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Measurement of lysosomal enzyme activities: A technical standard of the American College of Medical Genetics and Genomics (ACMG)

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ABSTRACT

Assays that measure lysosomal enzyme activity are important tools for the screening and diagnosis of lysosomal storage disorders (LSDs). They are often ordered in combination with urine oligosaccharide and glycosaminoglycan analysis, additional biomarker assays, and/or DNA sequencing when an LSD is suspected. Enzyme testing in whole blood/leukocytes, serum/plasma, cultured fibroblasts, or dried blood spots demonstrating deficient enzyme activity remains a key component of LSD diagnosis and is often prompted by characteristic clinical findings, abnormal newborn screening, abnormal biochemical findings (eg, elevated glycosaminoglycans), or molecular results indicating pathogenic variants or variants of uncertain significance in a gene associated with an LSD. This document, which focuses on clinical enzyme testing for LSDs, provides a resource for laboratories to develop and implement clinical testing, to describe variables that can influence test performance and interpretation of results, and to delineate situations for which follow-up molecular testing is warranted.

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Background

Lysosomal structure and function

Lysosomes are membrane-bound cytoplasmic organelles that stem from transport vesicles budded from the *trans*-Golgi network to form endosomes. They partake in several cellular processes, including phagocytosis, autophagy, and apoptosis.[1]

The lysosomal membrane is a single phospholipid bilayer that contains >120 membrane proteins, including structural proteins (eg, LAMP2), receptor proteins, proton pumps, ion channels, transporters, and vesicle traffic and fusion proteins. The H+/ATPase proton pump ensures the lysosomal hydrolases have an optimal pH of 4.5 to 5.0 because these hydrolases are typically not active at the neutral pH found in the cytosol. The membrane proteins import other proteins, provide protection from proteolytic enzymes, and allow for the transport of small molecules such as cysteine and sialic acid. Na^+, K^+, and Ca^{2+} channels maintain concentration gradients that regulate lysosome movement, membrane trafficking, nutrient sensing, membrane repair, organelle membrane contact, and lysosome biogenesis. In addition to these ion channels, biogenesis of the lysosome is controlled by the kinase mTOR and the transcription factor TFEB, which regulates the expression of lysosomal genes, controls the number of lysosomes, and promotes degradation of lysosomal substrates.[2,3]

Lysosomes contain >60 hydrolytic enzymes required to break down and recycle macromolecules, namely proteins, lipids, and carbohydrates. These enzymes are synthesized with approximately 20 amino acids at the N-terminal that signal to the endoplasmic reticulum where they undergo a variety of post-translational modifications, including N-linked glycosylation, and for a subset of enzymes, further proteolytic cleavage occurs to activate subunits; sulfatases have highly conserved cysteine residues converted to formylglycine at their active sites. Next, they are transported to the Golgi where they are further trimmed or modified by additional sugars (eg, N-acetylglucosamine) or mannose-6-phosphate (M6P). Most hydrolases are modified with M6P, which is recognized by Golgi receptors and ensures proper transport to the endosome/lysosome, whereas other hydrolases use alternate receptors such as sortilin and the lysosomal integral membrane protein LIMP-II.[4]

Clinical description and indications for testing

Lysosomal storage disorders (LSDs) comprise a group of >70 genetically distinct conditions. Although most LSDs result from pathogenic variants in genes encoding specific lysosomal hydrolases (eg, beta-glucocerebrosidase deficiency in Gaucher disease), others arise from pathogenic variants in genes encoding activator proteins (eg, atypical Gaucher disease due to saposin C deficiency), transport proteins (eg, Salla disease and Niemann-Pick type C), or proteins that post-translationally modify lysosomal enzymes (eg, mucolipidosis type II and multiple sulfatase deficiency). The consequences of these pathogenic genetic alterations are deficient enzyme activity and subsequent substrate accumulation that results in lysosomal dysfunction and dysregulation of several downstream pathways impacting autophagy, phagocytosis, and calcium homeostasis.[1]

Individuals with LSDs typically have no distinctive features at birth but experience progressive worsening over time as macromolecular substrates accumulate and cellular damage occurs. Symptoms vary by specific enzyme deficiency, but some degree of developmental delay and/or regression is seen in most individuals with an LSD. Prominent features of the mucopolysaccharidoses (MPSs) include coarse facial features, joint stiffness, organomegaly and dysostosis multiplex, whereas individuals with sphingolipidoses may have progressive hypotonia and seizures (metachromatic leukodystrophy, Krabbe disease), cherry-red spot (GM1- and GM2- gangliosidosis), hepatosplenomegaly (Gaucher disease, Niemann-Pick types A and B), or renal failure (Fabry disease).[1,2] There is a broad range of clinical severity and age of onset, with both early-onset and attenuated forms reported for most of the disorders. The degree of clinical severity is often correlated with the amount of residual enzyme activity, although meaningful differences in residual activity may not be evident from clinical enzyme testing. Table 1 summarizes the major LSDs along with their associated genes, enzymes, and phenotypes.

In addition to clinical features concerning for an LSD, enzyme testing may be prompted by abnormal results from newborn screening (NBS), urine screening studies (oligosaccharides, glycosaminoglycans, free sialic acid), or molecular testing (exome or genome sequencing). Sex is important when considering X-linked disorders such as MPS type II, which is almost exclusively seen in males. Ethnicity may play a role for certain disorders more common in specific ethnic groups, such as Gaucher disease, Niemann-Pick type A/B, and Tay-Sachs disease (TSD) among individuals of Ashkenazi Jewish descent and GM1-gangliosidosis in individuals of Roma ancestry.[5] A family history of an LSD may guide testing of additional relatives at-risk, and prenatal history such as nonimmune hydrops fetalis may trigger testing for several LSDs, including Gaucher disease and sialidosis.[6]

Incidence

The combined incidence of LSDs is estimated at 1:4000 to 1:9000 live births.[7] Individually, estimates range from 1:13,000 births for neuronal ceroid lipofuscinosis 1 to 1:4 million for sialidosis.[8] The worldwide incidence of Gaucher disease is 1:100,000 live births,[9] whereas in individuals of Ashkenazi Jewish descent the incidence may be as high as 1:450 births.[10] The combined incidence of early- and late-onset Pompe disease in the United States has been estimated to be 1:40,000; however, recent studies suggest a prevalence rate of approximately 1:25,000.[11] For some

