Effect of 6 years of enzyme replacement therapy on plasma and urine glycosaminoglycans in attenuated MPS I patients

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Enzyme-replacement therapy (ERT) is a new option for the clinical management of MPS I. However, no detailed data are available on the structural characterization of glycosaminoglycans (GAGs) in the urine and plasma of patients before ERT and during treatment regimens. Before ERT and over a two-week period of enzyme infusion, GAGs in urine and plasma were analyzed in two patients with the Hurler-Scheie form of MPS I subjected to ERT for 6 years. In both patients before ERT, high amounts of a GAG were found in the urine, composed in particular of a high molecular mass polymer (∼13,000-13,500) consisting of ∼75-78% iduronic acid and rich in 4-sulfated disaccharides (ΔDi4s) and attributable to DS. Furthermore, a high amount of this GAG was directly detected in the blood. Plasma GAGs in MPS I patients subjected to ERT were found to be comparable to those of normal subjects with the absence of heparan sulfate and of DS. On the contrary, a polysaccharide possessing a high molecular mass, ∼11,500-12,000, lower than the polymer extracted before ERT but slightly higher than the controls (∼11,000), was found in the urine of both patients. This macromolecule was characterized as a mixture of DS/chondroitin sulfate based on the high percentage of 4-sulfated disaccharide (4s/6s ratio of ∼3.1) and iduronic acid (∼60%). These results are indicative of the incapacity of ERT at the standard dose to definitively eliminate DS from the urine. Finally, a variable effect of ERT depending on each administration was also observed.

Keywords: dermatan sulfate/enzyme replacement therapy/glycosaminoglycans/heparan sulfate/mucopolysaccharidoses

Introduction

Mucopolysaccharidoses (MPS) are a group of inherited lysosomal storage disorders characterized by a deficiency in one of the lysosomal enzymes required to degrade glycosaminoglycans (GAGs) (Meikle et al. 1999; Fuller, Rozaklis et al. 2004). In all MPS subtypes, partially degraded GAG(s) accumulate in the lysosomes of affected cells and/or are eliminated in the blood and excreted in the urine (Martins et al. 2009). Mucopolysaccharidosis type I (MPS I) is an autosomal recessive disorder caused by deficient activity of α-L-iduronidase, the lysosomal enzyme that selectively cleaves L-iduronic acid from N-acetylgalactosamine in dermatan sulfate (DS) and N-acetylglucosamine in heparan sulfate (HS) molecule (Fuller, Rozaklis et al. 2004; Martins et al. 2009). Deficient enzyme activity leads to widespread accumulation mainly of DS and HS, ultimately resulting in a progressive multisystem disease with respiratory, cardiac, skeletal, ocular and sometimes neurological manifestations. MPS I is characterized by a wide clinical spectrum ranging from severe (Hurler syndrome, which occurs in infancy and is characterized by relentless cognitive decline) to attenuated (Hurler-Scheie and Scheie syndromes, occurring in childhood or later with slower progression and moderate-to-absent central nervous system involvement). Untreated patients with severe MPS I have a median survival time of 7 years, whereas patients with attenuated MPS I often survive to adulthood, albeit with moderate-to-severe disability (Martins et al. 2009).

MPS I patients, regardless of the severity of the clinical findings, have an increased concentration of GAGs both in the urine and plasma with an abnormal pattern due to the presence of high amounts of DS and of HS (Burlingame et al. 1981; Fuller, Meikle et al. 2004; Fuller, Rozaklis et al. 2004). In fact, various high molecular mass GAGs (Burlingame et al. 1981) and related fragments, i.e. oligomers (Fuller, Meikle et al. 2004; Fuller, Rozaklis et al. 2004; Mason et al. 2006), at very high concentration are produced in MPS urine, and high amounts of GAGs have been detected in the plasma of MPS patients after specific enzymatic treatment and high-performance liquid chromatography-electrospray ionization (HPLC-ESI)-tandem mass evaluation of generated disaccharides (Oguma et al. 2007). On the contrary, by means of agarose gel electrophoresis (Dietrich et al. 1993; Maccari et al. 2003), normal human urine mainly contains chondroitin sulfate (CS), about 85–90% of the total, HS in a percentage of about 10–15% and traces of hyaluronid acid and DS, in a total amount lower than ~100 µg/mg creatinine (Gabrielli et al. 2010). Moreover, the main urinary GAG, i.e. CS, is a fully sulfated macromolecule...
having sulfate groups in position 4 or 6 of the galactosamine unit at rather similar percentages (Maccari et al. 2003). Furthermore, normal human blood plasma contains low amounts, 0.1–6.0 µg/mL (Volpi and Maccari 2005), of a major under-sulfated CS (Juvani et al. 1975; Volpi and Maccari 2005) covalently bound to an inter-trypsin inhibitor (bikunin) with a chain molecular mass of ~6–8 kDa (Chi et al. 2008) mainly constituted by ~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).

