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RESEARCH REPORT

Plasmatic and Urinary Glycosaminoglycans Characterization in Mucopolysaccharidosis II Patient Treated with Enzyme-Replacement Therapy with Idursulfase

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Abstract We report the structural characterization of plasmatic and urinary GAGs in a patient affected by MPS II (Hunter syndrome) before and during the first 10 months of enzyme-replacement therapy (ERT). Plasmatic GAGs before ERT were rich in pathological DS consisting of iduronic acid (IdoA) and composed of ~90% ΔDi4s and trace amounts of disulfated disaccharides. DS was also characterized as the main (~90%) urinary GAG mainly composed of ~90% ΔDi4s with minor percentages of monosulfated and disulfated disaccharides, in particular ΔDi2,4dis. After 300 days of ERT, plasmatic DS strongly decreased but ~14% of IdoA-rich ΔDi4s was still detected. Similarly, urinary galactosaminoglycans were mainly composed of 78% ΔDi4s, ~11% ΔDi6s and ~4% ΔDi0s with the persistence of ΔDi2,4dis (~4%). About 40% of IdoA-formed ΔDi4s were also calculated, thus confirming that pathological DS is still present in excreted urinary GAGs during ERT. By considering the % of IdoA, we observed rather similar kinetics of excretion in fluids from the beginning of the treatment. Immediately after the first enzyme infusion, a large amount of abnormal DS is removed from tissues reaching the blood compartment and eliminated via the urine, and this process lasts for about 2 weeks. After this, the percentage of IdoA-rich material present in biological fluids remains fairly constant over the following 9 months of treatment. To date, these are the first data regarding plasmatic and urinary kinetics directly measured on products released by the activity of the recombinant enzyme Idursulfase, iduronate-2-sulfatase, evaluated using specific and sensitive analytical procedures.

Abbreviations

ΔDi0s	ΔUA-(1→3)-GalNAc
ΔDi2,4dis,ΔDi-dis B	ΔUA-2s-(1→3)-GalNAc-4s
ΔDi2,6dis,ΔDi-dis D	ΔUA-2s-(1→3)-GalNAc-6s
ΔDi4,6dis,ΔDi-dis E	ΔUA-(1→3)-GalNAc-4,6dis
ΔDi4s	ΔUA-(1→3)-GalNAc-4s
ΔDi6s	ΔUA-(1→3)-GalNAc-6s
CETAB	Cetyltrimethyl-ammonium bromide
CS	Chondroitin sulfate
DMB	1,9-Dimethylmethylene blue
DS	Dermatan sulfate
ERT	Enzyme-replacement therapy
GAG	Glycosaminoglycan
GlcA	Glucuronic acid
HS	Heparan sulfate
IdoA	Iduronic acid
MPS	Mucopolysaccharidoses

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Introduction

Mucopolysaccharidoses (MPS) are a group of rare genetic disorders of glycosaminoglycan (GAG) catabolism caused by a deficiency in the activity of a single specific lysosomal enzyme required for GAG degradation (Neufeld and Muenzer 2007; Meikle et al. 1999; Fuller et al. 2004a; Martins et al. 2009). These diseases are biochemically characterized by an accumulation of intact and partially degraded polysaccharides within lysosomes and in biological fluids (Burlingame et al. 1981; Fuller et al. 2004a; Fuller et al. 2004b; Martins et al. 2009; Coppa et al. 2010). The GAG accumulation results in progressive cellular damage, which can affect multiple organ systems and lead to organ failure, cognitive impairment, and reduced life expectancy.

Contrary to other MPS, MPS II, also known as Hunter syndrome, is an X-linked recessive disorder generally affecting only males, although rare female patients with MPS II have been described due to an X-autosome translocation and nonrandom X-chromosome inactivation in a carrier female (Tuschl et al. 2005). MPS II is caused by a deficiency of the lysosomal enzyme iduronate-2-sulfatase that selectively catalyzes the hydrolysis of O-sulfate esters from iduronate-2-sulfate belonging to heparan sulfate (HS) and dermatan sulfate (DS) macromolecules (Coppa et al. 1973; Sjöberg et al. 1973). MPS II affects multiple organs and physiological systems and has a variable onset age and variable rate of progression (Martin et al. 2008). MPS II is presently classified into two clinically distinct forms, mild and severe. Deficient enzyme activity leads to common features including facial dysmorphism, organomegaly, joint stiffness and contractures, pulmonary dysfunction, myocardial enlargement and valvular dysfunction, and neurological involvement (Martin et al. 2008). In patients with neurological involvement, intelligence is impaired, and death usually occurs in the second decade of life, whereas those patients with minimal or no neurological involvement may survive into adulthood with normal intellectual development (Martin et al. 2008).

Enzyme-replacement therapy (ERT), along with hematopoietic stem cell transplantation (HSCT), has emerged as a new treatment for MPS disorders, including Hunter syndrome, and they are the standard of care worldwide for a number of MPS diseases (Wraith 2009). The rationale for ERT is to provide the patient with active enzyme supplied exogenously through regular infusions. Idursulfase (Elaprase®, Shire Human Genetic Therapies, Inc., Cambridge, MA), a recombinant form of human iduronate-2-sulfatase, has been commercially available since 2006 (Okuyama et al. 2010; Muenzer et al. 2007; Muenzer et al. 2006). Benefits in patients with no baseline cognitive impairment were found in their walking and respiratory capacities, decreased liver and spleen volume, and in the reduction of urinary GAG levels (Okuyama et al. 2010; Muenzer et al. 2007; Muenzer et al. 2006). However, apart from a general aspecific reduced urinary excretion of GAGs observed during these clinical trials with idursulfase performed using a generic dye-binding assay with 1,9-dimethylmethylene blue (DMB) (Coppa et al. 1987; de Lima et al. 2007), no detailed analyses of plasma and urinary GAG composition and structure in MPS II patients during ERT have been published or are available.

In a previous study (Coppa et al. 2010), urinary and plasmatic GAGs were analyzed in two patients with the Hurler–Scheie form of MPS I subjected to ERT for 6 years, before and during treatment regimens. ERT at the standard dose was found to be unable to definitively eliminate DS from the urine, and a variable effect on GAGs was also observed depending on each administration. In this new research, we performed an accurate quantitative and structural characterization of high molecular mass GAGs present in the urine and plasma of a 3-year-old subject affected by the severe form of MPS II, before and during the first 10 months of ERT with Idursulfase, with the prospect of gaining a better understanding of the effect of therapy on the metabolic fate of these complex macromolecules.

