

Identification of Infants at Risk for Developing Fabry, Pompe, or Mucopolysaccharidosis-I from Newborn Blood Spots by Tandem Mass Spectrometry

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Objective To assess the performance of a tandem mass spectrometry (MS/MS) technology in a newborn screening laboratory to simultaneously measure α -galactosidase, acid- α -glucosidase, and α -L-iduronidase for the detection of infants at risk to develop Fabry, Pompe, or mucopolysaccharidosis (MPS)-I diseases.

Study design Enzyme activity was assayed from a 3.2-mm punch from 100 000+ anonymous newborn blood spots. Punches with low enzyme activity were further evaluated by nucleotide sequence analysis of the responsible gene. Confirmation of affected infants was dependent on identification of mutations compatible with diminished enzyme activity.

Results The technology for simultaneously measuring multiple enzyme activities by MS/MS was successful. The confirmation of diagnosis for Fabry, Pompe, or MPS-I, by DNA sequencing estimated the prevalence of Fabry disease at 1/7800 males (95% CI 1/17 800-1/3600); Pompe disease at 1/27 800 newborns (95% CI 1/90 000-1/10 200); and MPS-I at 1/35 500 newborns (95% CI 1/143 000-1/11 100). These estimates of prevalence are 2 to 4 times greater than the prevalence estimated by clinical diagnosis. The combined prevalence for the 3 disorders was 1/7500 newborns (95% CI 1/13 500-1/4500).

Conclusions MS/MS for the simultaneous assay of multiple lysosomal enzymes can be successfully introduced into a routine newborn screening laboratory. The technology has a positive predictive value equal to, or better, than methods currently used for the detection of nonlysosomal disorders. Using newborn blood spots, the combined prevalence of Fabry, Pompe, and MPS-I is estimated at 1/7500 newborns based on low-enzyme activity and confirmation by mutation analysis. (*J Pediatr* 2013; ■: ■-■).

More than 50 lysosomal storage diseases (LSDs), with an estimated clinical prevalence of 1/7000 to 1/9000 in European populations, have been described.^{1,2} LSD symptoms and severity vary, from central nervous system degeneration in infancy in severe Krabbe disease or mucopolysaccharidosis (MPS)-I, to minor somatic complications in Gaucher disease, type 1. Each of the disorders, however, has a wide spectrum of clinical presentations depending on the effect mutations have on the residual enzyme activity. Even with the same mutation, differences in factors controlling gene expression and the environmental milieu will influence disease manifestation. Because of the variable clinical presentation and nonspecific symptoms of the LSDs, there is often delay in clinical recognition and diagnosis. This delay in diagnosis may postpone the timely initiation of therapy for the prevention of serious central nervous system or somatic involvement.³

Since the recognition by Chamoles et al^{4,5} that lysosomal enzymes retain activity in dried blood spots on filter paper, our group has been designing novel substrates that can be used in diagnostic and newborn screening laboratories for the detection of LSD patients.⁶ We have designed substrates and internal standards for the quantitative measurement of lysosomal enzyme activities by tandem mass spectrometry (MS/MS). These include methods for the detection of Gaucher (acid β -glucosidase), Fabry (α -galactosidase [GLA] A), Pompe (acid α -glucosidase [GAA]), MPS-I (α -L-iduronidase [IDUA]), MPS-II (α -L-iduronide-2-sulfatase), Niemann-Pick diseases types A and B (acid sphingomyelinase), MPS-IVA (galactose-6-sulfate sulfatase), MPS-VI (N-acetylgalactosamine-4-sulfatase), and Krabbe (galactocerebrosidase) diseases.⁷⁻¹³

CDC	Centers for Disease Control and Prevention
GAA	α -glucosidase
GLA	α -galactosidase
IDUA	α -L-iduronidase
LSD	Lysosomal storage disease
MPS	Mucopolysaccharidosis
MS/MS	Tandem mass spectrometry
PCR	Polymerase chain reaction
QC	Quality control

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Many of these enzyme activity assays can be “multiplexed” by incubating samples with a cocktail containing substrates and internal standards in a common buffer, measuring the products by MS/MS,¹⁴⁻¹⁸ and calculating enzyme activities. To evaluate the robustness of the MS/MS method, we tested a multiplexed assay for Fabry disease, Pompe disease, and MPS-I in the Washington State Newborn Screening Laboratory on over 100 000 anonymous newborn dried blood spots. These 3 disorders were selected on the belief that they would be clinically acceptable to the newborn screening community for early detection and intervention.

