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Author manuscript

*N Am J Med Sci (Boston)*. Author manuscript; available in PMC 2016 June 10.

Published in final edited form as:

*N Am J Med Sci (Boston)*. 2013 ; 6(4): 186–193. doi:10.7156/najms.2013.0604186.

## Enzymatic Screening and Diagnosis of Lysosomal Storage Diseases

Chunli Yu<sup>1,\*</sup>, Qin Sun<sup>2</sup>, and Hui Zhou<sup>3,6</sup>

<sup>1</sup>Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, NY 10029

<sup>2</sup>Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030

<sup>3</sup>Newborn Screening and Molecular Biology Branch, Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA 30341

### Abstract

Lysosomal storage diseases (LSDs) are a group of more than 50 genetic disorders. Clinical symptoms are caused by the deficiency of specific enzyme (enzymes) function and resultant substrate accumulation in the lysosomes, which leads to impaired cellular function and progressive tissue and organ dysfunction. Measurement of lysosomal enzyme activity plays an important role in the clinical diagnosis of LSDs. The major enzymatic testing methods include fluorometric assays using artificial 4-methylumbelliferyl (4-MU) substrates, spectrophotometric assays and radioactive assays with radiolabeled natural substrates. As many effective treatment options have become available, presymptomatic diagnosis and early intervention are imperative. Many methods were developed in the past decade for newborn screening (NBS) of selective LSDs in dried blood spot (DBS) specimens. Modified fluorometric assays with 4-MU substrates, MS/MS or LC-MS/MS multiplex enzyme assays, digital microfluidic fluorometric assays, and immune-quantification assays for enzyme contents have been reported in NBS of LSDs, each with its own advantages and limitations. Active technical validation studies and pilot screening studies have been conducted or are ongoing. These studies have provided insight in the efficacy of various methodologies. In this review, technical aspects of the enzyme assays used in clinical diagnosis and NBS are summarized. The important findings from pilot NBS studies are also reviewed.

### Keywords

lysosomal storage diseases (LSDs); newborn screening (NBS); lysosomal enzyme; tandem mass spectrometry (MS/MS)

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<sup>6</sup>Disclosure: The discussion and conclusions in this article are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention. One of the authors (HZ) receives financial support from the CDC Foundation through an agreement with Genzyme Corporation, a Sanofi Company.

\*To whom correspondence should be addressed: Chunli Yu, M.D., FACMG, Associate Professor, Director, Biochemical Genetics Division, Mount Sinai Genetic Testing Laboratory, Department of Genetics and Genomic Sciences, Icahn School of Medicine Mount Sinai, One Gustave L. Levy Place, Box 1497, New York, NY 10029, Tel: 212-241-6964; Fax: 212-241-0139, chunli.yu@mssm.edu.

## Introduction

Lysosomes are subcellular organelles responsible for degrading complex macromolecules and recycling cellular debris. They contain a variety of acid hydrolases functioning at acidic pH 4–5. These hydrolytic enzymes are translated and modified in the endoplasmic reticulum, and then transported from the Golgi to the lysosomes via a mannose-6-phosphate receptor dependent mechanism. Lysosomal enzymes function in a coordinated manner to break down complex sugars, lipids, glycolipids, glycosaminoglycans (GAGs), nucleic acids and proteins. Deficiency of a lysosomal enzyme or any component that is required for proper lysosomal function results in the accumulation of macromolecules and distortion of the lysosomes, leading to progressive cellular dysfunction of the affected tissues and organs, and resultant clinical abnormalities.

Lysosomal storage diseases (LSDs) are mainly caused by mutations in the genes encoding a specific lysosomal enzyme. Some LSDs result from deficiencies in activator proteins (e.g., GM2 activator deficiency), lysosomal membrane proteins (e.g., Danon disease), transporters for substrates (e.g., Salla disease), proteins for lysosomal enzyme post-translational modification (e.g., multiple sulfatase deficiency) and proteins for targeting enzymes to the lysosomes (e.g., I-cell disease) [1–4]. Most LSDs are autosomal recessive conditions, with the exception of X-linked Fabry, Hunter (mucopolysaccharidosis type II, MPS-II) and Danon disease. The incidence of LSDs as a group is estimated as 1:7,000 to 1: 8,000 [5]. With the emergence of LSDs newborn screening (NBS) programs, the population frequency of LSDs will be more accurately predicted and could be higher than the current literature.

