# Mental retardation in mucopolysaccharidoses correlates with high molecular weight urinary heparan sulphate derived glucosamine

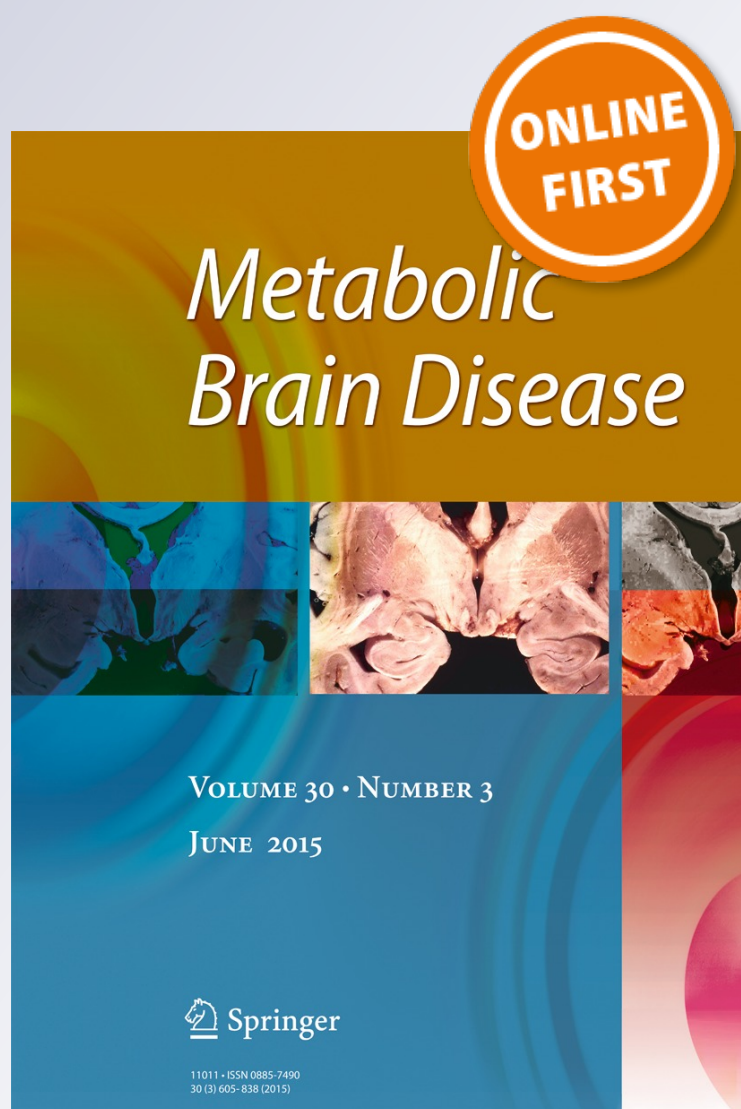## G. V. Coppa, O. Gabrielli, L. Zampini, F. Maccari, V. Mantovani, T. Galeazzi, L. Santoro, L. Padella, R. L. Marchesiello, et al.

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# Mental retardation in mucopolysaccharidoses correlates with high molecular weight urinary heparan sulphate derived glucosamine

G. V. Coppa[1] · O. Gabrielli[1] · L. Zampini[1] · F. Maccari[2] · V. Mantovani[2] · T. Galeazzi[1] ·
L. Santoro[1] · L. Padella[1] · R. L. Marchesiello[1] · F. Galeotti[2] · N. Volpi[2]

**Abstract** Mucopolysaccharidoses (MPS) are characterized by mental retardation constantly present in the severe forms of Hurler (MPS I), Hunter (MPS II) and Sanfilippo (MPS III) diseases. On the contrary, mental retardation is absent in Morquio (MPS IV) and Maroteaux-Lamy (MPS VI) diseases and absent or only minimal in the attenuated forms of MPS I, II and III. Considering that MPS patients affected by mental disease accumulate heparan sulfate (HS) due to specific enzymatic defects, we hypothesized a possible correlation between urinary HS-derived glucosamine (GlcN) accumulated in tissues and excreted in biological fluids and mental retardation. 83 healthy subjects were found to excrete HS in the form of fragments due to the activity of catabolic enzymes that are absent or impaired in MPS patients. On the contrary, urinary HS in 44 patients was observed to be composed of high molecular weight polymer and fragments of various lengths depending on MPS types. On this basis we correlated mental retardation with GlcN belonging to high and low molecular weight HS. We demonstrate a positive relationship between the accumulation of high molecular weight HS and mental retardation in MPS severe compared to attenuated forms. This is also supported by the consideration that accumulation of other GAGs different from HS, as in MPS IV and MPS VI, and low molecular weight HS fragments do not impact on central nervous system disease.