Methods

The screening of ~110 000 newborns was performed in the Washington State Newborn Screening Laboratory. Institutional Review Board approval was obtained from the Washington State Department of Health with the stipulation that the screening be done anonymously after all required newborn screens had been performed. Punches were obtained from blood spots that had been stored at 18°C for 8-10 months from collection.

Retention of 80% of initial enzyme activity was validated under these conditions. Two 3.2-mm punches were collected for each anonymous newborn screening sample. The first punch was used to determine enzyme activity. The second punch was used for sequence analysis for samples with enzyme activities in the affected range. MS/MS was used for quantitating enzyme activity for GLA A, acid GAA, and IDUA as reported by Duffey et al.¹⁴

Cocktails were prepared from vials containing substrate and internal standard from the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia.¹⁹

A 3.2-mm punch from each newborn dried blood spot was placed into a well of a 96-deep-well plate, and 30 μ L of ammonium formate buffer (0.1 M, pH 4.4) containing 0.48 mM IDUA substrate, 3.1 μ M IDUA internal standard, 0.2 mM GAA substrate, 2.0 μ M GAA internal standard, 0.6 mM GLA substrate, 1.2 μ M GLA internal standard, and 8 μ M acarbose (Sigma Aldrich, St Louis, Missouri) was added. In addition to the newborn dried blood spots, each 96-deep-well plate contained 6 wells with a blank filter paper punch and 2 wells each of the 3.2-mm punches from quality control (QC) dried blood spots from the CDC.¹⁵ The blanks and QC samples were in the first and last columns of each plate. All pipetting was performed using a Rainen Liquidator 96-tip pipetter. The plates were sealed with aluminum plate sealing film (VWR International LLC, Radnor, Pennsylvania) and incubated at 37°C for 16 hours (overnight) in an orbital, shaking incubator at 225 rpm.

In the morning, the plates were quenched by the addition of 100 μ L of ammonium acetate buffer (0.1 M, pH 5.5), and 400 μ L ethyl acetate was added to the wells and mixed by aspirating the liquid 20-40 times using the Rainen Liquidator (Rainen Instrument LLC, Oakland, California). The plates were covered with foil, centrifuged for 5 minutes at 3000 g, and 200 μ L of the top ethyl acetate layer was transferred to

a new 96-shallow-well plate. The ethyl acetate was evaporated in a stream of air with an SPE Dry 96 Dual Argonaut sample concentrator system (Biotage LLC, Charlotte, North Carolina), with a flow rate of 40-80 psi of air and heating <35°C (typically <30 minutes), and the residue was resuspended in 100 μ L of the mass spectrometry mobile phase (80% acetonitrile, 20% water, 0.2% formic acid). The plates were placed on an orbital shaker for 5 minutes, covered with aluminum foil, and placed into the injector tray for MS/MS analysis.

A Waters Acquity TQD Ultra Performance (Waters Corporation, Milford, Massachusetts) tandem quadrupole MS/MS was used for analysis. Ten microliter of each 100 μ L sample was injected using a Waters 2777C sample manager via flow injection. The injection fluid was 80/20 acetonitrile/water with 0.2% formic acid at 0.10 mL/min for 1.10 minutes and then 0.5 mL/min for 0.40 minutes. The enzyme activity was calculated from the abundance ratio of product to internal standard with subtraction of the average of the 6 blanks. Enzyme activity was reported in μ mol/h/L of blood, assuming each punch contained 3.2 μ L of blood.

