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# Glycosaminoglycan levels in dried blood spots of patients with mucopolysaccharidoses and mucolipidoses

Francyne Kubaski [a,b,c], Yasuyuki Suzuki [d], Kenji Orii [e], Roberto Giugliani [c,f,g], Heather J. Church [h], Robert W. Mason [a,b], Vũ Chí Dũng [i], Can Thi Bich Ngoc [i], Seiji Yamaguchi [j], Hironori Kobayashi [j], Katta M. Girisha [k], Toshiyuki Fukao [e], Tadao Orii [e], Shunji Tomatsu [a,e,*]

[a] Nemours/Alfred I. duPont Hospital for Children, Wilmington, DE, United States
[b] Department of Biological Sciences, University of Delaware, Newark, DE, United States
[c] INAGEMP, Porto Alegre, Brazil
[d] Medical Education Development Center, Gifu University, Japan
[e] Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Japan
[f] Medical Genetics Service, HCPA, Porto Alegre, Brazil
[g] Department of Genetics, UFRGS, Porto Alegre, Brazil
[h] Willink Biochemical Genetics Unit, Genomic Diagnostics Laboratory, Manchester Centre for Genomic Medicine, Central Manchester University Hospitals NHS Foundation Trust St Mary's Hospital, Manchester, UK
[i] Vietnam National Children's Hospital, Department of Medical Genetics, Metabolism & Endocrinology, Hanoi, Vietnam
[j] Department of Pediatrics, Shimane University, Shimane, Japan
[k] Department of Medical Genetics, Kasturba Medical College Manipal, Manipal University, India

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ABSTRACT

Mucopolysaccharidoses (MPSs) and mucolipidoses (ML) are groups of lysosomal storage disorders in which lysosomal hydrolases are deficient leading to accumulation of undegraded glycosaminoglycans (GAGs), throughout the body, subsequently resulting in progressive damage to multiple tissues and organs. Assays using tandem mass spectrometry (MS/MS) have been established to measure GAGs in serum or plasma from MPS and ML patients, but few studies were performed to determine whether these assays are sufficiently robust to measure GAG levels in dried blood spots (DBS) of patients with MPS and ML.

*Material and methods:* In this study, we evaluated GAG levels in DBS samples from 124 MPS and ML patients (MPS I = 16; MPS II = 21; MPS III = 40; MPS IV = 32; MPS VI = 10; MPS VII = 1; ML = 4), and compared them with 115 age-matched controls. Disaccharides were produced from polymer GAGs by digestion with chondroitinase B, heparitinase, and keratanase II. Subsequently, dermatan sulfate (DS), heparan sulfate (HS-0S, HS-NS), and keratan sulfate (mono-sulfated KS, di-sulfated KS, and ratio of di-sulfated KS in total KS) were measured by MS/MS.

*Results:* Untreated patients with MPS I, II, VI, and ML had higher levels of DS compared to control samples. Untreated patients with MPS I, II, III, VI, and ML had higher levels of HS-0S; and untreated patients with MPS II, III and VI and ML had higher levels of HS-NS. Levels of KS were age dependent, so although levels of both mono-sulfated KS and di-sulfated KS were generally higher in patients, particularly for MPS II and MPS IV, age group numbers were not sufficient to determine significance of such changes. However, the ratio of di-sulfated KS in total KS was significantly higher in all MPS patients younger than 5 years old, compared to age-matched controls. MPS I and VI patients treated with HSCT had normal levels of DS, and MPS I, VI, and VII treated with ERT or HSCT had normal levels of HS-0S and HS-NS, indicating that both treatments are effective in decreasing blood GAG levels.

*Conclusion:* Measurement of GAG levels in DBS is useful for diagnosis and potentially for monitoring the therapeutic efficacy in MPS.

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## 1. Introduction

Mucopolysaccharidoses (MPSs) are a group of lysosomal storage disorders (LSDs) caused by a deficiency of lysosomal hydrolases

* Corresponding author at: Department of Biomedical Research Nemours, Alfred I. duPont Hospital for Children, 1600 Rockland Rd., Wilmington, DE. 19899-0269, United States.
*E-mail address:* stomatsu@nemours.org (S. Tomatsu).