## Introduction

Mucopolysaccharidoses (MPS) are a group of inherited lysosomal storage disorders of glycosaminoglycan (GAG) catabolism caused by the deficient activity of specific enzymes (Neufeld and Muenzer 2007). As a consequence, an accumulation of partially degraded GAGs occurs within lysosomes, tissues, and biological fluids. According to the nature of the hexosamine (Hex), GAGs are classified into two groups, glucosaminoglycans such as heparan sulfate (HS), keratan sulfate (KS), hyaluronic acid, and heparin, and galactosaminoglycans, chondroitin sulfate (CS) and dermatan sulfate (DS) (Coppa et al. 2011a). In fact, CS and DS are formed of variously sulfated repeating disaccharides composed of $N$-acetyl-galactosamine (GalNAc) and uronic acid (glucuronic or iduronic acid) while $N$-acetyl-glucosamine (GlcNAc) and glucuronic/iduronic acids are the main monosaccharides of HS/heparin. KS is composed of galactose and GlcNAc sulfated in different positions (Jackson et al. 1991).

From the clinical point of view, MPS are characterized by a wide variety of symptoms with different degrees of involvement at the level of many organs and apparatuses. In general, mental retardation is characteristically and constantly present in the severe forms of MPS I (Hurler disease, always present in a severe form), MPS II (Hunter disease with mental retardation), MPS III (Sanfilippo disease) and MPS VII (Sly disease). On the contrary, mental retardation is typically absent in MPS IV (Morquio disease) and VI (Maroteaux-Lamy disease) and absent or only minimal in the attenuated forms of MPS I (Hurler/Scheie, slowly progressive with central nervous system (CNS) involvement, and Scheie, always with no mental

✉ N. Volpi
volpi@unimo.it

1  Pediatric Division, Department of Clinical Sciences, Polytechnic University of the Marche, Ospedali Riuniti, Presidio Salesi, Ancona, Italy

2  Department of Life Sciences, University of Modena and Reggio Emilia, Via Campi 213/D, 41125 Modena, Italy

retardation), II (attenuated phenotype always with no mental problems or an intermediate form with slowly progressive CNS involvement) and III (a slowly progressive form). From a metabolic point of view, HS is one of the main accumulating GAGs in MPS I, II, III and VII (Neufeld and Muenzer 2007) due to the deficiency of a lysosomal enzyme responsible for its degradation. In fact, the absence of $\alpha$-L-iduronidase in MPS I and iduronate-2-sulfatase in MPS II leads to accumulation of both DS and HS. In all four MPS III subtypes only the lysosomal degradation of HS is impaired due to a deficiency of either heparan $N$-sulfatase, acetyl-$\alpha$-glucosaminidase, acetyl-CoA:$\alpha$-glucosaminide $N$-acetyltransferase or $N$-acetylglucosamine-6-sulfatase (Neufeld and Muenzer 2007). The absence of galactose-6-sulfate sulfatase in MPS IVA and $\beta$-galactosidase in MPS IVB respectively produce a storage of KS and CS or just KS, while DS is accumulated in MPS VI for the lacking of $N$-acetylgalactosamine-4-sulfatase (Neufeld and Muenzer 2007).

