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Newborn Screening for Lysosomal Storage Disorders: Clinical Evaluation of a Two-Tier Strategy

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ABSTRACT. *Objective.* **To evaluate the use of protein markers using immune-quantification assays and of metabolite markers using tandem mass spectrometry for the identification, at birth, of individuals who have a lysosomal storage disorder.**

Methods. **A retrospective analysis was conducted of Guthrie cards that were collected from newborns in Denmark during the period 1982–1997. Patients whose lysosomal storage disorder (LSD; 47 representing 12 disorders) was diagnosed in Denmark during the period 1982–1997 were selected, and their Guthrie cards were retrieved from storage. Control cards (227) were retrieved from the same period. Additional control cards (273) were collected from the South Australian Screening Centre (Australia).**

Results. **From 2 protein and 94 metabolite markers, 15 were selected and evaluated for their use in the identification of LSDs. Glycosphingolipid and oligosaccharide markers showed 100% sensitivity and specificity for the identification of Fabry disease, α-mannosidosis, mucopolysaccharidosis (MPS) IVA, MPS IIIA, Tay-Sachs disease, and I-cell disease. Lower sensitivities were observed for Gaucher disease and sialidosis. No useful markers were identified for Krabbe disease, MPS II, Pompe disease, and Sandhoff disease. The protein markers LAMP-1 and saposin C were not able to differentiate individuals who had an LSD from the control population.**

Conclusions. **Newborn screening for selected LSDs is possible with current technology. However, additional development is required to provide a broad coverage of disorders in a single, viable program.** *Pediatrics* **2004;114: 909–916;** ***genetic disease, mass spectrometry, oligosaccharide, glycolipid, protein marker.***

ABBREVIATIONS. LSD, lysosomal storage disorder; MPS, mucopolysaccharidosis; GC, glucosylceramide; LC, lactosylceramide; CTH, ceramide trihexoside; CV, coefficient of variation.

Lysosomal storage disorders (LSDs) represent a group of >45 distinct genetic diseases, each one resulting from a deficiency of a particular lysosomal protein or, in a few cases, from nonlysosomal proteins that are involved in lysosomal biogenesis. Most LSDs are inherited in an autosomal recessive manner, with the exceptions of Fabry disease, Danon disease, and mucopolysaccharidosis (MPS) type II, which display X-linked recessive inheritance. Some LSDs have been classified into clinical subtypes (eg, the Hurler/Scheie definition of MPS I, the infantile/juvenile/adult-onset forms of Pompe disease), but it is clear that most LSDs have a broad continuum of clinical severity and age of presentation. With the advent of molecular biology/genetics and the characterization of many of the LSD genes, it is now recognized that the range of severity may in part be ascribed to different mutations within the same gene. However, genotype/phenotype correlations are imprecise, and other factors, including genetic background and environmental factors, presumably play a role in disease progression.

Although each LSD results from mutations in a different gene and consequent deficiency of enzyme activity or protein function, all LSDs share one common biochemical characteristic, in that the disorder results in an accumulation of substrates that are normally degraded within lysosomes. The particular

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substrates stored and the site(s) of storage vary with disease type. The nature of the substrate is used to group the LSD into broad categories, including MPS, lipidoses, glycogenoses, and oligosaccharidoses.[1] These categories show many clinical similarities within groups as well as significant similarities between groups. Common features of many LSDs include bone abnormalities, organomegaly, central nervous system dysfunction, and coarse hair and facies.

Treatment of some LSDs is possible. Cystinosis is treated with cysteamine,[2] and a number of LSDs, including MPS I, MPS VI,[3–5] and Wolman disease,[6] have been responsive to bone marrow transplantation. Furthermore, patients who have metachromatic leukodystrophy and Krabbe disease and receive a transplant before clinical signs are evident have been reported to develop less central nervous system pathology than patients who do not receive a transplant or patients who receive a transplant after clinical signs are present.[7] Enzyme replacement therapy has been used to treat nonneuropathic Gaucher disease for >10 years with considerable success. More recently, enzyme replacement therapy for Fabry disease and MPS I has become available,[8,9] and clinical trials of this type of therapy for MPS II, MPS VI, and Pompe disease are in progress. Enzyme replacement therapy is likely to be limited to those LSD types that do not develop central nervous system pathology. However, when the procedure is applied early, bone marrow and more recently cord blood stem cell transplantation have been reported to benefit a number of LSD types that have been shown to develop central nervous system pathology.[4–7,10] It is probable that within the next few years, therapies will be available for many of the LSDs.

