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Oxidative profile exhibited by Mucopolysaccharidosis type IVA patients at diagnosis: Increased keratan urinary levels

Bruna Donida[a], Desirèe P. Marchetti[a], Carlos Eduardo Diaz Jacques[a], Graziela Ribas[b,e], Marion Deon[c,e], Paula Manini[d], Helen Tais da Rosa[a], Dinara Jaqueline Moura[d], Jenifer Saffi[d], Roberto Giugliani[e,f], Carmen Regla Vargas[a,c,e,*]

[a] Programa de Pós-Graduação em Ciências Biológicas, Bioquímica, UFRGS, Porto Alegre, RS, Brazil
[b] Programa de Pós Graduação em Saúde da Criança e do Adolescente, UFRGS, Porto Alegre, RS, Brazil
[c] Programa de Pós-Graduação em Ciências Farmacêuticas, UFRGS, Porto Alegre, RS, Brazil
[d] Laboratório de Genética Toxicológica, UFCSPA, Porto Alegre, RS, Brazil
[e] Serviço de Genética Médica, HCPA, Porto Alegre, RS, Brazil
[f] Departamento de Genética, e Programa de Pós-Graduação em Genética e Biologia Molecular, Instituto de Biociências, UFRGS, Porto Alegre, RS, Brazil

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ABSTRACT

Morquio A disease (Mucopolysaccharidosis type IVA, MPS IVA) is one of the 11 mucopolysaccharidoses (MPSs), a heterogeneous group of inherited lysosomal storage disorders (LSDs) caused by deficiency in enzymes need to degrade glycosaminoglycans (GAGs). Morquio A is characterized by a decrease in *N*-acetylgalactosamine-6-sulfatase activity and subsequent accumulation of keratan sulfate and chondroitin 6-sulfate in cells and body fluids. As the pathophysiology of this LSD is not completely understood and considering the previous results of our group concerning oxidative stress in Morquio A patients receiving enzyme replacement therapy (ERT), the aim of this study was to investigate oxidative stress parameters in Morquio A patients at diagnosis. It was studied 15 untreated Morquio A patients, compared with healthy individuals. The affected individuals presented higher lipid peroxidation, assessed by urinary 15-F2t-isoprostane levels and no protein damage, determined by sulfhydryl groups in plasma and di-tyrosine levels in urine. Furthermore, Morquio A patients showed DNA oxidative damage in both pyrimidines and purines bases, being the DNA damage positively correlated with lipid peroxidation. In relation to antioxidant defenses, affected patients presented higher levels of reduced glutathione (GSH) and increased activity of glutathione peroxidase (GPx), while superoxide dismutase (SOD) and glutathione reductase (GR) activities were similar to controls. Our findings indicate that Morquio A patients present at diagnosis redox imbalance and oxidative damage to lipids and DNA, reinforcing the idea about the importance of antioxidant therapy as adjuvant to ERT, in this disorder.

1. Introduction

Morquio A disease (Mucopolysaccharidosis type IVA, MPS IVA, OMIM #253000) is an autosomal recessive inborn error of glycosaminoglycan (GAG) catabolism due to a defect in *N*-acetylgalactosamine-6-sulfatase enzyme (GALNS; E.C.3.1.6.4) [42]. This enzyme deficiency is responsible for keratan sulfate and chondroitin 6-sulfate accumulation in body fluids and tissues. Keratan sulfate, the main accumulated metabolite, contains a repeating disaccharide unit with alternating D-galactose and *N*-acetyl-D-glucosamine residues. Keratan sulfate accumulates principally in the cartilage, cornea and heart valves of Morquio A patients and excess of tissue accumulation leads to disruption in the cartilage and subsequent increase of keratan sulfate levels in blood and urine [34]. The Morquio A incidence ranges among different populations from 1 per 76,000 live births in Northern Ireland [40] to 1 per 640,000 live births in Western Australia [41]. However, within this

Abbreviations: 8-OHdG, 8-hydroxy-2′-deoxyguanosine; Cr, creatinine; DI, damage index; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Endo III, endonuclease III; ELISA, enzyme-linked immunoassay; ERT, enzyme replacement therapy; FU, fluorescence units; GAGs, glycosaminoglycans; GALNS, *N*-acetylgalactosamine-6-sulfatase; GCL, glutamate cysteine ligase; GCLC, catalytic subunit of GCL; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, glutathione oxidized; H_2O_2, hydrogen peroxide; IEM, inborn errors of metabolism; LPS, lipopolysaccharide; LSDs, lysosomal storage disorders; MPSs, mucopolysaccharidoses; mRNA, messenger ribonucleic acid; $OH^{\cdot}$, hydroxyl radical; ROS, reactive oxygen species; SEM, standard error of the mean; SOD, superoxide dismutase; TLR4, Toll Like Receptor 4; TNB, tionitrobenzoic acid

* Correspondence to: Serviço de Genética Médica, HCPA, Rua Ramiro Barcelos, 2350, 90.035-003 Porto Alegre, RS, Brazil.
E-mail addresses: donida.bruna@gmail.com (B. Donida), dinaram@ufcspa.edu.br (D.J. Moura), jenifers@ufcspa.edu.br (J. Saffi), rgiugliani@hcpa.edu.br (R. Giugliani), crvargas@hcpa.edu.br (C.R. Vargas).

