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## Comparison of Liquid Chromatography–Tandem Mass Spectrometry and Sandwich ELISA for Determination of Keratan Sulfate in Plasma and Urine

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**Abstract:**

**Background and aim:** Mucopolysaccharidosis IVA (MPS IVA) leads to skeletal dysplasia through excessive storage of chondroitin-6-sulfate and keratan sulfate (KS). KS is synthesized mainly in cartilage and released into circulation, making it a critical biomarker for MPS IVA to evaluate clinical course and effectiveness of therapies. Therefore, an accurate and sensitive method is required to measure KS levels.

**Material and methods:** Using sandwich ELISA and liquid chromatography tandem mass spectrometry (LC/MS/MS) assays, we measured KS levels in blood and urine from MPS IVA patients and healthy controls to evaluate comparability of results. Blood (patients, n = 110; controls, n = 364) and urine (patients, n = 103; controls, n = 326) specimens were obtained.

**Results:** Plasma and urine KS measurements in patients were age-dependent and higher than age-matched controls. We observed a moderate correlation ( $r = 0.666$ ;  $P < 0.001$ ) between urine KS measurements and a weak correlation ( $r = 0.333$ ;  $P = 0.002$ ) between plasma KS measurements by ELISA and LC/MS/MS methods in patients. No correlation was found between plasma KS measurements in controls. The difference between KS measurements assayed by LC/MS/MS and ELISA was greater in controls than in patients. A moderate correlation between blood and urine KS measurements in the same individual was observed.

**Conclusion:** These findings indicate that both methods to measure blood and urine KS are suitable for diagnosis, monitoring therapies, and longitudinal assessment of the disease course in MPS IVA, but the LC/MS/MS method measures over 10 times more KS present in body fluids.

**Keywords:** biomarker, clinical severity, correlation, monitor therapy, MPS IVA, Mucopolysaccharidosis IVA

*Biomarker Insights* 2011;6 69–78

doi: [10.4137/BMI.S7451](https://doi.org/10.4137/BMI.S7451)

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## Introduction

Mucopolysaccharidosis IVA (MPS IVA, Morquio A disease) is an autosomal recessive lysosomal storage disease characterized by deficiency of N-acetylgalactosamine-6-sulfate sulfatase (GALNS, E.C.3.1.6.4, MIM# 253000). GALNS is one of several enzymes required for the breakdown of the glycosaminoglycans (GAGs), chondroitin-6-sulfate (C6S) and keratan sulfate (KS). Deficiency of GALNS activity results in the build-up of C6S and KS in lysosomes leading to progressive skeletal dysplasia.<sup>1-4</sup> Consequently, excessive undegraded KS synthesized in cartilage cells is released into circulation and is thus an important biomarker for assessing MPS IVA.

In healthy individuals, blood KS levels rise progressively during the first 4 years of life and remain elevated until 12 years of age. At that time, KS levels decline markedly and after 15 years of age the levels continue to fall gradually until they stabilize around age 20.<sup>5,6</sup> Elongation of the long bones during growth occurs by a process of endochondral ossification in which new cartilage is laid down before it is degraded and replaced by bone. The decline of KS levels after 13 years of age is consistent with the fact that the growth rate in normal children decreases after age 13. In MPS IVA patients, plasma KS levels peak between 5 and 10 years of age while urine KS levels peak between ages 0 to 5.<sup>7,8</sup> Blood and urine KS levels are higher in MPS IVA patients than in age-matched controls under 20 years of age. Urine KS levels remain higher in MPS IVA patients than controls after 20 years of age, but plasma KS levels tend to normalize by age 20.<sup>6-8</sup>

Analysis of blood and urine KS concentrations is a useful tool in assessing the clinical status of patients with MPS IVA. It will be also useful in evaluating response to treatments such as enzyme replacement therapy (ERT), hematopoietic stem cell transplantation (HSCT), substrate reduction therapy and gene therapy. Currently, the clinical trials for MPS IVA by ERT are underway and investigational trials for HSCT are also in progress.<sup>9,10</sup> Gene therapy is under development.<sup>11</sup> Thus, a biomarker to monitor the efficacy of therapies is indispensable as the secondary clinical endpoint. KS is also an important biomarker in other disorders where cartilage catabolism is present, such as osteoarthritis, rheumatoid arthritis, and other types of MPS.<sup>12-16</sup>

Various methods have been established for the analysis of blood and urine KS levels, each with different benefits. Thin-layer chromatography<sup>17</sup> (TLC) is quick and inexpensive, yet provides poor quantitation and accuracy. Colorimetric analysis by use of dimethylmethylene blue<sup>18,19</sup> is not satisfactory in selectivity. Inhibition enzyme-linked immunosorbent assay<sup>12</sup> offers accurate, reproducible results, yet requires multiple laborious steps. The sandwich ELISA method<sup>6,15</sup> allows a faster and simpler method that provides high specificity to KS. Most recently, a liquid chromatography tandem mass spectrometry (LC/MS/MS) method has been developed,<sup>8,20</sup> which is highly specific and sensitive to measure KS. Overall, the sandwich ELISA and LC/MS/MS methods appear most fit for accurately measuring large sample sizes. Therefore, it is important to examine the correlation between values measured by two different assay systems. In this study, human plasma and urine KS levels from MPS IVA patients and healthy controls were determined by sandwich ELISA and LC/MS/MS methods to evaluate whether results from each method are comparable. We also examine plasma and urine levels measured in the same individual to test for correlation.

