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Multiplex tandem mass spectrometry enzymatic activity assay for the screening and diagnosis of Mucolipidosis type II and III

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ABSTRACT

Mucolipidosis type II and III (MLII/III) is caused by defects in the mannose-6-phosphate system, which is essential to target most of the lysosomal hydrolases to the lysosome. MLII/III patients present with marked elevations in the activities of most lysosomal enzymes in plasma, but their profiles in dried blood spots (DBS) have not been well described. In the current study, we measured the activities of 12 lysosomal enzymes in DBS, among which acid sphingomyelinase, iduronate-2-sulfatase, and alpha-*N*-acetylglucosaminidase were significantly elevated in MLII/III patients when compared to random newborns. This sets the stage for using DBS to diagnose MLII/III. Furthermore, given an increasing number of lysosomal storage disorders are being included in the recommended uniform screening panel, our results also indicate that population-based newborn screening for MLII/III can be implemented with minimal efforts.

1. Introduction

Mucolipidosis type II (MLII, I-cell disease, OMIM 252500), type III alpha/beta (MLIII alpha/beta, pseudo-Hurler polydystrophy, OMIM 252600), and type III gamma (MLIII gamma, OMIM 252605) are a group of rare autosomal recessively inherited lysosomal storage disorders (LSDs), with an estimated combined prevalence of 0.22 to 2.70 per 100,000 live births [1].

MLII/III is associated with significant morbidity and mortality and presents as a broad clinical continuum with MLII and MLIII at the severe and mild end, respectively. MLII patients typically present prenatally or in early infancy and succumb to death between 5 and 8 years old [2]. Symptoms include coarse facial features, severe gingival hyperplasia, restricted joint mobility, dysostosis multiplex, organomegaly, cardiorespiratory insufficiency, umbilical hernias, failure to thrive, and mental and motor developmental abnormalities [2]. MLIII is generally attenuated and has a much broader phenotypic spectrum than MLII, with MLIII gamma patients being the least severely affected. The average age-of-onset for MLIII is 5 years old, with the characteristic but less pronounced and more slowly progressive symptoms, including dysmorphic features, skeletal changes, limited joint mobility, short stature, and carpal and/or tarsal tunnel syndrome. Survival varies from childhood to late adulthood [1–3].

MLII/III is caused by deficiency of *N*-acetyl-glucosamine-1-phosphotransferase (GlcNAc-PTase, EC 2.7.8.15), which transfers GlcNAc-1-P from UDP-GlcNAc to mannose residues on high mannose- or hybrid-type N-linked glycans on lysosomal acid hydrolases. The GlcNAc residue is then excised by *N*-acetylglucosamine-1-phosphodiester alpha-*N*-acetylglucosaminidase ("uncovering enzyme", EC 3.1.4.45) to expose the mannose-6-phosphate (Man-6-P) ligand, recognizable by Man-6-P receptors in the trans-Golgi network. Upon recognition, the proteins are transported to the *endo*-lysosomal compartment. The Man-6-P system is essential for targeting endoplasmic reticulum-derived lysosomal proteins to the lysosome, although alternative pathways exist for certain lysosomal proteins, including beta-glucosidase [4]. Without the Man-6-P system, the newly synthesized lysosomal proteins are missorted into extracellular space and their respective substrates accumulate in the lysosome, resulting in the characteristic "inclusion cells", along with the bone, connective tissue, and neurological symptoms typically observed in MLII/III patients.

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GlcNAc-PTase is an $\alpha_2\beta_2\gamma_2$ hexamer. *GNPTAB* encodes the α and β subunits, which are responsible for the catalytic function and substrate recognition, and their deficiency leads to MLII or MLIII alpha/beta [5,6]. *GNPTG* encodes the γ subunit, which facilitates the phosphorylation of a subset of lysosomal enzymes, and its deficiency leads to MLIII gamma [7]. Genotype-phenotype correlation has been described for *GNPTAB*, with frameshift or nonsense variants associated with MLII and missense variants with some residual activity associated with MLIII alpha/beta [1,5]. In contrast, all pathogenic variants in *GNPTG*, even null mutations, inevitably lead to the mild MLIII gamma phenotype [1]. No disease has been associated with deficiency of the "uncovering enzyme", but in theory it can present as a phenocopy of MLII/III [2].