Table 1 LSDs

Disorder	Subtype	OMIM	Gene	Enzyme/Protein	Phenotype[a]
MPS					
MPS I	Hurler (Ih)	607014	*IDUA*[b]	α-L-iduronidase	Coarse facies, corneal clouding, intellectual disability, stiff joints, dysostosis multiplex, hepatosplenomegaly
	Hurler-Scheie (Ih/s)	607015			Corneal clouding, ±intellectual disability, stiff joints, dysostosis multiplex, hepatosplenomegaly
	Scheie (Is)	607016			Corneal clouding, normal intellect, stiff joints, aortic regurgitation
MPS II[c]	Hunter	309900	*IDS*	Iduronate 2-sulfatase	Coarse facies, no corneal clouding, intellectual disability, stiff joints, dysostosis multiplex, hepatosplenomegaly
MPS III	Sanfilippo A (IIIA)	252900	*SGSH*	N-sulfoglucosamine sulfohydrolase	Behavioral aberration, severe central nervous system degeneration
	Sanfilippo B (IIIB)	252920	*NAGLU*	N-acetyl-α-D-glucosaminidase	
	Sanfilippo C (IIIC)	252930	*HGSNAT*	Acetyl-CoA:α-glucosaminide N-acetyltransferase	
	Sanfilippo D (IIID)	252940	*GNS*	N-acetylglucosamine-6-sulfatase	
MPS IV	Morquio A (IVA)	253000	*GALNS*	Galactosamine-6-sulfatase	Corneal clouding, normal intellect, skeletal dysplasia
	Morquio B (IVB)	253010	*GLB1*	β-Galactosidase[d]	
MPS VI	Maroteaux-Lamy	253200	*ARSB*[b]	Arylsulfatase B	Coarse facies, corneal clouding, normal intellect, stiff joints, dysostosis multiplex, hepatosplenomegaly
MPS VII	Sly	253220	*GUSB*	β-Glucuronidase	Hydrops fetalis; coarse facies, ±intellectual disability, dysostosis multiplex, hepatosplenomegaly
Oligosaccharidoses					
Aspartylglucosaminuria		208400	*AGA*	Aspartylglucosaminidase	Intellectual disability, skeletal abnormalities, angiokeratoma
Fucosidosis		230000	*FUCA1*	α-Fucosidase	Type 1 - regression, severe neurologic deterioration Type 2 - intellectual disability, angiokeratoma
Galactosialidosis		256540	*CTSA*	Cathepsin A	Hydrops fetalis, coarse facies, cherry-red spot, dysostosis multiplex, hepatomegaly, angiokeratoma
α-Mannosidosis		248500	*MAN2B1*	α-D-mannosidase	Coarse facies, sensorineural deafness, intellectual disability, dysostosis multiplex, ataxia
β-Mannosidosis		248510	*MANBA*	β-Mannosidase	Deafness, intellectual disability, angiokeratoma
Schindler		609241	*NAGA*	α-N-acetylgalacto saminidase	Type I - infantile-onset neuroaxonal dystrophy Type II - adult-onset with angiokeratoma and mild intellectual disability

(continued)

Table 1 Continued

Disorder	Subtype	OMIM	Gene	Enzyme/Protein	Phenotype[a]
Sphingolipidoses					
GM1-gangliosidosis	Type I	230500	*GLB1*[b]	β-Galactosidase[d]	Hydrops fetalis, coarse facies, cherry-red spot, hepatosplenomegaly, skeletal dysplasia, rapid psychomotor deterioration, and early death
	Type II	230600			Seizures, mild skeletal changes, slowly progressive generalized neurodegeneration, and survival into childhood
	Type III	230650			Mild skeletal changes and central nervous system findings, such as dystonia, gait or speech disturbance
GM2-gangliosidosis	Tay-Sachs	272800	*HEXA*[b]	Hexosaminidase A	Cherry-red spot, startle reaction, progressive neurodegenerative, early death
	Sandhoff	268800	*HEXB*	Hexosaminidase A and B	
	AB variant	272750	*GM2A*	GM2 activator[e]	
ML					
ML I	Sialidosis	256550	*NEU1*	Neuraminidase	Hydrops fetalis, seizures, coarse facies, cherry-red spot, intellectual disability, dysostosis multiplex, hepatosplenomegaly
ML II	I-cell	252500	*GNPTAB*	N-acetylglucosamine-1-phosphotransferase	Hydrops fetalis, coarse facies, corneal clouding, intellectual disability, stiff joints, dysostosis multiplex, hepatosplenomegaly, cardiomegaly
ML III alpha/beta	Pseudo-Hurler polydystrophy	252600			
ML III gamma		252605	*GNPTAG*		
ML IV		252650	*MCOLN1*	Mucolipidin[f]	Corneal clouding, intellectual disability
Glycosphingolipidoses					
Fabry[c]		301500	*GLA*[b]	α-Galactosidase A	Renal failure, cerebrovascular accident, acroparesthesia, angiokeratoma
Farber		228000	*ASAH1*	Ceramidase	Hoarseness, joints with nodular, erythematous swellings
Gaucher	Type I	230800	*GBA*	β-Glucocerebrosidase	Hepatosplenomegaly, pancytopenia, bone pain
	Type II	230900			Hydrops fetalis, collodion skin, hepatosplenomegaly, pancytopenia, neurodegeneration, early death
	Type III	231000			Hepatosplenomegaly, pancytopenia, supranuclear gaze palsy, neurodegeneration, cardiovascular calcifications (IIIc)
	Atypical, due to saposin C deficiency	610539	*PSAP*	Sphingolipid activator protein[g]	Hepatosplenomegaly, pancytopenia, supranuclear gaze palsy, neurodegeneration
Krabbe		245200	*GALC*[b]	Galactosylceramidase	Irritability, spasticity, neurodegeneration, dysmyelination, early death
	Atypical, due to saposin A deficiency	611722	*PSAP*	Sphingolipid activator protein[g]	
Metachromatic leukodystrophy	Arylsulfatase A deficiency	250100	*ARSA*[b]	Arylsulfatase A	Neurodegeneration, dysmyelination
	Saposin B deficiency	249900	*PSAP*	Sphingolipid activator protein[g]	