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context it is important to mention the study developed by Leadley et al. [28], which shows that definitions for prevalence and incidence are not well understood in rare diseases field. Since overestimation and underestimation of prevalence data can occur, guidelines and quality tools for estimating the prevalence of rare diseases are recommended. Clinical manifestations of Morquio A patients include: skeletal dysplasia, restricted growth and short stature, joint hypermobility, valvular heart disease, pulmonary disease, corneal clouding, hearing loss, and poorly formed teeth [33]. The characteristic signs and symptoms of Morquio A syndrome appear at around two or three years of age [33].

The affected enzyme in Morquio A syndrome is encoded by the *GALNS* gene, which is located on chromosome 16q24.3 and split into 14 exons spanning approximately 50 kb [53]. Until April of 2017, 328 different mutations have been identified in the *GALNS* gene in the Human Gene Mutation Database (www.hgmd.cf.ac.uk [51]).

It is already described that the large number of mutations in *GALNS* are consistent with the broad spectrum of phenotypes observed in Morquio A patients, ranging from mild to severe based on the residual *GALNS* activity [14,34,35]. In almost all cases, Morquio A patients have no neurological impairment, differently from most MPS types [42].

Since 2014, enzyme replacement therapy (ERT) for Morquio A is approved in many countries, including Brazil. This treatment is based on the intravenous administration of a recombinant enzyme, similar to the native GALNS, aiming to reduce the high levels of GAGs accumulated inside the lysosomes [22]. Morquio A patients submitted to ERT showed improvements in 6-minute-walk test and presented a decrease in keratan sulfate excretion levels [22]. However, it is important emphasize that recent works are showing that ERT do not reverse most of the features present before the treatment and also not shown good clinical efficiency in Morquio A patients [43,56].

Reactive species, that are formed and degraded in cells during normal aerobic metabolism, are required for the correct functioning of the organism. However, an imbalance in the rate of synthesis and detoxification of these reactive species cause a disruption of redox metabolism with oxidative damage to biomolecules like proteins, lipids and DNA [20]. The involvement of reactive species is described in more than a hundred human diseases, including some IEM, and in the majority of published works about IEM, the accumulated metabolites are indicated as main responsible for the increase of reactive species [3,10,17,44,48]. An abnormal accumulation of GAGs not degraded within the lysosomes can lead to an increase of reactive oxygen species (ROS), which has a great impact on lysosomal function since lysosomes lack hydrogen peroxide degrading enzymes and have high content of iron, making them organelles extremely susceptible to oxidative damage [49,50]. A destabilization of lysosomal membranes can cause an overflow of lysosome contents into the cytoplasm which may trigger peroxidation cascades causing, at the end, cell apoptosis or necrosis [49,50].

We have been focusing our research on the mechanisms underlying the pathophysiology of Morquio A and, in a previous study we demonstrated that oxidative and inflammatory imbalance occurs in Morquio A patients even after eight months of ERT [13]. Therefore, the aim of the present study was to evaluate antioxidant defenses and oxidative damage to lipids, proteins and DNA in Morquio A patients without treatment.

2. Materials and methods

2.1. Subjects

The study was performed with 15 Morquio A patients with ages varying between 5 and 43 years (18.1 ± 11.25 years old, mean ± standard deviation) and with 39 healthy individuals with ages ranging between 7 and 32 years (21.31 ± 5.97 years old, mean ± standard deviation). At the moment of diagnosis, patients presented the classic symptoms, usually including short stature, skeletal deformities (pectus carinatum and genu valgum almost always present), limited ambulation, restrictive airway disease, heart valves problems and corneal clouding. Diagnosis was confirmed by evaluation of GAGs in urine (increased both total content and keratan sulfate) and measurement of GALNS in leukocytes (deficient activity) [32,55].

The research was carried out according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) and informed consent was obtained from all participants. The study was approved (project number 13-0246) by The Ethics Committee of the *Hospital de Clínicas de Porto Alegre* (HCPA), RS, Brazil.

