

## Heparan sulfate levels in mucopolysaccharidoses and mucopolipidoses

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**Summary:** Glycosaminoglycans are accumulated in both mucopolysaccharidoses (MPS) and mucopolipidoses (ML). MPS I, II, III and VII and ML II and ML III patients cannot properly degrade heparan sulphate (HS). In spite of the importance of HS storage in the metabolic pathway in these diseases, blood and urine HS levels have not been determined systematically using a simple and economical method. Using a new ELISA method using anti-HS antibodies, HS concentrations in blood and urine were determined in MPS and ML II and ML III patients. HS concentrations were determined in 156 plasma samples from MPS I ( $n = 23$ ), MPS II ( $n = 26$ ), MPS III ( $n = 24$ ), MPS IV ( $n = 62$ ), MPS VI ( $n = 5$ ), MPS VII ( $n = 5$ ), ML II ( $n = 8$ ) and ML III ( $n = 3$ ), and 205 urine samples from MPS I ( $n = 33$ ), MPS II ( $n = 33$ ), MPS III ( $n = 30$ ), MPS IV ( $n = 82$ ), MPS VI ( $n = 7$ ), MPS VII ( $n = 9$ ), ML II ( $n = 8$ ) and ML III ( $n = 3$ ). The ELISA method used monoclonal antibodies against HS. MPS I, II, III and VII and ML II and III patients had significant elevation in plasma HS, compared to the age-matched controls ( $p < 0.0001$ ). Eighty-three out of 89 (93.3%) of individual values in the above MPS types and ML were above the mean +2SD of the controls. In urine samples, 75% of individual values in patients with those types were above the mean +2SD of the controls. In contrast to the previous understanding of the HS

metabolic pathway, plasma HS levels in all five MPS VI and 15% of MPS IV patients were elevated above the mean +2SD of the controls. These findings suggest that HS concentration determined by ELISA, especially in plasma, could be a helpful marker for detection of the most severe MPS I, II, III, VI and VII and ML II, distinguishing them from normal populations.

Mucopolysaccharidoses (MPS) are a group of lysosomal storage disorders (LSDs) caused by deficiency of the lysosomal enzymes needed to degrade glycosaminoglycans (GAGs) such as dermatan sulphate (DS), heparan sulphate (HS), keratan sulphate (KS), chondroitin sulphate (CS), and hyaluronan (Neufeld and Muenzer 2001). In MPS, the undegraded or partially degraded GAGs are stored in lysosomes and/or secreted into the bloodstream and excreted in urine. Mucopolipidosis (ML) II and ML III are disorders of phosphorylation and localization of lysosomal enzymes, caused by deficiency of *N*-acetylglucosaminyl-1-phosphotransferase leading to accumulation of GAGs and sphingolipids intracellularly (Kornfeld and Sly 2001). In ML, a number of biochemical defects include deficiencies of multiple lysosomal enzymes as evidenced in cultured fibroblasts and abnormally high levels of GAGs and sphingolipids in the culture medium. Among LSDs, MPS I, II, III, VII, and ML patients cannot properly degrade HS, the accumulation of which in the brain leads to neurological symptoms.

The incidence for all types of MPS and ML is estimated as one in 20 000–50 000 live births (Meikle et al 1999). In general, MPS and ML patients are asymptomatic as newborns, with subsequent gradual onset of clinical signs that include short stature, bone deformity, visceral organ involvement and mental retardation during infancy or childhood. MPS are theoretically amenable to therapy by exogenous supply of enzymes that are deficient (enzyme replacement therapy, ERT). Clinical trials of ERT on MPS I, II and VI are in progress (Harmatz et al 2004; Kakkis et al 2001; Muenzer and Fisher 2004; Muenzer et al 2002; Wraith et al 2004, 2005). If these treatments are to be successful, methods must be developed to establish an early diagnosis and to accurately monitor the clinical course.

The structural complexity of the HS molecule (David 1993; Lindahl et al 1998; Yanagishita and Hascall 1992), as well as the extent of accumulation of HS, leads to varied clinical consequences in MPS and ML patients. Although stored HS is key to neurological pathology in MPS and ML patients, no simple and economical method has been developed to systematically measure and evaluate blood HS levels as they relate to clinical signs in a large number of patients.

In this study, a new ELISA assay for HS has been evaluated for blood and urine samples to assess whether HS determination by ELISA can be useful in distinguishing MPS and ML patients from normal individuals.