(continued)

Table 1 Continued

Disorder	Subtype	OMIM	Gene	Enzyme/Protein	Phenotype[a]
Niemann-Pick	Type A	257200	*SMPD1*	Sphingomyelinase	Hydrops fetalis, cherry-red spot, hepatosplenomegaly, intellectual disability, neurodegeneration, early death
	Type B	607616			Hepatosplenomegaly, no neurologic findings
	Type C	257220	*NPC1*	NPC intracellular cholesterol transporter 1[h]	Hepatosplenomegaly, supranuclear gaze palsy, cataplexy, neurodegeneration
CLN					
CLN1		256730	*PPT1*	Palmitoyl-protein thioesterase 1	Progressive dementia, seizures, and progressive visual failure
CLN2		204500	*TPP1*	Tripeptidyl peptidase 1	
CLN3 (Batten)		204200	*CLN3*	Battenin[i]	
CLN10		610127	*CTSD*	Cathepsin D	
CLN13		615362	*CTSF*	Cathepsin F	
Lysosomal transporter defects					
Cystinosis		219800	*CTNS*	Cystinosin[j]	Cysteine crystal accumulation in cornea and kidney, renal Fanconi
Sialuria	Infantile sialic acid storage disorder	269920	*SLC17A5*	Sialin[k]	Hydrops fetalis, coarse facies, hepatosplenomegaly, hypotonia, cerebellar ataxia, intellectual disability
	Salla	604369			Hypotonia, cerebellar ataxia, intellectual disability
Other LSDs					
Lysosomal acid lipase deficiency	Wolman	278000	*LIPA*	Lysosomal acid lipase	Hepatosplenomegaly, steatorrhea, adrenal calcification, early death
	Cholesteryl ester storage disease				Hepatosplenomegaly, liver failure
Multiple sulfatase deficiency		272200	*SUMF1*	C-α-formylglycine-generating enzyme	Coarse facies, corneal clouding, intellectual disability, dysostosis multiplex, hepatosplenomegaly, ichthyosis, neurodegeneration, dysmyelination
Pompe (Glycogen storage disorder II)		232300	*GAA*[b]	α-Glucosidase	Cardiomegaly, hypotonia, proximal muscle weakness
Danon[c]		300257	*LAMP2*	Lysosomal associated membrane protein 2[l]	Cardiomyopathy, proximal muscle weakness

CLN, neuronal ceroid lipofuscinoses; *LSD*, lysosomal storage disorder; *ML,* mucolipidoses; *MPS,* mucopolysaccharidoses.
[a]Phenotype description is not comprehensive but includes common or unique features for each condition with an associated OMIM number.
[b]Pseudodeficiency has been described; see Table 2 for additional information.
[c]Disorder is X-linked.
[d]MPS IVB and GM1 are allelic conditions.
[e]GM2 activator binds GM2 for degradation by β-hexosaminidase A.
[f]Mucolipidin regulates lysosomal exocytosis.
[g]Prosaposin is a precursor of several small glycoproteins that assist in the hydrolysis of sphingolipids.
[h]NPC1 mediates intracellular cholesterol trafficking.
[i]Battenin is necessary for lysosomal function.
[j]Cystinosin transports cystine out of lysosomes.
[k]Sialin exports free sialic acids from lysosomes.
[l]LAMP2 is a lysosomal membrane that imports proteins and provides protection from proteolytic enzymes.

conditions, estimates were revised after the implementation of NBS, underscoring the importance of reevaluating incidence numbers as more states implement NBS for LSDs. For example, NBS for Krabbe disease in New York identified 5 newborns with early infantile Krabbe disease out of 2 million infants screened or an incidence for early infantile Krabbe disease of 1:394,000. This is lower than the previous Krabbe disease estimate of 1:100,000 but does not include potential late-onset forms.[12] The incidence of Fabry disease hemizygotes before NBS was estimated at 1:120,000;[7] however, recent studies evaluating NBS data found Fabry disease to be the most common LSD, with an incidence of 1:1852 to 1:7057 males.[13,14] The implementation of NBS for several LSDs suggests the incidence of most conditions is higher than previously estimated.[15]

Modes of inheritance

Most LSDs follow an autosomal recessive mode of inheritance, whereas a minority are X-linked, including Fabry disease, Danon disease, and MPS type II. The vast majority of females heterozygous for a pathogenic variant in *IDS* (MPS type II) are asymptomatic; however, a few with highly skewed X-inactivation toward the deficient allele have exhibited symptoms.[16] Females with pathogenic variants for Fabry or Danon diseases may exhibit symptoms regardless of their X-inactivation status.[17,18]

Treatment of LSDs

Although once considered incurable, treatment strategies have been or are being developed for many of the LSDs. The most successful approach to date is enzyme replacement therapy, which has been applied to Gaucher disease, Fabry disease; Pompe disease; MPS types I, II, IVA, and VI; Wolman or cholesteryl ester storage disease; neuronal ceroid lipofuscinosis 2, and Niemann-Pick type A/B. Other strategies include hematopoietic stem cell transplantation (HSCT), substrate reduction or chaperone therapy (eg, Miglustat [N-butyl-deoxynojirimycin] for Gaucher disease), and gene therapy.[2] In general, clinical outcome is optimized when treatment is initiated as early as possible and ideally before the development of symptoms. However, because of the insidious yet inexorable nature of these disorders, there is often a significant lag time between the initial onset of symptoms and the time of diagnosis. In addition to disorder-specific therapies, individuals with an LSD should be cared for by a multidisciplinary team of experts, including cardiologists, ophthalmologists, neurologists, and orthopedic specialists. Individuals with an LSD also benefit from ongoing symptomatic and supportive care, including analgesics for bone pain, antiepileptic medications for seizures, physical therapy to optimize mobility, and nutrition monitoring.