## Materials and Methods

### Sample collection

Blood (plasma) samples were obtained from 474 individuals (110 MPS IVA patients, 364 healthy controls) and urine samples were obtained from 429 individuals (103 MPS IVA patients, 326 healthy controls) after informed consent was obtained from each individual according to IRB protocol at Saint Louis University. One hundred twenty-one individuals provided both blood and urine samples (91 MPS IVA patients, 30 healthy controls). Both plasma and urine samples were used for measurement of KS without dilution (2–20  $\mu$ l) for ELISA and LC/MS/MS methods.

### Sandwich ELISA method

Plasma and urine KS analyses were carried out as described previously.<sup>5</sup> Briefly, analyses were performed with a commercially available KS-ELISA kit (Code: 280565) from Seikagaku (Tokyo, Japan). Antibody pre-coated plates were made by adding antibody solution to each well of the microtiter plate. The anti-KS monoclonal antibody (5-D-4) was



biotinylated using NHS-Biotin from Pierce Chemical (Rockford, IL, USA). Washing buffer was added to the microplate by multi-pipette then discarded. Samples of KS standards and diluted unknown samples were added to the wells and incubated at 37 °C. The plate wells were then washed with the washing buffer. Next, horseradish peroxidase conjugated streptavidin and biotinylated antibody were added to the plate and incubated at 37 °C. After washing the plate, substrate solution tetramethylbenzidine was added to the plate and incubated at room temperature. The reaction was then stopped with 1 N HCl. The absorbance was measured at 450 nm with a microplate spectrophotometer. KS concentration was read by applying the absorbances of each sample to the calibration curve.

### LC/MS/MS method

Plasma and urine KS analyses were carried out by the method previously described.<sup>8,20</sup> Briefly, LC/MS/MS (API 4000 mass spectrometer equipped with a turbo-ion spray: Applied Biosystems, Foster City, CA) was used to analyze the disaccharides produced from KS. KS was digested to disaccharides by keratanase II (Seikagaku Corporation, Tokyo, Japan). The disaccharides were analyzed by LC/MS/MS using multiple reactions monitoring in negative ion mode. Separation was achieved by LC on a Hypercarb column (2.0 mm internal diameter (i.d.) × 150 mm, 5 μm) with gradient elution by acetonitrile–0.01 M ammonium bicarbonate (pH 10). The flow rate of the mobile phase was 0.2 ml/min. The disaccharide compositions of Galβ1→4GlcNAc(6S) and Galβ1(6S)→4GlcNAc(6S) were recognized. The C-6 position of the GlcNAc residue or both the Gal and GlcNAc residues was sulfated. Samples with KS concentrations were assayed in duplicate using the appropriate dilution.

### Data analysis

The correlation between ELISA and LC/MS/MS estimates was tested by a simple linear regression analysis. We interpreted correlation strength as outlined by Johnston.<sup>21</sup> Briefly, correlations are interpreted based on *r* values as follows: 0.0 to 0.2, very weak to negligible correlation; 0.2 to 0.4, weak correlation; 0.4 to 0.7, moderate correlation; 0.7 to 0.9, strong correlation. Data points that were greater than three standard deviations from the mean for

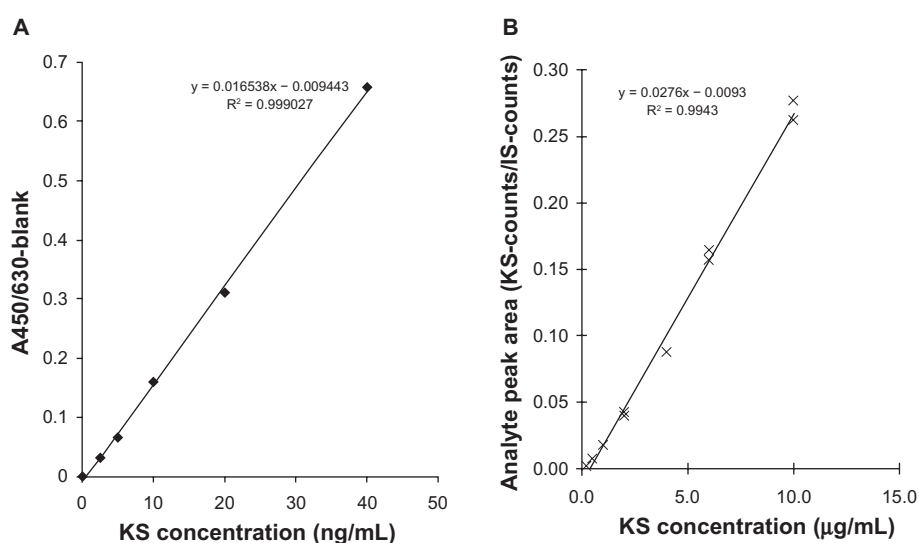
each assay were considered outliers. Analysis was performed using SPSS for Windows (version 17.0, SPSS Inc., Chicago, IL, USA).