Methods

Materials

Microcon YM-3 filters of 0.45 μm with an MM cutoff of 3,000 Da were from Millipore (Billerica, MA, USA). Chondroitinase ABC [E.C. 4.2.2.4], chondroitinase B [no EC number], and proteinase K [3.4.21.64] were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMB (research grade) was purchased from Serva (Heidelberg, Germany). Unsaturated hyaluronic acid and chondro/dermato disaccharides were from Seikagaku Corporation (Tokyo City, Japan). HS from beef spleen, CS from bovine trachea, and DS from beef mucosa were purified as previously reported (Volpi and Maccari 2002; Volpi and Maccari 2009). Highly pure (>98%) first European pharmacopoeia CS Standard manufactured by Bioiberica (www.bioiberica.com/) and approved in 2004 as Chemical Reference Substance (CRS) by the European Pharmacopeia Commission was used for quantitative evaluation. All the other reagents were analytical grade generally supplied by Sigma-Aldrich.

MPS II Patient

MM is a male born at 41 weeks of gestation, after a normal pregnancy and delivery. At birth, weight was 4.035 kg

(90th percentile), length 51 cm (50th percentile) and head circumference 35 cm (50th percentile). He is the second child with healthy nonconsanguineous parents, with no family history of genetic diseases or mental retardation. The neonatal period and early psychomotor development were normal. In particular, he acquired the sitting position at 7 months and he began to walk at 14 months. At 8 months of age he suffered from catarrhal otitis. Afterwards, he underwent a surgical inguinal hernia. The patient was first referred to the Pediatric Division of the Polytechnic University of the Marche, at the age of 2 years and 9 months as his pediatric neurologist, who was following him for his speech delay, sleep disorders and hyperactivity, noticed macrocrania and a slightly coarse face. On physical examination his weight was 19.1 kg (>97th percentile), length 96 cm (50th percentile) and head circumference 55 cm (>97th percentile). He showed coarse facial features including frontal bossing, depressed nasal bridge and anteverted nostril. Macroglossia, hypertrophic tonsils and mucopurulent coryza were also present. The abdomen was protuberant, with an evident umbilical hernia. The lower liver margin was 3 cm below the ribs while the spleen was unremarkable. Thick skin, hirsutism and wiry hair were also noticed. He had mild dorsolumbar gibbus, claw hands, mild elbow flexion contractures and unstable gait. Ecocardiographic evaluation revealed a moderate mitral valve insufficiency. Following neuropsychological evaluation, an I.Q. of 45 was found. He was hyperactive, aggressive, with poor socialization and absence of expressive language. Audiometry demonstrated hearing loss requiring hearing aids. The bone X-ray survey showed the typical findings of multiple dysostosis: hip hypoplasia, ovoid shape of lumbar vertebral bodies, V-shaped distal ulnar and radial extremities, short and broad metacarpal bones and enlargement of the sella turcica. Urinary GAGs excretion was 1018 μg/mg creatinine (normal value for age: 14–90 μg/mg), and electrophoretic urinary GAGs characterization on acetate of cellulose showed the presence of bands corresponding to DS and HS. Plasma iduronate sulfatase activity was 0.8 nM/ml/4 h (normal value: 12.9–28.0). Molecular analysis revealed the P120R mutation on Xq28. On the basis of the above results and of the clinical evaluation, the diagnosis of mucopolysaccharidosis type II (Hunter syndrome), severe form, was made. After 10 months of ERT, on physical examination his weight was 23.0 kg (>97th percentile), length 107 cm (90th percentile) and head circumference 56 cm (>97th percentile). Cardiovascular abnormalities and hearing loss had stabilized, hepatomegaly had been resolved and joint stiffness had improved. On the contrary, the neuropsychological evaluation did not show any significant improvement.

Patient Sample Collection

Daily morning urine samples were collected from our subject before and during the first 10 months of ERT and frozen at −20°C for analytical investigation.

Blood was collected from the patient before any ERT and treated as reported below. Blood samples were withdrawn immediately before, and at 1 and 3 h after enzyme infusion, and at different times and days after treatment, over ten consecutive months of ERT, collected in tubes containing citrate as an anticoagulant, and the plasma obtained was stored at −20°C for analytical investigation.

Structural Characterization of Plasma and Urine GAGs

The adopted methodology for the determination of the structure and properties of biological fluid GAGs has been reported in detail in (Coppa et al. 2010).

After extraction and purification from 500 μl of plasma (see Coppa et al. 2010), the crude retained GAG fraction was dissolved in 50 μl distilled water. Two aliquots of 10 μL were freeze-dried, dissolved in 30 μL Tris-Cl 100 mM pH 8.0 and treated with 20 μL chondroitinase ABC or 20 μL chondroitinase B. Unsaturated CS/DS disaccharides were separated and quantified by HPLC and postcolumn derivatization with 2-cyanoacetamide and fluorimetric detection (Volpi 2004). The percentage determination of each disaccharide was obtained by means of specific calibration curves performed with authentic standards manufactured by Seikagaku Corporation.

For the quantitation of total urinary GAGs, 100 μL of urine was subjected to DMB assay performed according to (Coppa et al. 1987, 2010). The final quantitative data were normalized for mg creatinine and expressed as μg GAGs/mg creatinine. Furthermore, 5 mL of urine samples from the Patient before any ERT and during treatment was treated with 5% CETAB as previously reported in detail (Dietrich et al. 1993; Maccari et al. 2003; Coppa et al. 2010) and, after extraction, GAGs were dissolved in 200 μl distilled water for further analyses. 40 μl of human urine extracts, not treated or degraded with chondroitinase ABC or B, was separated and quantified by agarose-gel electrophoresis (20 μl layered on gel) and densitometric scanning by means of specific calibration curves as reported elsewhere (Volpi 1993; Volpi and Maccari 2002, 2009) performed by layering on each gel increasing amounts, from 0.5 to 4 μg of the first European pharmacopoeia CS Standard manufactured by Bioiberica (www.bioiberica.com/) (Volpi 2007). The final quantitative data were normalized for mg creatinine and expressed as μg GAGs/mg creatinine. Another 20 μl containing unsaturated disaccharides was separated and quantified by HPLC and

UV detection at 232 nm. The percentage determination of each disaccharide was obtained by means of specific calibration curves performed with authentic standards manufactured by Seikagaku Corporation.

Results

Plasma GAG Composition

It is known that normal human plasma contains low amounts of a major undersulfated CS (Juvani et al. 1975; Volpi and Maccari 2005) covalently bound to an inter-trypsin inhibitor (bikunin) with a chain molecular mass of approx. 6–8 kDa (Chi et al. 2008) mainly constituted by approx. 40–60% nonsulfated disaccharide and 40–60% 4-sulfated disaccharide with trace amounts of 6-sulfated disaccharide (Juvani et al. 1975; Volpi and Maccari 2005; Coppa et al. 2010) (see as an example Fig. 1a, + chondroitinase ABC), and totally composed of glucuronic acid (GlcA) with no trace of iduronic acid (IdoA) typical of DS (see as an example Fig. 1b, + chondroitinase B). As a consequence, for an accurate evaluation of plasmatic CS/DS species, GAGs were subjected to treatment with chondroitinases ABC and B for the determination of disaccharide species and presence of IdoA.