2.2. Samples preparation

Urine and heparinized blood samples were obtained from patients and healthy individuals at the same time. The urine samples were collected in sterile flask, aliquoted and frozen at − 80 °C until analysis. Whole heparinized blood was centrifuged at 1000 × *g* for 10 min and plasma was removed by aspiration, aliquoted and frozen at − 80 °C until biochemical determinations. An aliquot of whole blood was separated for comet assay. Erythrocytes were washed three times with cold saline solution (0.153 mol/L sodium chloride) and the lysates were prepared by addition of 1 mL of distilled water to 100 μL of washed erythrocytes. The lysates were frozen at − 80 °C until determination of GSH and antioxidant enzymes activities. For these determinations, the 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. Urine 15-F2t-isoprostane levels

15-F2t-isoprostane, a product of arachidonic acid metabolism and a biomarker of lipid peroxidation, was measured by a competitive enzyme-linked immunoassay (ELISA) (Oxford Biomed, EA 85), according the kit's instructions. First, the urine samples were mixed with dilution buffer. In this assay, the 15-F2t-isoprostane in the urine samples competes with the 15-F2t-isoprostane conjugated to horseradish peroxidase for the binding to a specific antibody fixed to the microplate. The concentration of 15-F2t-isoprostane was determined spectrophotometrically at 630 nm by the intensity of color developed after the substrate had been added. Results were expressed as picograms of isoprostanes per mg of urinary creatinine (pg/mg Cr).

2.3.2. Total plasmatic level of sulfhydryl groups

The plasmatic concentration of sulfhydryl groups was performed as previously described. [1]. The method is based on the reduction of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) by sulfhydryl groups into a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as μmol TNB/mg protein.

2.3.3. Urinary di-tyrosine levels

In order to determine the levels of protein oxidation in urine, the intensity of di-tyrosine fluorescence was measured according to method previously described [26]. In brief, 50 μL of thawed urine was added to 950 μL of 6 mol/L urea in 20 mmol/L sodium phosphate buffer pH 7.4. After 30 min, the concentration was measured using a fluorometer (excitation 315 nm, emission 410 nm). Results were expressed as fluorescence units per mg of urinary creatinine (FU/mg Cr).

2.3.4. Alkaline comet assay in leukocytes

The alkaline comet assay, that measures single and double DNA strand breaks, was performed as previously described and in accordance with general guidelines for the comet assay [47,52]. Aliquots of 100 μL from whole blood were suspended in agarose and spread into a glass microscope slide pre-coated with agarose. Slides were placed in

ice-cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris pH 10.0–10.5 with 10% DMSO and 1% Triton X-100) to remove cell proteins and leaving DNA as "nucleoids". After the lysis-buffer procedure, the slides were covered with fresh buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 15 min to allow DNA unwinding and then, electrophoresis was performed for 15 min (25 V; 300 mA; 0.9 V/cm). Slides were neutralized with 0.4 M Tris (pH 7.5), washed in bi-distilled water and stained using silver nitrate staining protocol [39]. After drying at room temperature overnight, the samples were analyzed using an optical microscope. One hundred cells (50 cells from each of the two replicate slides) were selected and analyzed. Cells were visually scored according to tail length and received scores from 0 (no migration) to 4 (maximal migration). Therefore, the damage index (DI) was calculated, ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4) [7,52]. The slides were analyzed under blind conditions at least by two different individuals.

A digestion step with a bacterial repair enzyme was included in the alkaline comet assay before electrophoresis, in accordance to Dizdaroglu et al. [12]. The enzyme used was endonuclease III (Endo III, also known as Nth) which converts oxidized pyrimidines (including thymine glycol and uracil glycol) to strand breaks. It was obtained from New England Biolabs (NEB, Ipswich, MA). After lysis, the slides were washed for 5 min each in enzyme buffer (40 mM HEPES-KOH, 1 M KCl, 5 mM EDTA, 2.5 mg/mL bovine serum albumin fraction V-BSA, and pH 8.0). The suspension was added to the slide, covered with coverslip, and incubated for 45 at 37 °C. Subsequent steps were the same as in the alkaline version of comet assay. DNA damage with Endo III was calculated as the score obtained with enzyme minus the score without enzyme (basal).

2.3.5. Urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels

Urinary 8-OHdG levels, a product of DNA oxidative damage due to the hydroxyl radical attack at the C8 position of deoxyguanosine, were determined by Highly Sensitive 8-OHdG Check ELISA kit (JaICA, Fukuroi, Japan). The 8-OHdG Check ELISA kit is a competitive *in vitro* enzyme-linked immunosorbent assay for quantitative measurement of the oxidative DNA adduct 8-hydroxy-2′-deoxyguanosine. This assay employs a microplate with 96 wells pre-coated with 8-OHdG, a monoclonal antibody specific for 8-OHdG (primary antibody), an HPR-conjugated antibody (secondary antibody) and colorimetric detection substrate whose absorbance was measured in an ELISA microwell reader at 450 nm. The sample 8-OHdG concentrations (ng/mL) were calculated using a polynomial equation from the relative absorbance of standard curve. After creatinine correction, the results were expressed as ng/mg Cr.

2.3.6. Erythrocyte reduced glutathione (GSH) content

In order to measure GSH levels, the main intracellular antioxidant, lysates of erythrocytes were processed as described previously [5] and the fluorescence measured (excitation = 350 nm, emission = 420 nm) was compared to a calibration curve prepared with GSH solutions. Results were expressed as nmol/mg protein.