## METHODS

*Subjects:* Plasma LSD samples from 234 patients and urine LSD samples from 222 patients were collected along with 450 plasma and 400 urine samples from age-matched, normal healthy controls (Table 1). Blood was collected into a tube with EDTA. All the patients

**Table 1 Plasma and urine HS concentrations by ELISA****A Plasma HS ( $\mu\text{mL}$ )**

Age ( $\chi$ , years)	Mean HS	SD	p-value	Maximum	Minimum	N	Mean age (years)
Group A: MPS I, II, III, VII, ML (all ages)	37.9	42.2	0.00029**	203	1.9	89	8
Newborns <sup>a</sup>	16.6	9.1	<0.0001***	29.1	6.5	4	0
0 < $\chi$ $\leq$ 5	26.8	25.6	<0.0001***	150	4.9	46	1.9
5 < $\chi$ $\leq$ 10	40.2	42.1	<0.0001***	147	2.8	17	6.7
10 < $\chi$ $\leq$ 15	35.6	47.9	<0.0001***	131	4.4	5	11.5
15 < $\chi$ $\leq$ 20	75.9	66.8	<0.0001***	188	4.9	6	17.7
$\chi \geq 20$	69.2	54.9	<0.0001***	203	1.9	11	31.8
<i>Type of MPS and ML</i>							
MPS I	24.9	29.5		150	1.9	23	4.7
MPS II	79.8	49.9		203	4.4	26	13.6
MPS III	18.2	10.5		37.7	9.8	24	5.5
MPS VII	14.6	12.2		30	3.5	5	12.1
ML	19.8	21.7		82.9	3.1	11	5.6
<i>Group B: MPS IV and VI</i>							
MPS IV	6.1	2.8		12.4	0.7	62	14.1
MPS VI	15.9	6.4		25.5	8.7	5	3.7
Group C: Other LSD	10	6.8		50.7	0.2	78	18.6
<i>Group D: Control (all ages)</i>							
Newborns <sup>a</sup>	2.4	1.3		6.3	0.3	100	0
0 < $\chi$ $\leq$ 5	5.1	2		9.6	1.6	100	1.7
5 < $\chi$ $\leq$ 10	4.3	1.5		7.2	1.2	50	7.1
10 < $\chi$ $\leq$ 15	5.7	2.9		9.8	2	50	11.5
15 < $\chi$ $\leq$ 20	6.4	2.7		9.6	3	50	18.2
$\chi \geq 20$	5.5	1.9		9.9	0.5	100	35.2

<sup>a</sup>Within 1 month after birth

\*\* p-value calculated comparing the values of group A and group D

\*\*\* p-value calculated comparing the age-matched subgroups in group A and group D

**B Urine HS (mg/g creatinine)**

Age ( $\chi$ , years)	Mean HS	SD	p-value	Maximum	Minimum	N	Mean age (years)
Group A: MPS I, II, III, VII, ML (all ages) <sup>a</sup>	50	40.6	0.00033**	238	4.4	116	7.3
0 < $\chi$ $\leq$ 5	59.7	47.3	<0.0001***	238	6.1	61	2
5 < $\chi$ $\leq$ 10	46.1	33.6	<0.0001***	176	8.6	29	6.4
10 < $\chi$ $\leq$ 15	38.6	14.8	<0.0001***	59	14.5	8	11.7
15 < $\chi$ $\leq$ 20	30.1	12.8	<0.0001***	56	17.8	6	18
$\chi \geq 20$	27.6	17.8	<0.0001***	65	4.4	12	27.7

(Continued on next page)

**Table 1** (Continued)

Age ( $\chi$ , years)	Mean HS	SD	<i>p</i> -value	Maximum	Minimum	N	Mean age (years)
<i>Type of MPS and ML</i>							
MPS I	63.4	60.5		238	13.2	33	4.6
MPS II	44.2	18.9		90	6.1	33	10.3
MPS III	43.1	21.7		97	10.7	30	5.3
MPS VII	33.2	27.5		100	4.4	9	14.2
ML	60.1	48.6		151	8.6	11	5.9
<i>Group B: MPS IV and VI</i>							
MPS IV	26.2	19.7		105	3.5	82	13.3
MPS VI	24.4	13.1		44	2.3	7	5
<i>Group C: Other LSD</i>							
	22.6	24.7		98	0.07	17	15.3
<i>Group D: Control (all ages)</i>							
Newborn <sup>b</sup>	13.1	7.7		26.3	2.2	42	0
0 < $\chi$ ≤ 5	13.4	7.1		29.9	0.6	108	1.7
5 < $\chi$ ≤ 10	11.1	6.9		27.9	1	50	6.3
10 < $\chi$ ≤ 15	12.5	8.1		27.7	2.1	50	12.2
15 < $\chi$ ≤ 20	14.9	6.1		23.9	8.7	50	16.2
$\chi$ ≥ 20	10.5	6.9		28.8	0.7	100	34.9

<sup>a</sup>There is no newborn patient in group A

<sup>b</sup>Within 1 month after birth

\*\**p*-value calculated comparing the values of group A and group D

\*\*\**p*-value calculated comparing the age-matched subgroups in group A and group D

were diagnosed as having below 5% of the normal enzyme activity. One specimen of urine and/or plasma was taken from each patient for the analysis. LSD patients were classified as groups A, B and C as follows: group A, MPS I, II, III, VII and ML II and ML III; group B, MPS IV and VI; group C, the other LSD patients. The control population was designated as group D. The MPS and ML disorders in group A involve direct metabolic pathway of HS. The patients showing severe clinical signs involving the central nervous system (CNS) were defined as severe. Some patients over 5 years of age in group A presented attenuated forms, without central nervous system involvement (non-CNS). Patients below 5 years of age and presenting CNS involvement were also classified as severe forms.