Plasmatic GAG before any ERT treated with chondroitinase ABC (Fig. 2a, + chondroitinase ABC) was found to be composed of ~70% ΔDi4s, ~24% ΔDi0s ~5% ΔDi6s and trace amounts of disulfated disaccharides (Table 1). The charge density was calculated to be 0.77 with a 4s/0s ratio of 2.88. Furthermore, after degradation with chondroitinase B (Fig. 2b, + chondroitinase B), a single disaccharide species was detected, ~90% ΔDi4s, with ~6% ΔDi6s, and trace amounts of disulfated disaccharides. As a consequence, the plasmatic polysaccharide in our MPS II patient before any ERT was constituted by ~34%

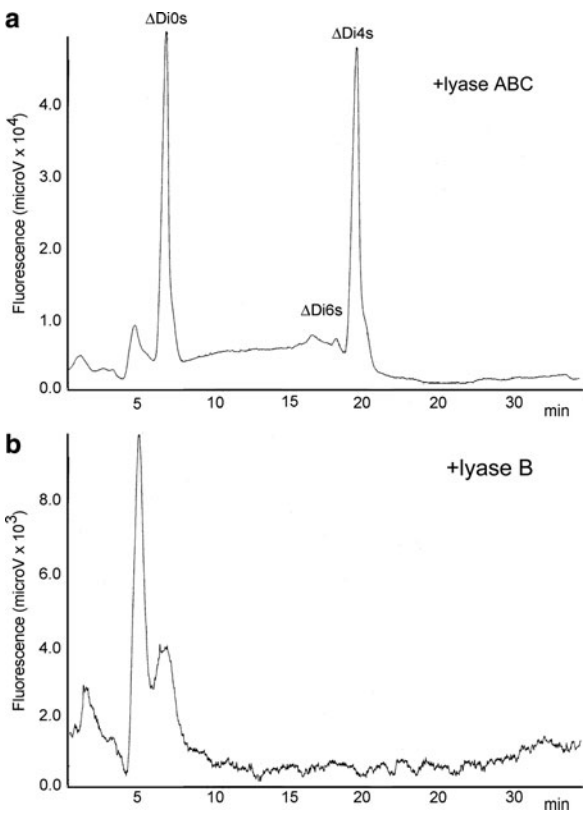

Fig. 1 HPLC separation and postcolumn derivatization with fluorescence detection of unsaturated nonsulfated and variously sulfated disaccharides of the galactosaminoglycans extracted from plasma of a 3-year-old healthy pediatric subject subjected to chondroitin ABC lyase (A, +lyase ABC) or chondroitin B lyase (B, +lyase B) digestions. ΔDi0s, ΔUA-(1→3)-GalNAc. ΔDi4s, ΔUA-(1→3)-GalNAc-4s. ΔDi6s, ΔUA-(1→3)-GalNAc-6s

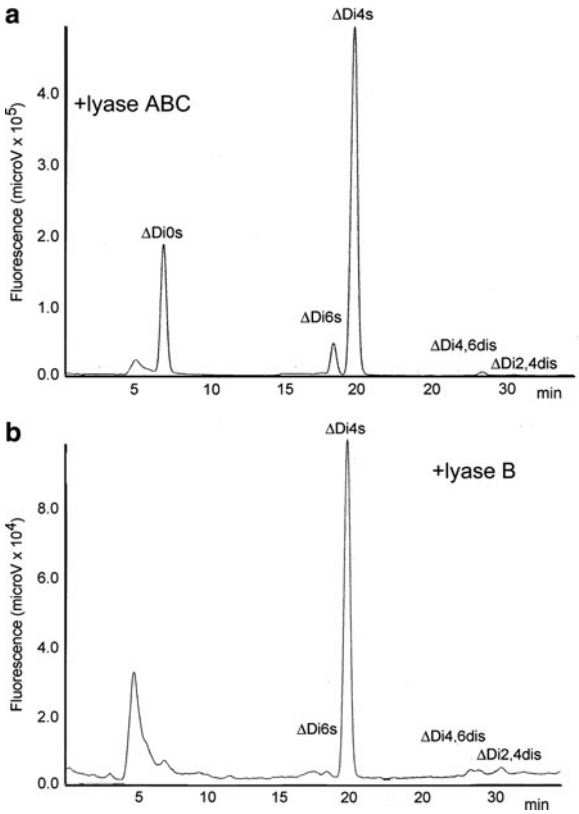

Fig. 2 HPLC separation and postcolumn derivatization with fluorescence detection of unsaturated nonsulfated and variously sulfated disaccharides of the galactosaminoglycans extracted from plasma of the MPS II patient before any enzyme-replacement therapy (day 5) subjected to chondroitin ABC lyase (A, +lyase ABC) or chondroitin B lyase (B, +lyase B) digestions. ΔDi0s, ΔUA-(1→3)-GalNAc. ΔDi4s, ΔUA-(1→3)-GalNAc-4s. ΔDi6s, ΔUA-(1→3)-GalNAc-6s. ΔDi2,4dis, ΔDi-dis B, ΔUA-2s-(1→3)-GalNAc-4s. ΔDi2,6dis, ΔDi-dis D, ΔUA-2s-(1→3)-GalNAc-6s. ΔDi4,6dis, ΔDi-dis E, ΔUA-(1→3)-GalNAc-4,6dis

Table 1 Structural characterization of plasmatic galactosaminoglycans of the MPS II patient before and after 300 days of continuous ERT

	Patient MPS II	
	Before ERT	After 10 months of ERT
ΔDi0s (%)	24.2	36.2
ΔDi6s (%)	4.8	1.2
ΔDi4s (%)	69.6	62.6
ΔDi2,6dis (%)	0.0	0.0
ΔDi4,6dis (%)	1.1	0.0
ΔDi2,4dis (%)	0.3	0.0
Charge density	0.77	0.64
4s/0s Ratio	2.88	1.73
IdoA/GlcA Ratio (%)	34/66	14/86

The results are the mean of three different analyses. The coefficient of variation % was always found to be lower than 15% for all analyses. The charge density was calculated considering the presence and the percentage of carboxyl and sulfate groups for each disaccharide. 4s/0s ratio: ratio between 4-sulfated disaccharide, ΔDi4s, and the non-sulfated disaccharide, ΔDi0s. The percentages of the two different uronic acids have been calculated by means of HPLC after treatment with chondroitinases ABC and B. ΔDi0s, ΔUA-(1→3)-GalNAc. ΔDi4s, ΔUA-(1→3)-GalNAc-4s. ΔDi6s, ΔUA-(1→3)-GalNAc-6s. ΔDi2,4dis, ΔDi-dis B, ΔUA-2s-(1→3)-GalNAc-4s. ΔDi2,6dis, ΔDi-dis D, ΔUA-2s-(1→3)-GalNAc-6s. ΔDi4,6dis, ΔDi-dis E, ΔUA-(1→3)-GalNAc-4,6dis