For samples with enzyme activities in the affected range, genotype was determined by analysis of the nucleotide sequence of the appropriate gene. Genomic DNA was isolated from the duplicate 3.2-mm punch using a QIAamp DNA micro kit (Qiagen, Carlsbad, California). The Vector NTI Advance 11 computer program from Invitrogen (Carlsbad, California) was used to develop primers for amplification. Exons and the flanking intron boundaries of the GAA and GLA genes were amplified by polymerase chain reaction (PCR) using Tsg DNA Plus Polymerase (D102; Lamda Biotech, St. Louis, Missouri) following the supplier's instruction. The exons and the flanking introns of IDUA were amplified using the GC-RICH PCR system (#12140306001; Roche Applied Science, Mannheim, Germany) following the supplier's instructions. The PCR amplicons were sequenced using a 3130 Genetic Analyzer (Applied Biosystems, Foster City, California). Unless otherwise noted, primers used for PCR and sequencing reactions were identical. The Mutation Surveyor DNA Variant Analysis software from SoftGenetics (State College, Pennsylvania) was used to confirm sequencing genotypes. GenBank entries NM_000152.3 (GAA), NM_000169.2 (GLA), and NM_000203.3 (IDUA) were used as references for coding regions, whereby nucleotide A of the translation initiation codon ATG constituted numbering +1 of the complementary DNA sequences. We followed the standard naming convention of the Human Genome Variation Society such that methionine encoded by the translation mutation codon was designated as position 1 in amino acid numbering.

Dried blood spots used for QC were obtained from the CDC.¹⁹ Four different samples were obtained: (1) unprocessed cord blood (high QC); (2) 50% cord blood in leukocyte-reduced base pool (medium QC); (3) 5% cord blood in leukocyte-reduced base pool (low QC); and (4) leukocyte-reduced base pool (blank). Two 3.2-mm punches from each QC blood spot were included in each 96-well assay plate. A continuous 95% CI was calculated for each QC

Table I. Calculated CV for interday enzyme variation based on QC samples supplied by the CDC¹³

QC Samples	GLA			GAA			IDUA		
	Mean*	SD	CV	Mean*	SD	CV	Mean*	SD	CV
Low	1.24	0.12	9.73	1.58	0.20	12.42	1.41	0.13	9.52
Medium	9.18	0.76	8.23	10.07	1.00	9.94	3.75	0.32	8.44
High	18.01	1.36	7.53	15.47	1.54	9.94	6.23	0.51	8.21

CV, coefficient of variation.

*Activity is reported as $\mu\text{mol/h/L}$ blood.

sample and any plate where the QC sample was outside the 95% CI was reason to repeat or exclude the plate from the final analysis. The interday coefficient of variation for each control sample was typically less than 10% (Table I).

Results

Over 100 000 newborn blood spots were assayed for the enzymes GLA, GAA, and IDUA. Based on experience during method development, arbitrary enzyme activity cutoffs of $\leq 19\%$ of the daily mean value for GLA, $\leq 15\%$ of the daily mean value for GAA, and $\leq 32\%$ of the daily mean value for IDUA were selected for further analysis by DNA sequencing.

GLA activity was measured in 108 905 samples (Figure, A). Enzyme activity was distributed as a log-normal curve; and mean activity was $10.2 \mu\text{mol/h/L}$ blood. This activity is similar to that using a MS/MS method to quantitate GLA enzyme activity reported by Dajnoki.²⁰ Based on our prior studies of activity in blood spots from males affected with Fabry disease,¹⁴ a cutoff of $1.91 \mu\text{mol/h/L}$ blood was used ($\leq 19\%$ of the mean value). Sixteen samples were at or below $1.91 \mu\text{mol/h/L}$ blood. Seven samples had nucleotide changes within the *GLA* gene consistent with Fabry disease (Table II). Consistent with the X-linked inheritance of the disease, all were males. Samples from 3 female and 6 male infants had enzyme activities between 1.91 and $1.12 \mu\text{mol/h/L}$ but no detectable mutations in the *GLA* gene. Based on this data, the estimated prevalence of Fabry disease in a US population of mixed ethnic background of approximately 54 800 male infants was 1/7800 (95% CI 1/17 800-1/3600). The positive predictive value for any sample, male or female, was 0.43 (95% CI 0.21-0.70); and the false positive rate was estimated at 1/12 100 (95% CI 1/6300-1/23 300).