Biochemically, MLII/III can be diagnosed by elevated activities of many, but not all, lysosomal hydrolases in plasma or serum, whereas the activities are within normal limits or slightly reduced in leukocytes [8–11]. In cultured fibroblasts from MLII/III patients, there is a characteristic pattern of multiple lysosomal enzymes being deficient, whereas the activities of the same enzymes are elevated in the culture medium [2]. In addition, GlcNAc-PTase activity can be measured in fibroblasts or leukocytes [10,11]. A battery of storage materials, including oligosaccharides, glycosaminoglycans (GAGs), and sulfatides, can be found elevated in urine or dried blood spots (DBS) [12–16]. However, biochemical testing cannot definitively differentiate MLII from MLIII, therefore molecular analysis of *GNPTAB* and *GNPTG* are indicated for stratification [11,17].

Currently, there is no curative or disease-modifying treatments for MLII/III. Stem cell transplant has been attempted with no apparent benefit [1,17]. However, novel therapies, including gene therapy and enzyme replacement therapy, for monogenetic conditions are advancing rapidly and treatment for MLII/III may be available in the near future [17,18]. Given that optimal therapeutic outcome can only be secured if initiated before or right after the onset of irreversible symptoms, newborn screening for MLII/III should be considered when the treatment becomes available [19,20].

Measurement of enzyme activity in DBS is the dominant testing modality to screen neonates for LSDs. Currently, Mucopolysaccharidosis type I (MPS-I, OMIM 607014, 607015, 607016), Pompe disease (OMIM 232300), and MPS-II (OMIM 309900) are on the Recommended Uniform Screening Panel (RUSP). Other LSDs, including Krabbe disease (OMIM 245200), Gaucher disease (OMIM 230800, 230900, 231000, 608013, 231005), and Fabry disease (OMIM 301500), are being screened for in certain states in the US and/or are being evaluated in ongoing pilot studies [21]. Neonates with reduced enzymatic activity are deemed screen-positives and follow up testing including biomarker and/or molecular analysis is indicated [22,23].

Given the biochemical derangement, we hypothesized that MLII/III patients could be identified through the detection of elevated activity (ies) of one or multiple lysosomal enzyme(s) in DBS. This is because the activities of most of the lysosomal enzymes are elevated in plasma but relatively unaltered in leukocytes in MLII/III [11,24]. Indeed, an MLIII patient was identified through newborn screening based on raised actives of two lysosomal enzymes in DBS [25]. In the current study, we demonstrated increased activities of multiple lysosomal hydrolases in DBS from 15 MLII/III patients as compared to that from random newborns, setting the stage for using DBS to diagnosis MLII/III. Our results also have great implication for the newborn screening of MLII/III. To the best of our knowledge, this study had the largest cohort of MLII/III patient DBS, with the largest battery of lysosomal enzyme activities measured.

2. Materials and methods

2.1. Materials

DBS samples from 1 MLII, 13 MLIII alpha/beta, and 1 MLIII gamma patients were collected with informed consent as part of the Longitudinal Studies of the Glycoproteinoses (NCT01891422) and sent to the Clinical Biochemical Laboratory at the Greenwood Genetic Center for clinical testing. The DBS were stored at −20 °C for four years prior to being sent to the University of Washington for the current research study in a de-identified manner.

DBS from 508 de-identified random newborns were issued by the Washington State Department of Health after being stored at room temperature for 30–60 days. The study was approved by the Washington State Institutional Review Board.

The reagents for the enzymatic assays were acquired from PerkinElmer, Inc. and were synthesized as described [26–28]. The first 6-plex assay measured the activities of alpha-L-iduronidase (IDUA; EC 3.2.1.76; MPS-I), alpha-1,4-glucosidase (GAA; EC 3.2.1.20; Pompe disease), alpha-galactosidase (GLA; EC 3.2.1.22; Fabry disease), galactosylceramidase (GALC, EC 3.2.1.46; Krabbe disease), acid sphingomyelinase (ASM, EC 3.1.4.12; Niemann-Pick A/B disease), and beta-glucosidase (GBA; EC 3.2.1.45; Gauche disease). The second 6-plex assay measured the activities of tripeptidyl peptidase I (TPP1; EC 3.4.14.9), iduronate-2-sulfatase (IDS; EC 3.1.6.13), alpha-*N*-acetylglucosaminidase (NAGLU, EC:3.2.1.50, MPS-IIIB), galactosamine-6-sulfatase (GALNS, EC 3.1.6.4, MPS-IVA), arylsulfatase B (ARSB, EC 3.1.6.12, MPS-VI), and beta-glucuronidase (GUSB, EC 3.2.1.31, MPS-VII).