The effectiveness of these therapies, particularly for those LSDs that involve central nervous system and bone pathologies, will rely heavily on the early diagnosis and treatment of the disorder, before the onset of irreversible pathology. An additional consideration, critical to bone marrow transplant therapy, is that early diagnosis of LSDs will allow clinicians to take advantage of the window of opportunity presented by the naturally immature immune system in the neonate to maximize the chance of successful engraftment. Early detection of these disorders has the added advantage of permitting genetic counseling for the parents, with the option of prenatal diagnosis in subsequent pregnancies. In the absence of a family history, the only practical way to identify affected individuals presymptomatically is through a newborn screening program.

LSDs are rare disorders with prevalence values ranging from ~1:50 000 births to <1:4 000 000 births.[11] However, when considered as a group, the combined prevalence is substantially higher. We have previously estimated the prevalence of LSD in Australia to be 1:7700 births, excluding the neuronal ceroid lipofuscinoses. The prevalence of this latter group of LSD has been reported to be as high as 1 per 12 500 births in the United States.[12] In Finland, prevalence values of 1 per 13 000 births for infantile and 1 per 21 000 births for juvenile forms have been reported.[13] Clearly, the neuronal ceroid lipofuscinoses will contribute significantly to the overall prevalence of LSD. It is equally certain that additional LSDs will be identified as our understanding of lysosomal biology and the clinical manifestations resulting from lysosomal dysfunction improve. A conservative estimate of the prevalence of LSDs in the Australian population would be 1 in 5000 births. The cost of screening for LSDs individually would, in most cases, be prohibitive as a result of the low prevalence. However, screening for multiple disorders as a group with a total prevalence rate of ~1:5000 births could be economically justified.

We have previously identified a number of potentially useful protein markers of LSDs[14–16] that may enable the identification of the majority of individuals with LSDs into a high-risk group. More recently, we have developed tandem mass spectrometry–based methods for the determination of many of the stored oligosaccharide and glycosphingolipid substrates in LSDs.[17,18] Here we report on the evaluation of these markers using retrospective newborn Guthrie cards.

METHODS

Patient Samples

Statens Serum Institut, Denmark, has established a biological specimen bank of Guthrie cards collected from newborns since 1981.[19] These cards have been stored at −20°C since they were used for screening. Guthrie cards were retrieved from LSD patients (n = 47) who were born during the period 1982–1997. Control cards (n = 227, matched for age of the cards) were retrieved from the same period. Additional control, newborn Guthrie cards (n = 273) were obtained from the South Australian Newborn Screening Centre during 2001. The dried blood spots from Denmark were collected on S&S 2992 paper, and the samples from Australia were on S&S 903. A correction factor (0.79), determined using radiolabeled tracer in whole blood, was used to correct for the lower blood volume of the Denmark samples. All cards were deidentified. The use of the cards was approved by the Women's and Children's Hospital Ethics Committee and the Copenhagen-Frederiksberg Scientific Ethics Committee.

Production and Labeling of Antibodies

The anti–LAMP-1 monoclonal antibody (BB6) has been reported previously.[20] The anti-saposin C monoclonal antibody (7B2) was generated by the method of Zola and Brooks[21] after immunizing mice with recombinant saposin C[22] using a standard complete/incomplete Freunds adjuvant protocol.[23] Polyclonal antibodies were produced in rabbits against both recombinant LAMP-1[24] and saposin C[22] using standard complete/incomplete Freunds adjuvant protocols.[23] Affinity-purified polyclonal antibodies against LAMP-1 and saposin C were labeled with europium and samarium, respectively, using DELFIA labeling kits (Wallac, Melbourne, Australia).

Determination of LAMP-1 and Saposin C in Dried Blood Spots

The protein markers LAMP-1 and saposin C were determined by immune quantification in a dual assay using time-resolved fluorescence. The individual assays have been described previously.[14,16] In this study, the assays were combined into a single well by using both europium and samarium labels. Briefly, microtiter plates were coated (16 hours, 4°C) with a combination of anti–LAMP-1 monoclonal antibody (BB6) and anti–saposin C monoclonal antibody (7B2) at 5.0 mg/L in 0.1 mol/L $NaHCO_3$. The plates were washed twice with DELFIA wash buffer, lyophilized, and stored (desiccated, 4°C) for up to 8 weeks. Dried blood spots (3 mm) were punched from Guthrie cards and placed in the coated wells with assay buffer (200 μL) that contained europium-labeled

anti–LAMP-1 polyclonal antibody (200 μg/L) and samarium-labeled anti–saposin C polyclonal antibody (200 μg/L). The plate was shaken (1 hour, 20°C), incubated (16 hours, 4°C), then washed (×6) with DELFIA wash buffer. DELFIA enhancement solution (200 μL) was added to each well, and the fluorescence was determined on a DELFIA 1234 research spectrophotometer. Protein marker concentrations were calculated by comparison with blood-spot calibration curves prepared for each analyte as previously described.[25]

Derivatization of Oligosaccharides for Mass Spectrometry

Dried blood spots (3 mm) were punched from the Guthrie cards and derivatized with 1-phenyl-3-methyl-5-pyrazolone.[18] N-Acetylglucosamine-6-SO_4(*d3*) (200 pmol), synthesized as previously described,[26] and methyllactose (100 pmol) were included with each blood spot as internal standards.

