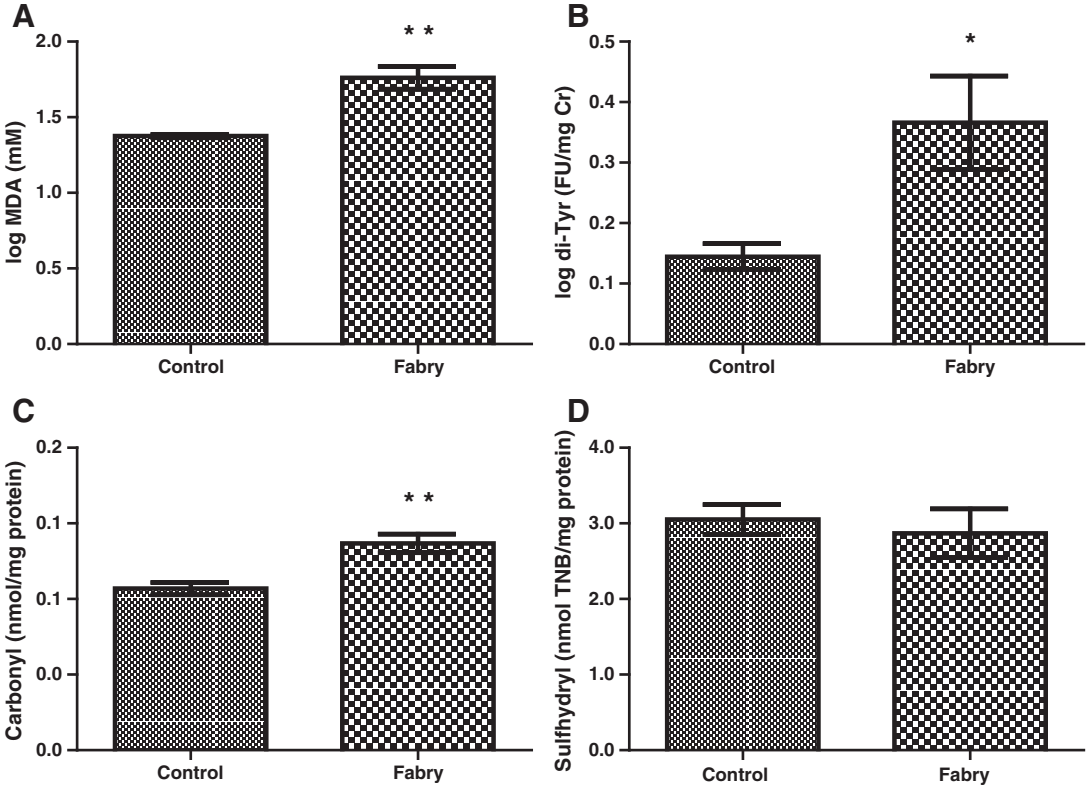

Fig. 2. Oxidative damage to lipids [MDA (A)] and proteins [di-Tyr (B), carbonyl groups (C), sulfhydryl groups (D)] in Fabry patients under ERT ($n = 12–14$) and controls ($n = 6–13$). Data represent mean ± SEM. $^{*}p<0.05$, $^{**}p<0.01$ (Student's t test for unpaired samples) compared to the control group.