2.2. Methods

Two different 6-plex enzymatic activity assays were carried out with two 3-mm DBS punches using a previously published protocol [29]. In short, a 3 mm DBS punch was incubated overnight at 37 °C with the respective assay cocktail which contained the substrates and internal standards for the six lysosomal enzymes tested. The sample was then quenched, followed by liquid-liquid extraction, before being analyzed by ultra-performance liquid-chromatography tandem mass spectrometry (UPLC-MS/MS). The enzymatic activity was calculated based on the ratio between the enzymatic product and its corresponding internal standard [29].

Statistical analysis was carried out using GraphPad Prism 8.

3. Results

Results of the 12 enzyme activities in DBS from 15 MLII/III pediatric and adult patients and $>$ 500 random, presumably normal newborns are summarized in Fig. 1. Only one of the 6-plex assays was performed in each newborn control. For MLII/III patients, activities from all of the 12 enzymes were measured using two 3-mm DBS punches.

Among the 12 enzymes, activities of ASM, IDS, and NAGLU were significantly elevated ($p < 0.0001$) in MLII/III patients when compared to the random newborns with no overlaps. The mean activities for ASM, IDS, and NAGLU were 20-, 11-, and 17-fold higher in the MLII/III patients than the random newborns, respectively. For IDUA, GALNS, and GUSB, the activities were significantly higher ($p < 0.0001$) in MLII/III patients with some overlaps, with the mean activities being 1.5 to 2-fold higher in the affected cohort. For GAA, GLA, GALC, GBA and TPP1, the activities were significantly reduced ($p < 0.0001$) in MLII/III patients with some overlaps, with the mean activities being 2 to 4-fold lower in the affected cohort. There was no statistical difference in ARSB activity between the affected and control cohorts (Fig. 1).

Among the 15 MLII/III patients, there was 1 MLII and 1 MLIII gamma patient. No statistical study can be performed to assess whether the activities of the secreted lysosomal enzymes can stratify MLII from MLIII patients.

4. Discussion

This study presented data from the largest cohort of MLII/III patient DBS, with the largest battery of lysosomal enzyme activities measured. Our results demonstrated that in DBS from MLII/III patients, ASM, IDS,