The MPS IV and VI patients in group B, who had no direct metabolic pathway for HS, were free of CNS symptoms. The MPS IVA patients were defined as attenuated if above 125 cm and severe if below 125 cm of the final height (−9SD compared with normal adult height) throughout ages. Group C were other types of LSD patients who may or may not have had neurological signs.

Of the 234 plasma samples, 89 were obtained from group A (age range 0–55 years) (MPS I, *n* = 23, 3 attenuated, 20 severe; MPS II, *n* = 26, 12 attenuated, 14 severe; MPS III, *n* = 24, 1 attenuated, 23 severe; MPS VII, *n* = 5, 3 attenuated, 2 severe; ML II-severe, *n* = 8; ML III-attenuated, *n* = 3); 67 were from group B (age range 2–65 years) (MPS IVA, *n* = 60, 13 attenuated, 47 severe; MPS IVB, *n* = 2; MPS VI, *n* = 5, 5 severe); and

the rest, 78, were from group C (age range 0–55 years). Of the 222 urine samples, 116 were from group A (age range 0–40 years) (MPS I,  $n = 33$ , 4 attenuated, 29 severe; MPS II,  $n = 33$ , 11 attenuated, 22 severe; MPS III,  $n = 30$ , 2 attenuated, 28 severe; MPS VII,  $n = 9$ , 7 attenuated, 2 severe; ML II-severe,  $n = 8$  and ML III-attenuated,  $n = 3$ ); 89 were from group B (age range 0–65 years) (MPS IVA,  $n = 75$ , 15 attenuated, 60 severe; MPS IVB,  $n = 7$ ; MPS VI,  $n = 7$ , 7 severe); and 17 were from group C (age range 0–60 years).

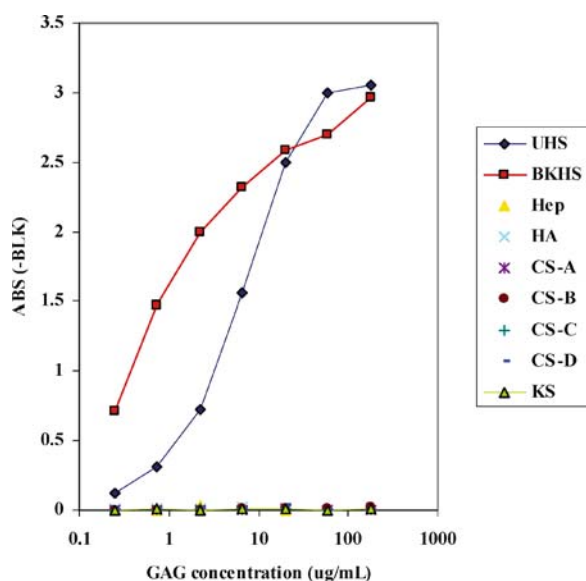
The urine and blood samples from the patients were collected at the following institutes: Shimane University, Gifu University, Royal Manchester Children's Hospital, Javeriana University, Federal University of Rio Grande do Sul, University of Mainz, University of Vienna, University of Graz, and University of Hamburg. The samples were sent to the Department of Pediatrics, St Louis University, for further analysis. Written informed consent was obtained from each individual at entry to the study at each institute. The study protocol was approved by the Institutional Review Board at St Louis University.

*Sandwich ELISA assay:* All reagents described for HS-ELISA were available from Seikagaku Corporation or Associates of Cape Cod, Inc. (Falmouth, MA, USA) (HS-ELISA kit no. 280564). The DS, HS, KS, hyaluronic acid, heparin, chondroitin sulphate A and chondroitin sulphate C were obtained from Seikagaku in an AMPS kit (no. 400610). The kit utilizes monoclonal antibodies against HS. The ELISA method was essentially the same as described previously (Tomatsu et al 2004).

The absorbance was measured at 450 nm (reference absorbance 630 nm) with a microplate spectrophotometer. The HS concentration was read by applying the absorbance of each sample to the calibration curve. Typical assays yielded a quadratic-line [ $\log(\text{STD conc.}) - \log(\text{subtracted absorbances})$ ] HS calibration curve ( $R = 0.999$ ) between 0.25  $\mu\text{g/mL}$  and 8  $\mu\text{g/mL}$ . Intra-assay coefficients of variation (CV%) determined with three different standard HS samples (STD-HS samples; 1, 2, 4  $\mu\text{g/mL}$ ) were 2.1%, 1.5% and 3.2%, respectively. Inter-assay CVs determined with three STD-HS samples, as above, using three different kits were 1.0%, 1.0% and 1.2%, respectively. This HS-ELISA detected HS from human urine and bovine kidney but did not detect any HA, CS-A, DS, CS-C, CS-D or KS (Figure 1). Heparitinase I pretreatment of samples eliminated detection. Further detailed information of the validation of the kit is available via the World Wide Web (<http://www.seikagaku-hit.com/english/02tech/gag/03he.htm>).

*HPLC assay for urine samples:* In 14 urine samples, HS concentrations were determined by both ELISA and HPLC as previously described (Yoshida et al 1989). The unsaturated disaccharide isomers formed by sample digestion with heparitinase I were detected by HPLC, in which the mono-, di- and tri-sulphated disaccharide isomers were eluted with increasing salt. Total urine HS as well as each  $\Delta\text{di-mono-HS}$ ,  $\Delta\text{di-di-HS}$  and  $\Delta\text{di-tri-HS}$  were calculated.