More than 50 LSDs have been described and are classified into sphingolipidoses, mucopolysaccharidoses (MPS), oligosaccharidoses (glycoproteinosis), mucopolipidoses, neuronal ceroid lipofusinoses, and other categories based on the accumulated substrates. The clinical spectrum is very broad and highly variable partly due to the different substrates accumulated and levels of residual enzyme function underlying the disease-causing mutation. Classic features highly suggestive of LSDs include progressive mental retardation, developmental delay or regression, other neurological symptoms including ataxia and seizures, coarse features, organomegaly, abnormal eye findings (cherry-red spot, corneal clouding), and skeletal abnormalities (dysostosis multiplex). Besides supportive treatments, enzyme replacement therapy, hematopoietic stem cell transplantation (HSCT), chemical chaperon therapy and substrate reduction therapy have become increasingly available for treating specific LSDs [6–8]. Early diagnosis of these conditions, preferably pre-symptomatic detection, is critical to ensure early treatment and to avoid or reverse adverse clinical outcomes.

Lysosomal enzyme testing has been the gold standard for providing definitive diagnoses, which can be further confirmed by identifying disease-causing mutations. Many enzymes can be assayed in blood (leukocytes or serum/plasma) using commercially available synthetic 4-methylumbelliferone (4-MU) substrates. Other methods use spectrometric or radioactive substrates [1, 2, 4]. The selection of a specific enzyme or a panel of enzymes for testing is based on the clinical presentations, MRI findings, ultra-structural findings from

biopsied material and available biomarker results (GAGs, oligosaccharides pattern, sialic acid, etc.) [1, 4].

This differential diagnostic process requires a significant amount of expertise and experience in these diseases. Sometimes patients go through years of diagnostic odyssey before the correct diagnosis is made.

The concept of using dried blood spot (DBS) extracts for lysosomal enzyme testing, as pioneered by Nester Chamoles and colleagues in the early 2000s, opened up the potential for NBS of LSDs [9]. Although these modified fluorometric methods for DBS specimens are simple and inexpensive to set up, the multiplex capacity is limited as the enzyme reactions all produce the same end product 4-MU for measuring enzyme activity. Michael Gelb and colleagues have developed a series of specific substrates and internal standards for tandem mass spectrometry enzyme assays with (LC-MS/MS) or without (MS/MS) liquid chromatography. The enzyme function assays can be performed separately for one disease and can also be efficiently multiplexed for the detection of multiple LSDs [10–16]. Other technology developments include the microfluidic fluorometry platform for multiple enzymes by Advance Liquid Logic, Inc [17–19] and immune assays of LSD proteins by John Hopwood and colleagues [20, 21]. The technical aspects of lysosomal enzyme assays for clinical diagnostic and NBS are reviewed in this paper. The current status of NBS and ongoing pilot studies of selective LSDs are also summarized.

## Lysosomal enzyme testing for clinical diagnosis

Lysosomal enzyme tests are available to diagnose essentially all LSDs identified to date. The one disorder not diagnosed by enzyme assay is Niemann-Pick disease type C, for which testing is based on Filipin staining for abnormal cholesterol morphology under light microscopy, and demonstration of abnormal cholesterol esterification in cultured fibroblasts. Most lysosomal enzyme assays are performed in mixed leukocytes extracted from whole blood. Cultured fibroblasts are necessary for the diagnosis of Niemann-Pick C and I-cell disease. In I-cell disease, multiple enzyme deficiencies are demonstrated in cultured fibroblasts in contrast to the elevated enzymes in serum or plasma [4]. Lysosomal enzyme activity can also be measured in serum or plasma when the enzymes are abundant.

Historically radiolabeled natural substrates have been used for many lysosomal enzyme assays. Such radioactive methods are still preferred in testing  $\beta$ -galactocerebrosidase (Krabbe disease), acid sphingomyelinase (Niemann-Pick disease types A and B) and lysosomal acid lipase (Wolman disease and cholesteryl ester storage disease), due to the high specificity of these substrates.

The 4-MU derived artificial substrates have been widely used for measuring the vast majority of lysosomal enzymes. Under optimal conditions (pH, detergent, inhibitors, co-factors, etc.), a portion of the total substrates is hydrolyzed by the corresponding enzyme to release 4-MU, which is highly fluorogenic at pH 10–11. The fluorescence intensity measured by the fluorometer is proportional to the enzyme function and is calculated for enzyme activity [4, 22]. The 4-MU enzyme assays are simple and highly sensitive; therefore

they demonstrate great clinical utility in the screening and diagnosis of LSDs. Many 4-MU based substrates have been developed to replace radioactive enzyme assays, such as MPS-II, MPS-III, MPS-IVA, Krabbe and Niemann-Pick A and B, etc [22–27].