2.3.7. Erythrocyte glutathione peroxidase (GPx) activity

Erythrocyte GPx activity was measured by using a commercially available kit (Cayman Chemical Company, 703102). GPx catalyzes the oxidation of glutathione (GSH) to GSSG (oxidized glutathione). In the presence of glutathione reductase (GR) and NADPH, the oxidized GSSG is converted to its reduced form, with concomitant oxidation of NADPH to NADP +. The decrease in absorbance of NAPDH was measured at 340 nm after every minute until 5 min. One unit of GPx is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to NADP + per minute at 25 °C and the results were expressed in nmol/min/mL.

2.3.8. Erythrocyte glutathione reductase (GR) activity

GR activity was determined by the method of Carlberg and Mannervik [8], which is based on GSSG reduction using NADPH as a cofactor. Erythrocyte samples were added to a reaction medium containing 0.2 mol/L sodium phosphate buffer, pH 7.5, 6.3 mmol/L EDTA and 0.4 mmol/L NADPH. At 340 nm, the consumption of NADPH was monitored for 10 min. One unit of GR represents one μmol of NADPH consumed per minute, and its specific activity is expressed as mU/mg protein.

2.3.9. Erythrocyte superoxide dismutase (SOD) activity

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 was expressed as U/mg protein.

2.3.10. Keratan sulfate

The measurement of urinary keratan sulfate was performed according to the technique described by Martell et al. [32]. The keratan sulfate-derived disaccharides Galβl-4GlcNAc(6S) and Gal(6S)βl-4GlcNAc(6S) were detected in urine by LC-MS/MS assay using keratanase II digestion. The results were expressed in μg keratan/mg Cr.

2.3.11. Urinary creatinine (Cr)

Creatinine was determined using *Creatinine K* kit of Labtest® (Labtest Diagnóstica, Lagoa Santa, MG, Brazil), by reaction 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/dL.

2.3.12. Protein determination

Plasma and erythrocyte protein levels were determined respectively, by Biuret method – using Labtest® kit (Labtest Diagnóstica, Lagoa Santa, MG, Brazil) and by Lowry method [29], both measured in spectrophotometer.

2.4. Statistical analysis

All results were expressed as mean ± standard error of the mean (SEM). Normal distribution was tested by the D'Agostino & Pearson omnibus normality test. Unpaired Student's *t*-test was used for all comparisons between the two groups. As keratan values didn't present Gaussian distribution, they were analyzed by Mann-Whitney test for unparametric data. Correlations between parameters were performed by Pearson's correlation test. Differences were considered significant when $p < 0.05$. All analyses and graphs were done using the GraphPad Prism® (GraphPad Software Inc., San Diego, CA, USA - version 5.0) software.

3. Results

3.1. Oxidative damage to lipids and proteins

Results showed that Morquio A patients had increased levels of urinary isoprostanes, biomarker of oxidative damage to lipids (1310 ± 13.1 pg/mg Cr) (Fig. 1A) ($p < 0.05$) when compared to controls (926 ± 93.03 pg/mg Cr). When oxidative damage to proteins was analyzed, the results revealed that plasma sulfhydryl groups in Morquio A patients (41.16 ± 1.76 μmol TNB/mg protein) did not differ statistically from control group (41.66 ± 0.9183 μmol TNB/mg protein) ($p > 0.05$) (Fig. 1B). Likewise, no significant difference was