*Data analysis:* The mean HS value and standard deviation in each disease type were calculated. The Mann–Whitney test was used to compare HS levels in the patient samples with the control samples according to age. The correlation between blood and urine HS levels was analysed using Pearson's correlation. The correlation in blood (or urine) HS concentrations assayed by ELISA versus HPLC was evaluated using a regression plot. All



**Figure 1** Specificity of the HS immunoassay by studying the cross-reactivity with GAGs. Solutions of the following compounds at 0.25–180  $\mu\text{g/mL}$  were analysed by ELISA. Hyaluronic acid (HA), chondroitin sulphate A (CS-A), chondroitin sulphate B (CS-B), chondroitin sulphate C (CS-C), chondroitin sulphate D (CS-D), keratan sulphate (KS) were treated by heparitinase I. This HS assay detects HS from human urine (UHS, standard material) and bovine kidney (BKHS). The assay does not detect heparin (Hep), HA, CS-A, CS-B, CS-C, CS-D or KS

data analyses were performed with Statview statistical software (StatView-J 4.5; Abacus Concepts, Inc, Berkeley, CA, USA).

## RESULTS

### Blood HS concentrations

*Group A:* The age groups within group A were divided into newborn (<4 weeks), 0–5 years, 5–10 years, 10–15 years, 15–20 years, and >20 years for comparison with the age-matched controls (Table 1A). Four newborn patients (<4 weeks) in group A, consisting of two MPS I, one MPS VII and one ML II, had plasma HS of 21.3, 6.5, 29.1, and 9.5  $\mu\text{g/mL}$ , respectively. These plasma concentrations were significantly higher than those of the control newborns (mean value 2.4  $\mu\text{g/mL}$ ; 100% sensitivity and specificity). The mean value in normal controls was higher at 0–5 years (5.1  $\mu\text{g/mL}$ ). Throughout the age groups, HS was higher in group A patients compared with controls ( $p < 0.0001$ ).

The plasma HS concentration was evaluated in each type of MPS and ML, compared to the age-matched controls (Table 1A). The mean plasma HS in MPS I patients was 24.9  $\mu\text{g/mL}$ , and 22 out of 23 (95.7%) patients (2 of 3 attenuated; all 20 severe) had plasma HS

values above the mean +2SD of the age-matched controls (Figure 2A). MPS II patients had the highest mean plasma HS among all types of MPS and ML patients (79.8 µg/mL), and 25 of 26 (96.2%) patients (all of 12 attenuated; 13 of 14 severe) exhibited plasma HS values above the mean +2SD of controls (Figure 2B). The mean value for MPS III patients was 18.2 µg/mL, and the plasma HS values in all 24 patients (1 attenuated and 23 severe) were above the mean +2SD of the age-matched controls (Figure 2C). Two MPS VII patients with a severe form showed plasma HS values above the mean +2SD of controls, while none of three MPS VII patients with an attenuated form had plasma HS values above the mean +2SD of controls (Figure 2E). Seven of eight ML 2 patients (and none of three ML 3) exhibited HS values above the mean +2SD of controls (Figure 2E). Overall, HS plasma levels in 18 of 22 (82.6%) attenuated and 65 of 67 (97%) severe patients of group A were above the mean +2SD of the age-matched controls, indicating that severe patients had a higher frequency of elevated plasma HS ( $p < 0.029$ ). Taken together, the sensitivity with the cut-off above +2SD of normal values was 93.3% and the specificity was 95% in all MPS I, II, II and VII and ML II and ML III patients.