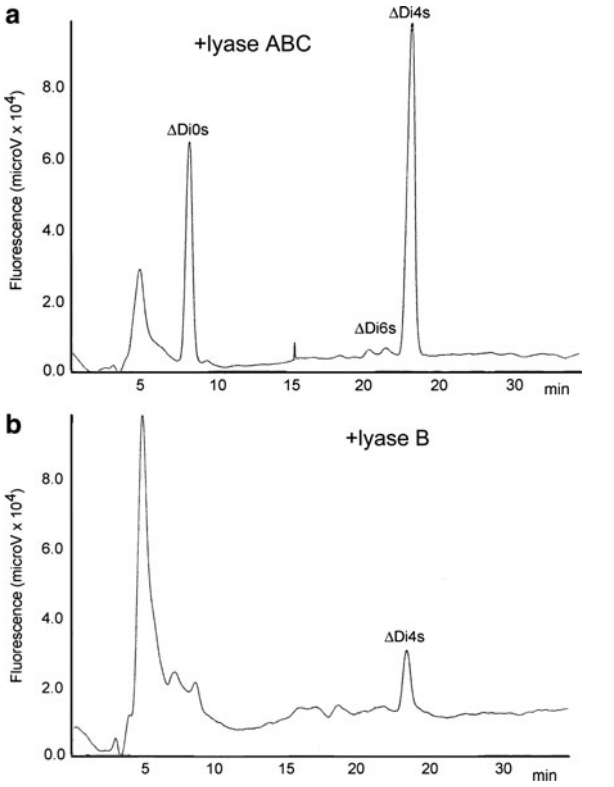

Fig. 3 HPLC separation and postcolumn derivatization with fluorescence detection of unsaturated nonsulfated and variously sulfated disaccharides of the galactosaminoglycans extracted from plasma of the MPS II patient after 300 days of continuous treatment (day 300) subjected to chondroitin ABC lyase (A, +lyase ABC) or chondroitin B lyase (B, +lyase B) digestions. ΔDi0s, ΔUA-(1→3)-GalNAc. ΔDi4s, ΔUA-(1→3)-GalNAc-4s. ΔDi6s, ΔUA-(1→3)-GalNAc-6s. ΔDi2,4dis, ΔDi-dis B, ΔUA-2s-(1→3)-GalNAc-4s. ΔDi2,6dis, ΔDi-dis D, ΔUA-2s-(1→3)-GalNAc-6s. ΔDi4,6dis, ΔDi-dis E, ΔUA-(1→3)-GalNAc-4,6dis

IdoA mainly associated with ΔDi4s disaccharide with the remaining 66% being formed by expected undersulfated CS composed of GlcA with typical disaccharides ΔDi0s and ΔDi4s.

After 300 days of continuous ERT, plasmatic GAG was found to be composed of ~63% ΔDi4s, ~36% ΔDi0s and ~1% ΔDi6s (Fig. 3a, + chondroitinase ABC) with a decrease in the charge density (0.64) and 4s/0s ratio (1.73) compared to the polysaccharide before treatment showing a progression towards the more physiological undersulfated CS (Table 1). To confirm this, treatment with chondroitinase B (Fig. 3b, + chondroitinase B) showed a strong decrease in the ΔDi4s disaccharide associated with IdoA being ~14% of the disaccharides produced by ABC.

The previous structural plasmatic GAG evaluation showed, along with the physiological undersulfated CS, the presence of an abnormal polysaccharide characterized as DS due to the presence of a high percentage of ΔDi4s disaccharide associated with IdoA, as expected for MPS II disorder. Over the 10-month period, ERT reduced the percentage of this abnormal polymer up to 85%, as illustrated in Fig. 3, but was unable to totally remove it from the blood. In fact, after 10 months of treatment, ~13–15% of this DS was still detected. Finally, as is evident from Fig. 4, a strong increase in the plasmatic IdoA-composed polysaccharide percentage was measured immediately after the first enzyme infusion, after 1 h, with a maximum after 3 h, then a continuous decrease in the 8–15 days following treatment. After this, a constant plasmatic content of IdoA-formed GAGs percentage was observed. We can suppose that, in our patient, immediately after the first enzyme administration, a large amount of abnormal DS is removed from tissues reaching the blood compartment, and this process lasts for approximately the following 2 weeks following the first infusion.

Urine GAG Composition

Urine GAGs collected before ERT and at different days and times during therapy over a period of 10 months were extracted by means of a conventional method (Dietrich et al. 1993; Maccari et al. 2003; Buzzega et al. 2010) and

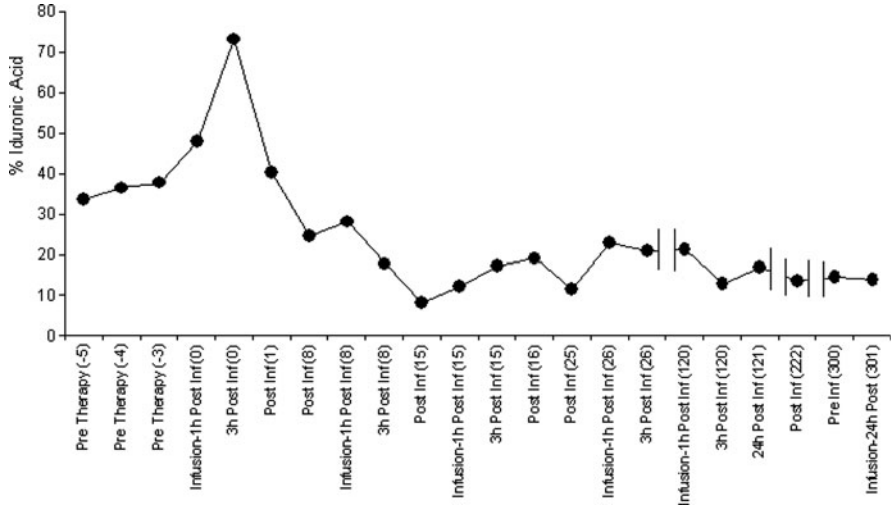

Fig. 4 Percentage of iduronic acid-composed plasmatic galactosaminoglycans depending on the days of enzyme-replacement therapy measured by HPLC separation of disaccharides obtained by treatment with chondroitin ABC and B lyases (see Figs. 2 and 3 as examples). Days and times of plasma collection are the same as indicated in Fig. 5 for urine samples characterization