To date, the therapeutic approach towards MPS I patients has offered different treatment options represented respectively by hematopoietic stem cell transplantation for the severe form and by enzyme replacement therapy (ERT) for the attenuated forms (Martins et al. 2009). Recombinant human α-L-iduronidase, laronidase (Aldurazyme by BioMarin Pharmaceutical Inc., Novato and Genzyme Corp.), is now available for MPS I at the standard infusion dose of 100 U/kg of body weight per week (Martins et al. 2009). Generally, clinical benefits of ERT for MPS I include decreased hepatomegaly, improved respiratory function, increased walking ability, increased joint range of movement, decreased left ventricular hypertrophy, improved growth and improved quality of life (Martins et al. 2009).

A reduced urinary excretion of GAGs has been observed during ERT with recombinant human α-iduronidase in MPS I subjects (Martins et al. 2009) by performing a generic dye-binding assay with 1,9-dimethylmethylen blue (DMB) (de Lima et al. 2007) or by evaluating unsaturated GAG disaccharides by HPLC-ESI (Tomatsu, Montaño, Oguma, Dung, Oikawa, de Carvalho et al. 2010). However, these approaches are incapable of identifying individual GAGs and related structures and characteristics, in particular high molecular mass fractions, useful for the characterization of specific modifications. To our knowledge, no detailed analyses of urinary GAG composition in MPS I patients during ERT have been published yet nor is there any data available in these subjects regarding the behavior of GAGs in plasma even if plasmatic GAG disaccharides have been quantified (Tomatsu, Montaño, Oguma, Dung, Oikawa, Gutiérrez et al. 2010).

Two patients, a 5-month-old boy (Patient M) and his 5-year-old sister (Patient F), affected by an attenuated form of MPS I were admitted to our Pediatric Division in Ancona, Italy, in March 2003 and subjected to ERT from August 2003 (Gabrielli et al. 2010). When diagnosed at the age of 4 years and 6 months, Patient F showed a typical clinical picture of attenuated MPS I. Elevated levels of urinary GAGs were found to have a value of 805 µg/mg creatinine (normal value for the age of 22–86 µg/mg). After 5 years of therapy, her dysostosis multiplex, cardiac function and corneal clouding (visual acuity: 10/10) had stabilized but had not improved. On the other hand, the liver and spleen volume had normalized, the skin had become less thick and shoulder flexion and extension had moderately improved.

Patient M showed no clinical findings associated with MPS I other than a substantially elevated urinary GAG level of 652, with cellulose acetate electrophoresis revealing a predominance of HS and DS, at 5 months of age. After 5 years of ERT, Patient M was completely normal showing a mild corneal clouding as the only sign of MPS I (Gabrielli et al. 2010).

In this study, we quantitatively and qualitatively analyzed high molecular mass GAGs present in the urine and plasma of our two clinically stabilized patients after 6 years of continuous treatment with recombinant human α-L-iduronidase over a 2-week period compared with pretreatment analytical data. The aim of this research was to gain a better understanding of the metabolic fate of these macromolecules, GAGs, during ERT. Finally, results from this study may be useful to the development of appropriate analytical procedures and protocols for a more accurate follow-up of the enzymatic treatment and for the evaluation of new possible therapeutic interventions.

Results

Urinary and plasma GAG characterization before ERT

Two milliliters of urine from the two patients before ERT was extracted by means of a conventional method (Dietrich et al. 1993; Maccari et al. 2003; Buzzega et al. in press) and analyzed by agarose gel electrophoresis (Figure 1A) and densitometric evaluation (Figure 1B) performed by scanning a single polysaccharide band, and quantified versus specific calibration curves constructed with increasing absolute amounts (from 0.5 to 5.0 µg) of standard DS (see Volpi 2007 and Volpi and Maccari 2006 for analytical details). As is evident from electrophoresis, a single band having a migration time similar to standard DS was observed for both patients. The DS nature of this species was also confirmed by HPLC separation of disaccharides (see below) and by agarose gel electrophoresis after treatment with chondroitinase ABC (not shown). Quantitative determination produced lower values than DMB (513.3 vs ~805 µg/mg creatinine) for Patient F and of 336.5 (DMB value of ~652 µg/mg creatinine) for Patient M. 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, ~36% and ~48% of total GAGs (for Patient F and Patient M, respectively) were calculated to be fragments having molecular mass lower than ~1500. In particular, a great percentage of these low molecular mass fragments may be formed of HS that was demonstrated to be present in urine as oligomers (Burlingame et al. 1981; Fuller, Meikle et al. 2004; Fuller, Rozeklis et al. 2004). Furthermore, these fragments were not detected by electrophoresis and densitometric acquisition even after specific chondroitinase ABC treatment that was able to totally remove CS/DS (not shown) confirming their low molecular mass nature, lower than ~1500.