Extraction of Glycosphingolipids

Dried blood spots (3 mm) were extracted with isopropanol (200 μL) that contained 200 pmol each of the stable isotopes of glucosylceramide C16:0 (GC) and lactosylceramide C16:0 (LC), GC (d3), and LC (d3), respectively, as internal standards. The blood spots were removed, the isopropanol was evaporated under a stream of nitrogen, and the glycosphingolipids were redissolved in methanol that contained 5 mmol/L NH_4COOH (100 μL).

Mass Spectrometry

Mass spectrometric analysis of oligosaccharides and glycosphingolipids was performed using a PE Sciex API 3000 triple-quadruple mass spectrometer with a turbo-ionspray source and LC-Tune/Multiview data system (PE Sciex, Concord, Ontario, Canada). Samples (20 μL) were injected into the electrospray source with a Gilson 233 autosampler using a carrying solvent of 50% CH_3CN/0.025% HCOOH in water (oligosaccharides) or methanol (glycosphingolipids) at a flow rate of 100 μL/min. For all analytes, nitrogen was used as the collision gas at a pressure of 2×10^{-5} torr. Neutral oligosaccharides and glycosphingolipids were analyzed in +ve ion mode, and sulfated oligosaccharides were analyzed in −ve ion mode. Determination of oligosaccharides and glycosphingolipids was performed using the multiple-reaction monitoring mode. Fourteen different glycosphingolipid and ceramide species, in addition to 80 species of oligosaccharides, were monitored (data not shown). Many species of oligosaccharide were not detectable in blood. The ion pairs for the glycosphingolipid and oligosaccharide species that gave the greatest sensitivity for the detection of each LSD are shown in Table 1. Each ion pair was monitored for 100 ms, and the measurements were repeated and averaged over the injection period. Determination of oligosaccharides was achieved by relating the peak heights of the PMP-oligosaccharides to the peak height of the PMP-MeLac (+ve ion mode) or the PMP-N-acetylglucosamine-6-SO_4(*d3*) (−ve ion mode). Determination of glycosphingolipids was achieved by relating the peak height of GC to the peak height of GC (*d3*) and the peak heights of LC and ceramide trihexoside (CTH) to the peak height of LC (*d3*).

Quality Control and Statistical Analysis

Intra- and interassay coefficients of variation (CVs) for the protein analytes were determined with low and high blood-spot QC standards prepared as previously reported.[25] Interassay CVs for each of the oligosaccharide and glycosphingolipid analytes were determined using 3 separate QC blood spots for 10 repeats over 5 days. Intra-assay CVs were calculated from 9 repeats of a single QC blood spot. The Mann-Whitney *U* test was used to measure the difference between control and patient groups for each analyte. Pearson correlation coefficients were used to determine the correlation between analyte concentration and age of the Guthrie card. Discriminant analysis was performed using SPSS for Windows (Chicago, IL).

RESULTS

Protein Markers in Dried Blood Spots

Intra-assay CVs for the LAMP-1 and saposin C assays were <12% and 15%, respectively, whereas interassay CVs were <15% and 18%, respectively. Determination of the lysosomal proteins LAMP-1 and saposin C in dried blood spots showed no significant correlation between the concentration of each protein marker and the age of the blood spots as determined by the Pearson correlation coefficients. There was no significant difference between LAMP-1 concentration in the Denmark control group and the SA control group as determined by the nonparametric analysis, including all data sets (Mann-Whitney *U* test). However, the difference between the saposin C concentrations in these groups, although small (median values: 117 μg/L and 129 μg/L, respectively), was significant ($P < .01$; Fig 1). Furthermore, only I-cell disease showed a significant elevation in the LAMP-1 protein marker concentration ($P < .05$) and only Fabry disease showed a significant elevation in the saposin C marker concentration ($P < .01$; Fig 1,