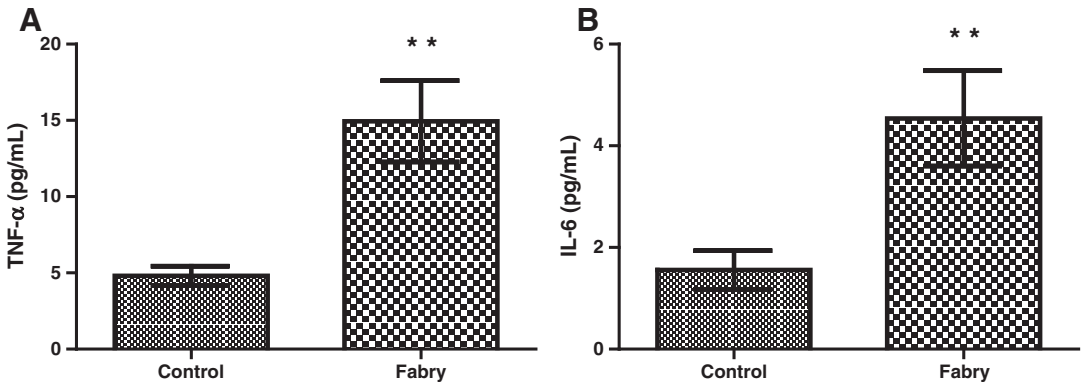

Fig. 3. Pro-inflammatory cytokines [TNF-α (A) and IL-6 (B)] in Fabry patients under ERT ($n = 9–12$) and controls ($n = 9–12$). Data represent mean ± SEM. $^{**}p < 0.01$ (Student's *t* test for unpaired samples) compared to the control group.

(as Gb3 in Fabry disease) exacerbates the pathologic process as shown by Shen et al. [11]. The researchers found, by an *in vitro* study in cultured cells, an increase of ROS and adhesion molecules in response to Gb3.

Many studies have shown the involvement of oxidative stress in the pathophysiology of more than a hundred of human diseases [23], including cancer [24–27], inflammatory processes [23] and inborn errors of metabolism (IEM) [28–33]. Recent studies of our group showed evidences that oxidative stress may be involved in the pathophysiology of mucopolysaccharidosis type II (MPS II) and ERT seemed to protect against lipid and protein oxidative damage in patients [32,33].

The aim of this study was to investigate oxidative stress parameters, pro-inflammatory cytokines and Gb3 levels in Fabry patients treated with ERT, in order to establish a possible relationship between them.

We found decreased levels of antioxidant defenses (erythrocyte GSH content and GPx activity) in FD patients when compared to controls. The low GSH content implies low GPx activity and may lead to higher susceptibility to oxidative damage. GSH is the most important non-enzymatic antioxidant, being preferentially oxidized by reactive species and then preserving more important biomolecules [34]. This deficient scavenger content of GSH, added to the increased SOD/CAT ratio verified in this study indicates that probably hydrogen peroxide (H_2O_2) is more available to oxidize biological molecules in Fabry patients. Furthermore, the low GPx activity confirms this hypothesis of H_2O_2 excess, since it is also the substrate of GPx. In the presence of Fe^{2+}, H_2O_2 yields the highly toxic hydroxyl radical (OH^-), by the called Fenton reaction. This radical attacks all types of biomolecules around it, being extremely dangerous to the cell [34]. Hence, the low GSH content and GPx activity and high SOD/CAT ratio presented by Fabry patients probably contribute to the oxidative stress process.

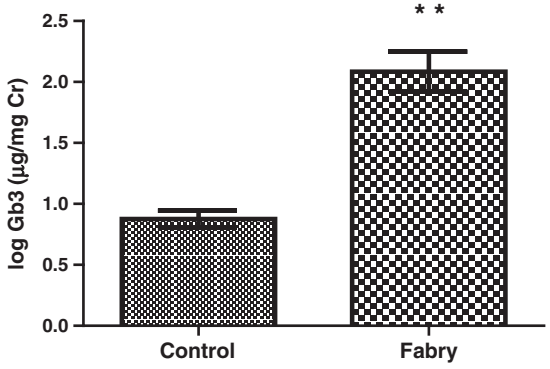

Fig. 4. Gb3 urinary levels in Fabry patients under ERT and controls. Data represent mean ± SEM. $^{**}p < 0.01$ (Student's *t* test for unpaired samples) compared to the control group.