### Results and Discussion

Cartilage contains several proteoglycans that are necessary for its normal function. Of these cartilage proteoglycans, KS contributes to the structures of aggrecan, fibromodulin, and lumican.<sup>22</sup> The size of KS and sulfation of galactose increase with age.<sup>8,23</sup> When KS is not degraded properly, it is stored mainly in chondrocytes, where it is synthesized.<sup>24</sup> The accumulation of undegraded KS in MPS IVA patients leads to disruption of cartilage cells resulting in systemic skeletal dysplasia.<sup>25</sup> Although pathological examination of bone and cartilage cells is useful for the diagnosis and staging of MPS IVA, it is not feasible to obtain biopsy samples from MPS IVA patients. It has been suggested that measurements of KS concentrations in plasma and urine samples provide useful information about the diagnosis and the clinical status of MPS IVA patients and the efficacy of treatments.<sup>6–8,26,27</sup>

Two current techniques for measuring plasma and urine KS concentrations are based upon the sandwich ELISA and LC/MS/MS methods. One advantage to using LC/MS/MS is that this method provides higher sensitivity. While the current version of ELISA kit used in the study could not detect below 2.5 ng/ml of KS, LC/MS/MS could detect 0.2 ng/ml of KS. Consequently, plasma KS in mice was measurable by the LC/MS/MS method.<sup>26,27</sup> However, this ELISA method is advantageous over LC/MS/MS since it does not require large, costly equipment, making it affordable to be used in most labs. The results of these two methods have never been compared quantitatively. In this study, we have compared measurements obtained by both methods.

We found that optimized KS calibration curves gave a good standard curve ( $R^2 = 0.99$ ) between 2.5 ng/mL and 40 ng/mL in ELISA (Fig. 1A) and 0.2 μg/ml and 10 μg/ml in LC/MS/MS (Fig. 1B). Plasma and urine KS measurements in MPS IVA patients were age-dependent and appreciably higher than age-matched controls (Tables 1 and 2; Figs. 2 and 3). Plasma KS concentrations measured by ELISA increased until age 10 years, at which point they fell gradually until normalizing around age 20



**Figure 1.** Standard curve for the KS ELISA and LC/MS/MS. **A)** Standard curve for the KS ELISA. For validation, two different KS concentration quality control samples were prepared (control KS was purchased from Seikagaku Co. Code.400760). The absorbances (minus blank) were plotted using KS (2.5, 5.0, 10.0, 20.0, 40.0 ng/mL) standards. **B)** Standard curve for the KS LC/MS/MS. For validation, two different KS concentration quality control samples were prepared (control KS was purchased from Seikagaku Co. Code.400760). The corrected KS counts (KS counts in the analyte peak area divided by IS counts) were plotted using KS (0.2, 0.5, 1.0, 2.0, 4.0, 6.0, 10.0  $\mu$ g/mL) standards (IS, chondrosine, was purchased from Seikagaku Co. Code.400430).

years. Plasma KS measurements by LC/MS/MS were highest in the first 5 years of life, and declined gradually until reaching a plateau near 20 years of age. Urine KS concentrations were highest during the first 5 years of life. After 5 years of age, urine KS levels

declined until stabilizing around 20 years of age. This age-dependent pattern was observed with both ELISA and LC/MS/MS urine KS measurements.

Plasma and urine KS levels in the same individual were also compared. A moderate correlation was