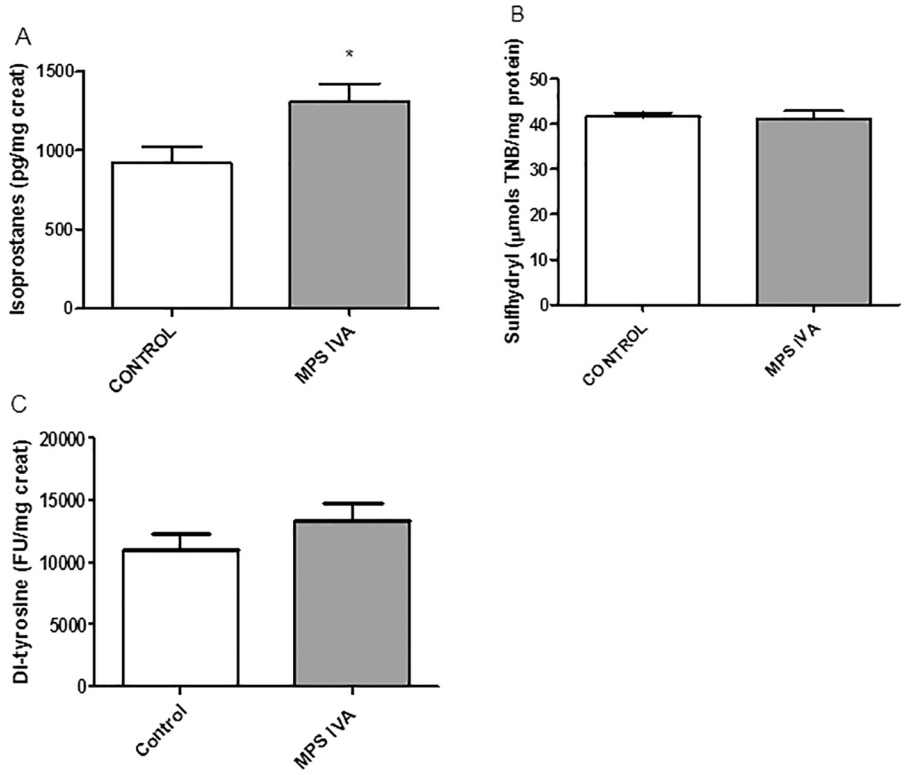

Fig. 1. Oxidative damage to biomolecules. Damage to lipids [isoprostanes in urine (A)] and proteins [sulfhydryl groups in plasma - SH (B); di-tyrosine in urine (C)] in Morquio A patients (n = 13–15) and controls (n = 12–14). Data represent mean ± SEM. *$p < 0.05$ (Student's *t*-test for unpaired samples) compared to the control group.

observed in the urinary content of di-tyrosine when Morquio A patients were compared to healthy individuals (13,414 ± 1355 FU/mg Cr and 10,995 ± 1371 FU/mg Cr, respectively) (Fig. 1C). All data were expressed as the mean ± SEM.

3.2. DNA damage

Morquio A patients showed significant increase in basal DNA damage (73.5 ± 7.90 arbitrary units) when compared to controls (35.0 ± 5.15 arbitrary units) ($p < 0.01$) (Fig. 2A). After Endo III enzyme (that indicates pyrimidines bases damage) treatment, Morquio A patients also showed an increased DNA damage (117.0 ± 20.13 in patients *versus* 59.00 ± 7.91 arbitrary units in controls) ($p < 0.05$) (Fig. 2B). The urinary excretion of 8-OHdG (biomarker of purines bases oxidative damage) was significantly higher in patients (9.86 ± 0.66 ng/mg Cr) when compared to controls (6.21 ± 0.61 ng/mg Cr) ($p < 0.001$) (Fig. 2C). All data were expressed as the mean ± SEM.

3.3. Antioxidant defenses

The concentration of erythrocyte GSH, the main non-enzymatic antioxidant in cells, was significantly increased in Morquio A patients (0.139 ± 0.024 nmol/mg protein) when compared to control group (0.079 ± 0.008 nmol/mg protein) ($p < 0.05$) (Fig. 3A). The GPx activity was significantly increased in patients (330.4 ± 15.15 nmol/min/ml) when compared to controls (265.7 ± 18.71 nmol/min/ml) ($p < 0.05$) (Fig. 3B), while there was no significant difference in GR activity between patients (5.74 ± 0.57 mU/mg protein) and controls (4.75 ± 0.30 mU/mg protein) ($p > 0.05$) (Fig. 3C). Also, SOD activity was not different between Morquio A patients and controls (3.9 ± 0.24 U/mg protein and 4.45 ± 0.38 U/mg protein, respectively) (Fig. 3D). All data were expressed as the mean ± SEM.

3.4. Correlation between lipid peroxidation and DNA damage

Urinary isoprostanes levels in Morquio A were positively correlated with DNA damage index (Fig. 4) ($r = 0.637$ $p < 0.05$).

3.5. Keratan sulfate levels

It was found significant difference in urinary keratan sulfate levels between the two studied groups. Morquio A patients presented higher values than control individuals (17.54 ± 3.85 µg/mg Cr and 0.04 ± 0.03 µg/mg Cr, respectively) (Table 1).

4. Discussion

Morquio A is one of > 50 rare genetic disorders included in the group of LSDs. These diseases, in most cases, are caused by deficiencies in lysosomal enzymes that can lead to the accumulation of lipids, proteins, glycosaminoglycans or other molecules inside the lysosomes [19]. The initial response in order to contain the abnormal accumulation of undegraded substances within the lysosomes is the increase of number and size of these organelles [19]. However, the precise biochemical and cellular mechanisms by which the intralysosomal accumulation leads to cell and tissue dysfunction is still not completely clear [19]. In the case of MPSs, one possible hypothesis to explain cellular damage is that GAGs degradation products are structurally similar to lipopolysaccharide (LPS), an endotoxin of gram-negative bacteria which binds and activates the Toll Like Receptor 4 (TLR4). This activation leads to a secretion of several proinflammatory cytokines inducing a response of the innate immune system [24]. Elevated levels of many genes involved in TLR signaling were already found in MPS animals [46]. Furthermore, in MPS VI and VII animal models, chondrocytes with GAGs accumulation showed elevated nitric oxide levels and proinflammatory cytokines, which can induce the expression of matrix metalloproteases that may directly contribute to cartilage degeneration through their proteolytic activity [2]. Also, the pro-