By performing strong-anion exchange (SAX)-HPLC separation of disaccharides produced by chondroitinase ABC treatment (Figure 2), the urinary galactosaminoglycans from MPS subjects before ERT were found to be mainly composed of ~80% ΔDi4s, ~10% ΔDi6s and ~7–8% ΔDi6s (Table I). Minor but significant percentages of disulfated disaccharides, in particular ΔDi2,4dis (1–2%) typical of DS (Volpi 2009; Volpi and Maccari 2009), were also observed (Table I). As a consequence, a polysaccharide rich in 4-sulfated groups on the hexosamine (4s/6s ratio of ~7–10) and having a charge density of ~0.94–0.97 (Table I) was detected in MPS I subjects. Moreover, the use of chondroitinase B specific for iduronic ac-
id typical of DS (Volpi and Maccari 2009) showed the presence essentially of ΔDi4s corresponding to about 75–78% of the amount of the same disaccharide obtained after chondroitinase ABC digestion in both patients (Table I), confirming that excreted urinary GAGs having a molecular mass greater than ∼1500 are essentially composed of 75–80% DS with 20–25% CS.

To date, no information about mass values is available for high molecular mass polysaccharides accumulated in MPS subjects. This parameter may give us further useful information about these anomalous GAGs produced by modified metabolic processes. Polyacrylamide gel electrophoresis (PAGE) analysis of urinary GAG from both patients before ERT (along with control subjects matched for age, Figure 3) showed an average molecular mass of 13,500–14,000 and was found to be fairly similar for the two patients and comparable with high-performance size exclusion chromatography (HPSEC) results (Table I). Finally, molecular mass values for GAGs of the two patients before ERT were found to be higher than the corresponding parameters of control (∼11,000, Table I) confirming the presence of a nonphysiological polymer.

Figure 4A presents the electrophoresis of plasma GAGs extracted from Patient F before any ERT treatment (plasma from Patient M was not available) showing two main bands. The lower band having a migration time rather similar to standard HS showed, on the contrary, no metachromasia typical of natural sulfated polyanions. Furthermore, it was resistant to chemical treatment with nitrous acid (not shown), which is able to specifically degrade HS/heparin (Conrad 2001). As a consequence, this band was assigned as not belonging to HS (or other GAGs characterized by agarose electrophoresis) and not further analyzed. On the contrary, the band having greater mobility was confirmed to be a DS by separation of disaccharides by reverse-phase (RP)-HPLC-fluorescence detection (Fd) (Volpi 2009) (Figure 5A). This GAG was mainly composed of ∼64% ΔDi4s, ∼8% ΔDi6s and ∼8% ΔDi0s with a 4s/6s ratio of ∼8. Additionally, significant percentages of disulfated disaccharides, in particular ΔDi2,4dis (∼13%) typical of DS, were also detected producing a high charge density of ∼1.12 (Table II). Chondroitinase B generated essentially ΔDi4s and disulfated disaccharides confirming the prevalent DS nature of this polymer. Finally, quantitative evaluation performed by agarose gel electrophoresis (Figure 4B) showed a DS/CS amount of ∼18.2 µg/mL plasma (Table II).

Urinary and plasma GAG characterization during ERT

Figure 6 illustrates the electrophoretic pattern of the urinary GAGs from the two patients over a 2-week period of ERT.
A high amount of a single band was detected in the urine of Patient M at the day labeled 0 when the enzyme was infused and remained high on days 1, 2 and 3 following treatment. However, on days 4 and 5 the urine showed no evident GAG, which was, on the contrary, detected on the 6th and 7th days after enzyme administration. As a consequence of
the second enzyme infusion on day 7, GAGs disappeared from the urine on day 10 with their reappearance on day 11 up to day 14 (Figure 6B). A similar profile was observed for Patient F in which a GAG band in high quantity disappeared from the urine on day 6 following the first enzyme infusion and on days 9 and 10 showed a decreased urinary concentration (Figure 6A). Furthermore, on the contrary of the urine of patients before ERT (see Figure 1B), a second band possessing lower mobility and the same migration behavior of HS standard was observed (Figure 6A and B) and more accurately determined by densitometric scanning (Figure 6C). This macromolecular species was detected in all urine samples taken during ERT administration and confirmed to be HS by specific nitrous acid treatment (Conrad 2001).