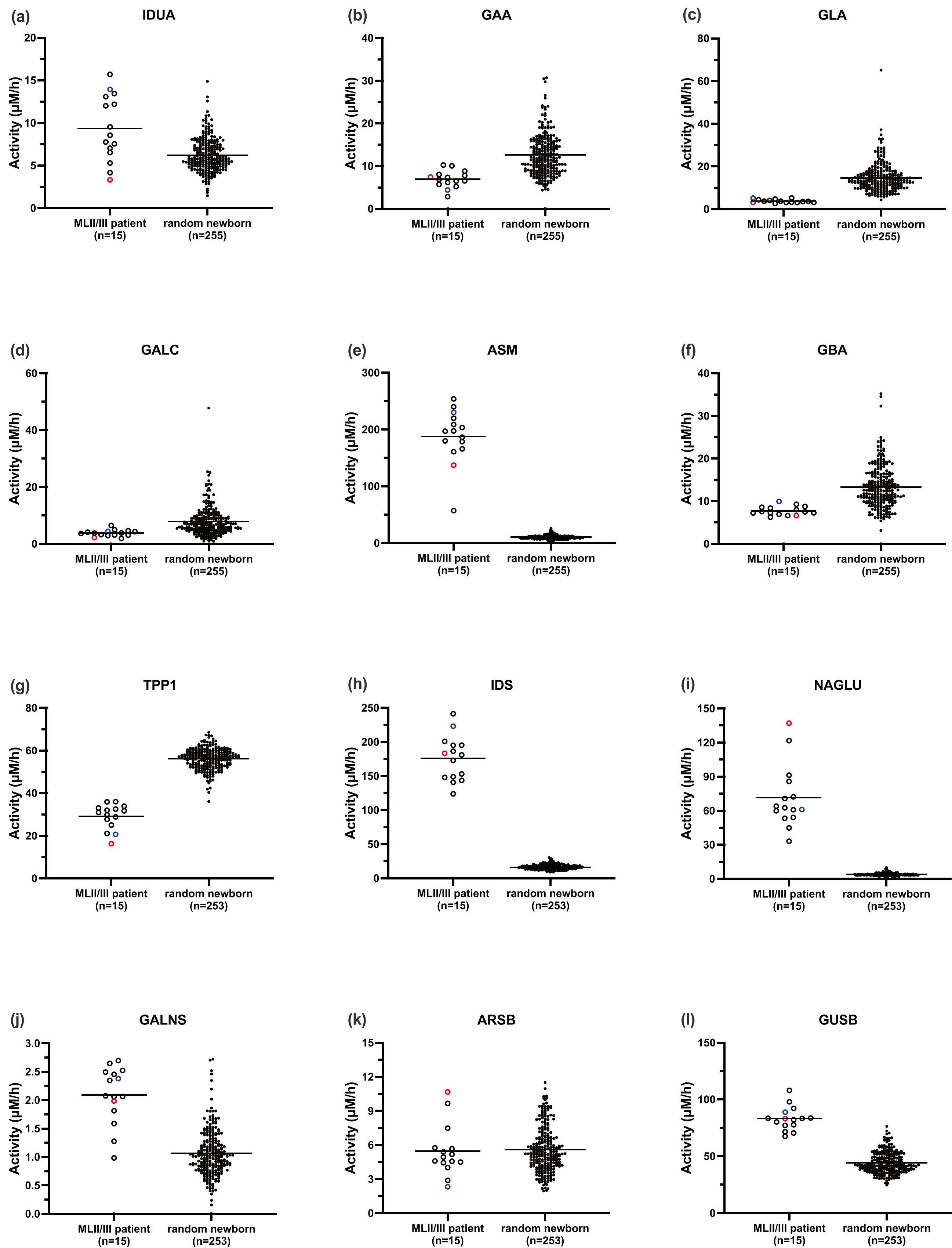

Fig. 1. Activities of (a) IDUA, (b) GAA, (c) GLA, (d) GALC, (e) ASM, (f) GBA, (g) TPP1, (h) IDS, (i) NAGLU, (j) GALNS, (k) ARSB, (l) GUSB in 15 MLII/III patients (black dots: MLIII alpha/beta, red dot: MLII, blue dot: MLIII gamma) and random newborns. The solid line in each group indicates the mean activity.

and NAGLU activities were significantly increased among the 12 targeted lysosomal enzymes.

While diagnosing MLII/III based on lysosomal enzyme activities in serum or plasma has been well established, their profiles in DBS have not been described in detail. Elevated total β-hexosaminidase, β-hexosaminidase A, IDUA, GUSB, IDS, and ARSB activities in DBS has been reported [16,25,30–34]. Interestingly, Verma et al. measured the activities of 18 lysosomal enzymes in DBS, ASM and IDS included, but only reported raised β-hexosaminidase A, IDUA, and GUSB activities in MLII/III [32]. Of note, none of these previous reports had a MLII/III cohort as large as the current study, and the majority of which used fluorometry-based methods. The difference between MLII/III patients and normal controls were also more significant in the current study, which may be ascribed to the nature of the respective MS/MS and fluorometry assays [35]. Furthermore, MS/MS and digital microfluidic-based multiplex assays have become the mainstay for LSDs newborn screening. Arunkumar et al. used an MS/MS-based 5-plex assay to diagnose MLII/III patients with DBS [16]. Chien et al. reported using an MS/MS-based 8-plex assay to identify an MLIII patient based on increased IDS and NAGLU activities through newborn screening [25].

Alternatively, Fuller et al. quantified the abundance of multiple lysosomal proteins in DBS from MLII/III patients, and found the amount of N-sulfamidase, ASM, arylsulfatase A, IDS and NAGLU protein was significantly elevated, consistent with the current study [36].