TABLE 1. Analytes Measured by Tandem Mass Spectrometry

Analyte	Q1*	Q3†	Charge	Int. Std.	Intra-Assay CV	Interassay CV
GC C16:0	700	264	+1	GC C16:0 (*d3*)	7	6–9
LC C16:0	862	264	+1	LC C16:0 (*d3*)	5	6–9
CTH C16:0	1024	264	+1	LC C16:0 (*d3*)	18	8–11
UA	525	175	+1	MeLac	25	20–26
HNAc	552	175	+1	MeLac	18	20–25
HNAcS	630	256	−1	HNAcS (*d3*)	38	6–10
HNAc-UA	726	331	−1	HNAcS (*d3*)	26	22–29
HNS-UA	764	173	−1	HNAcS (*d3*)	25	23–29
UA-HNAc-UA	904	525	+1	MeLac	36	22–34
HNAc-UA-HNAc-UA	1107	525	+1	MeLac	30	28–36
H2/HNAc	876	175	+1	MeLac	24	17–24
H3/HNAc	1038	175	+1	MeLac	26	20–28
H4/HNAc	1200	175	+1	MeLac	35	16–21
H2/NANA	964	175	+1	MeLac	41	19–34
H4	997	175	+1	MeLac	16	15–20
GC C16:0 (*d3*)	703	264	+1	–		
LC C16:0 (*d3*)	865	264	+1	–		
MeLac	687	175	+1	–		
HNAcS (*d3*)	633	259	−1	–		

S indicates sulphate; HN, hexosamine; UA, uronic acid; H, hexose; NANA, N-acetylneuraminic acid; MeLac, methyllactose.
* Ion monitored in quadrapole 1.
† Ion monitored in quadrapole 3.

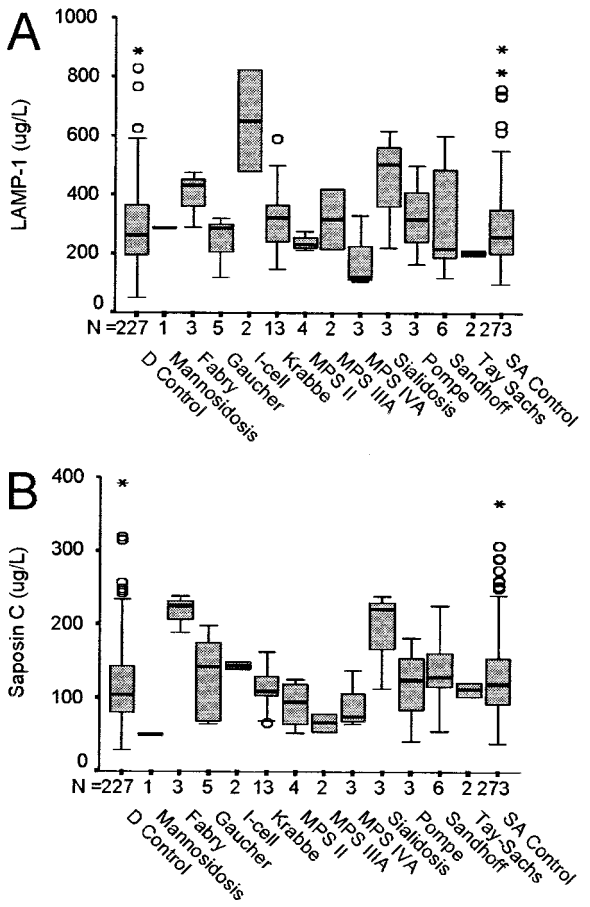

Fig 1. LAMP-1 and saposin C values in newborn blood spots from control and LSD-affected individuals. LAMP-1 (plot A) and saposin C (plot B) concentrations were determined in 3-mm blood spots from control and LSD-affected individuals in a dual immune-quantification assay as described in Methods. Center bars show the median level for each disorder, shaded area shows the 25th and 75th percentiles, and the upper and lower bars show the limits of the range. ○, Outliers (1.5–3.0 box lengths from the upper or lower edge of the box); ⋆, extreme outlier (>3.0 box lengths from the upper or lower edge of the box).

Table 2). A significant correlation was observed between LAMP-1 and saposin C in the Denmark and SA control populations with Pearson correlation coefficients of 0.65 ($P < .01$) and 0.70 ($P < .01$), respectively.