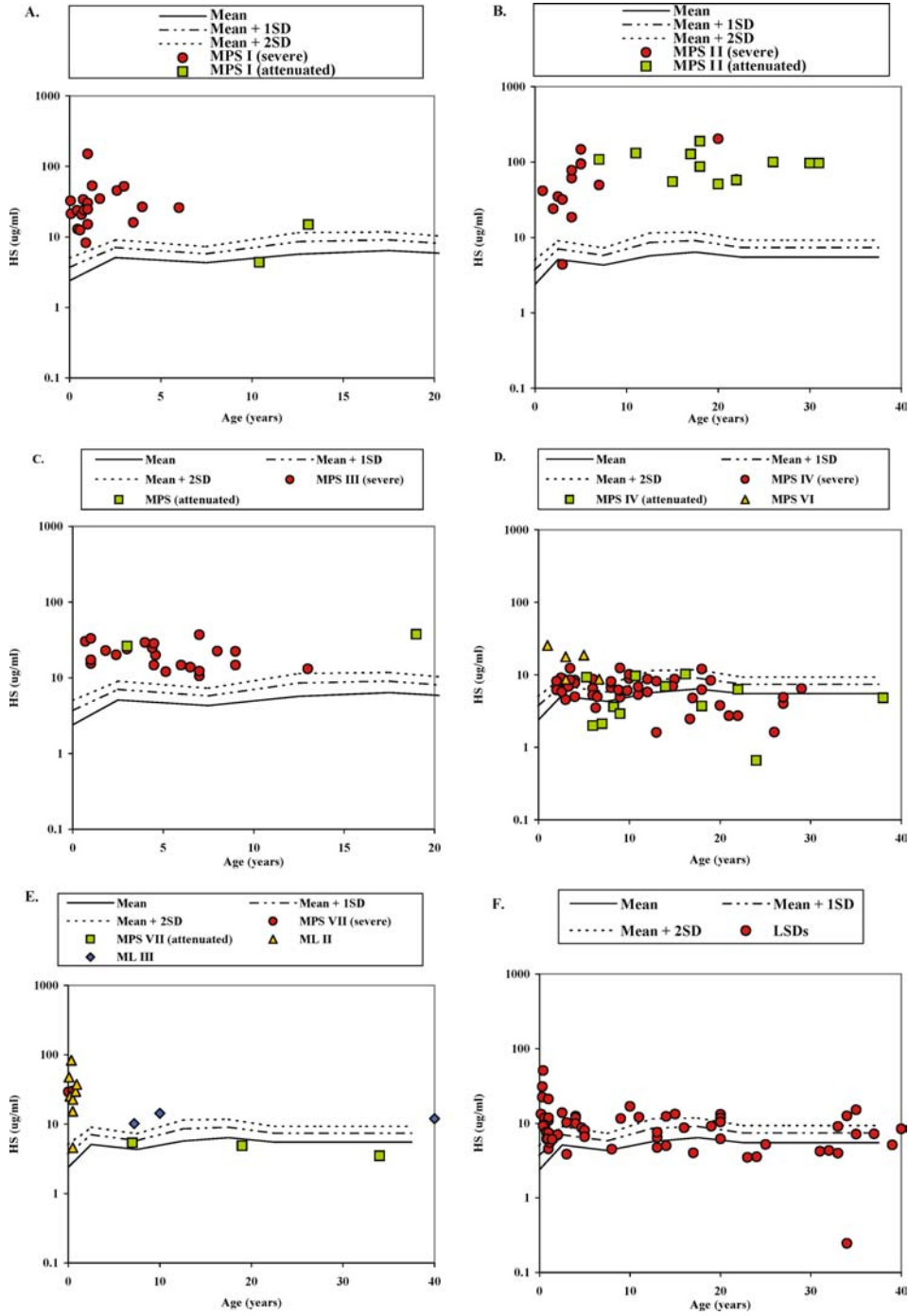
*Group B:* Nine of 60 (15%) MPS IVA patients (1 of 13 attenuated; 8 out of 47 severe) had plasma HS values higher than the mean +2SD of the age-matched controls (Figure 2D). One of two MPS IVB patients had plasma HS above the mean +2SD. Notably, plasma HS values in all five MPS VI patients (mean 15.9 µg/mL) were above the mean +2SD of controls (Figure 2D).

*Group C:* Thirty-three of 78 (42.3%) patients with other LSDs had plasma HS values above the mean +2SD of the age-matched controls (mean 10 µg/mL; Figure 2F). Twelve of 25 (48%) Fabry patients, 14 of 26 (53.8%) Gaucher patients, 2 of 3 GM<sub>1</sub>-gangliosidosis patients, 2 of 2 Niemann–Pick A patients and 1 of 5 Niemann–Pick B patients had plasma HS values above the mean +2SD. Plasma HS concentrations in one GM<sub>1</sub>-gangliosidosis patient (31 µg/mL) and one Niemann–Pick A patient (50.7 µg/mL) were elevated markedly.

### Urine HS concentrations

*Group A:* The age groups within group A were divided into 0–5 years, 5–10 years, 10–15 years, 15–20 years, and >20 years for comparison with the age-matched controls (Table 1). There was no newborn urine sample. The level of urine HS changed with age in control and patient populations. The highest mean value (59.7 µg/mL) was observed in the 0–5 year group. Throughout the age groups, the patient group A had significantly higher urine HS levels compared with the age-matched control group (Table 1B).

When each type of MPS and ML and the age-matched controls were compared, 23 out of 33 (70%) MPS I (2 of 5 attenuated; 22 of 29 severe; Figure 3A); 29 of 33 (87.8%) MPS II (8 of 11 attenuated; 21 of 22 severe; Figure 3B); 23 of 30 (76.7%) MPS III (23 of 28 severe; 0 of 2 attenuated; Figure 3C); 5 of 9 MPS VII (4 of 7 attenuated; 1 of 2 severe; Figure 3E); and 7 of 11 (63.6%) ML (7 of 8 ML II; 0 of 3 ML III; Figure 3E) were above the mean +2SD of the age-matched controls. Urine HS concentrations in 82 of 89 (92.1%) severe and 13 of 27 (48.1%) attenuated patients in group A were above the mean +2SD of the age-matched controls, indicating greater frequency of elevated urine HS in severe forms