Recently de Ruiter et al. (2013) reported a correlation between HS derived disaccharides in plasma and mental retardation in a large cohort of MPS III patients. Moreover, Coppa et al. (2013), using an original method recently developed for the diagnosis of MPS (Coppa et al. 2011a), described a 28-year-old patient with an attenuated phenotype of MPS IIIA with low urinary levels of HS, and corresponding glucosamine (GlcN) content, compared to patients with a severe phenotype. Finally, Bruyère et al. (2015) demonstrated in an animal model that long-chain HS constitutively activate integrin-based focal adhesions in MPSIII type B astrocytes or neural stem cells unless undigested saccharides are degraded by exogenous supply of the missing catabolic enzyme producing an abnormal organization of the rostral migratory stream in the brain of adult mice possibly resulting in the neurological disorders associated with Sanfilippo syndrome. On the basis of these data, we decided to critically evaluate the results obtained by the above method (Coppa et al. 2011a) in a cohort of patients affected by different types of MPS with the aim to evidence a possible correlation between mental retardation and HS derived urinary GlcN. Moreover, we were able to correlate mental retardation with the high molecular weight fraction of HS and with the mild and severe forms within each MPS type.

## Material and methods

All 44 patients were diagnosed by appropriate enzymatic assay. 14 patients suffered from MPS I, 11 from MPS II, 13 from MPS III, 5 from MPS IV and 1 from MPS VI. Clinical, neurological and neuropsychological evaluation of each patient was performed every six months for a period of at least 5 years to check the progression of the disease. Urine samples were obtained from all patients at the time of the diagnosis and stored at −20 °C until used. No patient was under any type of treatment at the time of collecting urine. High throughput determination of urinary Hexs was performed by HPLC according to previous methodology (Coppa et al. 2011a).

Separation of high and low molecular weight fractions of HS was performed according to the protocol illustrated below. After centrifugation at 5000×g for 15 min, 6 mL of ethanol were added to 500 µL of urine. After storing at −20 °C for 24 h, the samples were centrifuged at 5000×g for 15 min and the recovered precipitate dried at 60 °C for 24 h. The pellet in 1 mL of water was applied to a column (0.5 × 1.0 cm) packed with QAE Sephadex® A-25 anion-exchange resin (from Pharmacia Biotech, Uppsala, Sweden) equilibrated with a 50 mM NaCl solution. GAGs were eluted with 2 mL of a 2 M NaCl solution. Three volumes of ethanol saturated with sodium acetate were added to the recovered NaCl solution and stored at −20 °C for 24 h. The precipitate was recovered by centrifugation and dried at 60 °C for 12 h. The dried precipitate was dissolved in 500 µL of distilled water and filtered on Microcon YM-10 filters having a cut-off of 10,000 (from Millipore, Darmstadt, Germania). The filtered fraction of low molecular weight, lower than 10,000, was lyophilized. The ritentate of high molecular weight, higher than 10,000, was recovered from filters by adding 200 µL of water and lyophilized. GlcN was determined for the two fractions by HPLC, according to previous publications (Coppa et al. 2011a). Trace amounts of hyaluronic acid (and derived GlcN) are present in urine of healthy subjects and MPS under investigation (Coppa et al. 2011a, b). KS (and related GlcN) has been determined in normal urine in the order of ~0.2 µg/mg creatinine and of ~0.5 µg/mg creatinine for various MPS patients (Tomatsu et al. 2012). We determined GlcN values greater than ~3 µg/mg creatinine for healthy and MPS subjects (see Table 1) confirming that the quite total content of assayed urine GlcN specifically belong to HS.

After centrifugation at 5000×g for 15 min, 50 mL of ethanol were added to 5 mL of urine and stored at −20 °C for 24 h. The pellet was recovered by centrifugation, dried at 60 °C and applied to a column of 100 × 2 cm packed with Bio-gel P10 resin (from Bio-Rad Laboratories, Milano, Italy) fine polyacrylamide beads for size exclusion chromatography, 45–90 µm wet bead size, 1500–20,000 MW fractionation range. Urine samples were eluted with 10 mM of ammonium acetate and fractions of 2 mL were lyophilized. Fractions were assayed for GlcN content to obtain the molecular weight profile specific for HS.