The electrophoretic data were quantitatively confirmed by DMB assay on intact urine (Coppa et al. 1987; de Lima et al. 2007) and by SAX-HPLC of disaccharides (see below). By performing extraction procedures on 5 mL urine, a volume not normally used for assays routinely applied in clinical laboratories, we were able to determine, by means of the three different analytical approaches (Figure 7), a urinary GAGs content of ~20–100 µg/mg creatinine, which is fairly close to the normal value for age (Gabrielli et al. 2010). Agarose gel electrophoresis (and SAX-HPLC, not shown) detected ~64% for Patient F and ~52% for Patient M, before ERT, of total GAGs evaluated by DMB. As previously reported, these lower values may be due to the presence of GAG fragments and oligomers demonstrated to be present in MPS I subjects (Fuller, Meikle et al. 2004; Fuller, Rozaklis et al. 2004) and having molecular mass lower than ~1500 not detected by electrophoresis (Volpi 1993; Volpi and Maccari 2002). On the contrary, in both patients subjected to ERT, data obtained by DMB agree with those determined by electrophoresis (see Figure 7), which is probably indicative of a drastic decrease in lower molecular mass fragments and oligosaccharides during ERT.

The urinary GAGs from MPS subjects during ERT were mainly composed of ~61–62% ΔDi4s, ~20% ΔDi6s and ~13–15% ΔDi0s (Table I) showing a polysaccharide with a high 4s/6s ratio (~3.1). Minor but significant percentages of disulfated disaccharides, i.e. ΔDi2,6dis (2%), ΔDi4,6dis (2%) and ΔDi2,4dis (1%), were also detected (Figure 8) (Table I), resulting in an average charge density of ~0.90 for the chains. The use of chondroitinase B showed the presence essentially of ΔDi4s corresponding to ~55–60% of disaccharides produced by chondroitinase ABC in both patients (not shown), confirming that excreted urinary GAGs during ERT are a mixture of CS (~40%) and DS (~60%). On days 4, 5 and 10 of Patient M and day 6 of Patient F we were unable to detect any kind of sulfated polysaccharide (Figure 6).

PAGE analysis of urinary GAGs from Patient M on the days following infusion (Figure 9) shows an average molecular mass of 12,000, a value rather similar to results gained by HPSEC. The urinary GAG before ERT showed greater molecular mass values (~13,000–13,500) than the urine of the same patients during ERT (~11,500–12,000), but fairly similar to controls matched for age (Table I). This decrease in the GAG molecular mass value during ERT is also indicative of a modulation of the urinary polysaccharide pattern due to the enzyme infusion but, by considering the other structural and quantitative parameters evaluated, ERT still remains unable to completely remove DS from the urine.

Figure 10A presents the electrophoresis separation of plasma GAGs from Patient M over a 6-year period of ERT before and after laronidase infusion for two consecutive weeks, in comparison with two control subjects. As previously observed for the plasma of Subject F before ERT (Figure 4), the two main bands are separated in both the controls and Patient M

Table I. Structural characterization of the galactosaminoglycans component of urine of Patients F and M before and during ERT (also compared to controls)

<table>
<thead>
<tr>
<th></th>
<th>Control subjectsa</th>
<th>Patient F Before ERT</th>
<th>During ERT</th>
<th>Patient M Before ERT</th>
<th>During ERT</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg GAGs/mg Creatinineb</td>
<td>&lt;100</td>
<td>805</td>
<td>&lt;~160</td>
<td>652</td>
<td>&lt;100</td>
</tr>
<tr>
<td>ΔDi0s (%)</td>
<td>~2</td>
<td>6.9</td>
<td>13.2</td>
<td>7.9</td>
<td>14.8</td>
</tr>
<tr>
<td>ΔDi6s (%)</td>
<td>~48</td>
<td>8.3</td>
<td>20.3</td>
<td>10.7</td>
<td>19.9</td>
</tr>
<tr>
<td>ΔDi4s (%)</td>
<td>~50</td>
<td>80.9</td>
<td>61.9</td>
<td>79.2</td>
<td>60.9</td>
</tr>
<tr>
<td>ΔDi2,6dis (%)</td>
<td>nd</td>
<td>0.0</td>
<td>2.1</td>
<td>0.0</td>
<td>1.7</td>
</tr>
<tr>
<td>ΔDi4,6dis (%)</td>
<td>nd</td>
<td>1.6</td>
<td>1.7</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>ΔDi2,4dis (%)</td>
<td>nd</td>
<td>2.3</td>
<td>0.7</td>
<td>1.2</td>
<td>0.7</td>
</tr>
<tr>
<td>Charge density (%)</td>
<td>~0.98</td>
<td>0.97</td>
<td>0.91</td>
<td>0.94</td>
<td>0.90</td>
</tr>
<tr>
<td>4s/6s Ratio (%)</td>
<td>~1.05</td>
<td>9.75</td>
<td>3.05</td>
<td>7.40</td>
<td>3.06</td>
</tr>
<tr>
<td>IdoA/GlcA Ratio (%)</td>
<td>Trace/100</td>
<td>78/22</td>
<td>61/39</td>
<td>75/25</td>
<td>57/43</td>
</tr>
<tr>
<td>PAGEMM</td>
<td>~11,000</td>
<td>13,530</td>
<td>11,620</td>
<td>13,930</td>
<td>12,000</td>
</tr>
<tr>
<td>HPSEC Mw</td>
<td>~11,200</td>
<td>12,730</td>
<td>11,320</td>
<td>13,000</td>
<td>11,570</td>
</tr>
<tr>
<td>HPSEC Ms</td>
<td>~11,400</td>
<td>13,160</td>
<td>11,590</td>
<td>13,320</td>
<td>11,790</td>
</tr>
<tr>
<td>HPSEC Mz</td>
<td>~11,700</td>
<td>13,570</td>
<td>12,110</td>
<td>13,640</td>
<td>12,010</td>
</tr>
<tr>
<td>Dispersity index (Mw/Mn)</td>
<td>~1.020</td>
<td>1.034</td>
<td>1.024</td>
<td>1.025</td>
<td>1.019</td>
</tr>
</tbody>
</table>