NBS

NBS for LSDs became possible with the development of enzyme assays directly from dried blood spots (DBSs). Currently, MPS type I and Pompe disease appear on the US Health and Human Services' Recommended Uniform Screening Panel (https://www.hrsa.gov/advisory-committees/heritable-disorders/rusp/index.html), and they, along with MPS type II, Fabry disease, Gaucher disease, Krabbe disease, and Niemann-Pick type A/B, are included in NBS programs in ≥1 states. Pilot studies have been proposed or are underway for several other LSDs, including metachromatic leukodystrophy. NBS evaluates lysosomal enzyme activity directly from DBS using tandem mass spectrometry (MS/MS; specifically flow injection analysis-MS/MS or liquid chromatography-MS/MS) or digital microfluidics fluorimetry (DMF-F) platforms.[19] Confirmatory enzyme assays are performed in DBS, serum/plasma, leukocytes, and/or fibroblasts, typically in conjunction with biomarker testing and DNA sequencing.[20,21] With rare exception, individuals undergoing testing prompted by NBS are asymptomatic, which can make interpreting ambiguous enzyme or DNA results challenging. Of particular importance is the recognition of pseudodeficiency, which may appear as abnormally low enzyme activity by laboratory testing but is not associated with clinical disease. Pseudodeficiency historically refers to reduced enzyme activity in vitro due to decreased specificity toward an artificial substrate. More recently, the term has come to include a true partial reduction of enzyme activity but not to a level that results in accumulation of substrate. Regardless of the definition, evaluation of biomarkers may be useful in clarifying pseudodeficiency from actual disease. DNA variants associated with pseudodeficiency have been described for several of the LSDs and are summarized in Table 2. In addition, NBS may detect atypical or late-onset forms of an LSD that lack clear guidelines for treatment and management, as well as cases where results of follow-up testing remain unclear.

Methods

The laboratory technical standard was informed by a review of the literature, including any current guidelines, and expert opinion. Resources consulted included PubMed (search terms: lysosomal storage disorders; lysosomal enzyme assays; tandem mass spectrometry; 4-methylumbelliferone [OR fluorometry]; spectrophotometry; newborn screening; pseudodeficiency; arylsulfatase; mucolipidosis; mucopolysaccharidosis [OR iduronidase]; lipofuscinosis [OR palmitoyl-protein thioesterase OR tripeptidyl peptidase]; Danon [OR LAMP2]; Fabry [OR galactosidase]; Gaucher [OR glucocerebrosidase]; Krabbe [OR galactocerebrosidase]; Niemann-Pick [OR sphingomyelinase]; Pompe [OR glucosidase]; Tay-Sachs [OR hexosaminidase]; Wolman [OR lysosomal acid lipase]), the American College of

Table 2 Common pseudodeficiency alleles

Gene	Reference Sequence	Variant	GMAF	Effect of Variants in Enzyme Studies	References
ARSA	NM_000487.5	c.*96A>G	0.04992	Loss of polyadenylation signal decreases the amount of 2.1 kb mRNA by 90%; 3.7 kb and 4.8 kb mRNA species are not affected	Harvey et al,[22] Gieselmann et al[23]
		c.1055A>G p.Asn352Ser	0.22484	Loss of one of the N-glycosylation sites may result in aberrant targeting to the lysosome and reduces activity by approximately 50% c.[*96A>G;1055A>G];[*96A>G;1055A>G] reduces activity by ~90%	
ARSB	NM_000046.4	c.1072G>A p.Val358Met	0.28554	c.1072G>A reduces activity by approximately 30% c.1151G>A reduces activity by approximately 40%	Garrido et al[24]
		c.1151G>A p.Ser384Asn	0.01957	c.[1072G>A;1151G>A] reduces activity by approximately 80%	
GAA	NM_000152.4	c.1726G>A p.Gly576Ser	0.03714	c.[1726G>A];[1726G>A] reduces activity by approximately 85%	Tajima et al[25]
	NM_000152.3	c.2065G>A p.Glu689Lys	0.07808	c.[2065G>A];[2065G>A] reduces activity by approximately 50% and is a common allele in China and Japan c.[1726G>A;2065G>A];[1726G>A;2065G>A] is a common allele with activity levels in the affected range	Suzuki et al,[26] Kroos et al[27]
GALC	NM_000153.4	c.550C>T p.Arg184Cys	0.02716	Common and benign variants that attenuate GALC activity but do not cause disease	Orsini et al[12]
		c.742G>A p.Asp248Asn	0.07947		
		c.1685T>C p.Ile562Thr	0.44748	In cis with other variants, c.1685T>C further reduces activity by approximately 50% and is a common variant in Africa	Hosain et al[28]
GLA	NM_000169.2	c.937G>T p.Asp313Tyr	0.00212	c.937G>T reduces activity by approximately 40% at a neutral pH	Yasuda et al[29]
GLB1	NM_000404.3	c.1561C>T p.Arg521Cys	0.07288	c.1561C>T reduces activity by approximately 70% and is a common variant in Brazil	Caciotti et al[30]
		c.1783C>T p.Arg595Trp	0.00020	c.1783C>T reduces activity by approximately 50% and is a common variant in the Basque population	Gort et al[31]
HEXA	NM_000520.5	c.739C>T p.Arg247Trp	0.00040	c.739C>T and c.745C>T reduce activity by approximately 70%	Cao et al[32]
	NM_000520.4	c.745C>T p.Arg249Trp	0.00016[a]		
IDUA	NM_000203.4	c.235G>A p.Ala79Thr	0.01018	c.235G>A is a common variant in Africa Decreased DBS and WBC activity with normal urine GAGs	Wasserstein et al[33]
		c.246C>G p.His82Gln	0.00120	c.246G>A is a common variant in Africa Decreased DBS activity with normal urine GAGs	Donati et al[34]
		c.667G>A p.Asp223Asn	0.00260	Decreased DBS and WBC with normal urine GAGs	Wasserstein et al[33]
		c.898G>A p.Ala300Thr	0.00004[b]	c.898G>A has reduced activity with 4-MU substrate but normal fibroblast studies	Aronovich et al[35]
	NM_000203.5	c.965T>A p.Val322Glu	0.00180	Decreased DBS and WBC activity with normal urine GAGs	Wasserstein et al[33]

4-MU, 4-methylumbelliferone; *DBS*, dried blood spot; *GAG*, glycosaminoglycan; *GMAF*, global minor allele frequency; *gnomAD*, Genome Aggregation Database; *mRNA*, messenger RNA; *TOPmed*, Trans-Omics for Precision Medicine; *WBC*, white blood cell.

ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/). Accessed on March 17, 2021.

[a]GMAF unavailable for *HEXA* c.745C>T; used gnomAD.

[b]GMAF unavailable for *IDUA* c.898G>A; used TOPmed.

Medical Genetics and Genomics (ACMG) Standards and Guidelines for Clinical Genetics Laboratories, Clinical and Laboratory Standards Institute guidelines, Clinical Laboratory Improvement Amendments (CLIA) regulations, the Centers for Disease Control and Prevention Morbidity and Mortality Weekly Report on Good Laboratory Practices

for Biochemical Genetics Testing and Newborn Screening for Inherited Metabolic Disorders, OMIM, GeneReviews, Genetics Home Reference, and ClinVar. When the literature provided conflicting or insufficient evidence about a topic, the authors used expert opinion to inform the recommendations. Expert opinion included the coauthors of the document, members of the Biochemical Genetics Subcommittee of the Laboratory Quality Assurance Committee, as well as any experts consulted outside the workgroup and acknowledged in this document. Any conflicts of interests for workgroup members are listed at the end of the paper. The ACMG Laboratory Quality Assurance Committee reviewed the document providing further input on the content, and a final draft was delivered to the ACMG Board of Directors for review and approval to send out for member comment. The final draft of the document was posted on the ACMG website, and an email link was sent to ACMG members inviting all to provide comment. All members' comments were assessed by the authors, and our recommendations were amended as deemed appropriate. Member comments and author responses were reviewed by a representative of the Laboratory Quality Assurance Committee and the ACMG Board of Directors. The final document was approved by the ACMG Board of Directors.

Preanalytical Requirements

Specimen requirements

Most lysosomal enzymes can be assayed in cultured fibroblasts, leukocytes, serum, plasma, and/or DBS. Certain disorders such as Niemann-Pick type C, Farber disease, and sialidosis require freshly harvested fibroblasts for accurate measurement. Prenatal testing for many LSDs can be done on direct chorionic villus samples or cultured amniocytes, although few clinical laboratories offer prenatal enzyme testing. Molecular testing is preferred for prenatal diagnosis if the familial variant(s) have been identified.

Leukocytes

Most lysosomal enzyme assays are performed in leukocytes isolated from whole blood. Leukocytes are separated from red blood cells (RBCs) either by sedimentation in a chilled dextran-saline solution[36] or using a commercially available RBC lysis buffer.[37] In both procedures, the resulting pellets are washed to remove residual RBCs and either assayed immediately or stored frozen. Leukocyte lysates are prepared for assay by sonicating pellets in water, saline, or assay buffer to achieve a protein concentration appropriate for the enzyme (typically 0.5-2.0 mg/mL). Sonication should be performed in an ice bath to minimize heat generation. After low-speed centrifugation, the supernatant can be used for enzyme analysis.

Fibroblasts

Although fibroblast assays require invasive sample collection (ie, skin biopsy) and longer times for cell culture, they are also relatively unaffected by sample handling conditions and allow for multiple enzyme assays to be performed from a single sample collection. Additionally, because the number of cells can be controlled, assays in fibroblasts can be more robust than in other sample types. For fibroblast assays, cultured cells are washed, detached from the flask by scraping or trypsinization, and pelleted by gentle centrifugation. As with leukocytes, the resulting pellet can be assayed immediately or stored frozen.

DBS

DBS specimens are easy to collect and require minimal blood and sample preparation. Once dried, blood spots can be stored or shipped at room temperature with no to minimal impact on enzyme activity. A typical assay requires a 3.2 mm punch into a sample well followed by enzyme extraction with sodium phosphate buffer or water. Extracts are then incubated with a specific assay cocktail for times ranging from several hours to overnight depending on the assay. Once enzyme testing is completed, the residual DBS card can be used for additional biomarker analysis and/or DNA testing.[19]

Sample shipping, handling, and storage

Laboratories must establish specific requirements for sample type, collection volumes, and conditions of sample shipping and handling. Leukocyte assays typically require 5 to 10 mL of anticoagulated whole blood, although as little as 2 mL may suffice for some enzymes. In testing for disorders with cytopenia as a feature (eg, Gaucher disease), larger collection volumes may be required to obtain a sufficient leukocyte pellet for analysis. Blood should be refrigerated upon collection and sent to the testing laboratory via overnight delivery on cool packs or wet ice. Leukocytes should be isolated from whole blood as soon as possible, ideally within 24 hours of collection.[36] In most cases, isolated leukocyte pellets can be assayed immediately or stored at –20 °C for at least 1 month. For plasma or serum testing, whole blood should be centrifuged as soon as possible, and the plasma/serum should be immediately separated and frozen at –20 ºC. Samples should remain frozen until testing with minimal freezing and thawing. For DBS sampling, whole blood may be obtained by heel stick or venipuncture and then spotted on filter paper such as Whatman Protein Saver 903, completely filling in 2 to 5 circles. Samples must be dried completely before shipping but can be shipped at room temperature. Environmental conditions, including heat, humidity, or dampness, may result in loss of enzyme activity. DBS should be stored between –20 ºC and 4 ºC in the presence of a desiccant, where they remain stable for up to 6 months.[38]

Preanalytical variables

Samples should be processed after collection as soon as possible in order to maintain stability and minimize protein degradation. Enzyme activity can be reduced by extreme heat or cold, prolonged time between blood draw and leukocyte extraction, or excessive freeze-thaw cycles. Laboratories should evaluate and document enzyme stability under different conditions as part of comprehensive test validation. Note that enzyme replacement therapy and HSCT will affect enzyme activity results; HSCT may also affect genetic testing using DNA derived from blood. In these situations, other diagnostic testing may be warranted.