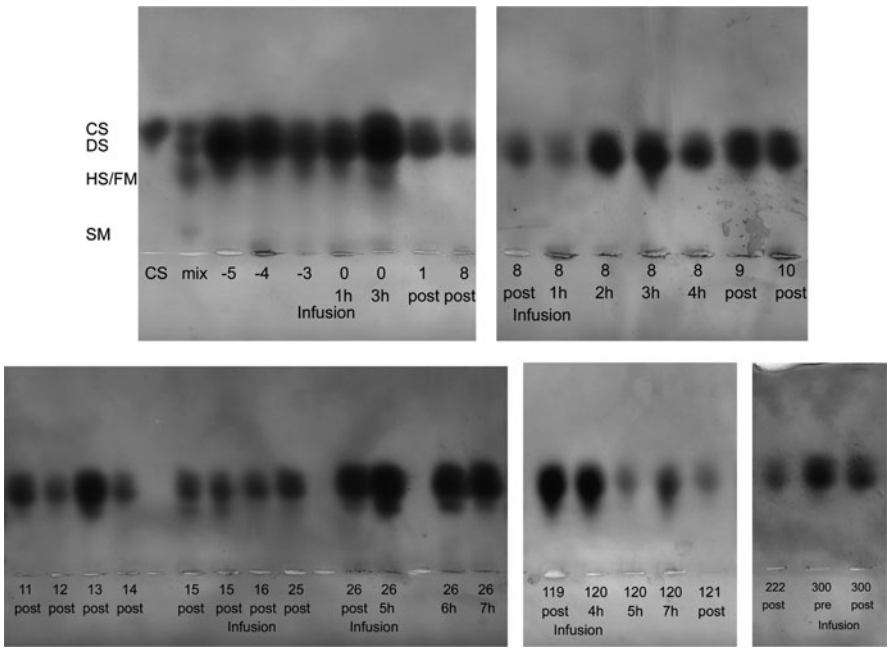

Fig. 5 Agarose-gel electrophoresis stained with toluidine blue of GAGs extracted from the urine of the MPS II patient before any enzyme-replacement therapy (-5, -4, -3) and on different days (the first line of label) and times (the second line of label) of treatment, also with the indication of the moment of enzyme infusion (the third line of label) up to 301 days. Post indicates a not fully specified time after enzyme infusion. Mix, GAGs standard. *CS* chondroitin sulfate, *DS* dermatan sulfate, *HS* heparan sulfate, *FM* fast-moving heparin, *SM* slow-moving heparin. Quantitative analysis was performed by means of specific calibration curves constructed by layering on each gel increasing amounts, from 0.5 to 4 μg, of the first European pharmacopoeia CS Standard (not shown in the figure)

analyzed by means of agarose-gel electrophoresis (Fig. 5) and densitometric evaluation for quantitative acquisition (then data were normalized for mg creatinine, Fig. 6a). Furthermore, total GAGs were determined directly in urine samples by a standard colorimetric assay accepted by the Guidelines for the management of MPS (Martins et al. 2009) by using DMB (Fig. 6a) (normal range of DMB values for ages ranging from 22 to 86 μg/mg creatinine). As is evident from the electrophoresis, a high content of a band having a migration position similar to standard DS was observed before and during ERT along with minor amounts of bands similar to standard CS (see −3, 0, 1 and 8 for example) and standard HS (see 0, 13, 15 and 26 for example). Evaluation of the total content of urinary GAGs

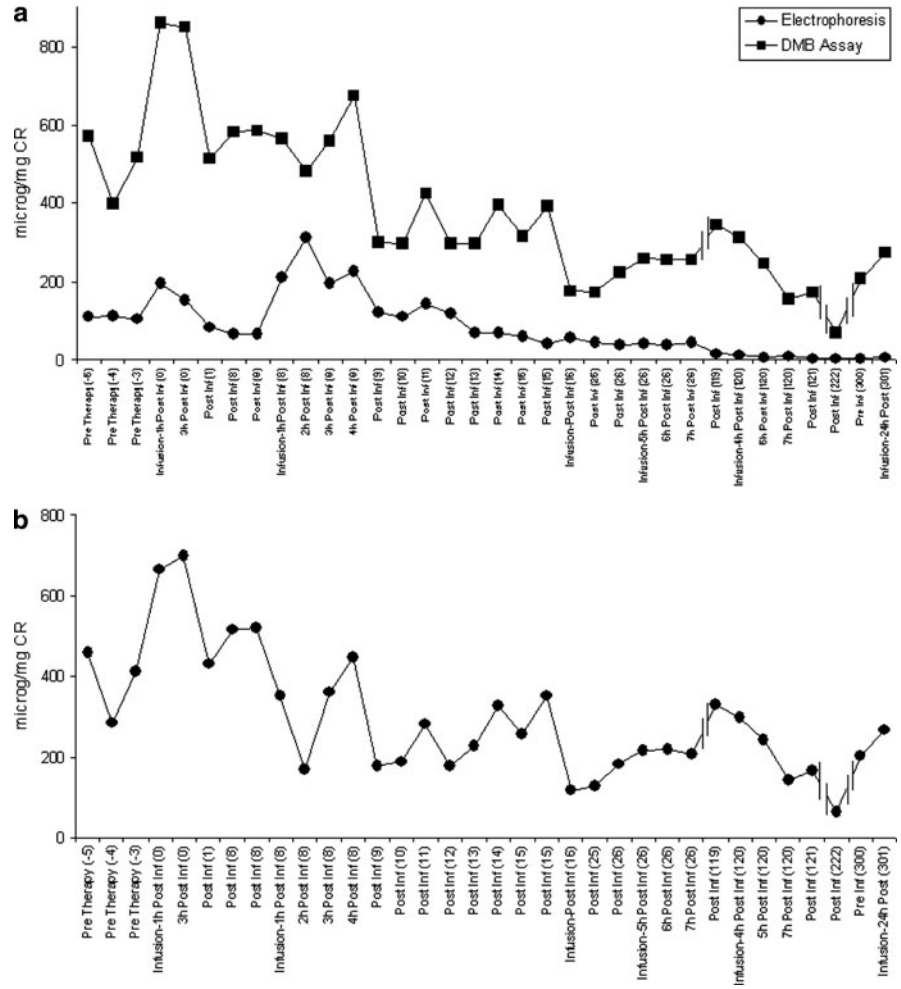

Fig. 6 (**a**) Total amounts of GAGs expressed as µg/mg creatinine in the urine of the MPS II patient before any enzyme-replacement therapy (-5, -4, -3) and on different days and times during enzyme-replacement treatment determined by agarose-gel electrophoresis (see Fig. 5) and DMB assay. (**b**) Amounts of urinary GAG fragments lower than ~1,500 Da expressed as µg/mg creatinine and determined by comparing DMB data with agarose-gel electrophoresis results. Days and times of urine sample collection are the same as indicated in Fig. 5

performed by DMB assay and agarose-gel electrophoresis normalized for mg creatinine is illustrated in Fig. 6a. A strong increase in the total urinary GAGs was measured immediately after the first infusion of enzyme by DMB assay (after 1–3 h, ~ +70%) and agarose-gel electrophoresis (~ +80% after 1 h and ~ +40% after 3 h) followed by lower values for the 8 days following treatment. Fairly similar behavior was observed after the second infusion with a strong increase in urinary GAGs during the first few hours immediately after administration followed by a constant decrease (Fig. 6a). Further infusions after 16, 26, 120 and 300 days from the first treatment produced less important modifications in the urinary GAGs levels. Furthermore, after 10 months of therapy, the total content of urinary GAGs performed by DMB assay still produced higher values than normal (normal range of 22–86 µg/mg creatinine) according to other studies showing a reduction but not a normalization of urinary DMB values after many months of ERT (Alcalde-Martín et al. 2010; Muenzer et al. 2011).