The results are the mean of three different analyses. The coefficient of variation % was always found to be lower than 15% for all analyses. 4s/6s ratio: ratio between 4-sulfated disaccharide, ΔDi4s and the sulfated disaccharide in position 6 of the N-acetyl-galactosamine unit. ΔDi6s; GlcA: glucuronic acid; IdoA: iduronic acid. The percentages of the two different uronic acids have been calculated by means of SAX-HPLC after treatment with chondroitinases ABC and B. The molecular mass values of control subjects have been determined by PAGE and HPSEC (not shown).

aData from references (Dietrich et al. 1993; Maccari et al. 2003; Buzzega et al. in press).

bDetermined by DMB assay.
Fig. 3. (A) PAGE analysis of GAGs extracted from 2 mL urine of Patients F and M before any enzyme replacement treatment. (B) PAGE analysis of GAGs extracted from 5 mL urine of control subjects matched for age with Patients F (C1) and M (C2). The calibration curve was constructed by using oligosaccharide standards of known molecular mass prepared from CS and having masses of 29,880, 16,750 and 4460. Gels were stained with toluidine blue (0.1% in acetic acid 1%) for 30 min. After decoloration with acetic acid 1%, molecular mass evaluation was performed after densitometric acquisition. HPSEC analysis of the GAGs extracted from 2 mL urine of Patient F (C) and Patient M (D) before any enzyme replacement treatment and from 5 mL urine of a control subject (E). The calibration curve was constructed by using oligosaccharide standards having mass values of 29,880, 16,750, 8700, 4460, 3700 and 2130 (see the third grade polynomial curve inside the HPSEC profile).
Fig. 4. Agarose gel electrophoresis stained with toluidine blue and Stains-All (A) and related densitometric scanning (B) of GAGs extracted from 500 µL plasma of Patient F before any enzyme replacement treatment. Mix, GAGs standard; CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate; SM, slow-moving heparin.

Fig. 5. Chromatogram showing the separation of unsaturated disaccharides generated by means of chondroitin ABC lyase digestion of galactosaminoglycans extracted from 500 µL plasma of Patient F before any enzyme replacement treatment, derivatized with AMAC, separated by RP-HPLC and detected by fluorescence detection. ΔD10s, ΔUA-(1→3)-GalNAc; ΔD14s, ΔUA-(1→3)-GalNAc-4s; ΔD6s, ΔUA-(1→3)-GalNAc-6s; 2,4dis, ΔD2,4dis, ΔD-dis B, ΔUA-2s-(1→3)-GalNAc-4s; 2,6dis, ΔD2,6dis, ΔD-dis D, ΔUA-2s-(1→3)-GalNAc-6s; 4,6dis, ΔD4,6dis, ΔD-dis E, ΔUA-(1→3)-GalNAc-4,6dis.
Table II. Structural characterization of the plasma galactosaminoglycan component of Patient F before and during ERT (also compared to controls)

<table>
<thead>
<tr>
<th>Patient F</th>
<th>Control subjects(\text{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before ERT</td>
</tr>
<tr>
<td>µg GAGs/mL Plasma</td>
<td>0.1–6.0</td>
</tr>
<tr>
<td>ΔD0s (%)</td>
<td>40–60</td>
</tr>
<tr>
<td>ΔD6s (%)</td>
<td>&lt;5</td>
</tr>
<tr>
<td>ΔD4s (%)</td>
<td>40–60</td>
</tr>
<tr>
<td>ΔD2,6dis (%)</td>
<td>nd</td>
</tr>
<tr>
<td>ΔD4,6dis (%)</td>
<td>nd</td>
</tr>
<tr>
<td>ΔD2,4dis (%)</td>
<td>nd</td>
</tr>
<tr>
<td>Charge density</td>
<td>~0.40–0.60</td>
</tr>
<tr>
<td>4s/6s Ratio</td>
<td>~8</td>
</tr>
<tr>
<td>IdoA/GlcA Ratio (%)</td>
<td>Trace/100</td>
</tr>
</tbody>
</table>

The results are the mean of three different analyses. The coefficient of variation % was always found to be lower than 15% for all analyses. 4s/6s ratio: ratio between 4-sulfated disaccharide, ΔD4s and the sulfated disaccharide in position 6 of the N-acetyl-galactosamine unit, ΔD6s; GlcA: glucuronic acid; IdoA: iduronic acid. The percentages of the two different uronic acids have been calculated by means of SAX-HPLC after treatment with chondroitinases ABC and B. nd = not detected.