Method Validation

As laboratory developed tests, lysosomal enzyme assays must be established and validated by each testing laboratory in accordance with the CLIA of 1988. This includes defining appropriate sample type(s), storage conditions and stability, assay conditions (eg, buffer and substrate concentrations, pH, protein concentration ranges, and incubation time and temperature), documenting assay performance (eg, imprecision, linearity, lower limit of detection, and analytic measurement range), establishing robust quality control (QC) procedures, and determining reference ranges. Assay interferents should be identified and documented, including effects of clinical status and medications (eg, pregnancy, liver dysfunction, oral contraceptives in Tay-Sachs carrier screening). Laboratories should implement procedures to address values outside of established criteria for assay performance.

Assay conditions should be designed to optimize discrimination between affected and unaffected individuals. A study of enzyme kinetic properties can guide selection of substrate concentration to ensure assays are maintained in substrate excess. Specific cofactors and/or inhibitors may be required depending on the enzyme. For leukocyte or fibroblast assays, protein concentrations should be defined and adjusted for clinical specimens either using a standardized protein concentration for all lysates (eg, 1 mg/mL protein) or using different concentrations within a predefined range.

QC specimens must be included in each batch of samples, including a positive control ideally from an affected individual (for specific instances of carrier screening [see later]) and a normal control from an unaffected individual. QC specimens can be prepared from pooled samples and stored as frozen aliquots; if there is insufficient positive control material from affected individuals, samples from unaffected individuals can be heat-inactivated to mimic an enzyme deficiency. For DBS-based assays, the Centers for Disease Control and Prevention Newborn Screening Quality Assurance Program offers QC material that includes 4 different levels of control for multiple enzymes (https://www.cdc.gov/labstandards/nsqap_resources.html). The low-activity controls were originally created by mixing leukocyte depleted blood with heat-inactivated, charcoal-stripped serum at a physiological hematocrit of 55% and then spotted onto filter paper.[38] For cultured cells, a concurrent normal control can be provided by the cell culture laboratory. Regardless of the source of positive control material, assay validation should include testing of authentic samples from affected individuals whenever possible. QC materials should be evaluated for performance and stability before use in clinical testing, with established acceptance criteria and procedures in place for handling any failed QC events. Performance of QC samples, including blanks, standards, and other controls, should be documented with each assay. Additional quality steps include testing samples in duplicate whenever possible and testing a control enzyme or additional enzyme unrelated to the enzyme under investigation, to assess sample integrity. The use of a second enzyme is particularly important when the measured enzyme of 1 or more samples in a batch is low. All QC results should be compiled at least monthly, with documented review by the laboratory director or designee.

Testing personnel

Qualifications for personnel performing lysosomal enzyme testing are the same as for all high-complexity testing and are specified in the Code of Federal Regulations sections 493.1441-493.1495. At a minimum, testing personnel must have an associate degree in laboratory science or certification in medical laboratory technology from an accredited program. Additional requirements and regulations determined by individual states may apply. Testing personnel must receive initial training as well as an annual assessment of competency. All training and competency assessments must be documented by the laboratory.

Proficiency testing

Participation in an ongoing proficiency testing (PT) program is required by CLIA and allows for continual monitoring and evaluation of testing quality. This can be achieved through external QC systems, including organized PT programs, although the number of external programs for lysosomal enzymes is limited. The European Research Network for Evaluation and Improvement of Screening, Diagnosis, and Treatment of Inherited Disorders of Metabolism provides clinical diagnostic laboratories with proficiency materials for the measurement of lysosomal enzymes in the form of lyophilized human fibroblasts; additional information can be found on their website: https://www.erndim.org/home/qascheme.asp. Because fibroblasts serve as the basis of this scheme, its usefulness may be limited to laboratories performing mainly cell-based enzyme analysis as opposed to testing in plasma or DBS. PT schemes for DBS testing are currently available through the Centers for Disease Control and Prevention Newborn Screening Quality Assurance Program for α-L-iduronidase, galactocerebrosidase, acid

α-glucosidase, α-galactosidase, β-glucocerebrosidase, and acid sphingomyelinase (https://www.cdc.gov/labstandards/nsqap_resources.html). For enzyme assays with no formal PT available, alternative quality assessment may be performed by various means, including split sample analysis with another clinical laboratory, testing internally blinded samples, and clinical correlation.[39,40] All proficiency samples must be incorporated into the regular clinical workflow and handled, analyzed, reviewed, and reported in the same manner as all other specimens in the laboratory.

Reference ranges

Ranges for unaffected, affected, inconclusive, and, if applicable, heterozygous individuals should be established or verified for each enzyme and sample type. The reference range can be set using an appropriate number of specimens from unaffected individuals and should be periodically reassessed by the laboratory. To determine the affected and heterozygote ranges, genuine samples should be used. Procurement of these specimens is often a challenge for the testing laboratory when establishing these LSD assays; therefore, cell lines or samples from affected and/or heterozygous individuals may be obtained from commercial vendors or sample exchanges with other laboratories. Some results (inconclusive) may fall in the area of overlap that occurs between affected, heterozygous, and unaffected individuals, and an unambiguous result cannot be obtained. It is the responsibility of each laboratory to determine how they choose to report results that fall into this zone.

Enzyme Analysis

Fluorometric assays

Most lysosomal enzyme assays use substrates based on the fluorophore 4-methylumbelliferone (4-MU). As a substrate for lysosomal glycosidases, 4-MU is covalently linked to a sugar moiety in the correct anomeric configuration for the enzyme of interest (eg, 4-MU-beta-D galactopyranoside). Alternatively, 4-MU may be linked to other groups such as fatty acids (4-MU-oleate or 4-MU-palmitate) to measure lysosomal acid lipase activity or free sulfate (4-MU-sulfate) to measure arylsulfatase B (ARSB) activity. Enzyme activity from the clinical specimen cleaves the sugar moiety (or other conjugate) releasing free 4-MU, which can be detected fluorometrically and related to enzyme activity using a standard curve of known 4-MU concentrations. These assays are simple to perform and identify individuals with an enzyme deficiency by their impaired cleavage of the 4-MU conjugate and correspondingly low fluorescence signal.[36]