In relation to oxidative damage to biomolecules, we found increased levels of MDA and carbonyl in plasma of Fabry patients when compared to controls. These data suggest that patients present lipid peroxidation and protein damage in significant higher levels. The continued oxidation and fragmentation of fatty acid side chains can produce aldehydes as MDA (formed from peroxidation of linolenic, arachidonic, or docosahexaenoic acids), that may lead to rupture of lysosomal (and other organelles) membrane and bind avidly to membrane proteins, inactivating enzymes and receptors and even attack DNA, forming mutagenic lesions [34,35]. Thus, the increased levels of MDA that we observed in Fabry patients must be a result of the lysosome destabilization and also may be acting in the maintenance of chain reactions with other molecules. Also, it is important to emphasize that the positive correlation between Gb3 and MDA levels that was found in this study suggest that Gb3 induces lipid peroxidation in FD. Moreover, it was verified a significant increase in MDA levels in Fabry patients at diagnosis moment and under ERT when compared to control group (data not shown). It allows us to suggest that the observed effect is due to the disease and could explain, at least in part, the pathophysiology of FD.

In this study, we verified a significant increase in plasma carbonyl groups as well as in urinary di-Tyr levels in Fabry patients. The alteration of proteins by formation of carbonyl groups can be recognized as "non-self" by the immune system and lead to autoimmune response [23]. Some studies have shown that possibly the FD has an autoimmune component in its pathophysiology [36–39]. The positive correlation found in this study between Gb3 and carbonyl levels suggests that Gb3 may be acting also as an inductor of protein damage in Fabry patients. We found no statistical difference between patients and controls in what concern to sulfhydryl groups. Although the amino acids containing this sulfur groups (cysteine and methionine) are readily oxidized, literature suggest that this parameter may give misleading data on the extent of oxidation in the case of chronic oxidant stress [40]. In this case, the repair mechanism may compete effectively and differences are not evident, as occurred in this study. On the other hand, the high carbonyl groups levels found in this study are indicative of severe damage to proteins since heavily carbonylated proteins tend to form high-molecular-weight aggregates that are resistant to degradation by the proteasomal system [41]. Moreover, it is important to emphasize that it was verified no significant correlation between proteinuria — as well as other renal function parameters (urea, creatinine and eGFR-MDRD) — and the oxidative stress parameters in these patients.

Flow cytometry studies revealed abnormalities in the number of immune cell in Fabry patients [42]. Also, there are evidences that vascular endothelium and leukocytes are under inflammatory activation in FD [11,43]. Our findings concerning to the inflammatory cytokines