\(\text{a}\)Data from references (Juvani et al. 1975; Volpi and Maccari 2005; Chi et al. 2008; Volpi 2009).

Discussion

So far, the only biochemical parameters used to monitor the efficacy of ERT in subjects affected by MPS have been performed on urine by means of a generic quantitative dye-binding assay using DMB and electrophoresis on cellulose acetate (Gabrielli et al. 2010) or by evaluating unsaturated disaccharides of urinary or plasma GAGs by HPLC-ESI (Tomatsu, Montaño, Oikawa, de Carvalho et al. 2010). To date, serum heparin cofactor II-thrombin complex has been proposed as a biomarker and tool to monitor the efficacy of MPS treatment (Randall et al. 2008) along with the determination of unsaturated disaccharides of urinary or plasma GAGs (Tomatsu, Montaño, Oikuma, Dung, Oikawa, de Carvalho et al. 2010).

However, these analytical approaches are unable to directly reveal structural modifications of GAGs. By using specific and sensitive analytical procedures, high amounts of a high molecular mass DS (~13,000–13,500) were found in the urine of MPS I-affected subjects but also in plasma being rich in iduronic acid, ΔD4s, ΔD2,4dis typical of DS, and with high charge density. It is worth mentioning that this specific disaccharide, ΔD2,4dis, never found in normal plasma, may be useful as a specific biomarker of MPS I and to monitor related therapies.

Patients M and F with MPS I subjected to ERT were reported to have, respectively, clinically prevented the disease and shown improvement of several clinical features (Gabrielli et al. 2010). As a consequence, we also expected a normal plasma and urine GAG profile. Indeed, plasma GAGs were found to be comparable to those of normal subjects with the absence of HS and DS. In fact, the total GAG amount was in a normal range (Juvani et al. 1975; Volpi and Maccari 2005; Chi et al. 2008), and the structure of the still present polymer was characterized as being similar to the physiologically circulating endogenous CS (Juvani et al. 1975; Volpi and Maccari 2005; Chi et al. 2008). On the contrary, a high molecular mass polysaccharide, ~11,500–12,000, slightly greater with respect to the controls (~11,000), was found in the urine of both patients. This macromolecule was a mixture of DS/CS having a high percentage of 4-sulfated disaccharide and iduronic acid (~60%) mainly associated with DS. Furthermore, minor but significant percentages (~5%) of disulfated disaccharides, never found in normal urine CS, were also detected (Dietrich et al. 1993; Maccari et al. 2003; Buzzega et al. in press).

From a quantitative point of view, the total amount of GAGs in Patients F and M subjected to ERT was found to be at normal levels for age according to previous analytical determination (Gabrielli et al. 2010). Additionally, a decrease of ~5.6–5.7 times was measured in urine GAGs before and during ERT, in accordance with the decrease observed for plasma polysaccharides (~4 times). Furthermore, fairly normal or borderline urinary GAG values were measured in other patients subjected to ERT by means of the DMB test (Wang et al. 2009).

Along with high molecular mass GAGs (Burlingame et al. 1981), also fragments and HS/DS oligosaccharides are excreted in MPS I urine (Fuller, Meikle et al. 2004; Fuller, Rozaklis et al. 2004). At the moment, we cannot exclude the presence of oligomers derived from HS and DS in the urine of ERT-treated patients due to the incapacity of electrophoresis to detect low molecular mass molecules. In any case, quantitative evaluation performed by comparing DMB assay and electrophoresis data on urine samples before and during ERT indicates the possible absence of these fragments during therapy. Further studies are in progress to address this specific point.

Interestingly, the presence of high molecular mass DS in the urine of MPS I patients subjected to ERT is not constant during the 2 weeks of observation, showing the incapacity of ERT at
the present standard dose to totally eliminate this polymer from the urine. Moreover, a different profile of urinary DS presence is observed for the two patients treated under the same conditions showing a possible variable effectiveness depending on the subject. It is known that there is an individual (Dietrich et al. 1993; Maccari et al. 2003) and daily variation (Maroclo et al. 2005) in GAG amount excreted by healthy subjects. Furthermore, we also observed daily urinary DS quantitative changes for both patients before ERT (Supplementary Figure 2). However, ERT was found unable to completely eliminate DS from the urine independently from daily variations during 2 weeks of observation as we qualitatively characterized a still abnormal nonphysiological polymer along with normal excreted CS in urine. As a consequence, we believe that daily variations observed for urinary DS from both patients are not related to normal quantitative daily changes but just to ERT as this therapeutic approach is the unique “catabolic mechanism” able in these subjects to remove pathological DS. At the moment, we do not know if these different urine profiles in ERT MPS I patients are related to the specific enzyme capacity or to some endogenous factor strictly dependent on each subject, or to other external factors capable of modifying the enzyme efficacy. Anyway, the present analytical procedures would also be useful to optimize and to monitor the current ERT regimens also considering the best benefit to risk profile (Martins et al. 2009).