We should consider that the DMB assay is able to detect high molecular mass GAGs along with fragments (Whitley et al. 1989; de Lima et al. 2007), contrary to agarose-gel electrophoresis, which is capable of evaluating molecules having a molecular mass greater than ~1500 (Volpi 1993; Volpi and Maccari 2002). As a consequence, as illustrated in Fig. 6b, by comparing the two data we can calculate the amount of urinary GAG fragments lower than ~1500 eliminated during ERT having a behavior comparable to total GAGs as well as to high molecular mass DS. In fact, a large amount of these low molecular mass fragments was measured immediately after the first enzyme infusion with a continuous decrease over the following 10 days then remaining fairly constant up to 10 months of treatment (Fig. 6b). Finally, after the first two enzyme administrations a constant decrease in the urinary content of high molecular

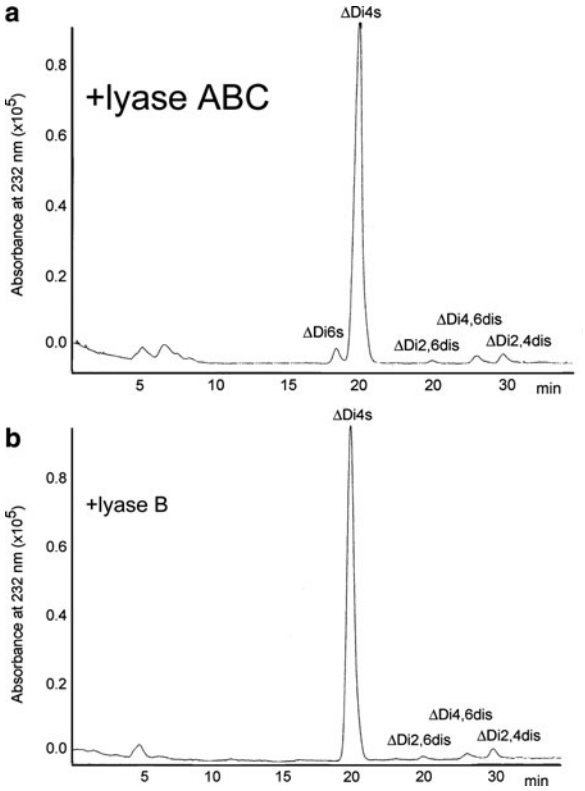

Fig. 7 HPLC separation and UV detection at 232 nm of unsaturated nonsulfated and variously sulfated disaccharides of the galactosaminoglycans extracted from the urine of the MPS II patient before any enzyme-replacement therapy (see day 5 of Fig. 5) subjected to chondroitin ABC lyase (**a**) or chondroitin B lyase (**b**) digestions. ΔDi0s, ΔUA-(1→3)-GalNAc. ΔDi4s, ΔUA-(1→3)-GalNAc-4s. ΔDi6s, ΔUA-(1→3)-GalNAc-6s. ΔDi2,4dis, ΔDi-dis B, ΔUA-2s-(1→3)-GalNAc-4s. ΔDi2,6dis, ΔDi-dis D, ΔUA-2s-(1→3)-GalNAc-6s. ΔDi4,6dis, ΔDi-dis E, ΔUA-(1→3)-GalNAc-4,6dis

mass GAGs was observed but with values still higher than normal subjects for age and, according to the results obtained in plasma, we can suppose that immediately after the first enzyme administrations, a high amount of abnormal DS is removed from tissues reaching the blood compartment and urines in particular during the 2 weeks subsequent to the beginning of therapy.

The main urinary GAG band detected in Agarose-gel electrophoresis before ERT was definitively characterized as DS by specific treatment with chondroitin ABC and B lyases, this last enzyme being able to degrade polymers consisting of iduronic acid typical of DS species (Volpi and Maccari 2009). Urinary galactosaminoglycans, i.e., CS and DS, in our MPS II patient before ERT (Fig. 7a) were found to be mainly composed of ~90% ΔDi4s, ~3% ΔDi6s and ~3% ΔDi0s (Table 2). Minor but significant percentages of disulfated disaccharides, in particular ΔDi2,4dis (2%) typical of DS (Volpi 2009; Volpi and Maccari 2009), were also observed (Table 2). As a consequence, a polysaccharide very rich in 4-sulfated groups on the

Table 2 Structural characterization of the galactosaminoglycans component of the urine of the MPS II patient before and after 300 days of continuous ERT

	Patient MPS II	
	Before ERT	After 10 months of ERT
ΔDi0s (%)	3.3	3.8
ΔDi6s (%)	3.0	11.5
ΔDi4s (%)	89.7	78.1
ΔDi2,6dis (%)	0.4	0.0
ΔDi4,6dis (%)	1.5	0.4
ΔDi2,4dis (%)	2.1	6.2
Charge density	1.00	1.03
4s/6s Ratio	29.9	6.8
IdoA/GlcA Ratio (%)	89/11	40/60

The results are the mean of three different analyses. The coefficient of variation % was always found to be lower than 15% for all analyses. The charge density was calculated considering the presence and the percentage of carboxyl and sulfate groups for each disaccharide. 4s/6s ratio: ratio between 4-sulfated disaccharide, ΔDi4s, and the sulfated disaccharide in position 6 of the *N*-acetyl-galactosamine unit, ΔDi6s. The percentages of the two different uronic acids have been calculated by means of SAX-HPLC after treatment with chondroitinases ABC and B. ΔDi0s, ΔUA-(1→3)-GalNAc. ΔDi4s, ΔUA-(1→3)-GalNAc-4s. ΔDi6s, ΔUA-(1→3)-GalNAc-6s. ΔDi2,4dis, ΔDi-dis B, ΔUA-2s-(1→3)-GalNAc-4s. ΔDi2,6dis, ΔDi-dis D, ΔUA-2s-(1→3)-GalNAc-6s. ΔDi4,6dis, ΔDi-dis E, ΔUA-(1→3)-GalNAc-4,6dis

hexosamine (4s/6s ratio of ~30) and having a charge density of ~1 (Table 1) was detected in the MPS II subject. Moreover, the use of chondroitinase B (Fig. 7b) showed the presence essentially of ΔDi4s corresponding to about 89% of the amount of the same disaccharide obtained after chondroitinase ABC digestion in our patient (Table 2), confirming that excreted urinary galactosaminoglycans are essentially composed of 90% DS with ~10% CS.

After 10 months of continuous ERT, galactosaminoglycans in urine were mainly composed of 78% ΔDi4s, ~11% ΔDi6s and ~4% ΔDi0s (Table 2) showing a polysaccharide still having a high 4s/6s ratio (~7) but lower than it was prior to therapy demonstrating a DS reduction of ~55–60%. Minor but significant percentages of disulfated disaccharides, in particular ΔDi2,4dis (~4%), were also detected (Fig. 8a) (Table 2), resulting in an average charge density of ~1.0 for the polysaccharide chains. The use of chondroitinase B (Fig. 8b) showed the presence essentially of ΔDi4s corresponding to ~40% of disaccharides produced by chondroitinase ABC, thus confirming that excreted urinary GAGs during ERT are a mixture of CS (~60%) and DS (~40%). Finally, Fig. 9 illustrates as an example the urinary galactosaminoglycan disaccharide profile of a normal subject after treatment with both lyases showing the absence of ΔDi4s produced by chondroitinase B.