GAA activity was measured in 111 544 samples. Enzyme activity was distributed as a log-normal curve; and mean activity was $17.6 \mu\text{mol/h/L}$ (Figure, B). A cutoff of $\leq 2.60 \mu\text{mol/h/L}$ ($\leq 15\%$ of mean) was used. Seventeen samples had enzyme activities at or below $2.60 \mu\text{mol/h/L}$ blood. Four samples were confirmed to have nucleotide changes consistent with possibly developing Pompe disease (Table II). Four samples had a single nucleotide change on one allele (carrier of Pompe), 3 were identified as carriers with an additional pseudodeficiency allele, and 6 samples were heterozygotes for a pseudodeficiency allele only. An

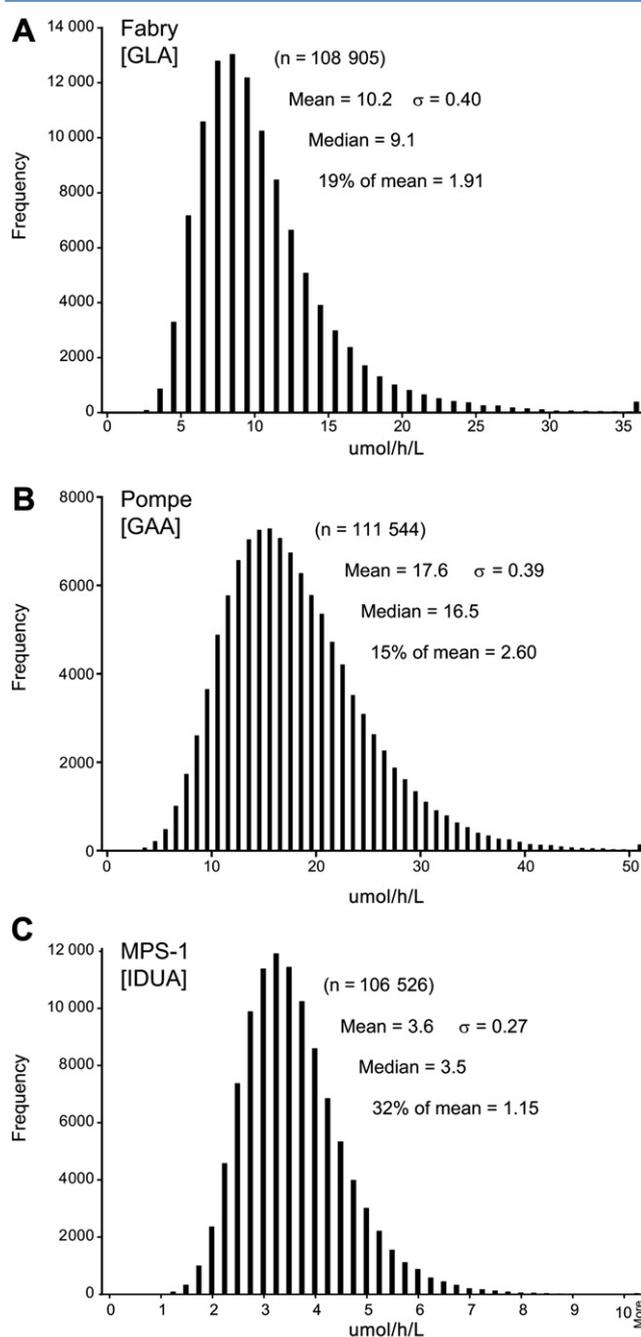


Figure. Distribution curves for the enzyme activity of **A**, GLA, **B**, GAA, and **C**, IDUA obtained from newborn dried blood spots.

estimate of the prevalence of infants with mutations who may eventually develop clinical symptoms of Pompe disease was 1/27 800 (95% CI 1/90 900-1/10 200). The positive predictive value was 0.24 (95% CI 0.08-0.50) and the false positive rate was 1/8600 (95% CI 1/5000-1/14 800).

