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RESEARCH REPORT

Newborn Screening for Lysosomal Storage Disorders in Hungary

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Abstract Even though lysosomal storage disorders (LSDs) are considered to be orphan diseases, they pose a highly relevant cause for morbidity and mortality as their cumulative prevalence is estimated to be 1:4,000. This is especially important as treatment in form of enzyme replacement therapy, substrate reduction therapy or stem cell transplantation is amenable for some LSDs. It is plausible that an early start of treatment might improve the overall prognosis and, even more important, prevent irreversible damage of key organs. To get a more precise insight into the real frequency of some LSDs in the general population, we screened 40,024 samples from the Hungarian newborn screening (NBS) program in Szeged for Fabry disease (FD), Gaucher disease (GD), Pompe disease (PD), and Niemann-Pick A/B (NPB) disease using tandem mass spectrometry. Altogether, 663 samples (1.66%) were submitted for retesting. Genetic confirmation was carried out for 120 samples with abnormal screening results after retesting, which identified three cases of GD, three cases of FD, nine cases of PD, and two cases with NPB. In some cases, we detected up to now unknown mutations – one in NPB and seven in PD – which raise questions about the clinical consequences of a NBS in the sense of late-onset manifestations. Overall, we conclude that screening for LSDs by tandem MS/MS followed by a genetic workup in identified patients is a robust, easy, valid, and feasible technology in newborn screening programs. Furthermore, early diagnosis of LSDs gives a chance to early treatment, but needs more clinical long-term data especially regarding the consequence of private mutations.

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Introduction

Newborn screening (NBS) nowadays has an important role in preventive medicine. Since the introduction of screening for phenylketonuria by Guthrie (Guthrie and Susi 1963) in the early 1960s, the number of diseases screened for has constantly increased. The main reason for this increase was the introduction of tandem mass spectrometry (MS/MS) in the 1990s (Millington et al. 1990). MS/MS has the potential to detect more than 30 inborn errors of metabolism via the measurement of acylcarnitines and amino acids in a single sample preparation. Due to this and the availability of reasonably priced machines, MS/MS is a standard tool in every modern screening laboratory. High sensitivity and selectivity of modern MS/MS machines increases the potential for target diseases to be included into a NBS further (Hubbard et al. 2009; Li et al. 2004).

Due to the availability of enzyme replacement therapies (ERT) for several lysosomal storage disorders (LSD), this group of diseases is currently under discussion to be included in newborn screening programs (Nakamura et al. 2011; Marsden and Levy 2010). However, many issues like phenotype prediction, follow-up, and late onset variants are still unsolved. Nonetheless, ERTs should be initiated before the onset of irreversible pathology. Thus, presymptomatic diagnosis of the respective disease is of high importance.

Several pilot studies for single diseases or multiple LSDs with fluorometric and mass spectrometric methods were carried out in recent years (Dajnoki et al. 2008; Orsini et al. 2009; Spada et al. 2006). Results suggest that some LSDs are quite frequent. Late onset Fabry disease was found in males with a frequency of 1:1,250 (Hwu et al. 2009) in Taiwan and with a frequency of 1:3,100 (Spada et al. 2006) in Italy and Pompe disease with a frequency of 1:35,000 in Taiwan (Chien et al. 2009), while others seems to be rather rare, e.g., Krabbe disease in New York (1:140,000) (Orsini et al. 2009; Kwon et al. 2009).

A method for screening of LSDs was published earlier (Li et al. 2004). NBS for five LSDs (Gaucher, Krabbe, Pompe, Fabry, and Niemann-Pick A/B) was described via the measurement of the respective enzyme activities in dried blood spots (DBS) from a single sample preparation with MS/MS (Blanchard et al. 2008).

In this paper, we report the application of the MS/MS method to screening for Pompe (OMIM 232300), Fabry (OMIM 301500), Gaucher (OMIM 230800, 230900, 231000), and Niemann-Pick A/B (OMIM 257200 and 607616), respectively, in newborn samples from the Hungarian newborn screening program in Szeged. Positively screened newborn samples were evaluated by molecular diagnostic testing.

Methods

Samples

Forty thousand and twenty-four samples were obtained from the Hungarian newborn screening program in Szeged, Hungary. It is one of the two screening centers in the country, each of which are responsible for testing approximately half of the territory/newborn population, resulting in 37,000–43,000 newborns screened annually. Blood sampling typically occurred at the 3rd (48–72 h) day of life on Ahlstrom 226 filter paper. All patient samples were stored at room temperature and were analyzed within 3 weeks after sampling. Sample integrity and enzyme activity was not impaired by this procedure (for GLA: Dajnoki et al. 2010; for GAA: Dajnoki et al. 2008; for GBA: Legnini et al. 2011a; for ASM: Legnini et al. 2011b).

The study was approved by the Human Investigation Review Board of the University of Szeged, Hungary. As newborn screening is mandatory and the study was carried out in an anonymous way, parental consent was not required.