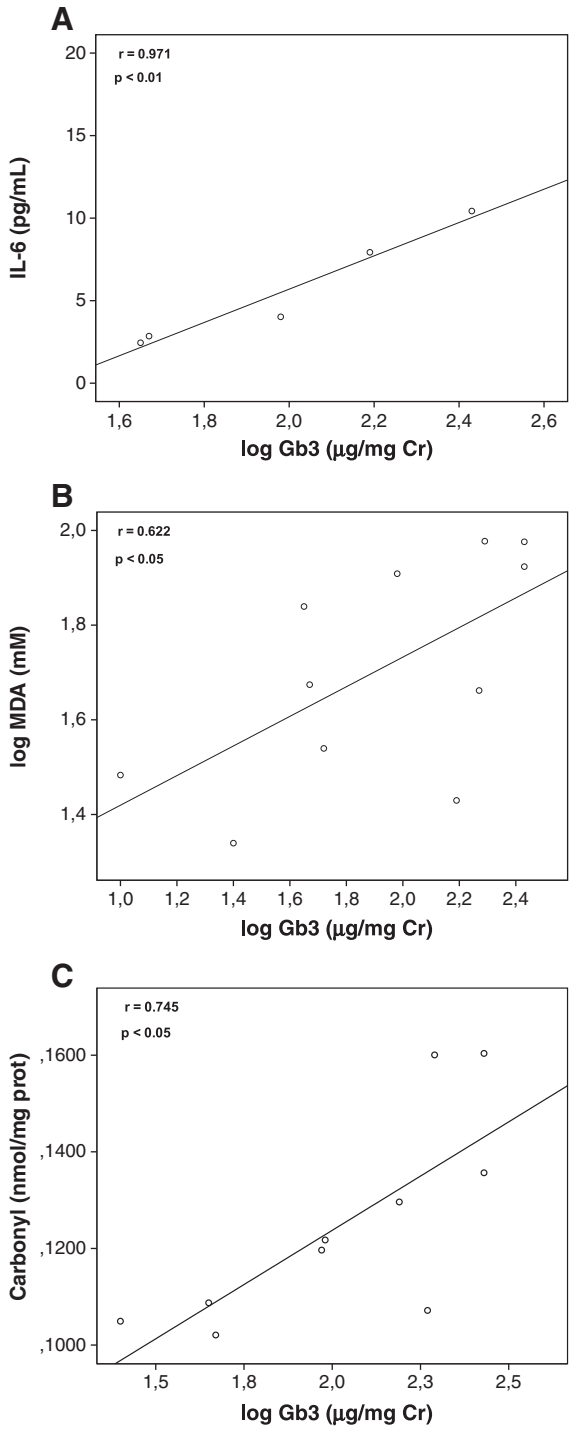

Fig. 5. Correlations between Gb3 and IL-6 (A), MDA (B) and carbonyl groups (C) in Fabry patients under ERT.

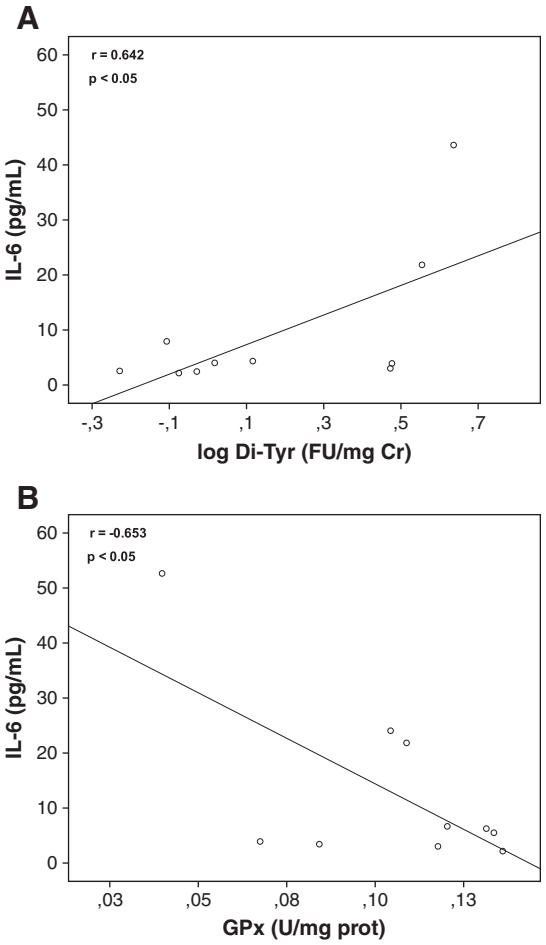

Fig. 6. Correlations between IL-6 and di-Tyr (A) and GPx activity (B) in Fabry patients under ERT.

IL-6 and TNF-α, both significantly increased in Fabry patients, provide evidence that the pro-inflammatory state occurs in these patients. Besides, IL-6 was positively correlated with di-Tyr, which indicates oxidative damage to proteins, as well as with Gb3 and negatively correlated with GPx activity. These findings may suggest that the inflammatory process is associated to protein damage and may be induced by Gb3 in Fabry patients.

The present study confirms the *in vitro* results from Shen et al. [11] in what concern to Gb3 and oxidative stress induction and provides new data to understand the pathophysiology of Fabry disease. Fabry patients presented high lipid and protein oxidative damage, decreased antioxidant defenses and increased inflammatory biomarkers. The most significant novelty in the present *in vivo* study is the presence of correlations between the oxidative stress parameters, pro-inflammatory cytokines and Gb3 levels. However, our results must be interpreted carefully, since only treated patients were evaluated.