**Table 2** HPLC analyses of urine from MPS and ML patients

No.	Type	Age (years)	HS (mg/g creatinine)		Ratio: ELISA/HPLC
			ELISA	HPLC	
1	I	2	49.1	94.6	0.52
2	I	2	89.3	115.7	0.77
3	II	4	112	150.0	0.75
4	II	13	51	140.2	0.36
5	IIIA	4	52.8	202.2	0.26
6	IIIA	3	72.5	183.6	0.39
7	IIIB	8	81.3	164.6	0.49
8	IIIC	10	60.8	219.7	0.28
9	IVA	10	35.6	9.5	3.7
10	IVA	2	41.6	11.1	3.7
12	ML	1	20	11.8	1.8
13	GM <sub>1</sub>	5	19.7	2.5	7.9
14	Fucosidosis	6	14.2	4.6	3.1
15	Normal	4	13.6	1.0	13.6
16	Normal	6	14.2	0.9	15.8
17	Normal	8	12.1	1.0	12.1
18	Normal	13	19.3	1.9	10.2

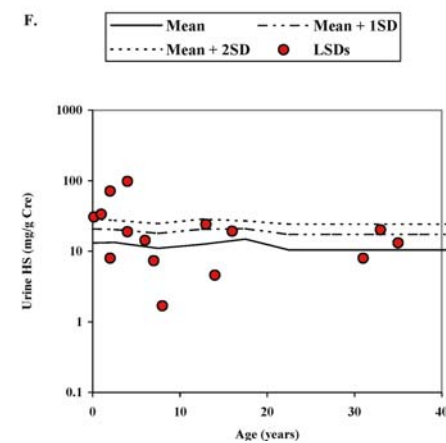
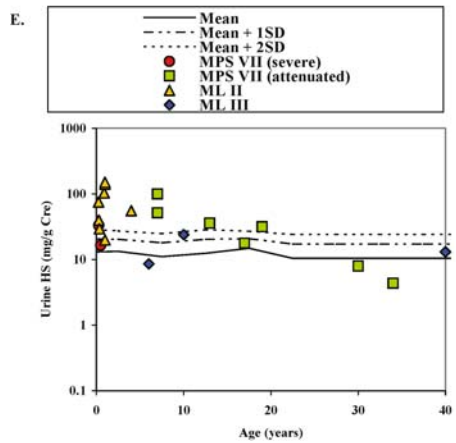
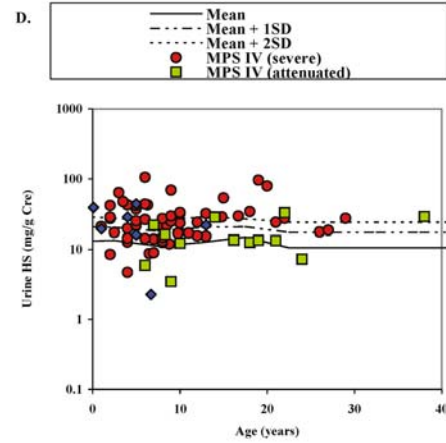
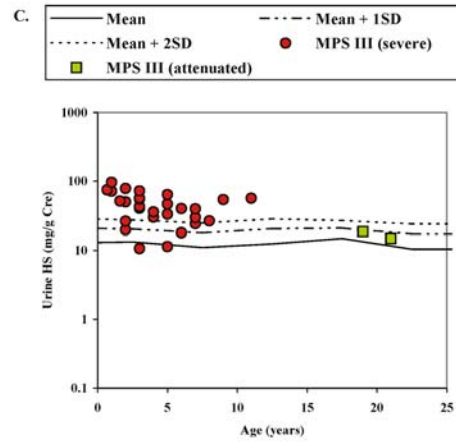
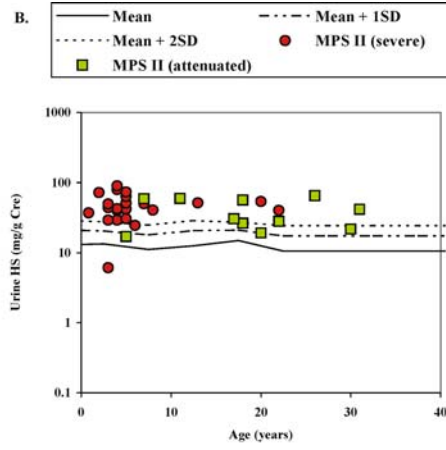
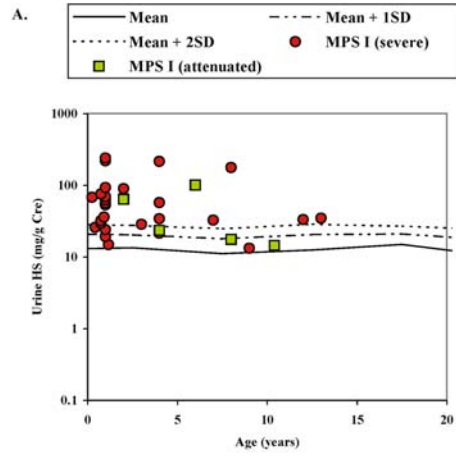
( $p < 0.0001$ ). Overall, 75% of individual values of group A patients (specificity) plotted above the mean +2SD of controls.

*Group B:* Twenty-nine of 75 (38.7%) MPS IVA patients (3 of 15 attenuated; 26 of 60 severe) had plasma HS values above the mean +2SD of controls (Figure 3D). Seven MPS IVA patients with a severe form had urine HS above 50 mg/g creatinine. Three of seven MPS VI patients showed urine HS levels above the mean +2SD (mean 24.4 mg/g creatinine) (Figure 3D).