Reagents

Vials of artificial substrates and internal standards for the respective enzymes were obtained from the Center of Disease Control and Prevention (CDC), Atlanta, USA. The vials contained substrate and internal standard in a molar ratio of about 50:1. Reagents were obtained lyophilized and dissolved in appropriate buffers as previously described (Zhang et al. 2008). Chemicals for preparation of buffers and inhibitor solutions were obtained from VWR International and Sigma-Aldrich, respectively.

Substrate and internal standard cocktails were used according to previously established protocols (Zang et al. 2008).

For reagent and assay preparation we used electronic multichannels pipettors (Brand, Wertheim, Germany) and Eppendorf single-channel pipettes (Eppendorf, Hamburg, Germany).

Polypropylene 96-well microtiter flat-bottomed plates 350 µl, deep well microtiter plates polypropylene 2.2 ml (Greiner Bio-One, Frickenhausen, Germany), and pipette tips 200 µl (Brand GmbH) have been used.

Sample Preparation

Sample preparation was performed as previously described (Li et al. 2004; Zhang et al. 2008). Briefly, 3.2 mm spots from the DBS and quality control material (CDC, Atlanta, USA) were punched into 96-well polypropylene plates (Greiner Bio-One) using a Wallac DBS Puncher (Perkin Elmer, Massachussets, USA). We added 80 µl of extraction buffer to each well, mixed gently and sealed the plates with a silicone plate sealer. Samples were extracted at 37°C and 750 rpm with shaking for 60 min in a Heidolph incubator (Incubator 1000, Titramax 1000). After incubation, 50 µl extract was aspirated with an electronic multichannel pipettor and 10 µl were dispensed in four different 96-well plates. Fifteen microliters of the respective enzyme assay cocktails were added to the plates containing the extract, sealed and incubated for 23 h at 37°C with shaking.

The enzyme reactions were stopped with 100 µl of 1:1 ethylacetate and methanol using bottle dispenser with an 8-channel stream splitter. One and twenty-five microliters of samples from the four plates were transferred into a single 96-deep-well plate to give a final volume of 500 µl. For subsequent liquid-liquid extraction 500 µl ethylacetate followed by 500 µl water was added with bottle dispenser and an 8-channel stream splitter.

Table 1 MS/MS parameter settings: M1/M3 transitions monitored

ID	M1	M3	DT	DP	FP	EP	CE	CXP
ASM-IS	370.6	264.3	200	20	250	10	25	15
ASM-P	398.6	264.3	200	20	250	10	30	15
GBA-P	482.6	264.3	200	45	250	12	45	15
GBA-IS	510.6	264.3	200	45	250	12	45	15
GLA-P	484.4	384.4	200	45	250	12	20	15
GLA-IS	489.4	389.4	200	45	250	12	15	15
GAA-P	498.4	398.4	200	45	250	12	21	15
GAA-IS	503.4	403.4	200	45	250	12	15	15

DT dwell time (ms), *DP* declustering potential (V), *FP* focusing potential (V), *CE* collision energy (V), *EP* entrance potential (V), *CXP* collision cell exit potential (V)

Ion source temperature: 150°C, Collision cell gas pressure setting: 4, Ion source potential: 5500 V

Samples were spun for 3 min at 3,000 rpm. Then, 300 µl of the upper phase were transferred into a new 96-well plate (Greiner Bio-One) and dried using a Minivap (Porvair Science, Leatherhead, UK). Samples were reconstituted in 100 µl of 1:19 methanol:ethylacetate and transferred into a 96-well filter plate (Pall Corporations, Port Washington, New York, USA) preloaded with 100 mg/well silica gel 60 (Merck, Darmstadt, Germany) washed with 800 µl of 1:19 methanol:ethylacetate prior to loading the samples. Enzymatic products and internals standards were eluted from the silica gel with two times 400µl of 1:19 methanol:ethylacetate into a fresh 96 deep-well plate. The solvents were evaporated using the Minivap evaporator and the residue was dissolved in 200 µl of 80:20:0.01 acetonitrile:water: formic acid (Merck; BDH Prolabo Chemicals, Darmstadt, Germany) prior to analysis with MS/MS.

MS/MS

An API 3000 triple-quadrupole MS/MS (PE Sciex, Perkin Elmer, Massachussets, USA; www.perkinelmer.com) in positive ion mode and analyst v. 1.5.1 software (ABSciex, Ontario Canada) was used for analysis. Analyses of products and respective internal standards were done in multiple-reaction monitoring mode. MS/MS parameters were optimized to give the highest sensitivity for internal standards with as few as possible in source fragmentation of substrate (Table 1). The MS/MS was calibrated with seven calibration solutions (CDC, Atlanta, USA) containing the substrate and internal standard in predefined ratio (P/IS: 0; 0.05; 0.1; 0.5; 1; 2; and 5).