In a typical 4-MU–based assay, samples are incubated with a specific substrate at acidic pH at 37 °C for 30 to 60 minutes for leukocyte lysates or longer for DBS extracts; exceptions include MPS types II, IIIA, IIIC, IIID, and IVA, where fluorogenic substrates require longer incubations and a second hydrolysis step to avoid underestimation of enzyme activity.[41,42] After incubation, reactions are stopped using a glycine-carbonate buffer at alkaline pH (eg, pH approximately 10), where 4-MU has maximal fluorescence and is stable for at least 1 hour. For measurement of lysosomal acid lipase activity, a reagent with a slightly lower pH (eg, 0.25 mol/L Trizma at pH 8) is used to stop the reactions because hydrolysis of the substrate (4-MU-oleate or 4-MU-palmitate) continues at a higher pH.[43,44] The fluorescence generated by free 4-MU is read at excitation wavelength 365 nm and emission wavelength 450 nm. The production of 4-MU is directly related to enzyme activity, which is typically expressed as nmol/h/mg protein (cell or tissue lysates) or nmol/h/mL (serum or plasma). Enzyme activity in DBS is expressed as μmol/h/punch or μmol/h/L (assuming 3 μL of blood volume for each 3.2 mm diameter punch[45]).

Blank samples should be analyzed with each batch to control for background interferences from reagents and nonenzymatic breakdown of substrate to product. Blanks contain all assay components except the clinical specimen, which is replaced with the same volume of water or buffer. Blank fluorescence should be recorded when preparing a new substrate lot and monitored with each assay. The laboratory should define tolerances for acceptable background signal and troubleshoot the causes and prepare new substrate when criteria are not met.

MS/MS

MS/MS-based assays use synthetic substrates that are structurally similar to their natural counterparts and yield products detectable by triple-quadrupole mass spectrometry using multiple reaction monitoring. Assay reagents and methods were optimized and refined into a standardized protocol in 2008,[46] and substrates and appropriate internal standards are commercially available for measuring α-galactosidase A, acid α-glucosidase, acid sphingomyelinase, β-galactocerebrosidase, β-glucocerebrosidase, and α-iduronidase activities in DBS samples.[47-49] Reagents and methods have also been developed for other disorders, including MPS types II, IIIA, IIIB, IIIC, IIID, IVA, VI, and VII; neuronal ceroid lipofuscinosis 1; and neuronal ceroid lipofuscinosis 2.[50-55]

Assays are performed on enzymes that have been extracted from DBS or leukocytes. Extracts are incubated overnight in individual reaction mixtures at optimal conditions (pH, buffer composition) for each enzyme. Reactions are then quenched using ethyl acetate:methanol (1:1), and mixtures are combined and purified by either solid phase extraction or liquid–liquid extraction (using ethyl acetate). These cleaning steps remove salts, detergents, and excess substrates to minimize ion suppression, contamination, and signal from in-source substrate fragmentation in the mass spectrometer. The final sample is dissolved in mobile phase (80% acetonitrile containing 0.2% formic acid) for rapid

flow injection MS/MS.[47] Online sample cleaning approaches have been proposed to eliminate manual sample preparation and facilitate high throughput screening,[56] but these require special hardware configurations that may not be practical for all laboratories.

MS/MS-based assays have several advantages over traditional methods. First, because each product is detected by a unique mass transition, assays can be multiplexed with additional analytes, including biomarkers, in a single multiple reaction monitoring analysis on the mass spectrometer. Although 4-MU assays have been multiplexed using DMF-F for NBS, expansion requires additional cartridges.[19,57] MS/MS assays also have higher sensitivity and dynamic range than 4-MU based fluorescent assays, which is reflected by a higher ratio of product signal in normal controls to blanks. This allows for a larger separation at the lower end of residual activity and potentially more accurate enzyme diagnosis.[58]

Spectrophotometric and other assays

Although most enzymes are currently measured by 4-MU or MS/MS-based assays, other methodologies, including spectrophotometric and radiolabeled assays, are still used by some laboratories. For example, spectrophotometric assays for arylsulfatase A (ARSA) and ARSB typically use the artificial substrate 4-nitrocatechol sulfate, which forms free 4-nitrocatechol upon enzyme hydrolysis. Metal ions such as barium salt are added to the assay mixture to chelate sulfate and phosphate, which otherwise inhibit sulfatases. Because both ARSA and ARSB have activity against the 4-nitrocatechol sulfate substrate, extra steps are needed to differentiate between the 2 enzymes. For ARSA measurements, ARSB is partially inactivated with 0.25 mM sodium pyrophosphate; ARSB activity is determined by subtracting the absorbance at 30 minutes incubation from that at 90 minutes because ARSA activity is essentially nil after 20 minutes.[36] Sulfatase activity is expressed as the amount of sulfate released per hour per mg of protein, which is correlated with the absorbance of free 4-nitrocatechol at 515 nm. Alternatively, ARSB can be measured using radioactive oligosaccharide substrate derived from chondroitin 4-sulfate or using an MS/MS assay with a synthetic substrate with an N-acetylgalactosamine-4-sulfate residue. The use of radiolabeled substrates has become increasingly uncommon in diagnostic laboratories, largely because of the added costs of handling and waste disposal, as well as implementation of MS/MS-based assays.

Test Interpretation and Reporting

Interpretation

Although the diagnosis of an LSD is based on a specific enzyme deficiency, it is important to note that the activity in affected individuals may not be 0. The degree of enzyme deficiency (ie, the amount of residual activity) depends both on the specific enzyme and the disorder, in addition to the assay performance (eg, the contribution of nonenzymatic substrate degradation). Individuals with late-onset disease may have higher levels of residual activity than those with early-onset disease, and individuals with pseudodeficiency may have decreased levels that fall in the affected or inconclusive range. Additional factors influencing enzyme activity should also be considered in establishing the final diagnosis. To control for preanalytical causes of enzyme deficiency, at least 1 other enzyme with a similar stability profile should be assayed (note this is a built-in feature of MS/MS and DMF-F multiplexed assays and other multienzyme panels). Because most enzymes can withstand several rounds of freezing and thawing, it is generally acceptable to perform repeat or additional testing on subsequent days if needed. Technical errors, such as failing to add an assay component or using the incorrect buffer, should be ruled out by repeat testing on any sample in which the enzyme activity is found to be low. A second sample should be requested if there is insufficient material for repeat testing.