The synthetic substrate p-nitrocatechol sulfate (NCS) can be used clinically for testing arylsulfatase A (ARSA) for metachromatic leukodystrophy (MLD) and arylsulfatase B (ARSB) for MPS-VI or Maroteaux-Lamy syndrome. Sulfatase activity is calculated by the amount of sulfate released per hour per mg of protein, which is correlated with the absorbance of free p-nitrocatechol at 515 nm. Metal ions are required to chelate free sulfate and phosphate, which would inhibit the sulfatase activities. Because NCS substrate can be hydrolyzed by both ARSA and ARSB, extra steps are needed to differentiate the two enzymes. When ARSA is measured, ARSB needs to be inactivated with 0.25 mM sodium pyrophosphate. ARSB activity is determined in a differential condition based on the different kinetics of the two enzymes and inhibition of ARSA with barium ion [28]. Generally more protein is required in the spectrophotometric enzyme method and the residual enzyme activity in MPS VI patients might not be measured accurately. In contrast, radioactive oligosaccharide substrate derived from chondroitin 4-sulfate containing N-acetylgalactosamine-4-sulfate is more specific and highly sensitive for ARSB assay. Ion exchange chromatography for the separation of radioactive product is required, and handling of radioactive hazard can be a limiting factor [29]. Recently, a synthetic substrate consisting of an N-acetylgalactosamine-4-sulfate residue glycosidically linked to a derivative of umbelliferone has been developed for tandem mass spectrometric measurement of specific ARSB activity in DBS [12, 13]. Not only has this approach improved substrate specificity in comparison to the fluorometric method, the high sensitivity and the potential of multiplexing the enzyme assays into high throughput are also obvious advantages.

Several considerations need to be taken into account when interpreting in vitro enzyme results. It should always be kept in mind that enzyme activities measured by artificial substrates might not necessarily represent their activities in vivo; both false positive and false negative results may occur. More than 30% of non-Jewish patients identified as a carrier for Tay-Sachs disease by heat inactivation hexosaminidase assay were actually carriers of two pseudodeficiency alleles p.Arg247Trp and p.Arg249Trp, which were not associated with neurological disease but were associated with reduced hexosaminidase A activity in vitro when synthetic substrate was used [30]. Similarly, the high prevalence of a pseudodeficiency allele p.G576S (14.5% allele frequency in the Chinese) was correlated with partial deficiency in acid  $\alpha$ -glucosidase (GAA) when measured by synthetic 4-MU-glucoside substrate from the Pompe newborn screening program in Taiwan [31]. In the case of pseudodeficiency, molecular testing is important for accurate diagnosis, and genetic counseling is highly recommended. False positive results due to the presence of pseudodeficiency alleles can also be clarified by testing with a different substrate or by the demonstration of elevated metabolites. For example, patient tested positive for MLD by enzyme assay must be confirmed with *ARSA* gene mutation and/or elevated urinary sulfatide excretion [4]. In vitro enzyme assays usually do not take into account other essential components inside the lysosome, such as cofactors, chaperones and activators that are needed for enzyme processing, stability or structure. A negative enzyme result does not necessarily exclude a diagnosis. In patients with high clinical suspicion, when an enzyme

was tested negative, diseases due to co-factors, activators, or other enzymes should be considered. Examples of this are  $G_{M2}$  activator deficiency, Saposin B deficiency or multiple sulfatase deficiency for MLD-like features [4]. In suspicion of multiple sulfatase deficiency, several sulfatases should be tested before molecular analysis of the *SUMF1* gene.

## Newborn screening technologies for LSDs

NBS for LSDs fell several decades behind other metabolic disorders due to the lack of screening methods. With more treatment options available for LSDs, and recent analytical developments for enzyme testing in DBS specimens, LSDs has become an active area in the NBS field. New York State was the first and so far is still the only state performing population screening for the lysosomal disorder Krabbe disease since 2006. In several other states, including Illinois, Missouri, New Mexico and New Jersey, legislations mandating NBS for selective LSDs have been passed. Many technical developments, validations and pilot population studies are currently ongoing in several states such as Illinois, Missouri, Washington, New York and Minnesota [32]. Most recently, Pompe disease was approved to be added to the Recommended Universal Screening Panel (RUSP) for all newborns by the Discretionary Advisory Committee on Heritable Disorders in Newborns and Children (DACHDNC) in May, 2013 [33]. Voluntarily and regional pilot studies and screening of LSDs are also ongoing in more than ten countries [32]. Several major technology platforms are reviewed here.