**Table 1.** Plasma KS level.

Age	Mean KS	SD	Maximum	Minimum	n	Average age
<b>Plasma KS (<math>\mu</math>g/ml) by ELISA method</b>						
Control (all ages)	0.128	0.076	0.365	0.003	212	19.2
>0, $\leq$ 5 y	0.089	0.067	0.353	0.003	90	0.9
>5, $\leq$ 10 y	0.237	0.088	0.365	0.048	12	8.7
>10, $\leq$ 15 y	0.185	0.067	0.328	0.086	11	13.1
>15 y	0.143	0.060	0.352	0.015	99	37.8
MPS IVA (all ages)	0.505	0.297	1.388	0.082	109	12.9
>0, $\leq$ 5 y	0.565	0.264	1.112	0.082	29	3.0
>5, $\leq$ 10 y	0.686	0.294	1.388	0.128	31	7.5
>10, $\leq$ 15 y	0.463	0.306	1.158	0.177	17	12.5
>15 y	0.297	0.174	0.717	0.089	32	27.2
<b>Plasma KS (<math>\mu</math>g/ml) by LC/MS/MS method</b>						
Control (all ages)	1.803	0.757	6.189	0.329	165	13.6
>0, $\leq$ 5 y	1.843	0.634	4.555	0.394	77	1.4
>5, $\leq$ 10 y	1.669	0.663	3.349	0.329	23	7.8
>10, $\leq$ 15 y	1.813	1.465	6.189	0.428	18	12.9
>15 y	1.801	0.604	3.717	0.676	47	36.7
MPS IVA (all ages)	5.723	3.072	14.076	0.630	85	12.1
>0, $\leq$ 5 y	7.445	3.042	13.891	1.738	25	3.0
>5, $\leq$ 10 y	6.281	3.101	14.076	0.630	25	7.1
>10, $\leq$ 15 y	5.476	2.652	9.632	1.788	14	12.7
>15 y	3.173	1.207	5.329	1.343	21	28.6

**Table 2.** Urine KS level.

Age	Mean KS	SD	Maximum	Minimum	n	Average age
<b>Urine KS (mg/g creatinine) by ELISA method</b>						
Control (all ages)	0.209	0.165	1.240	0.000	179	11.8
>0, ≤5 y	0.241	0.185	1.240	0.000	104	1.2
>5, ≤10 y	0.235	0.149	0.485	0.068	13	7.9
>10, ≤15 y	0.245	0.154	0.618	0.052	13	12.7
>15 y	0.127	0.079	0.418	0.029	49	35.1
MPS IVA (all ages)	7.741	6.600	27.200	0.083	100	13.3
>0, ≤5 y	12.384	6.610	25.700	2.600	23	3.2
>5, ≤10 y	10.464	6.676	27.200	0.431	31	7.7
>10, ≤15 y	4.612	3.372	13.900	0.083	17	12.6
>15 y	2.983	3.180	13.900	0.114	29	27.8
<b>Urine KS (mg/g creatinine) by LC/MS/MS method</b>						
Control (all ages)	8.943	7.640	34.589	0.774	134	6.5
>0, ≤5 y	11.275	7.967	34.589	1.992	94	1.7
>5, ≤10 y	4.380	1.180	5.835	2.521	7	8.7
>10, ≤15 y	3.903	2.256	9.547	1.205	19	11.8
>15 y	2.413	1.345	4.596	0.774	14	31.0
MPS IVA (all ages)	72.163	50.949	205.818	2.937	58	13.9
>0, ≤5 y	141.588	32.684	205.818	100.964	9	2.8
>5, ≤10 y	82.840	48.704	187.770	2.937	21	7.7
>10, ≤15 y	55.165	33.721	136.056	15.057	11	12.8
>15 y	33.216	19.144	70.882	3.644	17	28.1

observed between plasma and urine KS levels in MPS IVA patients when measured by LC/MS/MS ( $r = 0.469$ ;  $P = 0.004$ ; Fig. 4A) as well as by ELISA ( $r = 0.457$ ;  $P < 0.001$ ; Fig. 4B). The data were insufficient to establish age-dependent correlations between plasma and urine KS levels.