Depending on the clinical context, the finding of a single enzyme deficiency may prompt additional testing to address the possibility of a multiple enzyme deficiency. For example, the finding of decreased sulfatase activity (eg, ARSA) may prompt testing of other sulfatases (eg, ARSB, iduronate-2 sulfatase, or sulfamidase) to exclude multiple sulfatase deficiency, a disorder of post-translational sulfatase modification.[59] Decreased activity of either α-neuraminidase or β-galactosidase may prompt testing of the other enzymes to rule out galactosialidosis, a deficiency of their shared protective protein cathepsin A. The multiple enzyme deficiencies of mucolipidosis (ML) type II and III result from failure of newly synthesized lysosomal enzymes to acquire their M6P targeting signal, leading to their inappropriate secretion outside of cells. Although enzyme activities in ML types II and III are not significantly altered in leukocytes, they are broadly decreased in fibroblast cultures (β-glucocerebrosidase is a notable exception) and increased in culture media and plasma. In particular, assaying β-hexosaminidase, N-acetyl-α-D-glucosaminidase, and iduronate-2-sulfatase in plasma reveals abnormal elevations typical of ML type II and type III. It is important to distinguish between MPS type II and ML type II and type III, clinically similar conditions that involve a disruption of iduronate-2-sulfatase activity. Finally, increased plasma activity of ≥1 lysosomal enzymes has also been described in other conditions with abnormal glycosylation or glycoprotein trafficking, including congenital disorders of glycosylation, galactosemia (untreated), hereditary fructose intolerance, Lowe syndrome, diabetes, and cancer.[60]

On rare occasions, enzyme testing may be normal when an LSD is highly suspected. In this situation, it is important to consider whether cofactors and/or activators of these enzymes may be responsible for the clinical findings.

Specific examples include GM2 activator deficiency with normal hexosaminidase activity and defects in prosaposin, a precursor of sphingolipid activator proteins (eg, saposin A deficiency with normal galactosylceramidase activity, saposin B deficiency with normal ARSA activity, and saposin C deficiency with normal β-glucocerebrosidase activity).

Test interpretation should consider additional factors incorporating clinical history and, as applicable, results of other pertinent tests such as NBS, urine screening, biomarker testing, and/or DNA sequencing. A significant enzyme deficiency in the setting of consistent clinical findings, particularly with a positive family history, is essentially diagnostic for the disorder. Supportive data may also come from urine screening tests (eg, mucopolysaccharides, oligosaccharides, or sulfatides) and other biomarker studies (eg, lyso-globotriaosylsphingosine in Fabry disease, psychosine in Krabbe disease, and glucosylsphingosine in Gaucher disease).[61] Molecular testing results in the form of single-gene sequencing, next-generation sequencing panels, or exome or genome sequencing may be available either before or after enzyme testing. When DNA variants are identified first, enzyme testing, along with biomarker studies when available, may be useful to confirm molecular findings or help with clarifying variants of uncertain significance. Molecular testing after an enzymatic diagnosis can identify pathogenic variants to guide testing of other family members and may provide useful insight into management and prognosis. Molecular testing is also important for identifying variants associated with pseudodeficiency (Table 2), particularly in the setting of NBS or when clinical information is either unavailable or inconsistent with the measured enzyme deficiency.

Enzymatic testing for carrier screening is well established for TSD, with specific guidelines for test utilization, interpretation, and integration with molecular testing.[62,63] In particular, the interpretation of TSD carrier screening should be based on established reference ranges for normal, heterozygote, and inconclusive levels, as well as for Sandhoff disease. Possible influences of pregnancy, oral contraceptives, diabetes, liver dysfunction (due to alcoholism, hepatitis B virus, autoimmune disease, primary biliary cirrhosis), and other medical conditions should be considered.[63-67] Furthermore, the use of ethnic-specific reference ranges has been shown to reduce false-positive results in certain populations (eg, individuals of African and Central or South American descent).[68] DNA testing can be used to confirm the presence of pathogenic variants, identify pseudodeficiency alleles, clarify indeterminate enzyme results, and provide molecular information for prenatal diagnosis and carrier screening for other family members.

Enzymatic carrier screening for LSDs other than TSD is generally not reliable because of the wide overlap between unaffected and carrier status and should not be performed. For these disorders, molecular studies should be employed to determine carrier status.

Reporting

Test reports must contain appropriate patient and specimen information as given by the ACMG Standards and Guidelines for Clinical Genetics Laboratories, Section C8.5.6.7 https://www.acmg.net/ACMG/Medical-Genetics-Practice-Resources/Genetics_Lab_Standards/ACMG/Medical-Genetics-Practice-Resources/Genetics_Lab_Standards.aspx?hkey=0e473683-3910-420c-9efb-958707c59589? and as specified by CLIA. Reports should contain the enzyme(s) tested, activity level, unit of measure (eg, nmol/h/mg), and appropriate reference range. Affected, heterozygote, and inconclusive ranges should also be provided whenever possible and/or appropriate. The report should include an overall interpretation and specify that the result is consistent with unaffected, affected, indeterminate, or carrier status and discuss the significance of the result.[17] Recommendations for additional testing (eg, other enzyme assays, biomarker studies, molecular analysis to confirm a diagnosis or exclude pseudodeficiency) and follow-up, including genetic counseling and referral to a metabolic specialist, should be included when appropriate. Reports should include the methodology used and any known limitation of the assay as well as if the test is investigational, laboratory-developed, or FDA-cleared or -approved (Section C.8.5.6.7). Finally, the contact information for the reporting laboratory is required should the ordering provider have questions or concerns. Examples of clinical reports are provided in the Supplemental Appendix.

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Conflict of Interest

E.T.S., K.C.-O., T.W., and C.Y. direct clinical biochemical genetics laboratories that run the tests discussed in the current standard on a fee-for-service basis. C.Y. is an employee of Sema4 and has equity ownership in the form of incentive stock options and restricted stock units.

Additional Information

The online version of this article (https://doi.org/10.1016/j.gim.2021.12.013) contains supplementary material, which is available to authorized users.

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