In conclusion, our results indicate that pro-oxidant and pro-inflammatory states occur, are correlated and seem to be induced by Gb3 in Fabry patients. Possibly, these data may provide new targets to future therapeutic strategies in order to improve the quality of life of Fabry patients. Further research and clinical trials are needed to reveal how safe and effective would be the supplementation of antioxidants in combination with ERT in Fabry patients.

Conflict of interest disclosure

The authors declare that there is no conflict of interest associated with this manuscript.

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References

[1] R.J. Desnick, Y.A. Ioannou, C.M. Eng, α-Galactosidase A deficiency: Fabry disease, in: C.R. Scriver, W.A. Sly, A.L. Beaudet, D. Valle (Eds.), The Metabolic and Molecular Bases of Inherited Disease, eighth ed., McGraw-Hill Inc., New York, 2001, pp. 3733–3774.

[2] R.P. El Dib, G.M. Pastores, Enzyme replacement therapy for Anderson–Fabry disease, Cochrane Database Syst. Rev. 5 (2010) CD006663.
[3] D.F. Moore, F. Ye, M.L. Brennan, S. Gupta, B.A. Barshop, R.D. Steiner, W.J. Rhead, R.O. Brady, S.L. Hazen, R. Schiffmann, Ascorbate decreases Fabry cerebral hyperperfusion suggesting a reactive oxygen species abnormality: an arterial spin tagging study, J. Magn. Reson. Imaging 20 (2004) 674–683.
[4] P.F. Bodary, Y. Shen, F.B. Vargas, X. Bi, K.A. Ostenso, S. Gu, J.A. Shayman, D.T. Eitzman, Alpha-galactosidase A deficiency accelerates atherosclerosis in mice with apolipoprotein E deficiency, Circulation 111 (2005) 629–632.
[5] D.F. Moore, L.T.C. Scott, M.T. Gladwin, G. Altarescu, C. Kaneski, K. Suzuki, M. Pease-Fye, R. Ferri, R.O. Brady, P. Herscovitch, R. Schiffmann, Regional cerebral hyperperfusion and nitric oxide pathway dysregulation in Fabry disease: reversal by enzyme replacement therapy, Circulation 104 (2001) 1506–1512.
[6] B. Halliwell, Free radicals, antioxidants, and human disease: curiosity cause or consequence? Lancet 344 (1994) 721–724.
[7] M.C. Polidori, B. Frei, A. Cherubini, G. Nelles, G. Rordorf, J.F. Keaney Jr., L. Schwamm, P. Mecocci, W.J. Koroshetz, M.F. Beal, Increased plasma levels of lipid hydroperoxides in patients with ischemic stroke, Free Radic. Biol. Med. 25 (1998) 561–567.
[8] A. Terman, T. Kurz, B. Gustafsson, U.T. Brunk, Lysosomal labilization, IUBMB Life 58 (2006) 531–539.
[9] A. Terman, U.T. Brunk, Oxidative stress, accumulation of biological 'garbage', and aging, Antioxid. Redox Signal. 8 (2006) 197–204.
[10] U.T. Brunk, J. Neuzil, J.W. Eaton, Lysosomal involvement in apoptosis, Redox Rep. 6 (2001) 91–97.
[11] J.-. Shen, X.- Meng, D.F. Moore, J.M. Quirk, J.A. Shayman, R. Schiffmann, C.R. Kaneski, Globotriaosylceramide induces oxidative stress and up-regulates cell adhesion molecule expression in Fabry disease endothelial cells, Mol. Genet. Metab. 95 (2008) 163–168.
[12] H. Esterbauer, K.H. Cheeseman, Determination of aldehydic lipid peroxidation products: malondialdehyde and 4-hydroxynonenal, Methods Enzymol. 186 (1990) 407–421.