Four of seven MPS IVB patients had urine HS values above the mean +2SD of controls. Three of seven MPS VI patients exhibited urine HS levels above the mean +2SD (mean 24.4 mg/g creatinine) (Figure 3D).

*Group C:* Four of 17 (23.5%) other LSD patients had urine HS concentrations above the mean +2SD of the age-matched controls (Figure 3F). Notably, urine HS concentrations in one patient with infantile sialic acid storage disease (71.2 mg/g creatinine) and in one galactosialidosis patient (97.6 mg/g creatinine) were elevated markedly.

**Figure 2** Plasma concentrations of HS of patients with MPS, ML and other LSDs, and normal individuals. Results of the specimens from patients and normal individuals are plotted on a semilogarithmic scale with respect to age. (A) MPS I; (B) MPS II; (C) MPS III; (D) MPS IV and VI; (E) MPS VII and ML II and III; (F) other LSDs



### Blood vs urine

The blood and urine HS concentrations in an individual showed variable correlation depending on the type of MPS and ML. Individuals in group A with high plasma HS also had high urine HS. We observed a weak correlation between the blood and urine HS concentrations in group A patients ( $n = 34$ ,  $r = 0.575$ ,  $p = 0.0003$ ) (Figure 4). Plasma HS concentrations in MPS IV were relatively stable through the ages and almost the same as in normal controls, although urine HS concentrations varied. No significant correlation between blood and urine HS concentrations was observed in MPS IVA patients ( $n = 48$ ,  $r = 0.218$ ,  $p = 0.1379$ ).

### Urine HS by HPLC

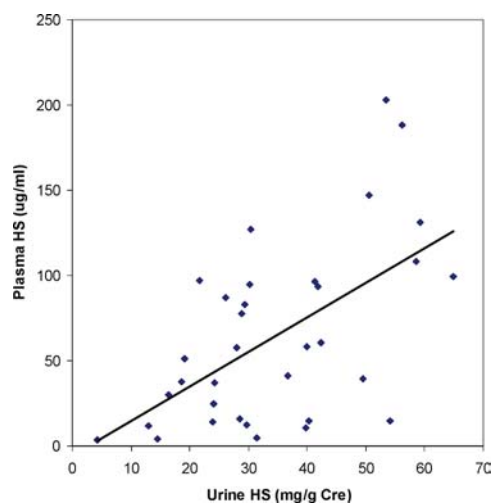
Since several MPS IVA patients had marked elevation of urine HS, total urine HS was also calculated in MPS and ML specimens, including two MPS IVA patients, by an HPLC method. The HPLC results of urine samples in MPS IVA patients were elevated HS (9.5 and 11.1 mg/g creatinine) compared with the normal controls ( $n = 4$ , mean 1.2 mg/g creatinine) (Table 2). The ratio of urine HS concentrations by ELISA to those by HPLC was different among MPS I, II, III and IVA patients and the normal controls. HPLC analysis for urine HS showed over 100 times higher concentrations in MPS I, II and III patients and 8 times higher concentrations in MPS IVA patients as compared to the controls. The ratio of urine HS by ELISA to that by HPLC among MPS I, II and III patients was between 0.26 and 0.77, while the ratios in the control group and MPS IVA patients were 10.2–15.8 and 3.7, respectively. The results for urine HS concentration by the HPLC method were compared with those by ELISA (ratio of ELISA to HPLC), showing that the ELISA-based method yielded higher HS concentrations in the normal control and MPS IVA samples, and lower levels in MPS I, II and III patients, than the HPLC method.

## DISCUSSION

Our findings with this ELISA method indicate that blood and urine HS concentrations in MPS I, II, III and VII, and ML patients (group A) are elevated compared to those in the age-matched controls as predicted from the metabolic pathway involved in these diseases. Plasma HS-ELISA showed 100% sensitivity and specificity for the newborn patients (age 1 month or less), and 96% sensitivity and 99.7% specificity for the patients 1 month to 5 years, when initial clinical symptoms are recognized. Overall, 93% of plasma HS values and 75% of urine HS values in group A patients were above the mean +2SD of controls. In MPS III as well as in other MPS patients, urine samples may show more excreted oligosaccharides (oligosaccharides smaller than tetrasaccharides), while blood samples have

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**Figure 3** Urine concentrations of HS of patients with MPS, ML and other LSDs, and normal individuals. Results of all specimens from each individual were plotted with respect to age. (A) MPS I; (B) MPS II; (C) MPS III; (D) MPS IV and VI; (E) MPS VII and ML II and III; (F) other LSDs



**Figure 4** Correlation between urine and plasma HS in affected samples ( $n = 34$ ). Linear regression analysis of these data yielded the equation:  $y = -5.821 + 2.028x$ , where  $x$  and  $y$  are the concentrations of urine and plasma HS, respectively. Pearson's correlation coefficient was 0.575 ( $p = 0.0003$ )

more undegraded HS (large molecules that cannot be excreted in the urine). The current ELISA method cannot detect the presence of these small molecules in the urine and hence some patients will not show elevated HS in urine.