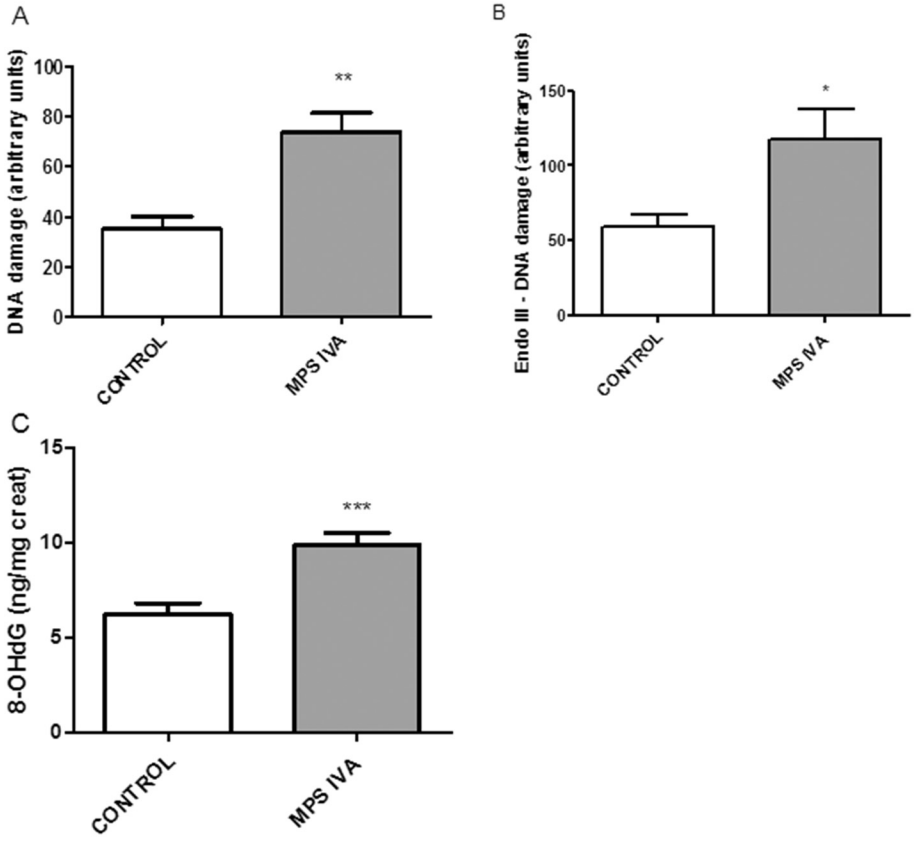

Fig. 2. Basal and oxidative damage to DNA. Basal DNA damage in leukocytes (A); DNA damage with Endo III in leukocytes (B); 8-hydroxy-2′-deoxyguanosine in urine - 8-OHdG (C) in Morquio A patients (n = 8–10) and controls (n = 9–12). Data represent mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ (Student's *t*-test for unpaired samples) compared to the control group.