## Results

After a 5-year follow-up of 44 patients (Table 1), no mental retardation was present in MPS IV and VI patients, in 7 MPS I

**Table 1** Single and total hexosamines values (from Coppa et al. 2011a) along with the contribution of GlcN belonging to high molecular weight (HMW) HS in healthy and various MPS types and related forms, mild and severe. Data are reported as mean ± standard errors. Significance is illustrated vs the healthy group and between mild and severe forms. Significance values, p, are referred comparing the various MPS forms vs healthy subjects, and severe vs mild subtypes belonging to the same MPS type

| | Subjects n | Age | GlcN μg/mg CR | GalN μg/mg CR | Total Hexs μg/mg CR | GlcN of HMW μg/mg CR | p vs Healthy | p Severe vs. Mild |
|---|---|---|---|---|---|---|---|---|
| Healthy | 83 | 4.0 ± 0.4 | 35.6 ± 2.0 | 28.3 ± 1.6 | 63.9 ± 3.5 | 3.4 ± 0.2 | | |
| MPS I | | | | | | | | |
| Mild | 7 | 9.2 ± 2.4 | 100.5 ± 14.5 | 98.2 ± 5.3 | 218.1 ± 19.0 | 55.1 ± 7.0 | 0.000 | |
| Severe | 7 | 1.7 ± 0.3 | 219.1 ± 31.4 | 290.9 ± 35.4 | 510.0 ± 65.4 | 113.9 ± 16.3 | 0.000 | 0.011 |
| MPS II | | | | | | | | |
| Mild | 4 | 4.2 ± 0.5 | 40.7 ± 11.7 | 43.5 ± 15.8 | 84.1 ± 27.4 | 23.4 ± 6.7 | 0.031 | |
| Severe | 7 | 2.9 ± 0.6 | 82.5 ± 4.0 | 101.71 ± 6.0 | 184.24 ± 7.2 | 47.5 ± 2.3 | 0.000 | 0.031 |
| MPS III | | | | | | | | |
| Mild | 1 | 28 | 84.0 | 16.0 | 100.0 | 49.7 | | |
| Severe | 12 | 5.7 ± 1.2 | 208.1 ± 26.2 | 53.2 ± 6.0 | 254.7 ± 30.8 | 119.1 ± 15.8 | 0.000 | nd |
| MPS IV | 5 | 5.0 ± 1.3 | 41.9 ± 3.7 | 22.5 ± 3.6 | 64.4 ± 7.3 | 3.0 ± 0.3 | 0.543 | |
| MPS VI | 1 | 2.5 | 212.9 | 377.7 | 590.6 | 8.9 | | |

and 4 MPS II patients. Mild mental retardation was present only in 1 of the 13 MPS III patients.

The results of the urinary Hex values of the 44 patients are reported in Table 1. The mean values of urinary GlcN are clearly elevated in MPS I, II and III patients affected by the severe phenotype, compared to those affected by the same disease but with an attenuated phenotype. Similar results are observed also as regards the total urinary Hex content (Coppa et al. 2011a). In MPS IV both urinary GlcN and total Hex levels were not substantially different from those observed in the mild form of MPS II. A very high level of total urinary Hex is present in the MPS VI patient and in the severe forms of MPS I and III (Coppa et al. 2011a). Overall, by considering the total levels of GlcN, no correspondence was observed between its urinary content and mental retardation (as well as for total Hex values).

Urine samples were fractionated on a preparative column able to separate high molecular weight (HMW) polymer from fractions of different low molecular weights (LMW). The assay and presence of GlcN in each recovered fraction enabled us to design a proper profile related to HS (Fig. 1). As can be seen, healthy pediatric subjects show a very high content of LMW fractions with a low percentage of HMW polymer. A fairly similar profile was observed for MPS IV and MPS VI patients (not shown). On the contrary, MPS I, II and III severe forms showed a very high increase of non-degraded HMW polysaccharide compared to the controls along with LMW fractions of various molecular weights (Fig. 1). Moreover, according to the quantitative data illustrated in Table 1, the total GlcN content strongly increases in the urine of MPS I, II and III patients compared to the controls as can be seen from the y-axes values (Fig. 1). Finally, the mild types of MPS I, II and III showed a fairly similar profile of severe forms (not shown) but with a lower total content of GlcN according to the data in Table 1 and a minor contribute of HMW component.