The magnitude of elevation of plasma HS in group A patients was greater at older ages, largely noted in MPS II patients, while that of urine HS was lower at older ages, corresponding to the increase in the number of attenuated patients and a trend of decreased urinary GAG excretion with age. The magnitude of elevation of blood and urine HS in group A patients correlated positively with clinical severity, especially in MPS I and VII and ML patients, while plasma HS concentrations in MPS II patients with an attenuated form were similar to concentrations in patients with a severe form. A large number of longitudinally collected samples analysed for each type of MPS are needed to understand the magnitude of correlation between elevated HS and clinical severity. Whether asymptomatic newborns can be detected by this method is an important question from a newborn screening viewpoint. Considerable evidence from both human (Wiesmann et al 1980) and animal models (Crawley et al 1997) suggests that biochemical storage begins early in gestation and is well advanced at birth in the absence of clinical signs. The increase of blood HS in all four newborns in group A suggests that the HS is stored well in advance of birth in human patients. Further studies of newborn populations will be required to verify this contention.

Elevation of HS in group A patients was expected. However, its elevation in some individuals within groups B and C was surprising, since there are well-known relationships between the types of MPS and specific GAGs that accumulate (Neufeld and Muenzer 2001). In the present study, elevated plasma HS was observed in 14.5% of MPS IVA and all MPS VI patients (group B), and elevated urine HS was also observed in about 40% of both types

of patients. MPS IVA patients with a severe form had elevated HS more often than patients with an attenuated form, and some patients with a severe form had marked elevation of urine HS, suggesting strong correlation between clinical severity and elevated HS. The mechanism by which HS is elevated in these disorders is not clear. Several hypotheses can be offered to explain elevation of blood HS: (a) the synthesis of HS may be stimulated by storage of other GAG(s); (b) the elevation of HS may be secondary consequence of the tissue damage produced by accumulation of the other GAGs; or (c) HS may be co-deposited with the other accumulated GAGs, which inhibits the interaction between HS and enzymes catabolizing HS. Knowledge of the correlation between HS and KS or DS concentrations in the urine and blood samples from these patients will be of great interest. Elevation of urine HS in MPS IVA patients was confirmed by HPLC, although there was a difference in the magnitude of elevation. The discrepancy in the ratio of urine HS by ELISA to that by HPLC among MPS I, II, III patients and MPS IVA patients or normal controls may arise because (1) the HS-ELISA kit detects HS with both epitopes of two different anti-HS antibodies specifically (one for nonsulphated glucosamine residue and the other for *N*-sulphated glucosamine), and (2) anti-HS monoclonal antibodies recognize oligosaccharides larger than penta- or hexasaccharides (Leteux et al 2001; Van den Born et al 1995) while HPLC can detect the disaccharides produced by heparitinase I digestion. Therefore, the ELISA method may miss tetrasaccharides or smaller oligosaccharides. In addition, the discrepancy may be related to the diversity of HS molecules, such as differences in sulphation, the composition of non-, mono-, di-, and tri-sulphated HS, and the molecular size of HS in different types of MPS and ML patients as described previously (Byers et al 1998). The main difference between the two methods is that, in the previous study, GAGs were digested by lysosomal hydrolase digestion, followed by the gradient-PAGE, while our study did not use a digestion step. Therefore, our method can recognize molecules larger than pentasaccharides, while the other method recognizes smaller molecules.

Interestingly, patients with GM<sub>1</sub>-gangliosidosis, galactosialidosis or infantile sialic acid storage disease also showed marked elevation in urine HS concentrations. It is well known that those disorders store predominantly oligosaccharide derivatives. This study suggests that sulphated oligosaccharides derived from HS also accumulate in these disorders. Another recent report also suggested that sialidosis patients had an increase in the HS disaccharide (Meikle et al 2004). Moreover, 40% of other LSD patients including Fabry, Gaucher, Niemann–Pick A and B and GM<sub>1</sub>-gangliosidosis patients had elevation of blood HS, although the magnitude of elevation in plasma was lower compared to that in MPS I, II, III, VI and VII and ML II and ML III patients.

There are established procedures for measuring HS. HPLC is a sensitive and accurate method and has provided a substantially wider range of effective analysis in urine samples from MPS and ML patients in our current study. However, HPLC is not appropriate for mass screening because of the high cost and the complicated procedure (Yoshida et al 1989) and still needs modification to assay blood HS accurately. Tandem mass spectrometry has been introduced to measure disaccharides from HS (Fuller et al 2004; Ramsay et al 2003) or oligosaccharides derived from HS (Oguma et al 2001), but the equipment and running expenses are high, with multiple pretreatment steps being required. Overall, the present HS assay based on sandwich ELISA is reproducible, sensitive and specific for HS, as well as simple and less costly (a few dollars per sample) compared to other methods.

Based on the simplicity and low cost of the method and the current results, we propose that measurement of plasma HS by ELISA can be a useful biomarker, not only for primary HS storage diseases (MPS I, II, III and VII, ML II and ML III) but also for secondarily elevated MPS VI individuals, to discriminate them from normal populations.

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