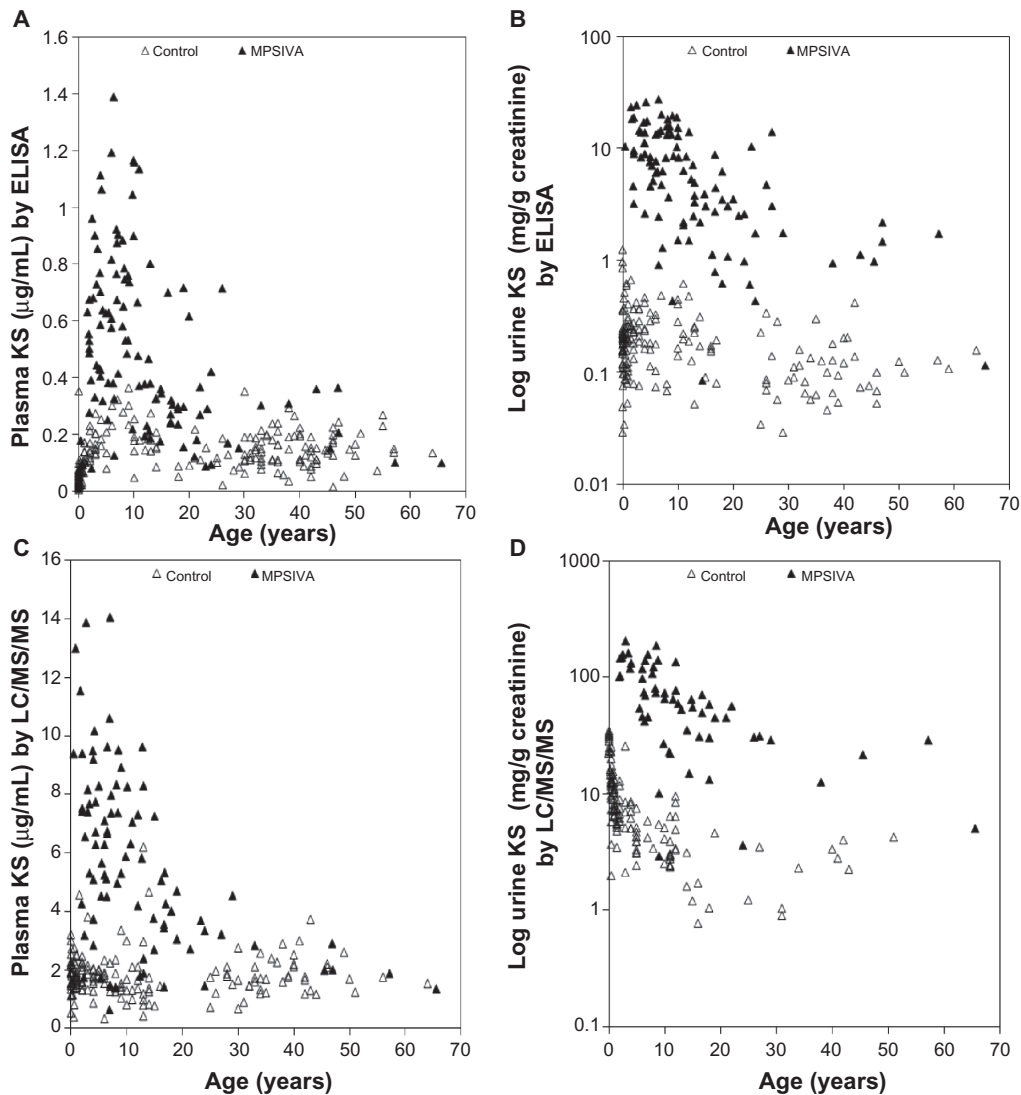
We observed a moderate correlation between urine KS measurements assayed by ELISA and LC/MS/MS methods in MPS IVA patients ( $r = 0.666$ ;  $P < 0.001$ ; Fig. 4C). The correlation between urine KS measurements by both methods was strong in patients over age 15 ( $r = 0.793$ ;  $P < 0.001$ ;  $n = 18$ ), and moderate in the 5–55 age group ( $r = 0.502$ ;  $P = 0.005$ ;  $n = 30$ ). We could not establish a correlation in the 0–5 age group as a result of insufficient data. A weak correlation ( $r = 0.333$ ;  $P = 0.002$ ; Fig. 4D) was observed between plasma KS measurements assayed by both methods for MPS IVA patients. Age-dependent correlations between plasma KS measurements assayed by each method were not significant since there were a limited number of observations in each age group.

Plasma KS measurements by ELISA and LC/MS/MS were also compared in healthy controls. The correlation between methods was negligible ( $r = -0.095$ ;  $P = 0.658$ ; Fig. 4E). The data were

insufficient to compare ELISA and LC/MS/MS urine KS measurements in healthy controls.

Urine KS levels measured by LC/MS/MS in MPS IVA patients were 9.3 times higher than those measured by ELISA, while KS levels measured by LC/MS/MS in healthy controls were 42.8 times higher than those by ELISA. Plasma KS levels measured by LC/MS/MS in MPS IVA patients were 11.3 times higher than by ELISA, and 14.1 times higher for controls. The largest difference between ELISA and LC/MS/MS measurement values in plasma and urine KS were observed in the 0–5 years of age group, all other age groups being fairly homogeneous.

Plasma and urine KS levels in MPS IVA patients measured by ELISA in this study are age-dependent and comparable to those reported previously.<sup>6,8</sup> Age-dependent urine KS levels in MPS IVA patients measured by LC/MS/MS have not been previously reported. We found that urine KS measurements by LC/MS/MS follow a pattern similar to those found by the ELISA method. Plasma KS measurements by LC/MS/MS were age-dependent and comparable to those previously reported.<sup>7</sup> There is currently no explanation why blood and urine KS levels follow different age-dependent patterns. One hypothesis is that the origin