[13] B. Kirschbaum, Correlative studies of urine fluorescence and free radical indicators, Clin. Nephrol. 58 (2002) 344–349.
[14] R.L. Levine, D. Garland, C.N. Oliver, A. Amici, I. Climent, A.G. Lenz, B.W. Ahn, Determination of carbonyl content in oxidatively modified proteins, Methods Enzymol. 186 (1990) 464–478.
[15] M.Y. Aksenov, W.R. Markesbery, Changes in thiol content and expression of glutathione redox system genes in the hippocampus and cerebellum in Alzheimer's disease, Neurosci. Lett. 302 (2001) 141–145.
[16] R.W. Browne, D. Armstrong, Reduced glutathione and glutathione disulfide, Methods Mol. Biol. 108 (1998) 347–352.
[17] H. Aebi, Catalase *in vitro*, Methods Enzymol. 105 (1984) 121–126.
[18] C. Auray-Blais, D. Cyr, A. Ntwari, M. West, J. Cox-Brinkman, D.G. Bichet, D.P. Germain, R. Laframboise, S.B. Melançon, T. Stockley, J.T.R. Clarke, R. Drouin, Urinary globotriaosylceramide excretion correlates with the genotype in children and adults with Fabry disease, Mol. Genet. Metab. 93 (2008) 331–340.
[19] O.H. Lowry, N.J. Rosenbrough, A.I. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[20] A.H. Futerman, G. Van Meer, The cell biology of lysosomal storage disorders, Nat. Rev. Mol. Cell Biol. 5 (2004) 554–565.
[21] S.U. Walkley, Pathogenic cascades in lysosomal disease — why so complex? J. Inherit. Metab. Dis. 32 (2009) 181–189.
[22] E.B. Vitner, F.M. Platt, A.H. Futerman, Common and uncommon pathogenic cascades in lysosomal storage diseases, J. Biol. Chem. (2010) 20423–20427.
[23] B. Halliwell, J.M.C. Gutteridge, Free Radicals in Biology and Medicine, fourth ed. Oxford University Press, New York, 2006.
[24] S. Toyokuni, Persistent oxidative stress in cancer, FEBS Lett. 358 (1995) 1–3.
[25] G. Mantovani, A. Macciò, C. Madeddu, L. Mura, G. Gramignano, M.R. Lusso, C. Mulas, M.C. Mudu, V. Murgia, P. Camboni, E. Massa, L. Ferreli, P. Contu, A. Rinaldi, E. Sanjust, D. Atzei, B. Elsener, Quantitative evaluation of oxidative stress, chronic inflammatory indices and leptin in cancer patients: correlation with stage and performance status, Int. J. Cancer 98 (2002) 84–91.
[26] R.V. Blackburn, D.R. Spitz, X. Liu, S.S. Galoforo, J.E. Sim, L.A. Ridnour, J.C. Chen, B.H. Davis, P.M. Corry, Y.J. Lee, Metabolic oxidative stress activates signal transduction and gene expression during glucose deprivation in human tumor cells, Free Radic. Biol. Med. 26 (1999) 419–430.
[27] M. López-Lázaro, A new view of carcinogenesis and an alternative approach to cancer therapy, Mol. Med. 16 (2010) 144–153.
[28] A.G. Barschak, A. Sitta, M. Deon, A.T. Barden, C.S. Dutra-Filho, M. Wajner, C.R. Vargas, Oxidative stress in plasma from maple syrup urine disease patients during treatment, Metab. Brain Dis. 23 (2008) 71–80.
[29] C.R. Vargas, M. Wajner, L.R. Sirtori, L. Goulart, M. Chiochetta, D. Coelho, Evidence that oxidative stress is increased in patients with X-linked adrenoleukodystrophy, Biochim. Biophys. Acta 1688 (2004) 26–32.