Glycosphingolipid Markers in Dried Blood Spots

Intra- and interassay CVs for the glycosphingolipid markers were within the range of 6% to 18% and are detailed in Table 1. There was no significant correlation between the concentration of the glycosphingolipids LC and CTH and the age of the control Guthrie cards. A significant but weak correlation was observed between the concentration of GC and the age of the control Guthrie cards (Pearson correlation coefficient: -0.259; $P < .01$). Linear regression analysis indicates that the loss of GC C16:0 over the period of Guthrie card collection would be in the order of 20%. Six of the LSD groups showed significant differences from the control group in 1 or more of the glycosphingolipid analytes (Table 2). The marker CTH C16:0 clearly resolves all of the Fabry patients from the control group (Fig 2A). GC C16:0 resolves 3 of the 5 Gaucher patients and 1 of the 2 I-cell patients from the control group (Fig 2B). When a combination of the GC C16:0 and LC C16:0 markers was used, it was possible to discriminate both I-cell patients as a result of the low LC C16:0 concentrations (Fig 2C). Krabbe disease, MPS IIIA, and sialidosis patient groups all showed significantly decreased concentrations of 1 or more glycosphingolipid markers. However, only the MPS IIIA–affected patients could be clearly differentiated from the control group (Fig 3).

Oligosaccharide Markers in Dried Blood Spots

Intra- and interassay CVs for the oligosaccharide markers were within the range of 6% to 41% and are detailed in Table 1. High CVs were observed for those analytes in which the concentration in control blood spots was relatively low. All of the LSD groups, with the exception of Pompe disease, showed significant differences from the control population, in the concentration of 1 or more of the oligosaccharide markers (Table 2). Figure 4A shows the discrimination of the MPS IVA patients from the control group using the N-acetylhexosamine-sulfate marker. The Tay-Sachs patients could be resolved from the control group using the N-acetylhexosamine-uronic acid disaccharide (HNAc-UA) marker (Fig 4B), and 2 of the 3 sialidosis patients could be resolved with the hexosamine sulfate-uronic acid (HNS-UA) marker (Fig 4C). The storage oligosaccharides in α-mannosidosis are made up of different combinations of hexose (H) and N-acetylhexosamine (HNAc) with the following compositions: H2/HNAc, H3/HNAc, and H4/HNAc. All of these were significantly elevated in the α-mannosidosis patient, although there was 1 control sample that had higher levels of these oligosaccharides (data not shown). Although the MPS II, Krabbe, Pompe, and Sandhoff patient groups showed significant increases or decreases in specific oligosaccharides, these did not provide clear discrimination of these patient groups from the control group. Discriminant analysis was performed on these groups; however, the resulting functions did not substantially improve the discrimination of the patient and control populations. The MPS IIIA patients had elevated levels of the trisaccharide UA-HNAc-UA and tetrasaccharide HNAc-UA-HNAc-UA, although these did not provide total discrimination of this group. The resolving power was increased when the glycosphingolipid marker LC C16:0 was also used as the MPS IIIA patients had a low concentration of this marker (Fig 3). Table 2 shows a summary of the sensitivity and specificity of selected markers for the individual LSD groups.

DISCUSSION

We have previously proposed a 2-tier screening strategy for LSDs.[14] This strategy involved a first-tier screen to identify a group at increased risk, using the lysosomal protein markers LAMP-1 and saposin C. This was followed by a second-tier screen, in which lysosomal substrates are quantified by tandem mass spectrometry to identify affected individuals. Previ-

TABLE 2. Sensitivity and Specificity of Selected Markers for the Identification of LSDs

Disorder	*n*	Markers	Mann-Whitney *U* Values	Sensitivity/Specificity
α-Mannosidosis	1	H2-HNAc	1†	100/99.6
Fabry disease	3	CTH C16:0/saposin C	0/41‡	100/100
Gaucher disease	5	GC C16:0	174‡	60/100
Krabbe disease*	13	UA/GC C16:0	548/844‡	-
MPS II*	4	HNS-UA	164	-
MPS IIIA	2	HNAc-UA-HNAc-UA/LC C16:0	5/0†	100/100
MPS IVA	3	HNAcS	1†	100/99.6
I-cell disease	2	GC/LC/LAMP-1	7†/16†/21†	100/100
Sialidosis	3	HNS-UA/CTH C16:0	150/65†	67/100
Pompe disease*	3	-		-
Sandhoff disease*	6	UA-HNAc-UA	296†	-
Tay-Sachs disease	2	HNAc-UA	1†	100/99.6

* No suitable marker identified.
† $P < 0.05$.
‡ $P < 0.01$.

ous studies on plasma samples from control and LSD-affected patients indicated that the first-tier screen would identify 70% to 90% of patients as high risk.[14,16] We have observed similar results when these studies were repeated on dried blood spots from LSD patients (unpublished observations). In this study, we proposed to evaluate the 2-tier strategy using retrospective, newborn Guthrie cards from LSD patients. Retrospective analysis of Guthrie cards for protein markers is limited as a result of the instability of proteins in these cards when stored at room temperature. The Statens Serum Institut in Denmark has stored all Guthrie cards at −20°C since 1980. The low temperature seems to stabilize the cards, making retrospective analysis possible. The patient group in this study was limited to newborns who were born in Denmark during the period 1982–1997.