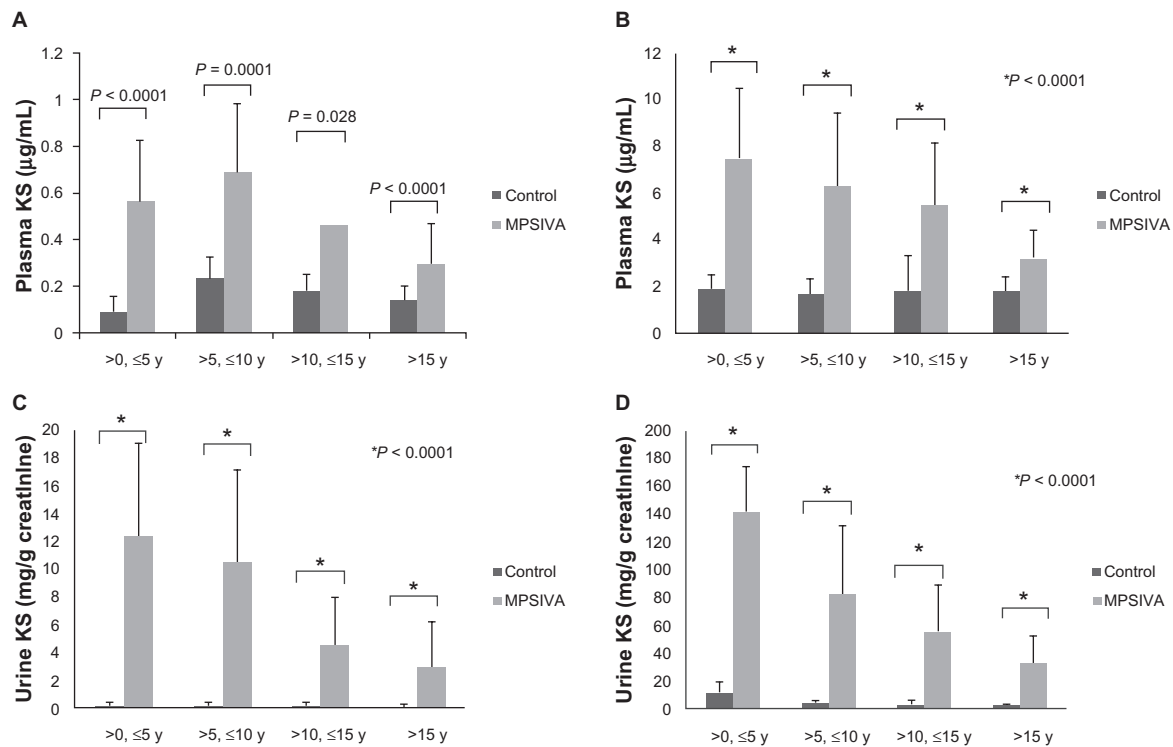
**Figure 2.** Concentrations of plasma and urine KS of patients with MPS IVA and normal individuals as measured by ELISA and LC/MS/MS. **A)** Plasma KS results by ELISA of 109 specimens from MPS IVA individuals and 212 normal individuals are plotted with respect to age. **B)** Urine KS results by ELISA of 100 specimens from MPS IVA individuals and 179 normal individuals are plotted with respect to age. **C)** Plasma KS results by LC/MS/MS of 85 specimens from MPS IVA individuals and 165 normal individuals are plotted with respect to age. **D)** Urine KS results by LC/MS/MS of 58 specimens from MPS IVA individuals and 134 normal individuals are plotted with respect to age.

of blood and urine KS is different. While blood KS is derived directly from cartilage cells, only smaller KS molecules are filtered in the kidney and excreted in urine. Another hypothesis is that urine concentration is reflected by age. Infants, who will not control urine concentration adequately, could have higher KS values corrected by lower creatinine level. Despite the difference in age-dependent patterns, our results reveal a moderate correlation between blood and urine KS levels measured in the same individual.

The moderate correlation between ELISA and LC/MS/MS urine KS measurements in MPS IVA patients indicates that results from each assay are comparable.

Plasma KS measurements by ELISA are less comparable to those by LC/MS/MS than urine KS measurements. Plasma KS measurements by ELISA and LC/MS/MS in healthy individuals showed no correlation. We could not conclude whether ELISA and LC/MS/MS urine KS measurements are correlated in controls because of data limitations. The reason for the variation in correlation strengths for each regression could be due to a difference in the ability of each assay to detect non-sulfated galactose (see below).

The GALNS enzyme catalyzes the hydrolysis of the 6-sulfate group of the D-galactose 6-sulfate unit of KS. Therefore, the level of GALNS enzyme activity