What is the origin of this high molecular mass DS still present in urine of ERT-treated patients? We can suppose that this
molecule has a systemic origin as it is not totally degraded by laronidase, excreted in plasma, filtered in the kidney and eliminated in the urine. Its absence in plasma may be due to its lower concentration due to ERT and to the capacity of liver cells to rapidly remove it from circulation (Guimarães and Mourão 1997; Masuda et al. 1999; Pecly et al. 2006). On the other hand, it is known that DS (and HS) are normally found in the kidney and urinary tract suggesting that the polysaccharide present in the urine of MPS I-treated patients may originate directly from these specific districts due to the incapacity of recombinant α-L-iduronidase to act in these areas.

In conclusion, we demonstrated that, despite a drastic improvement in clinical parameters and quality of life, a high molecular mass GAG is still present in the urine of MPS I patients subjected to ERT over a 6-year period. This result implies several important considerations. The urinary GAG found in treated patients was characterized as DS generally produced in MPS I subjects, even if present in lower amounts and having distinctive characteristics. Furthermore, the incapacity of ERT to totally remove catabolic products derived from the pathological condition of MPS I disorder focuses attention on the possibility that the present therapeutic regimen might be unable to effectively decrease and/or definitively remove the accumulation of lysosomal GAGs in specific districts. Additionally, a different fluctuation of the urinary GAGs is evident depending on the subject, showing that a more accurate and “personalized”

![Figure 7](https://example.com/fig7.jpg)
treatment regimen could possibly be adapted to the patient. Finally, specific and sensitive measurements and structural characterization of plasma GAGs and excretion would be valuable and even indispensable for a more accurate follow-up of ERT and monitoring of proposed therapies, for the evaluation of new therapeutic interventions and possibly for a more precise clinical delineation, in particular for MPS type I but also for other forms of MPS.

Materials and methods

Materials

Microcon YM-3 and 0.45 µm filters with a MM cut-off of 3000 Da were from Millipore (Billerica, MA). Chondroitinase ABC [E.C. 4.2.2.4], chondroitinase B [no E.C. number] and proteinase K [3.4.21.64] were purchased from Sigma-Aldrich (St. Louis, MO). 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). All the other reagents were of analytical grade, generally supplied by Sigma-Aldrich.

DMB assay was performed according to Coppa et al. (1987).

Patient sample collection

Urine samples were collected from both patients before any ERT and frozen at −20°C for analytical investigation. For two consecutive weeks of ERT, daily morning urine samples were collected from the two patients and frozen at −20°C for analytical investigation.
Blood was collected from Patient F before any ERT and treated as reported below. Blood samples were withdrawn immediately before and 1 h after enzyme infusion, over two consecutive weeks of ERT, collected in tubes containing citrate as an anticoagulant and the plasma obtained was stored at $-20^\circ$C for analytical investigation.

**Extraction and purification of plasma GAGs**

Five hundred microliters of human plasma was lyophilized and reconstituted with 500 µL of Tris–Cl 10 mM pH 7.5, and treated with 500 munits of proteinase K at 60°C for 12 h. After boiling for 5 min, the samples were centrifuged at 5000 × g for 5 min, filtered on 0.45 µm filters and lyophilized. After reconstitution with 500 µL bidistilled water, samples were filtered on YM-3 filters. The crude retained plasma GAG fraction was dissolved in 50 µL distilled water and further analyzed (see below) (Volpi 2009; Volpi and Maccari 2009).

**Extraction of human urine GAGs**

Two milliliters of urine from patients before any ERT and 5 mL from ERT subjects (or controls) were treated with 5% cetyltrimethylammonium bromide as previously reported in detail (Dietrich et al. 1993; Maccari et al. 2003; Buzzega et al. in press) and purified GAGs dissolved in 200 µL distilled water for further analyses (see below).