The results from the LAMP-1/saposin C first-tier screen showed almost no difference between the patient groups and the control group. Of the 12 disorders included in the study, only I-cell and Fabry disease showed significant differences from the control group (Table 2). Of the 2 I-cell patients, only 1 had a LAMP-1 concentration above the 95 centile of the control group, and only 1 of the 3 Fabry patients had a saposin C concentration above the 95 centile of the control group. The absence of any correlation between age of the blood spots and protein analyte concentration and the similarities between the mean LAMP-1 values of the Denmark controls and the SA controls indicate that the age of the blood spots has minimal effect on the protein concentrations determined for LAMP-1. The small but significant decrease in the saposin C concentrations in the Denmark control compared with the SA controls may reflect the lower stability of this protein compared with LAMP-1. It is unclear why there is no apparent increase in LAMP-1 or saposin C protein levels when there are obvious increases in a number of storage substrates in a range of disorders. However, it is noteworthy that both LAMP-1 and saposin C have a broad range in the newborn population with a large number of statistical outliers (Fig 1). This is thought to result from the elevated and variable white cell count in newborns, rather than any lysosomal disorder in these individuals, and may mask the elevation in the LSD-affected newborns. The significant correlation between LAMP-1 and saposin C in both the Denmark and the SA control groups also indicates a general increase in lysosomes/white cells rather than a storage disorder in the control individuals with high LAMP-1 and saposin C levels. The relationship between the protein markers and the storage substrates requires further investigation, particularly in the newborn period.

The glycosphingolipid and oligosaccharide markers determined by mass spectrometry showed clear differentiation between control and affected groups for most of the disorders examined (Table 2). Fabry and Gaucher patients both showed increases in their primary storage substrates CTH and GC, respectively. All of the Fabry patients showed an elevation of CTH, whereas only 3 of 5 Gaucher patients showed an elevation in the concentration of GC. All of the Gaucher patients were of the neuropathic type 2 (1 sample 4.8 years old; see Fig 2) or type 3 phenotypes. It is not clear what other factors may be affecting the glycolipid levels in these newborns. The I-cell patients also showed an increase in GC concentration, and these patients were further resolved from the control group by plotting GC values against LC values. We had previously observed that the ratio of GC to LC provides better definition between Gaucher and control groups[17] and believe that this relates to the downregulation of glycosphingolipid production in response to the gross accumulation of GC. That this effect was not observed in the Gaucher patients may reflect the early stage of the disorder and the relatively low level of GC accumulation. MPS IVA–affected individuals were clearly identified by the increase in the concentration of the N-acetylhexosamine-sulfate monosaccharide. This monosaccharide accumulates as a result of the action of β-hexosaminidase on the stored keratan sulfate saccharides.[27] MPS IIIA patients showed an increase in the concentration of the tetrasaccharide HNAc-UA-HNAc-UA, although this is not a primary storage substrate for MPS IIIA as it does not contain the N-sulfated glucosamine residue at the nonreducing terminus. Presumably, the accumulation of this oligosaccharide results from the altered turnover of glycosaminoglycan within the affected cells. Use of this marker alone did not provide 100% specificity and sensitivity for the identification of MPS IIIA;