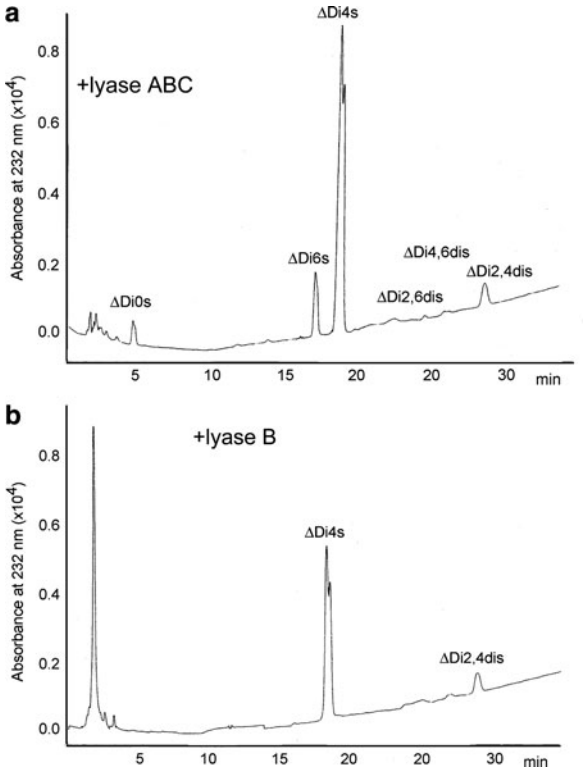

Fig. 8 HPLC separation and UV detection at 232 nm of unsaturated nonsulfated and variously sulfated disaccharides of the galactosaminoglycans extracted from the urine of the MPS II patient after 300 days of continuous treatment (see day 300 of Fig. 5) subjected to chondroitin ABC lyase (**a**) or chondroitin B lyase (**b**) digestions. ΔDi0s, ΔUA-(1→3)-GalNAc. ΔDi4s, ΔUA-(1→3)-GalNAc-4s. ΔDi6s, ΔUA-(1→3)-GalNAc-6s. ΔDi2,4dis, ΔDi-dis B, ΔUA-2s-(1→3)-GalNAc-4s. ΔDi2,6dis, ΔDi-dis D, ΔUA-2s-(1→3)-GalNAc-6s. ΔDi4,6dis, ΔDi-dis E, ΔUA-(1→3)-GalNAc-4,6dis

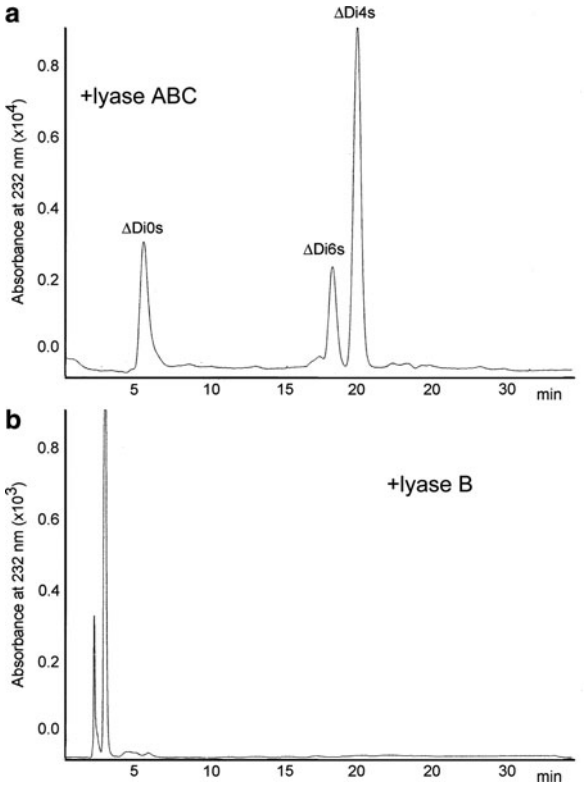

Fig. 9 HPLC separation and UV detection at 232 nm of unsaturated nonsulfated and variously sulfated disaccharides of the galactosaminoglycans extracted from the urine of a normal pediatric subject treated with chondroitin ABC lyase (**a**) or chondroitin B lyase (**b**) digestions. ΔDi0s, ΔUA-(1→3)-GalNAc. ΔDi4s, ΔUA-(1→3)-GalNAc-4s. ΔDi6s, ΔUA-(1→3)-GalNAc-6s

The urinary IdoA-composed polysaccharide percentage is reported in Fig. 10 depending on the days and hours before and during ERT. A trend fairly similar to that measured in plasma (see Fig. 4) was observed in the urine, in particular an increase in DS was measured after the first enzyme infusion followed by a progressive decrease up to the second administration, after which a new DS increase was evident, followed by a continuous progressive decrease. However, as in the case of plasma, after 300 days of treatment, ERT was unable to totally remove DS from the urine, being still present in a high percentage of (~40%).

Discussion

ERT with idursulfase has been commercially available since 2007 and early access programs have been established since 2005. However, limited information on the effects of ERT on principal accumulated macromolecules, GAGs, responsible for the pathological conditions is available to date. In recent clinical studies (Muenzer et al. 2011; Alcalde-Martín et al. 2010; Okuyama et al. 2010), urinary GAG excretion was found to decrease rapidly within the first 3 months of ERT treatment, simply by DMB assay evaluation.

In a previous paper (Coppa et al. 2010), we characterized the plasmatic and urinary GAGs of two patients with the Hurler–Scheie form of MPS I before ERT and after a 6-year period of treatment. This previous study showed, despite an evident clinical efficacy, the reduced capacity of ERT by infusion of recombinant human α-L-iduronidase at the standard dose to definitively eliminate pathological DS from the urine. In this new study, we applied a fairly similar analytical methodology to characterize plasmatic and urinary GAGs in a subject affected by the severe form of MPS II, before and during the first 10 months of ERT, with a recombinant form of human iduronate-2-sulfatase. The defect of the two enzymes responsible for MPS I and II gives rise to the accumulation of the same high molecular mass GAGs, i.e., urinary DS and HS (the latter mainly present in the form of low molecular mass fragments, see below). In fact, also in the MPS II patient before ERT, urinary galactosaminoglycans were mainly composed of ~90% ΔDi4s, with minor but significant percentages of