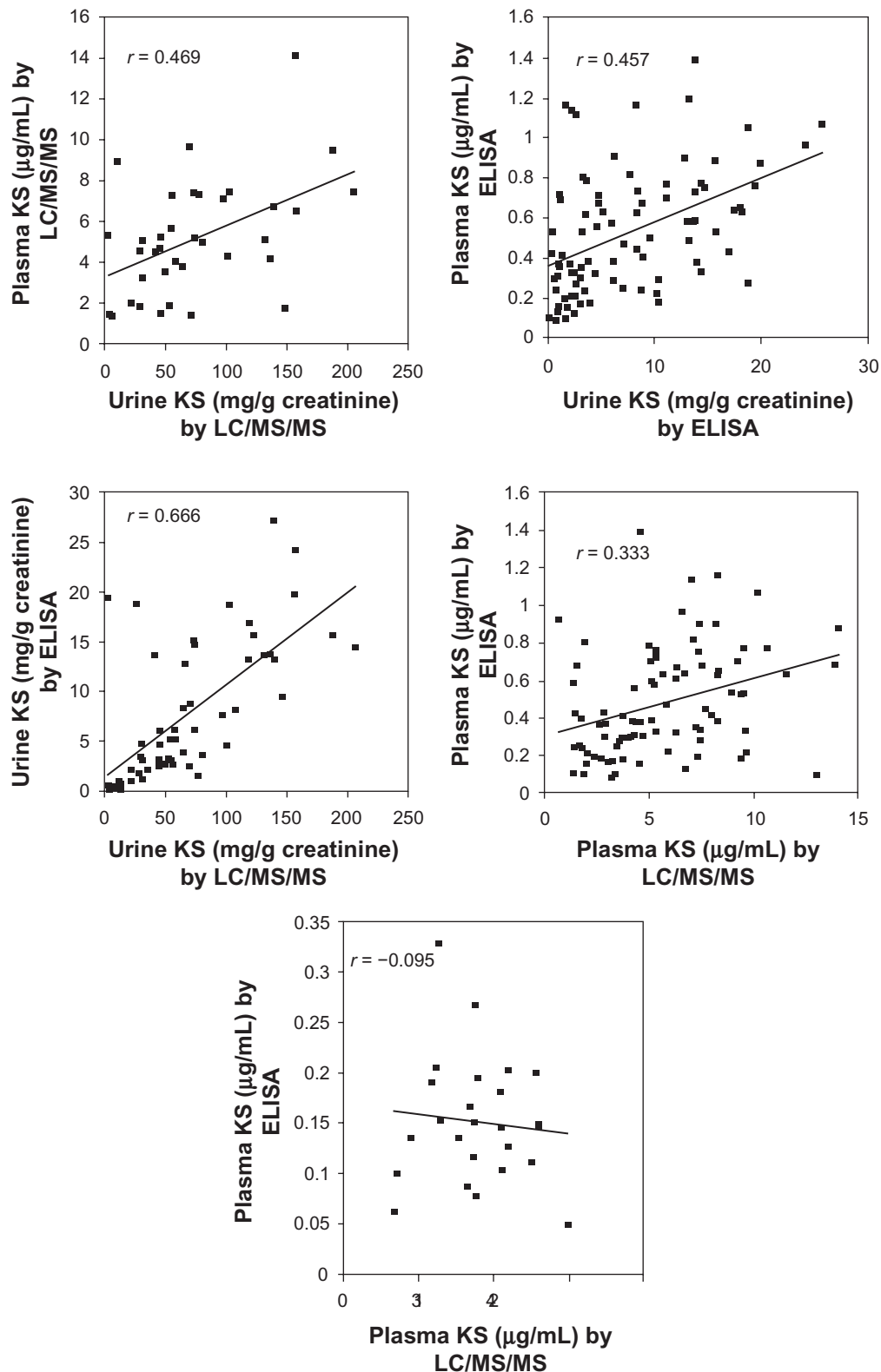
**Figure 3.** Comparison of plasma and urine KS levels between MPS IVA patients and normal individuals by ELISA and LC/MS/MS methods. **A)** Plasma KS results by ELISA. **B)** Plasma KS results by LC/MS/MS. **C)** Urine KS results by ELISA. **D)** Urine KS results by LC/MS/MS. Plasma and urine KS levels by both methods are significantly higher in MPS IVA patients at any age.

determines the ratio of Gal $\beta$ 1(6S) $\rightarrow$ 4GlcNAc(6S) to Gal $\beta$  $\rightarrow$ 4GlcNAc(6S) that is present in blood and urine. Thus, this ratio is higher in MPS IVA patients, where the GALNS enzyme is deficient, than in healthy individuals.<sup>8</sup> The ratio of Gal $\beta$ 1(6S) $\rightarrow$ 4GlcNAc(6S) present in blood varies by age. Briefly, when control subjects and MPS IVA patients in several age ranges (2–5 years, 5–10 years, 10–15 years, and over 15 years) were compared, their ratios of Gal $\beta$ 1(6S) $\rightarrow$ 4GlcNAc(6S) to Gal $\beta$  $\rightarrow$ 4GlcNAc(6S) were as follows: control subjects vs. MPS IVA patients, 17.5% vs. 19.7%; 17.3% vs. 21.3%; 17.6% vs. 28.5%; 23.6% vs. 23.1%, respectively.<sup>8</sup> The ratio of Gal $\beta$ 1(6S) $\rightarrow$ 4GlcNAc(6S) increases with age especially in MPS IVA patients. The compositional ratio of Gal $\beta$ 1(6S) $\rightarrow$ 4GlcNAc(6S) to Gal $\beta$  $\rightarrow$ 4GlcNAc(6S) present in urine has not been reported in previous and current studies.