## Fluorometric methods

The feasibility of detecting lysosomal enzyme deficiencies in DBS was first demonstrated by Chamoles and colleagues using modified 4-MU enzyme methods [9]. MPS-I, Hurler-like LSDs, Gaucher, Niemann-Pick disease A and B, Tay-Sachs and Sandhoff diseases were tested. The LSD enzymes on the DBS cards stored in the fridge or freezer were found to be stable for 21 days [34–36]. Even at room temperature,  $\alpha$ -L-iduronidase activity was stable for up to 20 days [9]. Retrospective studies of the DBS retrieved from affected patients stored at room temperature for months to years correctly identified patients with Gaucher, Niemann-Pick A and B,  $G_{M1}$  and  $G_{M2}$  [35–37]. By adding maltose [38], and later on more efficiently with acarbose [10], other glucosidases were inhibited and lysosomal acid  $\alpha$ -glucosidase activity could be accurately measured in DBS with 4-MU- $\alpha$ -D-glucopyranoside substrate for screening of Pompe disease or glycogen storage disease type II (GSD II). These DBS fluorometric assays are inexpensive and can be easily automated for NBS laboratories; however, the ability for multiplexing is limited.

The fluorometric method has been used in population screening in Taiwan for Pompe and Fabry diseases over the last several years. Due to the presence of a prevalent pseudodeficiency allele p.G576S (14.5% allele frequency in the Chinese), the false positive rate (FPR) was initially high for Pompe disease with a recall rate of 0.82%. In a retrospective study of the data from 473,738 newborn screens during a 4 year period, the ratio of neutral  $\alpha$ -glucosidase (NAG)/acid  $\alpha$ -glucosidase (GAA) was proven to be more efficient than GAA activity alone. A cutoff of NAG/GAA ratio >60 resulted in a satisfactory positive predictive value (PPV) of 63.4% [39]. Five out of six infants diagnosed from NBS,

even though clinically asymptomatic, had the infantile form of Pompe disease as evidenced by cardiac dysfunction and increased muscle glycogen storage. ERT was initiated within 30 days of life and resulted in normal cardiac function, normal growth, and significantly improved clinical outcomes compared to the cohort of clinically diagnosed patients treated with ERT [39, 40].  $\alpha$ -galactosidase A (GLA) and a reference enzyme  $\beta$ -galactosidase were measured fluorometrically with 4-MU substrates. GLA activity and the ratio of  $\beta$ -galactosidase/GLA were both used in screening for Fabry disease. Of the 92,288 male newborns, 638 or 0.7% screened positive with GLA <30% of normal mean and/or enzyme ratios >10. 73 were confirmed by mutation analysis to have Fabry disease, 86% of which carried a single late onset mutation c.936+919G>A (IVS4+919G>A) reported in cardiac variants. The incidence of Fabry disease in Taiwan was predicted to be about 1:1250 males [41]. A high incidence for Fabry disease was also reported by Spada et al to be 1:3100 males after screening 37,104 males in the Piedmont region of northern Italy. Similarly to Taiwan's data, the majority of Fabry patients identified from NBS in Italy had mutations consistent with late onset disease [42]. Both studies suggest that Fabry disease is clinically underdiagnosed, particularly the late onset variant form.

Digital Microfluidics technology was applied for DBS lysosomal enzyme testing by Advanced Liquid Logic, Inc. (Morrisville, North Carolina). This is a small tabletop instrument using the same testing principle as 4-MU enzyme assays. The 1.6  $\mu$ l extracts from DBS specimen and QC are loaded on a disposable microfluidic cartridge configured with an electronic circuit board for automated droplet handling and a built-in fluorometer. The calibration, incubation and fluorescence measurement are programmed in the software and performed on the cartridge. In the original prototype cartridge design, 12 samples could be tested on one cartridge for Pompe and Fabry diseases [17]. The feasibility was validated in a pilot study in 8012 screens in Illinois for Pompe, Fabry and Gaucher diseases; seven cases of Fabry and two cases of Gaucher disease were confirmed [43]. The most recent cartridge design has been scaled up for testing 44 DBS samples per cartridge for five LSDs Pompe, Fabry, Hunter, Gaucher and Hurler in less than 3 hours [19].