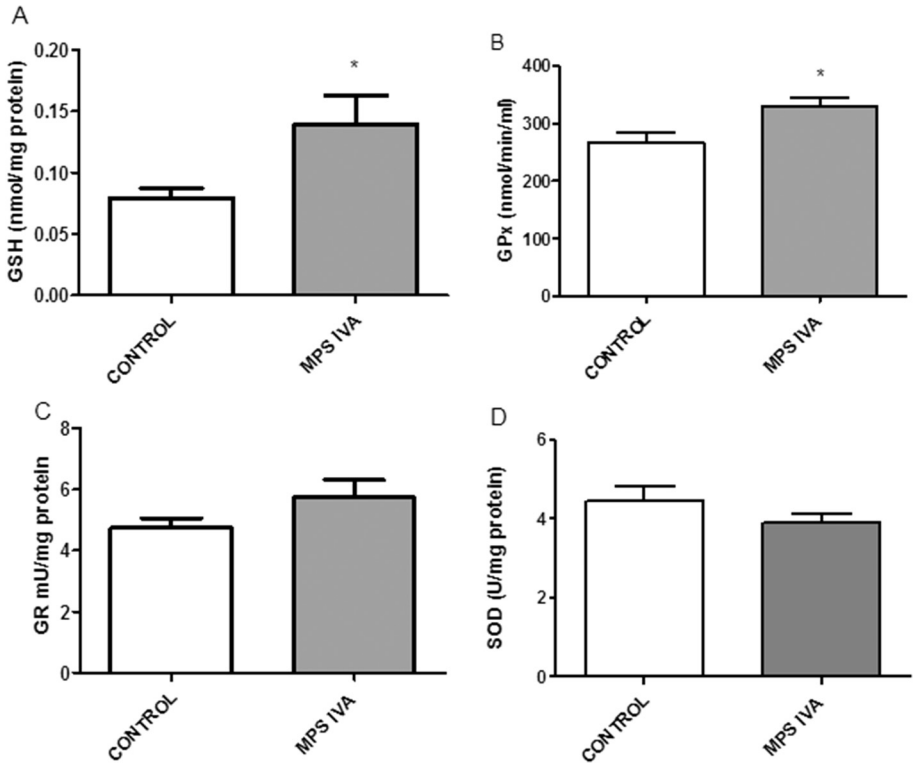

Fig. 3. Antioxidant defenses. Reduced glutathione - GSH (A), glutathione peroxidase - GPx (B), glutathione reductase - GR (C) and superoxide dismutase - SOD (D) in erythrocytes of Morquio A patients (n = 13–14) and controls (n = 12–14). Data represent mean ± SEM. $^{*}p < 0.05$ (Student's *t*-test for unpaired samples) compared to the control group.

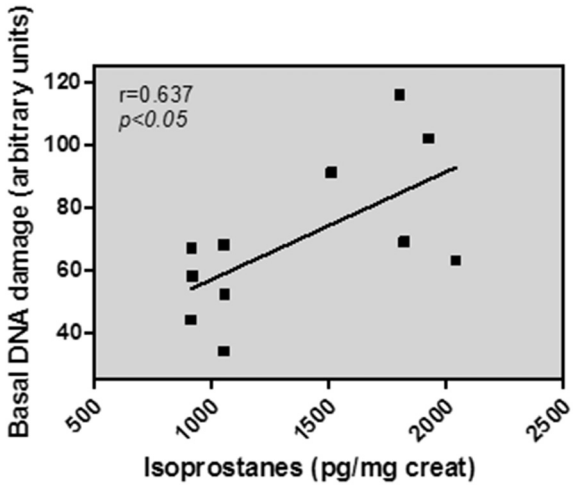

Fig. 4. Correlation between DNA damage and lipid peroxidation. Correlation between DNA damage in leukocytes and isoprostanes in urine of Morquio A patients (Pearson's correlation).

Table 1
Urinary keratan sulfate levels in controls and Morquio A patients.

	Control individuals (n = 15)	Morquio A patients (n = 15)
Keratan level (μg/mg Cr)	0.04 ± 0.03	17.54 ± 3.85***

Values correspond to mean ± SEM.
*** $p < 0.001$; Mann-Whitney nonparametric test.

inflammatory cytokines can induce the production of oxidants, prostaglandins, and mitochondrial ROS by macrophages, contributing to damage found in MPS patients [38].

Furthermore, the involvement of ROS is described in > 100 human diseases, including MPS and other IEM [3,10,17,44,48]. In LSD, such as Morquio A, oxidative stress can be even more harmful because lysosomes are very susceptible to oxidative stress. This is due to the fact that lysosomes have high iron content, which catalyzes Fenton reaction and yields the extremely reactive hydroxyl radical ($OH^{\cdot}$). This radical can attack biomolecules like lipids from lysosomal membrane, resulting in rupture of this organelle. The disruption of lysosomes can cause a release of hydrolases, undegraded metabolites and iron into the cytosol causing cell apoptosis or necrosis, and in last instance tissue injury. Also, the liberation of lysosomal content induces secondary ROS production in cytoplasm, suggesting that lysosomal damage causes further oxidative stress in a loop process [6,49,50,57].

Considering the relation between ROS and LSD described above, the aim of this work was analyze the oxidative damage to biomolecules and antioxidant defenses, as well as keratan sulfate urinary levels, in Morquio A patients without any treatment.

Initially, lipid peroxidation was investigated in Morquio A patients through urinary 15-F2t-isoprostane measurement. Patients affected by a Morquio A syndrome showed increased 15-F2t-isoprostane urinary levels when compared to controls. It should be emphasized that isoprostanes are prostaglandin-like compounds which are not formed by a cyclooxygenase-dependent oxidation of free arachidonic acid; in fact, they are formed by a free radical-catalysed peroxidation of arachidonic acid esterified in membrane phospholipids [36]. Also, 15-F2t-isoprostane is rapidly metabolized and excreted in urine, making it a well-established biomarker of oxidative stress [27]. The presence of large amounts of isoprostanes is highly harmful since it can compromise membrane integrity and fluidity which can lead to rupture of organelles such as lysosomes or even a rupture of the cell [16]. Comparing the results obtained in this study with the results of our previous study [13], it can be concluded that lipid peroxidation occurs both in diagnosis and treatment.