Based on the urinary GlcN profile reported in Fig. 1, the content of HMW polymer greater than 10 kDa and LMW fractions lower than 10 kDa were experimentally determined by using filters of 10 kDa cut-off. These values were utilized to calculate the GlcN content belonging to HMW and LMW HS and the data illustrated in Fig. 2. All forms of MPS I, II and III affected by severe mental retardation showed a significant strong increase in GlcN belonging to HMW HS polymer compared to healthy controls and corresponding mild forms (Table 1). HMW HS of mild forms was present in a lower content than the corresponding forms with severe retardation. MPS IV and VI (one patient) with no mental retardation showed a very low content of GlcN (Fig. 2). On the contrary, to demonstrate the specific correlation between mental retardation and HMW HS, we also measured the GlcN belonging to LMW fractions (Fig. 2). The MPS I mild form was found to be not significantly different from the controls ($p = 0.059$) as well as MPS II mild ($p = 0.147$) and MPS II severe ($p = 0.520$). Finally, the only MPS VI patient not affected by mental problems showed a very high content of LMW HS GlcN (Fig. 2).

According to scientific literature (Komosinska-Vassev et al. 2014), urine GAG excretion decreases with age and minor decline was also observed for related urinary Hex (Coppa et al. 2011a). Overall, changes in urinary GAGs is limited to few micrograms/mg creatinine during physiological human growth and development (Komosinska-Vassev et al. 2014). On the contrary, we observed a very huge increase in