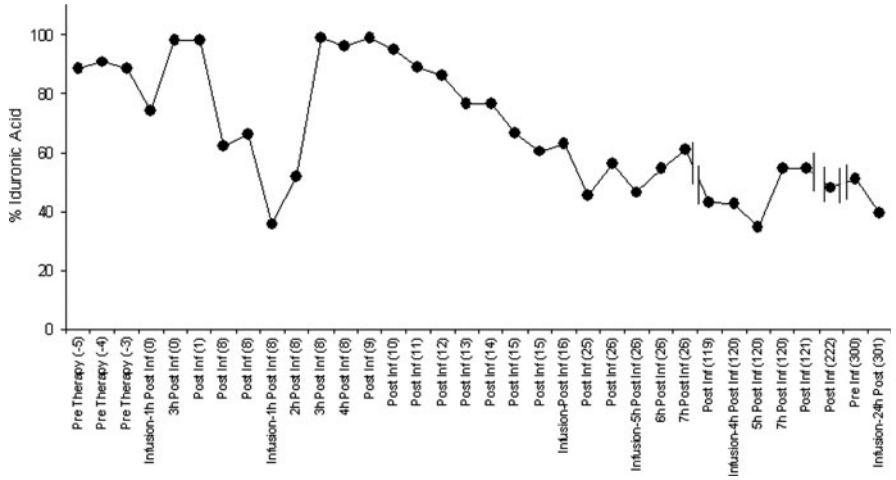

Fig. 10 Percentage of iduronic acid-composed urinary galactosaminoglycans depending on days of enzyme-replacement therapy measured by HPLC separation of disaccharides obtained by treatment with chondroitin ABC and B lyases (see Figs. 7 and 8 as examples). Days and times of urine sample collection are the same as indicated in Fig. 5

disulfated disaccharides and ~90% IdoA. This should explain the presence of many similar clinical features for MPS I and II. Furthermore, in a recent study (Coppa et al. 2011), we found a correlation between the amount of total urinary GAGs and the severity of MPS type I and II. As a consequence, we can suppose that in our MPS II patient affected by a severe form, ERT would be less effective to totally remove the high content of pathological DS.

Along with high molecular mass GAGs (Burlingame et al. 1981; Coppa et al. 2010), also fragments and HS/DS oligosaccharides are excreted in MPS I urine (Fuller et al. 2004b; Coppa et al. 2011). In fact, by using a specific analytical approach, we were able to detect in the urine a high content of glucosamine demonstrating that HS is mainly excreted as fragments and oligomers not determined by agarose-gel electrophoresis due to its incapacity to detect molecules having a molecular mass lower than approx.1500 (Volpi and Maccari 2002). Furthermore, the presence of a low content of HS having a molecular mass greater than ~1,500 is also evident in agarose-gel electrophoresis in some urine samples (see Fig. 5, the faint band having lower mobility).

As DS accumulated in MPS II is obviously composed of IdoA as opposed to physiological undersulfated CS present in plasma and CS in urine, we used the percentage of IdoA calculated in the total galactosaminoglycans to monitor the efficacy of ERT immediately after the first enzyme infusion and over the 10-month treatment period. Fairly similar kinetics of IdoA-rich material excretion was observed in plasma and urine from the beginning of treatment. A strong increase in the plasmatic IdoA-composed polysaccharide percentage was measured immediately after the first enzyme infusion, followed by a continuous decrease in the 8–15 days following the beginning of treatment. After this, an almost constant plasmatic content of the IdoA-formed GAGs percentage was observed. In the urine, an increase in DS excretion was measured after the first and second enzyme infusion followed by a continuous progressive decrease. We can suppose that immediately after the first enzyme administrations, a large amount of abnormal DS is removed from tissues reaching the blood compartment and eliminated via the urine, and thereafter only minimal changes have been observed. This process lasts for approximately 2 weeks after the first infusion. After this, the percentage of IdoA-rich material present in plasma and eliminated with the urine remains fairly constant over the following months of treatment. To date, these are the first data regarding plasmatic and urinary kinetics directly measured on products released by the activity of the recombinant enzyme Idursulfase, iduronate-2-sulfatase.

Plasma GAGs in MPS I subjects after 6 years of ERT were found to be comparable to those of normal subjects (Coppa et al. 2010), while a pathological high molecular mass DS was still found in the urine having a high percentage of 4-sulfated disaccharide and iduronic acid (~60%) mainly associated with DS. Interestingly, a rather similar trend was observed in the plasmatic and urinary GAGs in our MPS II subject after several months of ERT evaluated in this study. In fact, after 10 months' treatment, a high percentage (~40%) of DS was detected in the urine, and plasma still showed ~14% of IdoA-associated material. ERT performed by infusion of recombinant human α-L-iduronidase to treat MPS I or iduronate-2-sulfatase for MPS II at the standard doses does not totally remove DS from urine (and also plasma in our MPS II Patient) after prolonged treatment even if they are very effective in eliminating large amounts of this pathological GAG just after the first weeks following the beginning of enzyme infusion. In fact, after 10 months' treatment, a very low

content of high molecular mass polymer(s) was observed compared to pretreatment on the contrary of fragments still present in the urine in relatively high amounts.

According to the previous study on MPS I patients subjected to ERT, the presence of high molecular mass DS in the urine of the MPS II subject treated with iduronate-2-sulfatase was found to be inconstant over one continuous week of observation (see for example Fig. 6 for days 8, 9, 10, 11, 12, 13, 14 and 15 after infusion), thus confirming the incomplete effectiveness of ERT at the present standard dose to maintain a constant level of physiological GAGs, as we qualitatively characterized still abnormal levels of the pathological polymer along with normally excreted CS in the urine.

Contrary to this new research in which we measured ~14% of IdoA-associated material, in the previous study we were unable to detect any pathological DS in human plasma during ERT. This difference may be due to several reasons, such as different periods of treatment (10 months versus 6 years), or possible variable effects depending on the subjects. However, the incapacity of ERT, after 10 months' treatment, to totally remove pathological products derived from the condition of MPS II disorder in our patient (severe form) focuses attention on the possibility that the present therapeutic regimen might not be sufficient to totally remove the accumulation of lysosomal GAGs. Therefore, the present specific and sensitive measurements and structural characterization of human biological fluid GAGs, even if applied in a unique MPS II patient, would be extremely helpful for an accurate evaluation of the kinetics of catabolic products following possibly more effective and new therapeutic interventions, such as ERT for recent clinical trials (for MPS type VI) (Wraith 2009) or hematopoietic stem cell transplantation (HSCT).

Contributors

N.V. developed the applied methodologies. D.B., L.Z., F. M., L.S., F.G. and T.G. performed the experimental procedures and analyses. N.V., G.V.C. and O.G. designed and developed the experimental design, performed data analysis and wrote the manuscript.

All authors reviewed and approved the study.

Conflict of Interest

We declare that we have no conflict of interest.

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The Concise Sentence Take-Home Message

Before ERT and over 10 months of enzyme infusion, glycosaminoglycans in urine and plasma were analyzed in a patient with severe MPS II (Hunter syndrome) and new data and a better understanding of the metabolic fate of these macromolecules have been obtained.

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