### MS/MS and LC-MS/MS methods

Gelb and colleagues in the University of Washington pioneered tandem mass spectrometry assay development for LSD newborn screening. Novel substrates, internal standards and assays have been developed over the years for testing enzyme activity of Krabbe, Pompe, Niemann-Pick A and B, Gaucher, Fabry, MPS-I, MPS-II, MPS-III, MPS-IVA and MPS-VI [10, 13, 14, 16, 44–46]. In 2004, the development of multiplexed enzyme activity assays for Gaucher, Pompe, Krabbe, Fabry and Niemann-Pick A/B diseases in DBS specimens laid the foundation for many pilot NBS studies later on [10]. In the initial method, the extracts from a 5-mm DBS punch are used in 5 concurrent individual enzyme reactions, then combined and purified by liquid-liquid extraction (LLE) followed by solid phase extraction (SPE) to remove the salts, detergents and excess substrates prior to flow injection mass spectrometry analysis (MS/MS) [10]. The substrates chosen or synthesized for these assays are structurally closer to the natural substrates (Table 1) than artificial 4-MU substrates. In addition the enzyme products are specific to each enzyme reaction and quantified against internal standards with known concentrations for the measurements of enzyme activities.

The method was further optimized and refined into a standardized protocol by Genzyme Corporation (Cambridge, Massachusetts) in 2008 [47]. Manufactured with good manufacture practice (GMP) standards and registered with the Food and Drug Administration (FDA) by Genzyme Corporation, these Analyte Specific Regents (ASR) (substrates, internal standards and products) were produced in large quantity and distributed to the testing laboratory with no charge through the Newborn Screening Translation Research Initiative (NSTRI) at the Centers for Disease Control and Prevention (CDC). Quality control materials, standardized operational protocols for reagents and sample preparation, and mass spectrometric conditions, as well as technical training and supports were also provided by NSTRI at CDC [32].

Even with these training and standardization efforts, there have been general concerns for the implementation of those methods in the NBS laboratories, due to the complexity of the procedures. Several technical modifications were carried out focusing on simplifying the sample preparation processes. A specially configured online trapping and clean-up coupled with high performance liquid chromatography tandem mass spectrometry (LC-MS/MS) was reported by Marca et al [48]. After incubation, 5 reaction mixtures from separate reactions are stopped and combined together. After the centrifugation the supernatant is injected directly onto the mass spectrometer without manual purification. The sample mixture is first trapped on a preparation column and then switched to the C18 separation column, followed by mass spectrometry analysis in a total of 4 minutes run time. A similar approach was reported using a multi-dimensional ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) with turbulent flow chromatography (TFC) for online sample clean-up by Kasper et al [49]. The throughput of the analysis was improved approximately four times with this multiplexed LC system for alternate sample introduction and separation with cycling-in MS/MS function. In a collaborative study between Washington State NBS laboratory and Gelb's group at the University of Washington in 2010, Pompe, Fabry and MPS-I enzymes were analyzed with a single incubation from DBS followed by one step LLE and LC-MS/MS analysis. The substrates, internal standards, detergents, and inhibitors were multiplexed into one assay cocktail in ammonium formate buffer at pH 4.4. With chromatographic separation of substrates, internal standards and products, specific enzyme products were reliably measured for enzyme activity [50]. Since then, more multiplex approaches were developed and piloted in NBS studies. Orsini et al reported a 4+1 multiplex MS/MS assay trying to add more disorders to the existing NYS Krabbe screening. Samples from quadruplex reactions of Krabbe, Gaucher, Pompe and Fabry, and single reaction of Niemann-Pick A and B were combined and purified by automated LLE and SPE prior to flow injection MS/MS analysis [51]. The most comprehensive multiplex approach was reported by Gelb and colleagues with up to 9 lysosomal enzymes, using a similar approach from their 3-plex studies. In addition to Gaucher, Pompe, Krabbe, Fabry and Niemann-Pick A/B enzymes, MPS-I, MPS-II, MPS-IVA and MPS-VI enzymes were added. The assay was proven to be robust as 9-plex, or 6+3 plex separating into two groups with three sulfatases MPS-II, MPS-IVA and MPS-VI as a separate group [52].