[30] A. Sitta, A.G. Barschak, M. Deon, A.T. Barden, G.B. Biancini, P.R. Vargas, C.F. de Souza, C. Netto, M. Wajner, C.R. Vargas, Effect of short- and long-term exposition to high phenylalanine blood levels on oxidative damage in phenylketonuric patients, Int. J. Dev. Neurosci. 27 (2009) 243–247.
[31] G.S. Ribas, V. Manfredini, J.F. de Mari, C.Y. Wayhs, C.S. Vanzin, G.B. Biancini, A. Sitta, M. Deon, M. Wajner, C.R. Vargas, Reduction of lipid and protein damage in patients with disorders of propionate metabolism under treatment: a possible protective role of L-carnitine supplementation, Int. J. Dev. Neurosci. 28 (2010) 127–132.
[32] L. Filippon, C.S. Vanzin, G.B. Biancini, I.N. Pereira, V. Manfredini, A. Sitta, M.C.R. Peralba, I.V.D. Schwartz, R. Giugliani, C.R. Vargas, Oxidative stress in patients with mucopolysaccharidosis type II before and during enzyme replacement therapy, Mol. Genet. Metab. 103 (2011) 121–127.
[33] L. Filippon, C.A.Y. Wayhs, D.M. Atik, V. Manfredini, S. Herber, C.G. Carvalho, I.V.D. Schwartz, R. Giugliani, C.R. Vargas, DNA damage in leukocytes from pretreatment mucopolysaccharidosis type II patients; protective effect of enzyme replacement therapy, Mutat. Res. 721 (2011) 206–210.
[34] B. Halliwell, Reactive species and antioxidants. redox biology is a fundamental theme of aerobic life, Plant Physiol. 141 (2006) 312–322.
[35] H. Esterbauer, R.J. Schaur, H. Zollner, Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes, Free Radic. Biol. Med. 11 (1991) 81–128.
[36] D.F. Moore, E. Goldin, M.P. Gelderman, C. Robinson, J. Baer, M. Ries, A. Elkahloun, R.O. Brady, R. Schiffmann, Apoptotic abnormalities in differential gene expression in peripheral blood mononuclear cells from children with Fabry disease, Acta Paediatr. 97 (2008) 48–52.
[37] P. Rahman, D.D. Gladman, J. Wither, M.D. Silver, Coexistence of Fabry's disease and systemic lupus erythematosus, Clin. Exp. Rheumatol. 16 (1998) 475–478.
[38] C. Whybra, A. Schwarting, J. Kriegsmann, A. Gal, E. Mengel, C. Kampmann, F. Baehner, E. Schaefer, M. Beck, IgA nephropathy in two adolescent sisters heterozygous for Fabry disease, Pediatr. Nephrol. 21 (2006) 1251–1256.
[39] P. Martinez, M. Aggio, P. Rozenfeld, High incidence of autoantibodies in Fabry disease patients, J. Inherit. Metab. Dis. 30 (2007) 365–369.
[40] M.J. Davies, S. Fu, H. Wang, R.T. Dean, Stable markers of oxidant damage to proteins and their application in the study of human disease, Free Radic. Biol. Med. 27 (1999) 1151–1163.
[41] I. Dalle-Donne, G. Aldini, M. Carini, R. Colombo, R. Rossi, A. Milzani, Protein carbonylation, cellular dysfunction, and disease progression, J. Cell. Mol. Med. 10 (2006) 389–406.
[42] P. Rozenfeld, E. Agriello, N. De Francesco, P. Martinez, C. Fossati, Leukocyte perturbation associated with Fabry disease, J. Inherit. Metab. Dis. 155 (2009) 1–11, doi: 10.1007/s10545-009-1060-9.
[43] T. DeGraba, S. Azhar, F. Dignat-George, E. Brown, B. Boutière, G. Altarescu, R. McCarron, R. Schiffmann, Profile of endothelial and leukocyte activation in Fabry patients, Ann. Neurol. 47 (2000) 229–233.