The monoclonal antibody used for the ELISA in the present study involves sulfated hepta- or larger oligosaccharides of KS<sup>28</sup> and is specific for Gal $\beta$ 1(6S) $\rightarrow$ 4GlcNAc(6S), meaning both galactose and N-acetyl-glucosamine must be sulfated for recognition.<sup>29</sup> Hence, the ELISA method does not

reflect total KS quantification in a specimen. Meanwhile, the LC/MS/MS method provides more complete quantification of KS by utilizing keratanase II for KS digestion. Keratanase II recognizes both Gal $\beta$  $\rightarrow$ 4GlcNAc(6S) and Gal $\beta$ 1(6S) $\rightarrow$ 4GlcNAc(6S) disaccharides, in which N-acetyl-glucosamine is sulfated. All KS molecules digested by keratanase II turn into disaccharides. Thereby, in spite of the original size of KS molecule, it is measurable. Thus, the LC/MS/MS method is more efficient at measuring KS contained in a specimen than the ELISA method.

As previously noted, LC/MS/MS measurements were markedly higher than ELISA measurements. However, the difference between LC/MS/MS and ELISA measurements was greater in healthy controls than in MPS IVA patients. This is due to GALNS activity in healthy controls producing more Gal $\beta$  $\rightarrow$ 4GlcNAc(6S) component which is not detectable by ELISA. Conversely, a deficiency in GALNS activity in MPS IVA patient reduces the amount of Gal $\beta$  $\rightarrow$ 4GlcNAc(6S) component that is not measured by ELISA. The greatest gap between ELISA and LC/MS/MS was observed in urine measurements in controls. This suggests that the



**Figure 4.** Correlation in KS concentrations. **A)** Correlation between plasma and urine KS as measured by LC/MS/MS in MPS IVA patients ( $n = 36$ ,  $P = 0.004$ ). **B)** Correlation between plasma and urine KS as measured by ELISA in MPS IVA patients ( $n = 87$ ,  $P < 0.001$ ). **C)** Correlation between LC/MS/MS and ELISA methods by urine KS levels in MPS IVA patients ( $n = 55$ ,  $P < 0.001$ ). **D)** Correlation between LC/MS/MS and ELISA methods by plasma KS levels in MPS IVA patients ( $n = 83$ ,  $P = 0.002$ ). **E)** Correlation between LC/MS/MS and ELISA methods by plasma KS in healthy controls ( $n = 24$ ,  $P = 0.658$ ).





Gal $\beta$ 1(6S) $\rightarrow$ 4GlcNAc(6S) to Gal $\beta$  $\rightarrow$ 4GlcNAc(6S) ratio is lowest in the urine of healthy controls. Thus, the ELISA assay does not reflect the total urine KS level in healthy controls. The smallest gap was observed in MPS IVA urine measurements, suggesting that the ratio between Gal $\beta$ 1(6S) $\rightarrow$ 4GlcNAc(6S) and Gal $\beta$  $\rightarrow$ 4GlcNAc(6S) is high in patients urine. Overall, the gap between ELISA and LC/MS/MS measurements was highest in the 0–5 age group in controls and patients, and was fairly uniform in all other age groups. These results support the previous findings that the sulfation of KS increases with age.<sup>8,23</sup> Therefore, the ELISA method may be less effective at measuring total KS in children under age 5 years than in older individuals. The Gal $\beta$ 1(6S) $\rightarrow$ 4GlcNAc(6S) to Gal $\beta$  $\rightarrow$ 4GlcNAc(6S) ratio is a significant factor to consider when comparing ELISA and LC/MC/MC assay measurements, but each method is suitable for measuring urine and plasma KS levels in MPS IVA patients.

Our findings suggest that both blood and urine KS are a good biomarker for MPS IVA. Blood KS has a narrower ranged value and will be directly reflected for cartilage disruption and/or repair after therapy. Dried blood spot can be a quite useful tool for transport of samples and newborn screening. Meanwhile, urine KS has a broad ranged value and will be indirectly reflected for cartilage condition. Advantage to use urine KS is less invasive although creatinine has to be measured for correction. Why urine KS in MPS IVA patients over 20 years old can not be normalized remains unanswered. It will be of great interest to understand the origin of blood and urine KS and the metabolism pathway of KS from cartilage to urine.

In conclusion, we have suggested that both the ELISA and LC/MS/MS methods of measuring urine KS in MPS IVA patients yield comparable results, although plasma KS results are less comparable between methods. The ELISA method is less effective at measuring total KS. However, both methods have reproducibility, accuracy, and high throughput for MPS IVA patients. Overall, both methods should provide a useful tool to measure blood and urine KS to assess the clinical status of MPS IVA patients and to measure the response to treatments such as ERT, HSCT, SRT, and gene therapy.

## Acknowledgements

This study was supported by grants from the International Morquio Organization, the Austrian Research Society for Mucopolysaccharidoses and Related Diseases, and Bennett Foundation.

## Disclosure

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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