**Fig. 1** Urinary HS profile of three pediatric healthy subjects compared with MPS I, II and III patients depending on molecular weight and determined as its constituent GlcN performed by separation on Bio-gel P10 resin, 1500–20,000 MW fractionation range, and eluted with 10 mM ammonium acetate. x-axes represents fractions eluted from Bio-gel P10 column and y-axes reports the GlcN content determined by HPLC according to previous methodology (see Coppa et al. 2011a)
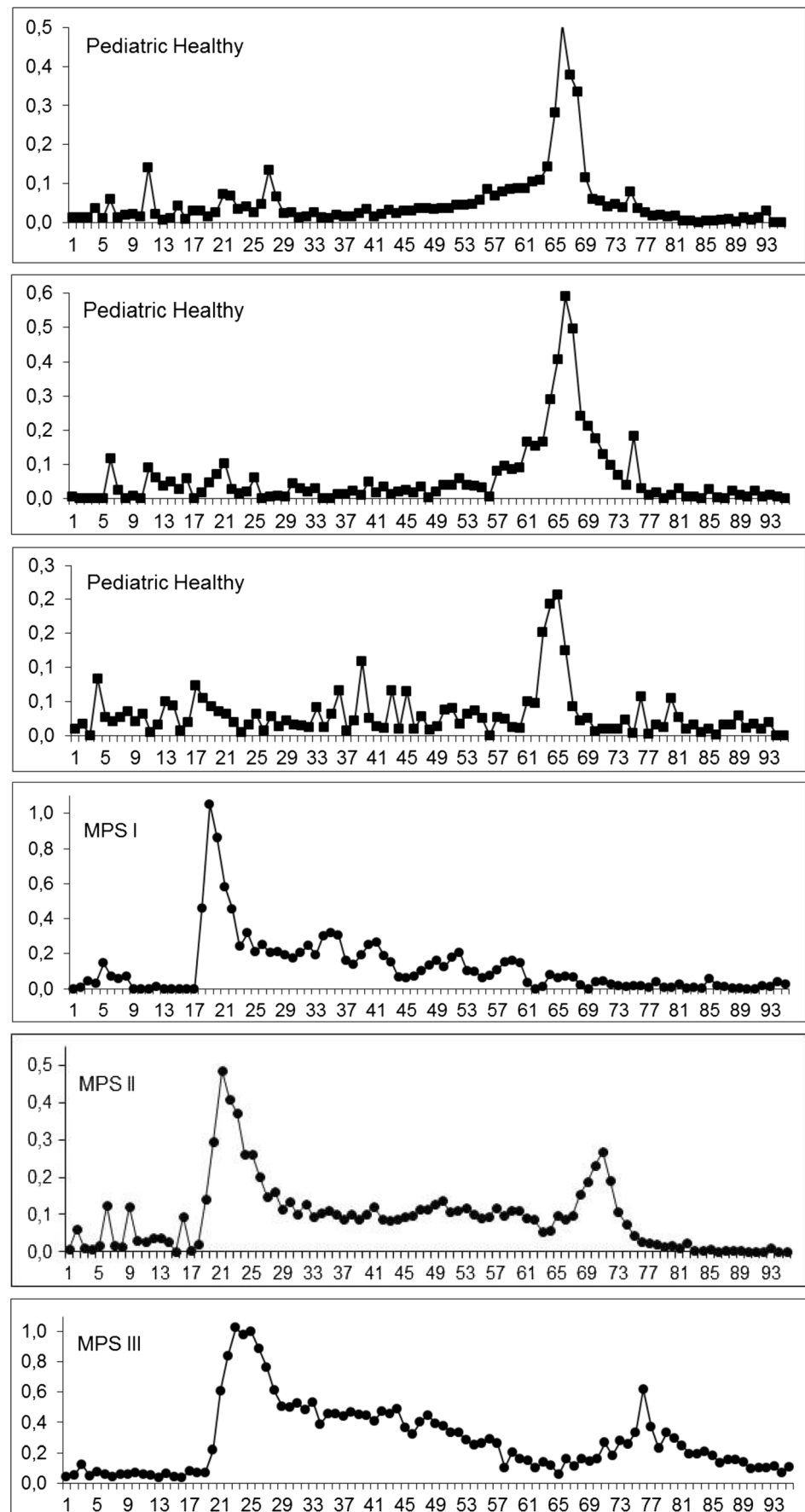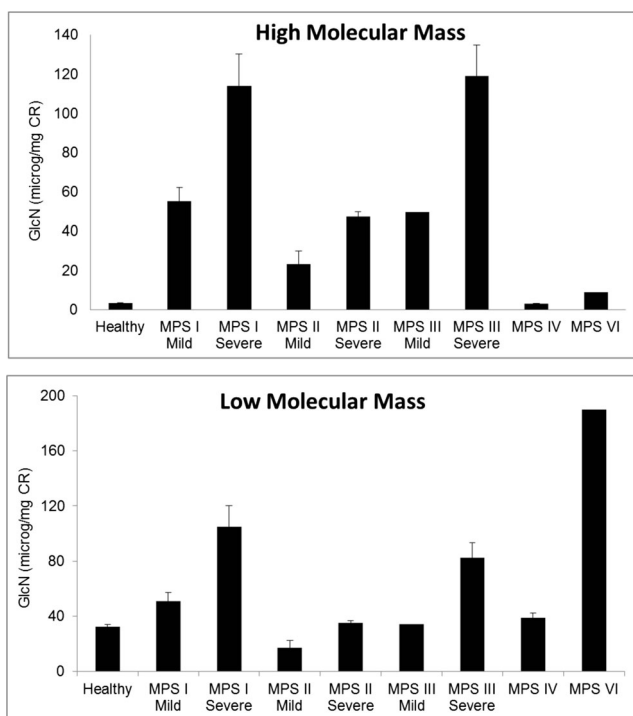
**Fig. 2** Urinary HS levels belonging to high molecular weight polymer (>10,000, *upper panel*) and low molecular weight fractions (<10,000, *lower panel*) determined as GlcN of controls and various MPS types and related mild and severe forms. *Data* are illustrated as mean ± standard errors

urinary GlcN of MPS subjects compared to healthy and in severe vs. mild forms (Table 1) confirming that the observed correlation between mental retardation and HMW urinary HS derived GlcN is not age-dependent.