IDUA activity was measured in 106 526 samples (Figure, C). The mean activity was $3.6 \mu\text{mol/h/L}$. The variance in IDUA activity was less than that for GLA or GAA, but also had a log-normal distribution. A cutoff of $\leq 1.15 \mu\text{mol/h/L}$

Table II. Summary of mutation data for those blood spots identified with low enzyme activity

Enzyme activity ($\mu\text{mol/h/L}$ blood)	cDNA	Amino acid
Fabry disease (n = 7):		
1.90	c.660G>A	p.Arg220Gln
1.66	c.1001G>A	p.Ser334Asn
1.34	c.70T>A	p.Trp24Arg
1.28	c.427C->A	p.Ala143Thr
1.21	c.758T>C	p.Ile253Thr
0.89	c.938A>G	p.Asp313Gly
0.58	c.644A>G	p.Asn215Ser
Unaffected with low GLA activity (n = 9):		
1.91-1.12	wt/ wt/wt	n = 6 n = 3
Pompe disease (n = 4):		
2.53	IVS1-13t>g IVS1-13t>g	
1.70	c.365T>A c.1925T>A	p.Met122Lys p.Val642Glu
1.57	IVS1-13t>g IVS1-13t>g	
1.43	IVS1-13t>g c.1-17C>T	p.Ser(-6)Phe
Unaffected with low GAA activity (n = 13):		
2.45-2.20	carrier/wt	n = 4
2.38-2.20	carrier/pseudo def	n = 3
2.44-1.49	pseudo def/wt	n = 6
MPS-I (n = 3):		
1.15	c.246C>G c.246C>G	p.His82Gln p.His82Gln
1.06	c.355G>T c.250G>A	p.Asp119Tyr p.Gly84Ser
1.05	c.208C>T c.208C>T	p.Gln70X p.Gln70X
Unaffected with low IDUA activity (n = 6):		
1.15-0.87	carrier/wt poor punch wt/wt	n = 1 n = 2 n = 3

wt, normal allele sequence; def, deficiency; cDNA, complementary DNA. Identified mutations were used to imply the clinical phenotype.

($\leq 32\%$ of mean) was used. Three samples were confirmed to have nucleotide changes consistent with MPS-I (Table II). One sample was identified as a carrier of a common mutation for MPS-I; 3 samples had no identified nucleotide change; and 2 had low activity attributed to a poor punch from the newborn card. The estimated prevalence of MPS-I from this study was 1/35 700 (95% CI: 1/143 000-1/11 100). The positive predictive value was 0.33 (95% CI 0.08-0.65), and the false positive rate was 1/17 750 (95% CI 1/7250-1/31 900).

The empirically set cutoffs, 19% of the mean for GLA, 15% of the mean for GAA, and 32% of the mean for IDUA, were further examined by finding distribution functions that would fit the experimental enzyme activities. All 3 distributions could be fitted with a log-normal probability density function according to formula,

$$f(X) = \frac{e^{-\frac{(\ln X)^2}{2\sigma^2}}}{X\sigma\sqrt{2\pi}}$$

where X is the activity relative to the experimental distribution median, and σ is the SD obtained by least squares fits. For GLA, the log-normal probability density function

predicted 6 counts at or below 19% of the mean (1.91 $\mu\text{mol/h/L}$). Likewise, the GAA distribution function predicted 6 counts at or below the 15% cutoff (2.60 $\mu\text{mol/h/L}$). The IDUA distribution function predicted 13 counts at or below the 32% cutoff (1.15 $\mu\text{mol/h/L}$). Thus, the experiment-based cutoffs are consistent with the statistical behavior of the dataset for each of the enzymes. Integration of the distribution curves from zero to the cutoff limits gives the predicted (theoretical) false positive rates which are $100 \times 6/108 905 = 0.006\%$ for GLA, $100 \times 6/111 544 = 0.005\%$ for GAA, and $100 \times 13/106 526 = 0.012\%$ for IDUA.

Discussion

Our goal was to answer 3 major questions with this study: (1) Can the multiplex MS/MS method for the detection of LSDs be performed as a routine procedure in a newborn screening laboratory?; (2) What is the estimated prevalence of each of the disorders?; and (3) What is the positive predictive value of the MS/MS method?

The multiplexed assay for Fabry, Pompe, and MPS-I was successfully integrated into the routine workflow of the Washington State Newborn Screening Laboratory. It required a dedicated MS/MS, nitrogen source, the availability of a 96-tip manual pipetter, and a dedicated technician. The existing software in the screening lab for data collection (Neometrix Neometrics Software; Natus Medical Incorporated, Hauppauge, New York) and analysis (Quanlynx; Natus Medical Incorporated) was satisfactory.