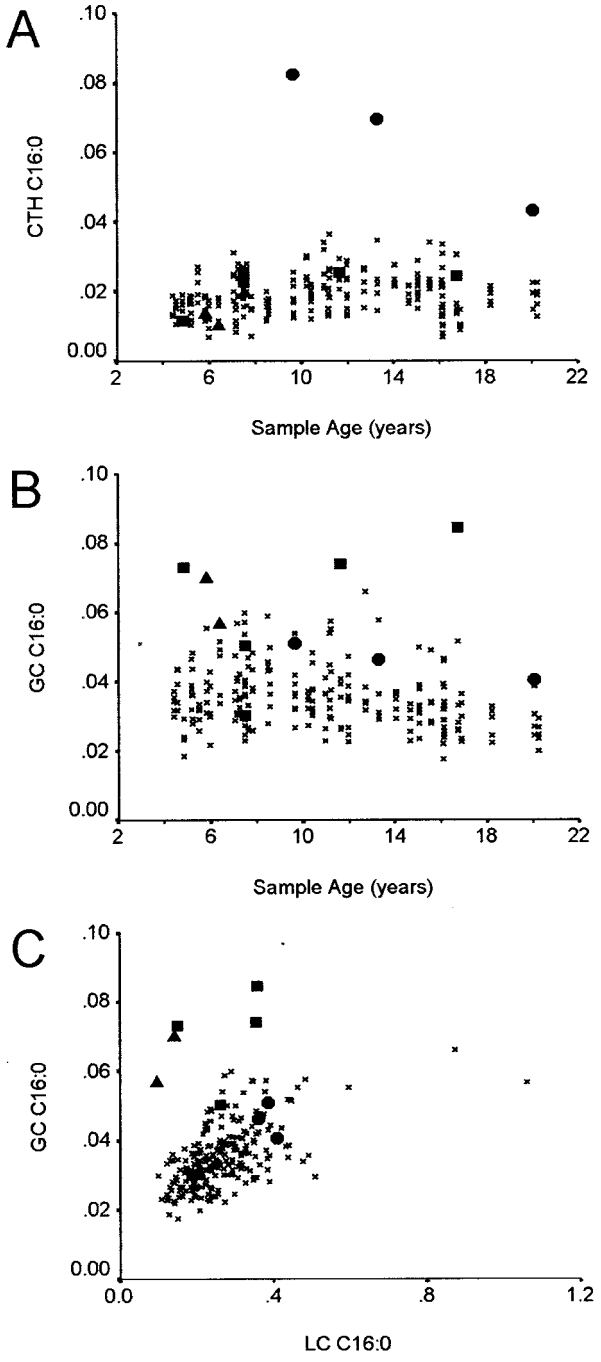

Fig 2. Glycosphingolipids levels in selected LSD groups. Glycosphingolipids were determined from 3-mm blood spots as described in Methods. CTH (A) and GC (B) were plotted against age of the blood spots. GC was also plotted against LC (C). x, control; ●, Fabry; ■, Gaucher; ▲, I-cell.

however, when combined with the LC marker, we were able to differentiate the MPS IIIA individuals from the control group (Fig 3). In Tay-Sachs disease, we observed the accumulation of the HNAc-UA disaccharide as a result of the β-hexosaminidase deficiency, and the α-mannosidosis–affected individual also showed elevated levels of the primary storage substrates. In 2 of 3 sialidosis patients, we observed an increase in the HNS-UA disaccharide but not in the N-acetylneuraminic acid–containing tetrasaccharide. The explanation for this is unclear at this stage but may relate to the rate of clearance of different oligosaccharides from circulation. We have observed that oligosaccharides resulting from lysosomal storage are present in urine at many times the concentration of plasma, so the kidneys seem to be very efficient at removing these oligosaccharides from circulation. This also relates to the inability to identify the Pompe disease–affected individuals from the accumulation of the H4 tetrasaccharide that is elevated in the urine of these patients[18] and results from the limited digestion of glycogen in circulation.[28] MPS II–, Krabbe-, and Sandhoff-affected individuals all showed significant differences in some analytes but were unable to be resolved from the control population. This may be related to the phenotype of these patients or to the particular storage substrates present. Additional work will be required to identify suitable markers for these disorders.

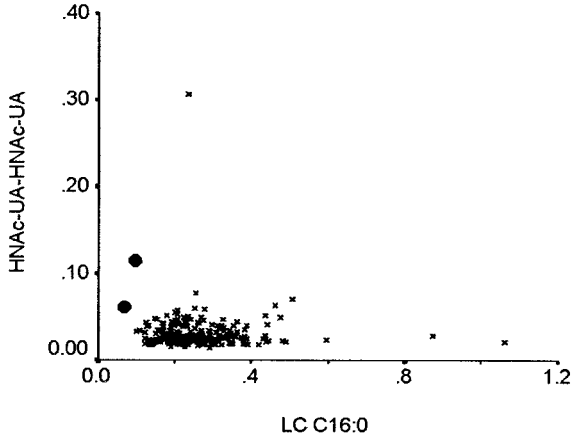

Fig 3. Selected marker levels in MPS IIIA and control blood spots. Oligosaccharides and glycosphingolipids were determined from 3-mm blood spots as described in Methods. The levels of the tetrasaccharide HNAc-UA-HNAc-UA were plotted against the levels of LC. x, control; ●, MPS IIIA.