## Discussion

Within MPS, mental retardation is present in patients affected by MPS I, II, III and VII (Neufeld and Muenzer 2007). All these types of MPS are characterized by urinary excretion of large amounts of non-degraded HMW HS (Neufeld and Muenzer 2007; Lehman et al. 2011) which is commonly detected by electrophoretic methods (Stone 1998; Coppa et al. 2011b). The clinical evaluation of large numbers of patients affected by the above reported forms of MPS demonstrated that a certain number of them present no or only mild mental retardation. On the basis of these observations we decided to evaluate the possible correlation between urinary HS derived GlcN and mental retardation in 44 patients affected by different types of MPS. However, we should consider that HS is a huge heterogeneous natural polysaccharide showing variable composition in terms of molecular weight (and structure). Healthy subjects mainly excrete HS in the form of fragments (see Lokeshwar et al. 2005 and this study) due to the activity of catabolic enzymes that are absent or impaired in MPS

patients. As a consequence, urinary HS in MPS subjects is composed of HMW polymer and fragments of various lengths. On this basis we also correlated mental retardation with GlcN belonging to high and low molecular weight HS. Our results confirm that patients affected by the severe form of the disease have GlcN values almost double compared with those of patients affected by the attenuated forms.

In this study, we have always observed a considerable increase in GlcN, ranging from 103 ÷ 139 %, in severe compared to mild forms for each MPS type and, as a consequence, associated with more severe mental retardation. However, urinary GlcN levels associated with HMW HS were found to be lower in MPS II compared to MPS I and III. Evidently, it is not possible to compare the absolute content of GlcN belonging to HMW HS for the various forms of MPS as well as the severe and mild subtypes belonging to different MPS due to the different enzymatic defects. In fact, the presence of enzymatic defects typical of each type of MPS leads to great differences in the content and structure of HS and in the composition and sulfation of derived disaccharides also observed comparing the brain tissues of MPS I and MPS III mice (Wilkinson et al. 2012). This is further highlighted by the several striking differences in CNS symptoms among the various MPS types. For example, the neurocognitive decline in MPS III patients is accompanied and even preceded by behavioral problems, hyperactivity, fearlessness and temper tantrums, and these symptoms are absent in MPS I severe subjects (Ahmed et al. 2014; Rumsey et al. 2014).

MPS IV showed a GlcN amount comparable to the controls lacking in enzymatic defect related to HS (Neufeld and Muenzer 2007). The only MPS VI subject, not affected by mental problems, was found to have a greater HMW HS content with respect to healthy subjects. However, this may be caused by a secondary accumulation of GAGs (Lamanna et al. 2011) described also for MPS disease not having the related enzymatic defect as in MPS VI. Anyway, a larger cohort of MPS VI patients is necessary in order to perform a statistical evaluation and to confirm this observation.

The enzymatic degradation of HS is an organized concerted process able to cleave large HS chains into smaller fragments by glycosidases, sulfatases and an acyl-transferase (Neufeld and Muenzer 2007). As a consequence, the urine samples of healthy subjects are rich in HS oligomers, as opposed to those of MPS patients in which HMW polymer and fragments of different lengths are present. Obviously, the elevated content of urinary HS and its pattern of chains of various lengths corresponds to non-degraded and accumulated molecules in cells and tissues, and in brain as expected (Wilkinson et al. 2012; Hopwood and Elliott 1985). Therefore, the reported different levels of urinary HMW HS may determine the severity of the CNS disease in MPS. In fact, as a consequence of the nature of the mutations in the relevant genes, mild forms still present residual enzyme activity (Neufeld and Muenzer 2007)

responsible for a partial degradation of HMW HS producing less severe mental implications.

In conclusion, we demonstrate a positive correlation between the accumulation of HMW HS and the subsequent cascade of pathophysiological events, including neuro-inflammation and apoptotic triggers, responsible for the CNS disease in MPS I, II and III severe forms. This is all the more conclusive considering that accumulation of only KS (and CS) as in MPS IV, DS (and CS) as in MPS VI, and LMW HS fragments do not lead to CNS disease. Additionally, present data obtained in MPS patients with mental retardation well confirm the neurological disorders produced by HMW HS demonstrated in an animal model of MPS IIIB (Bruyère et al. 2014). Finally, the present methodology able to correlate urinary HMW HS with mild and severe forms and mental retardation may be useful in the perspective of a possible prediction of a patient phenotype as well as in the evaluation of possible experimental treatments to animal models.

**Contributors** N.V. developed the applied methodologies. F.M., V.M., L.Z., T.G. and F.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 no conflicts of interest.

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