Consistent with prior reports, the detection of LSDs by newborn screening indicates a higher prevalence in the population than the prevalence estimates by clinical diagnosis. This increase in prevalence is primarily due to recognition of later-onset forms of the diseases. We detected 1/7800 newborn males to have low enzyme activity and nucleotide changes consistent with Fabry disease. We did not detect any female heterozygotes. We believe this was due to our cutoff of $<19\%$ of the population mean. This cutoff was based on data from affected males. This prevalence is considerably more frequent than the estimate of 1/40 000 based on clinical data.¹ Spada et al²¹ screened 37 164 newborn males in Northern Italy for Fabry disease using a fluorometric enzyme assay and identified $\sim 1/3100$ as affected males. Three of the 6 detected males carried the p.A143T mutation that is associated with a later-onset form of the disease. In Austria, Mechtler et al²² screened 34 736 infants by MS/MS and detected a prevalence of 1/3859 births ($\sim 1/1630$ males) with Fabry disease. Similar to the population in Italy, p.A143T was present in 3 of the 6 males identified, suggesting a founder effect for this mutation in the Northern Italy/Austrian region of Europe. Screening for Fabry disease has also been performed in Asia using a fluorometric enzyme assay.²³ In Taiwan, 1/1250 males were identified to have Fabry disease, with many having the genotype IVS4+919G>A. This mutation is common in the Asian population and is stated to have a late-onset phenotype in males, primarily with involvement of heart and kidney.²⁴

We detected 4 infants with Pompe disease, for an estimated prevalence of 1/27 800. Based on mutation data, we believe each of the 4 affected infants will have a late-onset phenotype. This value is twice as common as the estimate of 1/40 000 cited for the prevalence of both infantile and late-onset phenotypes of Pompe disease in the US population.²⁵ In Taiwan, a screening program using a fluorometric assay for GAA identified 5 affected infants in a population of 132 538, giving a prevalence of 1/41 000.²⁶ A complicating factor for screening for Pompe disease in the Asian population is the presence of a pseudodeficiency allele (p.G576S; E689K) that increased the false positive rate.²⁷ The 5 infants detected by screening and confirmed by clinical evaluation to have Pompe disease in Taiwan were placed on enzyme replacement therapy prior to the development of severe symptoms, and are reported to have normal cardiac function, growth, and age-appropriate developmental milestones.²⁶

The prevalence of 1/35 500 for MPS-1 identified by newborn screening is 3-fold greater than the prevalence estimated from clinical studies.^{1,2} Early diagnosis of MPS-I patients with the severe (Hurler) phenotype is needed to gain maximum benefit from hematopoietic stem cell transplant. For those affected with the Hurler phenotype, the greatest benefit in intellectual outcome is achieved if the procedure can be performed at less than 2.5 years of age.²⁸ The attenuated phenotype of MPS-I can benefit from enzyme replacement therapy to minimize the joint, organ, skin, and facial changes characteristic of the disorder.²⁹

In this study, the overall prevalence of disease identification for the 3 LSDs is 1 per 7500 newborns. Approximately two-thirds of the identified infants will have disease manifestations outside of early childhood. This raises the ethical question as to whether the early recognition of children at risk for significant disease after infancy is the appropriate venue for screening in the newborn period.³⁰ However, a significant number of patients with later-onset forms of LSDs have significant disease burden early in childhood and go mis- and undiagnosed for years.

The introduction of MS/MS into the newborn screening laboratory by Millington et al and Chace et al^{31,32} has revolutionized the ability to detect many genetic disorders prior to their symptomatic presentation and has prevented significant morbidity and mortality. The expansion of the use of MS/MS for the early detection of infants affected with various LSDs should prove to be just as clinically valuable as the detection of amino acid and organic acid disorders.

The compelling advantage of the MS/MS technology for the assay of enzyme activity is its ability to quantitate multiple reaction products from a single incubation and a single injection. High throughput fluorometric methods that measure enzyme activity and immunocapture methods that can quantitate residual enzyme protein are also being evaluated for newborn screening for lysosomal diseases. Data from their adaptation in newborn screening programs is not yet available. Although we have demonstrated the ability to simultaneously measure 3 enzymes from a single 3.2-mm dried blood spots, we anticipate expanding this technology to

include 9 or more assays for the detection of additional LSDs from a single 3.2-mm dried blood spots.³³ ■

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