Other factors are known to affect lysosomal enzyme activities in DBS. Hematocrit level, leukocyte counts, and DBS card oversaturation would have a global effect on all the enzymes tested, including those expected to be reduced or unaltered in MLII/III [37]. On the contrary, genetic variations would only affect an isolated enzyme. Therefore, it is important to recognize multiple enzymes with increased as well as reduced or unaltered activities in DBS from MLII/III (activity profiling) to improve the sensitivity and specificity of the assay. Furthermore, a MLII/III-like lysosomal enzyme activity pattern has also been described in conditions with defective glycosylation, including congenital disorders of glycosylation (CDGs), classic galactosemia, and hereditary fructose intolerance (HFI), although to a lesser extent [38,39].

Our study also demonstrated reduced activities of 5 lysosomal enzymes in DBS, including GBA which is sorted to the lysosome through an alternative pathway and is not expected to increase in MLII/III DBS [4]. We do not have a clear explanation for the reduction of GAA, GLA, GALC, and TPP1 activities. Although it is possible that alternative lysosomal sorting pathways exist for GAA, GLA, GALC, and TPP1, we note that it is hard to ascribe the reduction in blood to either plasma or leukocytes fraction.

We note that the results presented in this study should not be used as reference rages, since stability, hematocrit, age, and sex might be confounders. The DBS from MLII/III patients and random newborns were not stored under the same condition or duration. In addition, our samples from ML patients were not age-matched with the newborn controls. This could also bias our results, as hematocrit and leukocyte counts are age-dependent and can affect the DBS enzyme activities [37].

Currently, all the newborn screening DBS enzymatic activity assays aim to identify neonates with reduced activity. However, if the screening algorithm includes a cutoff for increased activity, MLII/III may be readily detected as a secondary finding [25]. As patients with the severe form of MLII/III often present in the neonatal period, early/timely diagnosis will help guiding the management and significantly reducing the health care cost from a diagnostic odyssey. This is becoming more relevant as an increasing number of LSDs are being included into the RUSP. MPS-II is the most recent addition, and IDS is one of the three enzymes presenting with markedly increased activity in DBS from MLII/III patients (Fig. 1). In addition, newborn screening for Niemann-Pick A/B, MPS-IIIB and Gaucher disease are either being evaluated in pilot studies or are actively being performed in certain states in the US or other countries [40,41]. With more lysosomal enzyme activities being measured as part of routine screening, instead of relying on an isolated enzyme, activity profiling can be used to identify neonates with MLII/III and even conditions causing hypoglycosylation, such as CDGs and HFI. Therefore, should treatment for MLII/III become available, newborn screening for this condition can be implemented with minimal additional pre-analytical and analytical efforts. However, cutoffs for elevated activities need to be established carefully and activity profiling should be considered. Post-analytical interpretive tools, such as Collaborative Laboratory Integrated Reports (CLIR) may be an asset in this regard [42].

In a real-world scenario, second-tier biomarker testing may be considered to reduce the false-positive rates for MLII/III screening. Elevated urinary and/or DBS GAGs and sulfatides has been reported for MLII/III, for which assays have also been described in DBS for first-tier or second-tier screening [12,15,22,43,44]. However, biomarker analysis was not performed in the current cohorts, since the levels of GAGs and sulfatides tend to be age-dependent and the DBS from our MLII/III cohort were not collected during the first weeks of life [15,44]. Therefore, the validity of using DBS biomarkers for second-tier testing needs to be fully assessed before its implementation.

5. Conclusions

We measured the activities of 12 lysosomal enzymes in DBS from 15 MLII/III patients and $>$ 500 random newborns and demonstrated significant elevation of ASM, IDS, and NAGLU activities in the affected cohort. This sets the stage for using DBS to diagnose MLII/III. Furthermore, given an increasing number of lysosomal storage disorders are being included into the newborn screening panel, our results also indicate that population-based screening for MLII/III can be implemented with minimal efforts.

Declaration of Competing Interest

M.H. G. is a consultant for PerkinElmer and a co-founder for Gelb-Chem, LLC. Awarded and filed patents filed by M.H. Gelb and co-workers at the University of Washington include US20140249054A1, US20160298166A1, US8802833B2, EP2191006B1, and EP2385950B1. The other authors declare that they have no conflicts of interest.

Data availability

No data was used for the research described in the article.

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