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responsible for the catabolism of glycosaminoglycans (GAGs) [1,2]. Mucolipidoses (ML) are related diseases caused by a deficiency of *N*-acetylglucosaminyl-1-phosphotransferase. This enzyme deficiency produces unphosphorylated lysosomal enzymes, which leads to inhibition of reuptake of enzymes and accumulation of GAGs and lipids. MPSs and ML are classified according to the enzyme deficiency (Table 1).

The MPSs and ML are progressive LSDs that share many clinical features such as: coarse faces, neurological impairment (MPS I, II, III, VII and ML II), skeletal dysplasia (all, but maybe mild in MPS III), hepatosplenomegaly, joint rigidity, and heart valvular disease [3]. MPSs are usually asymptomatic at birth, and the initial signs and symptoms appear with progression of the disease during the first one or two years of age. Mucolipidoses II (ML II; I-cell disease) is fatal during childhood or the first decade of life, and can even produce intra-uterine fractures, while ML III has a milder somatic phenotype with slower progression throughout childhood but leads to severe neurodegeneration with a fatal outcome during adulthood [2,4].

ML II and III are caused by impaired trafficking of several lysosomal enzymes [2,5]. The prevalence of ML is variable among different populations: 0.3 cases per 100,000 live births in Australia, 0.16 per 100,000 live births in Portugal, and 0.08 per 100,000 live births in the Netherlands [6,7]. The incidence in Quebec, Canada is very high, 1:6184, due to a founder effect [8]. The combined incidence of MPSs is 1:25,000 live births, and therefore more common than ML [9].

Enzyme replacement therapy (ERT) is available commercially in practice for MPS I, II, VI, and IVA [10–13]. Hematopoietic stem cell transplantation (HSCT) is recommended for MPS I [14,15]. Several studies indicate that HSCT will also improve outcomes for MPS II [16–18], MPS IVA [19,20], MPS VI [21] and MPS VII [22].

Levels of GAGs in patients with MPS have been studied for several decades. Initially, total urinary GAGs were measured using a variety of dye methods [23–34]. Although these methods were useful and cost-effective; they gave high false-positive rates [35], could not be easily applied to measure GAGs in blood and/or tissues due to the presence of proteins and other interferent molecules, and could not distinguish specific GAG(s) [36]. Measurement of total urinary GAG using a dimethylmethylene blue (DMMB) method did not distinguish a substantial number of MPS IVA patients from age-matched controls [37–41]. The development of ELISA methods in early 90's made it possible to measure HS and KS in blood and urine of MPS and ML patients [40,42–44]. We used an ELISA method to show that KS levels in blood are elevated not only in MPS IV, but also in other types of MPS and ML [44]. However, ELISA assays are expensive and cannot distinguish subclasses of HS and KS. Since 2001, protocols have been developed for GAG analysis using tandem mass spectrometry (MS/MS). Two main branches of GAG detection methods by MS/MS have been developed: detection of digested disaccharides (direct or labeled with aniline) [45–50] and chemically depolymerized GAGs by methanolysis and/or butanolysis [51–56]. Such MS/MS methods have been used to measure specific GAGs in blood and urine of MPS and ML patients [51,54,55,57–65]. MS/MS provides a sensitive, specific, and reproducible GAG analysis and allows measurement of several GAGs simultaneously, indicating its potential for use in mass screening, prognosis, and monitoring therapeutic effect in patients with MPS and ML. More recently, MS/MS methods have been developed to measure GAGs in dried blood spots (DBS) [57,61,66,67].

In this study, we have simultaneously determined levels of dermatan sulfate (DS), heparan sulfate (HS-0S, HS-NS), and keratan sulfate (mono, di-sulfated, and ratio di-sulfated in total KS) in DBS of control subjects and patients with MPS I, II, III, IV, VI, VII; and ML II and III by liquid chromatography tandem mass spectrometry (LC/MS/MS). We have also evaluated GAG levels in ERT and HSCT treated patients with some types of MPS.