This study has identified the limitations of the 2-tier strategy for newborn screening for LSD; additional primary markers will be required for complete success. We have reported that in Pompe disease, the determination of the α-glucosidase protein or activity can be performed on a dried blood spot using antibody capture techniques and is diagnostic for this disorder.[25,29] Chamoles et al[30,31] performed enzyme analysis for a number of disorders from dried blood spots and can differentiate affected from control populations. Thus, the use of deficient proteins/enzymes as markers for LSD is feasible. However, the challenge lies in the ability to multiplex these assays to enable the screening for multiple LSDs in a single procedure. The low prevalence of these disorders makes it unlikely that screening programs for individual disorders will be widely adopted, and performing multiple assays to cover a range of disorders will not be cost-effective.

An alternate approach is to use the mass spectrometric analysis of glycosphingolipids and oligosaccharides in a single-tier screen. We have demonstrated that this is feasible for a number of LSDs with

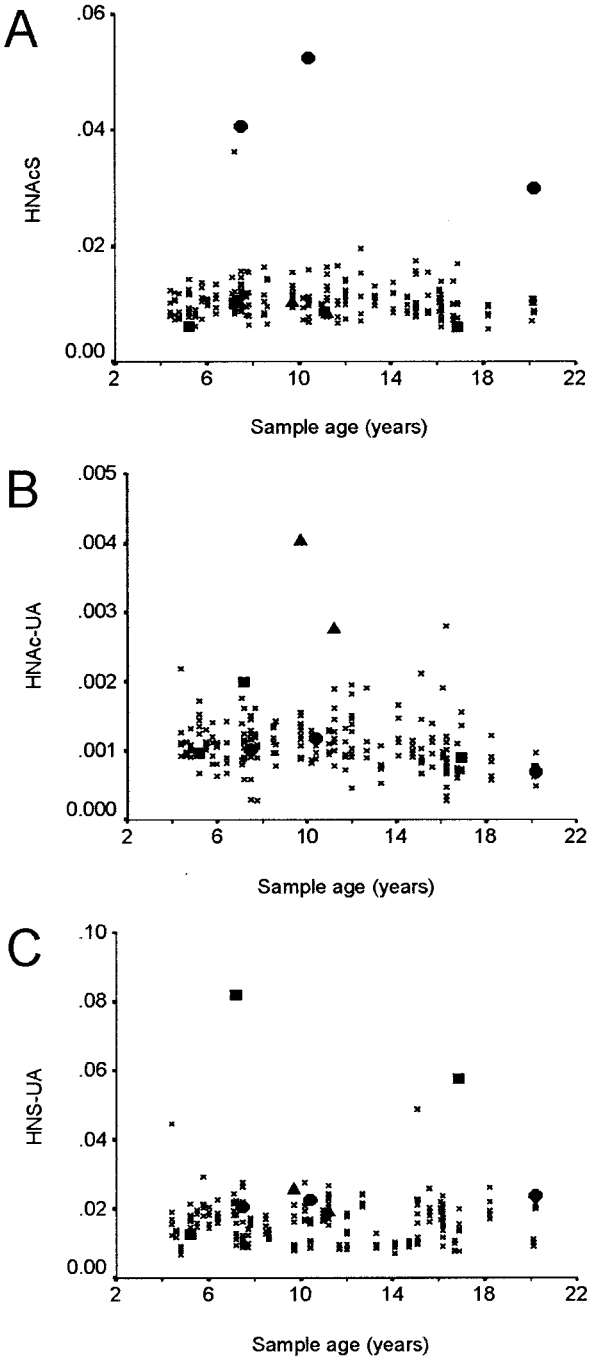

Fig 4. Oligosaccharide levels in selected LSD groups. Oligosaccharides were determined from 3-mm blood spots as described in Methods. N-Acetylhexosamine-sulfate (A), HNAc-UA (B), and HNS-UA (C) were plotted against age of the blood spots. x, control; ●, MPS IVA; ■, sialidosis; ▲, Tay-Sachs.

the current markers, although additional markers would be required to provide coverage of LSDs for which therapy is currently available. One limitation to this approach is the labor-intensive derivatization process for the determination of oligosaccharides. Although it is possible for 1 person to process 100 to 200 samples per day, this process would need to be automated for large-scale screening programs. A second limitation of this approach is the relatively low concentration of many of the oligosaccharides in circulation. We have identified many oligosaccharides that are elevated many-fold in the urine of LSD patients but only slightly elevated or not elevated in blood (unpublished data). If this approach is to achieve optimal results, then consideration should be given to the use of urine in a newborn screen. This has been achieved in some limited population studies such as the preclinical detection of neuroblastoma in Japan and Canada.[32,33] However, collection of an additional sample from all newborns would substantially increase the cost of screening for LSDs and would limit the implementation of such a program. Additional work to identify suitable markers and evaluate strategies will be required before newborn screening for LSDs is to be widely accepted.

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