Several large-scale newborn screening and pilot studies using MS/MS or LC-MS/MS methods are worth mentioning. New York State started screening Krabbe disease in 2006 by tandem mass spectrometry. An algorithm was used in which samples with enzyme activity

below the daily mean activity cut-off were further analyzed by second tier DNA analysis of a 30 kb deletion associated with infantile Krabbe disease. Patients with initial positives in DBS were followed up by the metabolic centers for confirmation studies of enzyme activity in leukocytes and molecular studies. Affected patients were clinically evaluated and followed up closely for neurological function [53]. Reportedly, of 550,000 newborns screened up to June 2008, four infants were confirmed as having high risk for the disease, 6 infants as having moderate risk and 15 infants as having low risk for the disease. Two of the four infants with high risk were found to be homozygous for the 30 kb deletion and went through bone marrow transplants before one month of age, one of whom died following the transplant [54]. In Washington State, over 100,000 newborns were screened for Fabry, Pompe and MPS-I using the triplex LC-MS/MS method in a pilot study. The prevalence of Fabry was 1:7,800 males, and the prevalence of Pompe and MPS-I were 1:27,800 and 1:35,500 newborns, respectively. These prevalence data were 2 to 4 times greater than the frequency estimated by clinical diagnosis [55]. In 2013, NYS initiated the pilot NBS program for Gaucher, Pompe, Niemann-Pick A and B and MPS-I in addition to Krabbe disease, so more population data will be revealed soon (personal communication).

### **Multiplexed immune quantification method**

Immune-quantitation of lysosomal enzymes and proteins for screening 11 LSDs was developed by Hopwood and colleagues. The enzyme and protein contents are measured instead of functional enzyme activity, on the basis that in many LSDs the pathogenic mutations result in reduced amounts of protein in addition to low enzyme activity [20]. 14 lysosomal enzymes and proteins extracted from one blood spot are captured by antibodies using microbead suspension array and then quantified by fluorescence detection. The pilot study successfully identified Fabry, Pompe, MPS-I, MPS-IIIA, MPS-IIIB, MPS-VI and MLD patient specimens [20, 21]. However, one false negative case was reported for MPS-II. This technology has not been utilized in other NBS laboratories except for a pilot study to compare different screening methodologies [56]. In addition to the cost and availability of the antibodies, there are general concerns that patients carrying mutations that result in enzyme deficiency without a reduction of protein content might be missed.

### **Quality management of lysosomal enzyme testing**

The catalytic function of lysosomal enzymes is determined by the rate of production of the end products in a specific assay system. Any component of the reaction system (e.g., substrate nature and concentration, buffer and pH, temperature, presence of activators or inhibitors, etc.) may impact measured enzyme activity [57]. In addition, inter-laboratory variance of numerical enzyme activity could be large. To ensure the quality of LSD enzyme testing performance, each testing laboratory should establish its own Quality Management (QM) system in compliance with the regulations set forth in Clinical Laboratory Improvement Amendments (CLIA-88). The framework of the QM plan could be based on guidelines from the Clinical and Laboratory Standards Institute (CLSI) and the College of American Pathologists (CAP). Duplicate testing is generally desirable for enzyme assays. For each run, appropriate blanks, a series of calibration, and at least one affected control and one normal control sample must be included for quality control purposes. If controls from



affected patients are not available, inactivated samples by heating or other method could be an alternative. However, biologically affected controls must be tested during the test validation and periodically tested to assure the quality of testing. For DBS LSD enzyme assays, artificial low enzyme controls for multiple LSD enzymes were created by mixing the leukocytes depleted blood with heat-inactivated, charco-stripped serum at the physiological hematocrit level of 55%, and then spotted on filter papers. Unprocessed cord blood was used to generate DBS specimens as normal enzyme controls. Mixing of the normal and low enzyme blood at different ratios served as medium activity controls [58]. These enzyme activity control specimens were generated, and validated by the NSTRI at CDC before distributing to the testing laboratories. The normal range, disease range, and, if appropriate, a carrier range should be established by the laboratory based on its own analysis. Laboratory reports include an interpretation of the result that reflects the presence or absence of the disease, possible limitations of the test, and recommendations for additional testing if applicable.