**Table 1**
Classification of MPS and ML.

| MPS | Enzyme deficiency | GAG or sphingolipid accumulated |
|---|---|---|
| MPS I | α-L-iduronidase (IDS) | DS & HS |
| MPS II | Iduronate-2-sulfatase (IDS) | DS & HS |
| MPS IIIA | Heparan-*N*-sulfatase | HS |
| MPS IIIB | α-*N*-acetylglucosaminidase | HS |
| MPS IIIC | Acetyl-CoA-α-glucosaminidase acetyltransferase | HS |
| MPS IIID | *N*-acetylglucosamine-6-sulfatase | HS |
| MPS IVA | *N*-acetylgalactosamine-6-sulfate sulfatase | C6S & KS |
| MPS IVB | β-Galactosidase | KS |
| MPS VI | *N*-acetylgalactosamine-4-sulfatase | DS |
| MPS VII | β-Glucuronidase | DS, CS, HS |
| MPS IX | Hyaluronidase | HA |
| ML II | *N*-acetylglucosaminyl-1-phosphotransferase | GAGs & sphingolipids |
| ML III | *N*-acetulglucosamine-1-phosphotransferase | |

DS: dermatan sulfate; CS: chondroitin sulfate; HS: heparan sulfate; HA: hyaluronan; KS: keratan sulfate.

## 2. Material and methods

### 2.1. Enzymes and standards

Chondroitinase B, heparitinase, keratanase II, chondrosine (internal standard-IS), and the unsaturated disaccharides: heparan ΔDi-0S [2-acetamido-2-deoxy-4-O-(4-deoxy-a-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose] (HS-0S), heparan ΔDi-NS [2-deoxy-2-sulfamino 4-O-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose] (HS-NS), chondro ΔDi-4S [2-acetamido-2-deoxy 3-O-(β-D-gluco-4-enepyranosyluronic acid)-4-O-D-sulfo-galactose] (Di-4S), mono-sulfated KS [Galβ1-4GlcNAc(6S)], and di-sulfated KS [Gal(6S) Galβ1-4GlcNAc(6S)] were all provided by Seikagaku Co (Tokyo, Japan). Stock solutions of HS-0S (100 μg/mL), HS-NS (100 μg/mL), Di-4S (250 μg/mL), mono- and di-sulfated KS (1000 μg/mL), and IS (5 μg/mL) were prepared in $ddH_20$ (Millipore Milli-Q Reference A+ System).

### 2.2. Samples

Whole blood was collected with EDTA by venipuncture and 150 μL of blood was spotted onto filter paper to create DBS. DBS from 106 untreated MPS and ML patients (MPS I = 7; MPS II = 21; MPS IIIA = 12, MPS IIIB = 17, MPS IIIC = 6, MPS III (undefined) = 2; MPS IVA = 28, MPS IVB = 2; MPS VI = 7; MLII = 3; ML III = 1), 18 treated MPS (MPS I with ERT = 6, MPS I with ERT + HSCT = 2, MPS I with HSCT = 1; MPS IIIA with HSCT = 1, MPS IIIB with HSCT = 2; MPS IVA with HSCT = 2; MPS VI with ERT = 2, MPS VI with HSCT = 1; MPS VII with HSCT = 1), and 115 control subjects. Diagnosis of MPS and ML was made with enzyme assay.

DBS from MPS patients were provided by Shimane University (Japan), Gifu University (Japan), St. Mary's Hospital (UK), and Kasturba Medical College Manipal University (India). Control samples were obtained from 15 volunteer subjects from Hospital de Clínicas de Porto Alegre (Brazil) and from subjects who had blood draws for clinical testing for non-metabolic disease from Shimane University (Japan). Informed consent was obtained at each Institute for all patient and control samples according to IRB approval at each institute.

All de-identified samples were shipped to Nemours/AIDHC and stored at −20 °C until the GAG assay was conducted. This study was approved by the Nemours IRB (protocol # 281498).

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