It is well established that lipid peroxides, in addition of its ability to cause membrane destabilization, can attack other molecules, like DNA, for example, forming mutagenic lesions [16]. Therefore, using the comet assay in leukocytes, it was investigated DNA damage in the studied patients. It was found that Morquio A patients had higher levels of DNA damage and, as expected, this damage was positively correlated with lipid peroxidation. In order to better understand if the mechanisms of DNA damage were oxidative, it was used an Endo III enzyme in comet assay. This enzyme recognizes oxidized pyrimidines bases and removes them, which increase the comet's tail [15]. The results obtained whit Endo III suggest that the damage found in Morquio A patients was oxidative and occurs in pyrimidine bases. Oxidative DNA damage was also investigated by 8-OHdG levels in urine of Morquio A patients and healthy individuals. When $OH^{\cdot}$ attacks the guanosine present in nuclear and mitochondrial DNA, 8-OHdG is formed and excreted into the bloodstream and urine [9]. In this work, it was verified that Morquio A patients had higher urinary excretion of 8-OHdG than control group. Combining this result with those obtained by enzymatic comet assay, we can presume that the Morquio A patients exhibit oxidative damage to purines and pyrimidines bases. Filippon et al. [18] also demonstrated that MPS II patients, before and during the ERT, had higher DNA damage than control individuals.

Surprisingly, when the antioxidant defenses of Morquio A patients were analyzed, it was observed an increase of GSH content and elevated GPx activity compared to control individuals. GSH is the most relevant non-enzymatic antioxidant in organism due to its ability to be preferentially oxidized by reactive species and thereby preventing the damage to important biomolecules [21]. Glutamate cysteine ligase (GCL - EC 6.3.2.2), that catalyzes the first step of GSH biosynthesis, can be induced by oxidative stress, as well as the GCL catalytic subunit (GCLC) mRNA and GCLC gene transcription, leading to an increase of GSH levels [30,37]. Therefore, the higher GSH levels found in Morquio A patients could be interpreted as a physiological response to oxidative injury. It was found no statistical differences in GR activity between Morquio A patients and controls. GR is an oxidoreductase (EC 1.8.1.7) that catalyzes the reduction of GSSG to GSH. This enzyme has an important role in glutathione metabolism linking the cellular NADPH pool with the thiol/disulfide pool and maintaining the intracellular reducing environment of high GSH and low GSSG level [11]. Corroborating the supposition of GCL induced by an oxidative stress, Morquio A patients had no change in GR activity, which shows that this enzyme is not being the source of increased GSH. Furthermore, the high level of lipid peroxides in Morquio A patients may be causing a biological response to retain oxidative injury through the increase of both GSH content and GPx activity. The fact that the enzymatic activity of SOD, and consequently the hydrogen peroxide (H_2O_2) content was not altered in Morquio A patients, also suggests that increased GPx activity could be a consequence of high lipid peroxides content, since GPx could be catalyzing the reduction of organic hydroperoxides in cells. Enhanced antioxidant defenses were not seen in our previous study with treated Morquio A patients [13]. Quite the opposite, treated patients showed decreased levels of GSH and also damage to proteins, which was not seen in the present study. A recent work demonstrated that all Morquio A patients develop anti-drug antibodies during ERT [45]. However, it is not known the exact extent of these antibodies; probably the inflammatory and oxidative chain reactions generated by the immune system are responsible for the increased protein damage. As well as Morquio A patients, Fabry patients without treatment presented high levels of GSH and no protein damage, while treated Fabry patients presented decreased GSH levels [4]. Probably, the high levels of GSH found in untreated patients can be masking the measurement of sulfhydryl content, once one third of sulfhydryl groups were represented by GSH.

Finally, urinary keratan sulfate was measured by a LC/MS/MS technique and, as expected, the values of this GAG were greatly

increased in Morquio A patients. Accumulated keratan sulfate in Morquio A disease is excreted in urine and it is an important biomarker of this disease [25]. Differently of blood samples, urinary KS levels remained high in Morquio A patients even after 20 years of age, and are distinguishable from unaffected individuals [23,31,54]. Also, the levels of blood and urine keratan sulfate are correlated with clinical severity during early and progressive stages of the Morquio A disease, and therefore, they are a good prognostic biomarker [25]. It is important to emphasize that urinary GAGs level of Morquio A patients under ERT did not recline to the level of healthy individuals [13]. Therefore, considering the results of this work it can be proposed that keratan are related with the oxidative damage to lipids and DNA found in not treated Morquio A patients. Also, it was found an important increase in GSH levels in Morquio A patients before ERT, which can be interpreted as an adaptive response to oxidative injury.

5. Conclusion

In conclusion, the results of the present work provide new data about the profile of oxidative stress in Morquio A patients contributing to a better understanding of the Morquio A syndrome pathophysiology. Furthermore, the present results reinforce the idea that antioxidants probably would be beneficial if they are used as an adjuvant therapy of ERT. This work provides new targets that can be studied as future therapeutic strategies to improve the quality of life of Morquio A patients.

Conflict of interest

All authors have read the journal's authorship agreement, and the article has been reviewed and approved by all named authors. Roberto Giugliani received educational grants and speaker honoraria, and has been investigator in clinical trials sponsored by BioMarin and other companies. The other authors declare that they have no conflict of interest.

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