For ongoing evaluation of test performance, the testing laboratory should participate in both internal and external quality assessments. The internal audit program monitors operations throughout the testing process and the quality system. External assessments include regulatory inspections and proficiency testing programs. Several external proficiency test programs are available for lysosomal enzyme assays. The European Research Network for Evaluation and Improvement of Screening, Diagnosis, and Treatment of Inherited Disorders of Metabolism (ERNDIM) serves as an external proficiency testing program for clinical diagnostic laboratories, providing lyophilized fibroblasts for eight LSD enzymes (<http://cms.erndimqa.nl/Home/Lysosomal-Enzymes.aspx>). Both numerical enzyme activity values and percentage of the normal mean were collected and curated. The National Tay-Sachs & Allied Diseases Association (NTSAD) offers an international Tay-Sachs carrier testing quality control program to evaluate laboratory performance of Tay-Sachs biochemical carrier screening in serum and leukocytes (<http://www.ntsad.org/index.php/ensuring-quality-results>). For laboratories testing lysosomal enzymes on DBS, the Newborn Screening Quality Assurance Program (NSQAP) at CDC provides QC materials, proficiency testing (PT) services, and technical support in collaboration with the NSTRI at CDC. A pilot proficiency testing program for Pompe and Krabbe diseases is available through NSQAP.

## Discussion and conclusions

Although the NBS of LSDs was initially driven by available treatments and available screening methods and reagents, a great deal of useful information has been gained from the existing pilot studies. As indicated in the screening from several populations in Taiwan (1:1,250) [59], Italy (1:3,100) [42] and Washington State in the United States (1:7,800) [55], Fabry disease was proven to be much more common than the previously estimated 1:40,000 from clinical diagnosis [5]. Fabry patients carrying late onset variant mutations were picked up by reduced enzyme activity in DBS in the newborn period. Some of these mutations are highly frequent in specific ethnic groups. For instance, the cardiac variant IVS+919G>A was common in Taiwanese (probably true for Chinese or Asian populations as well) [59]; and p. A143T was frequently present in positive patients in Italy [42] and Austria [60], suggesting a founder effect for this milder mutation in the Northern Italy/Austria region of Europe.

Demonstration of normalization of cardiac function and normal growth in the infantile Pompe patients from Taiwan identified from NBS with early initiation of ERT provided the most compelling evidence for the first time that NBS of certain LSDs is warranted for early intervention to ensure good clinical outcome [61, 62]. As more pilot studies and research are being conducted on other LSDs, more will be learned about the efficacy of screening methodologies (sensitivity, PPV and FPR), efficacy and risk of treatment, and best time for initiation of treatment. Besides Pompe disease which was recently approved to be added to the RUSP for NBS, several LSDs have also been proposed and reviewed by the DACHDNC, but have not yet been endorsed. This situation might change when more evidence become available. In coping with current screening activities and future NBS, a professional work group representing the American College of Medical Genetics (ACMG) was formed to address the diagnostic confirmation of lysosomal storage diseases, and a preliminary guideline on diagnostic confirmation algorithm and management strategies of presymptomatic LSD patients was published [8].

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**Table 1**

Summary of selective LSDs implicated for newborn screening-clinical and biochemistry features and comparison of enzyme methods