**DMB assay**

The determination is based on the reaction of GAGs with DMB, yielding a pink colored complex that can be measured spectrophotometrically (Coppa et al. 1987; Whitley et al. 1989; de Lima et al. 2007). Working solution was prepared with 10.66 mg of DMB, 3.33 mL of ethanol, 1.33 g of sodium formate and 1.33 mL of formic acid. The pH value was adjusted to 3.75 by adding more concentrated acid. The flask was made up to 1 L with distilled water. A standard DS (and CS) solution containing 100 mg/L was prepared in distilled water. The blank solution was prepared by mixing 500 µL of water and 2.5 mL of DMB solution. For the quantitation of urinary GAGs, 100 µL of urine, 400 µL of water and 2.5 mL of DMB solution were added in a quartz cell. The mixture was shaken and allowed to stand for 5 min. Afterwards, the absorption spectrum was recorded between 400 and 800 nm. The difference between the maximum and minimum absorbance (≈520 and 595 nm, respectively) was recorded. One hundred microliters of urine is an appropriate volume in order to obtain a difference of

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**(Fig. 9. (A) PAGE analysis of GAGs extracted from 5 mL urine of Patient M on different days, 0, 1, 2 and 3 (see Figure 6) during enzyme replacement treatment with laronidase. The calibration curve was constructed by using oligosaccharide standards of known molecular mass prepared from CS and having masses of 29,880, 16,750 and 4460. (B) HPSEC analysis of the GAGs extracted from 5 mL urine of Patient M on day 0. The calibration curve was constructed by using oligosaccharide standards having mass values of 29,880, 16,750, 8700, 4460, 3700 and 2130 (see the third grade polynomial curve inside the HPSEC profile).)**
absorbance within the middle range of the calibration curve (Andrade et al. 2008). If a particular urine sample is too diluted or concentrated, the volume can be increased or reduced, taking into account the appropriate dilution factor. Finally, the value was corrected with regard to the amount of creatinine, determined by an automated chemistry analyzer.

**Agarose gel electrophoresis of urinary and plasma GAGs**

The human urine GAGs were separated and quantified using polysaccharides standard by agarose gel electrophoresis and densitometric scanning by means of specific calibration curves as reported elsewhere (Volpi 1993; Volpi and Maccari 2002; Volpi and Maccari 2009). One to ten microliters of urine extracts was layered on gel.

The human plasma GAGs extracted from 500 µL plasma were separated by electrophoresis as above illustrated but further stained with Stains-All after toluidine blue staining to increase the sensitivity (Volpi and Maccari 2002).

**Qualitative and quantitative evaluation of unsaturated disaccharides of urinary GAGs**

The disaccharide products formed by chondroitinase ABC, or chondroitinase B, upon the urinary GAGs were identified by SAX-HPLC, as elsewhere reported (Volpi 1993). Quantitative determination of CS/DS was performed by using specific calibration curves constructed by separation of increasing amounts of unsaturated disaccharides generated by increasing amounts of CS/DS standard enzymatically degraded and derivatized with 2-aminoacridone (AMAC). According to a previous study (Volpi 2009), the calibration curves for all disaccharide standards showed a linear response from ∼1 µg to 30 µg with correlation coefficients generally greater than 0.990.

The sulfate-to-carboxyl ratio (charge density) was calculated considering the presence and the percentage of carboxyl and sulfate groups for each disaccharide.

**Characterization of unsaturated disaccharides of plasma GAGs**

After treatment with chondroitinases as above reported, the generated unsaturated disaccharides were derivatized with AMAC and separated by RP-HPLC and fluorimetric detection (Fd) according to published methodology (Volpi 2009).

**Molecular mass determination of urinary GAGs**

The molecular mass of urinary GAGs was determined by PAGE according to Edens et al. (1982). Twenty micrograms of the purified polysaccharides was layered on the gel, and the calibration curve was constructed by using oligosaccharide standards of known molecular mass prepared from CS.

The molecular mass and polydispersity of urinary GAGs were also determined by HPSEC by using CS fractions of known molecular mass (Volpi and Bolognani 1993).
Supplementary data
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

Contributors
N.V. developed the applied methodologies. D.B., L.Z., F.M., T. G. and F.P. 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 statement
None declared.

Abbreviations
AMAC, 2-aminoacridone; CS, chondroitin sulfate; DMB, 1,9-dimethylmethylen blue; DS, dermatan sulfate; ERT, enzyme replacement therapy; Fd, fluorescence detection; GAG, glycosaminoglycan; HPLC-ESI, high-performance liquid chromatography-electrospray ionization; HPSEC, high-performance size exclusion chromatography; HS, heparan sulfate; Mn, the number average molecular weight; MPS, mucopolysaccharidoses; Mw, the weight average molecular weight; Mw/Mn, dispersity index; Mz, the average molecular weight; MPS I, mucopolysaccharidosis type I; PAGE, polyacrylamide gel electrophoresis; PG, proteoglycan; ΔDIHA, ΔUA-(1→3)-GlcNAc; ΔDi0s, ΔUA-(1→3)-GalNAc; ΔDi4s, ΔUA-(1→3)-GalNAc-4s; ΔDi6s, ΔUA-(1→3)-GalNAc-6s; ΔDi2s, ΔUA-2s-(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; ΔDi2,4,6tris, ΔDi-tris, ΔUA-2s-(1→3)-GalNAc-4s,6s.

References