Diseases	Deficient Enzyme	Key Clinical Features	Accumulated Substrates and Affected Tissues	Substrates Used in Traditional Enzyme Methods	Substrates Used in MS/MS Enzyme Methods
Fabry Disease	$\alpha$ -Galactosidase A (GLA)	Extremity pain and paresthesias, angiokeratoma, hypohidrosis, corneal opacity, deteriorating renal function	Glycosphingolipids-globotriaosylceramide, and galabiosylceramide, blood group B substances in body fluid, blood vessels, heart, kidney and eyes	4-MU- $\alpha$ -galactoside *Both enzyme and DNA are recommended for detecting female Fabry patients	Lipidated- $\alpha$ -galactoside with N-linked $\epsilon$ -butyloxycarbamido group
Gaucher Disease	Acid $\beta$ -glucosidase (GBA)	Type I: Bone lesions, hepatosplenomegaly, anemia and thrombocytopenia, lung disease. Type II: Neurodegeneration and hepatosplenomegaly in infancy Type III: Intermediate between I and II	Glucosylceramide in all organs, including spleen, bone marrow and lymph nodes	4-MU- $\beta$ -D-glucoside	C12-glucosylceramide
Krabbe Disease	$\beta$ -Galactocerebrosidase (GALC)	Extreme irritability, rapid mental and motor degeneration, spastic quadriplegia, blindness without organomegaly	Galactosylceramide and galactosylsphingosine (psychosine) in myelinated sheath only	$^3$ H labeled Galactosylceramide 6-Hexadecanoylamino-4-MU- $\beta$ -D-galactoside	C8-galactosylceramide
MPS-I (Hurler, Hurler-Scheie and Scheie syndrome)	$\alpha$ -L-Iduronidase (IDUA)	Coarse facial features, umbilical or inguinal hernia, impaired mental development, corneal clouding, dysostosis multiplex, short stature, hearing loss, cardiomyopathy; only somatic features in mild form	Dermatan sulfate (DS) and heparan sulfate (HS) in all organs, specifically CNS, joint/skeletal, heart, eye, liver, spleen	4-MU- $\alpha$ -L-iduronide	Umbelliferyl- $\alpha$ -L-iduronide attached to a fourcarbon linker with terminal $\epsilon$ -butyloxycarbamido group
MPS-II (Hunter syndrome)	Iduronate-2-sulfatase (IDS)	Similar features as MPS-I: coarse facial features, stiff joints, short stature, progressive cognitive deterioration, dysostosis multiplex. No corneal clouding.	Dermatan sulfate, heparan sulfate in all organs, specifically CNS, joint/skeletal, heart, eye, liver and spleen	4-MU-1-iduronide-2-sulphate	Umbelliferyl- $\alpha$ -L-iduronide-2-sulfate attached to a five carbon linker with terminal $\epsilon$ -butyloxycarbamido group
MPS-IVA (Morquio syndrome, type A)	N-acetyl-galactosamine-6-sulfatase (GALNS)	Dwarfism, distinct skeletal dysplasia (genu valgum, pectuscarinatum, kyphosis, odontoid hypoplasia, pectuscarinatum), corneal clouding. Normal intellectual abilities.	Gal-6S from Keratan sulfate and GalNAc-6S from chondroitin 6-sulfate in joint/skeletal, heart, respiratory and eye	$^3$ H labeled 6-sulfo-N-acetylgalactosamine-glucuronic acid-6-sulfo-N-acetyl galactosaminitol 4-MU- $\beta$ -D-galactoside-6-sulphate (Determine $\beta$ -galactosidase activity to differentiate MPS IVB)	Umbelliferyl- $\beta$ -D-galactose-6-sulfate attached to a five-carbon linker with a terminal $\epsilon$ -butyloxycarbamido group

Diseases	Deficient Enzyme	Key Clinical Features	Accumulated Substrates and Affected Tissues	Substrates Used in Traditional Enzyme Methods	Substrates Used in MS/MS Enzyme Methods
MPS-VI (Maroteaux-Lamy syndrome)	N-acetylgalactosamine-4-sulfatase (Arylsulfatase B, ARSB)	Similar somatic features as MPS I: short stature, restricted joint movement, corneal clouding, dysostosis multiplex, and hepatomegaly. No neurological defects.	Dermatan sulfate in joint/skeletal, heart, respiratory and eye	p-nitrocatecol sulfate, subtraction of arylsulfatase A (ARSA) activity from total reaction <sup>3</sup> H labeled substrate derived from chondroitin 4-sulfate	Umbelliferyl-2-acetamido-D-galactose-4-sulfate attached to a six carbon linker with a terminal t-butylloxycarbonyl group
Niemann-Pick Disease, type A and B	Acid sphingomyelinase (ASM)	Type A: Massive hepatosplenomegaly with rapid neurodegeneration in infancy. Type B: hepatomegaly, restrictive lung disease and hyperlipidemia in childhood. Normal intelligence.	Sphingomyelin and cholesterol in CNS, spleen, lymph nodes, bone marrow, lung and liver	<sup>14</sup> C-labeled sphingomyelin 6-Hexadecanoyl/lanino-4-MU-phosphorylcholine	C6-sphingomyelin
Pompe Disease (Glycogen Storage Disease, type II)	Acid α-galactosidase (GAA)	Infantile form: Hypotonia, cardiomegaly and hypertrophic cardiomyopathy, hepatomegaly, severe muscle weakness, respiratory failure, macroglossia. Late-onset form: Progressive muscle weakness.	Glycogen in cardiac and skeletal muscles	4-MU-α-D-glucoside	Lipidated α-D-glucoside with N-linked t-butylloxycarbonyl group

Abbreviations: 4-MU, 4-methylumbelliferyl; CNS, central nervous system; MPS, mucopolysaccharidosis; Gal-6S, galactose-6-sulfate; GalNAc-6S, N-acetylgalactosamine-6-sulfate