Determination of Cutoff Values

After analysis of 1,000 samples, the lower 0.25th and 0.5th percentile of the distribution of measured values were calculated (Table 3). The definition of the cutoffs was based on the experience that affected patients have virtually no enzyme activity (Zhang et al. 2008), but they were also kept low in order to keep the potential recall rate low. Only in case of Fabry disease the cutoffs used were slightly above the 0.5th percentile. Afterward the 0.25th and 0.5th percentile for the respective enzyme activities were calculated from the total number of samples. An adjustment of the preliminary cutoff values was not necessary (data not shown). Samples with a first analysis result below the respective cutoff value were repeated twice in a subsequent measurement. If the mean of all three assays was below the cutoff, the sample was submitted for molecular confirmatory testing.

Molecular Genetics

DNA isolation: Genomic DNA was isolated from dried blood spots using the Qiagen QIAamp DNA Micro Kit. Lysis was carried out with six blood card punches by adding 300 µl Buffer ATL, 40 µl proteinase K, and 180 µl Buffer AL. Two lysates were pooled for subsequent isolation of DNA according to the manufacturer's instruction. In all cases the gene had been amplified for the entire exon regions as well as the exon-intron-boundaries. For Fabry disease also specific intronic areas have been analyzed. Following standard PCR amplification, sequencing of the resulting fragments was done with Sanger di-deoxy-termination technology (Applied Biosystems, Carlsbad, California, USA). PCR methods for sequencing of the GBA gene are as follows: To prevent sequencing of the pseudogene, all exons and exon-intron-junctions of GBA except exon 8 were amplified and sequenced with nested primers using GBA-XL1, -XL2, and -XL3 amplicons as template, respectively. Amplification of exon 8 was carried out with GBA-E08F_PCR and GBA-E08R_PCR using genomic DNA as template (Finckh et al. 1998). Standard procedures were used for PCR amplification; for cycle sequencing, additional sequencing primers GBA-E08F_CYC and GBA-E08R_CYC were used, respectively (Seeman et al. 1996). The fragments amplified were sequenced on an ABI 3730 automated sequencer using the BigDye Terminator v3.1 Sequencing Standard Kit. Genotypes were analyzed by comparing sequences from patients to NCBI Reference Sequence NM_000157.3 using the SequencePilot software. Primer sequences, amplicon sizes, and templates are listed in Table 2.

Results

Screening

A total number of 40,024 samples were screened. Abnormal low activities for GBA were found in 141 (0.35%), for

Table 2 Primers used for amplification and sequencing of the GBA gene

Primer	Sequence	Amplicon size	Template
GBA-XL1F	CTGGGATTTAGGAGCCTCTACC	3493	Genomic DNA
GBA-XL1R	AAGATTGACAGGCCCAAGGC		
GBA-XL2F	TCAAATGATATACCTGCCTTGG	2045	Genomic DNA
GBA-XL2R	AGTTTGGGAGCCAGTCATTT		
GBA-XL3F	AAGTTCCAGAAGCCTGTGTGC	3992	Genomic DNA
GBA-XL3R	CAGGACCTTGACGGCCAGCG		
GBA-E01F	TCACCCATACATGCCCTCCA	378	Amplicon XL1
GBA-E01R	TCCAGTGCCAGGATTCCAGA		
GBA-E02F	CCAGGTTTCTGTGGGCCTTG	291	Amplicon XL1
GBA-E02R	ACCAAAGGACTATGAGGCAGAAGG		
GBA-E03-04F	GGCCAAGGGGTGAGGAATTT	649	Amplicon XL1
GBA-E03-04R	GCCGACAGAATGGGCAGAGT		
GBA-E05F	GACTGGCAAGTGATAAGCAGAGTCC	356	Amplicon XL2
GBA-E05R	ATCCGGTTCAGCCATTAGCC		
GBA-E06F	GCTAATGGCTGAACCGGATGC	413	Amplicon XL2
GBA-E06R	AAGATTGACAGGCCCAAGGCTG		
GBA-E07F	TCCTGAACTCAAGTGATCCACC	432	Amplicon XL2
GBA-E07R	TTTGGGAGCCAGTCATTTGG		
GBA-E08F_PCR	CAAAATCTCCCCAAACCTCTCTAG	1358	Genomic DNA
GBA-E08R_PCR	GTGCCACTGCGTCCAGGT		
GBA-E08F_CYC	ACTAGAAGTTCCAGAAGCCTG		
GBA-E08R_CYC	CTTGGTGATGTCTACAATG		
GBA-E09F	TGCCTCTCCCACATGTGACC	374	Amplicon XL3
GBA-E09R	TGCCTCTTCCGAGGTTCCAC		
GBA-E10F	GTCCGTGGGTGGGTGACTTC	322	Amplicon XL3
GBA-E10R	TTGATGGTAAGAGGCACATCCTTAG		
GBA-E11F	ACTTCTTAGATGAGGGTTTCATG	465	Amplicon XL3
GBA-E11R	TTTAGTCACAGACAGCGTGTG		

Table 3 Cutoff values used for the screening procedure. Average enzyme activities, standard deviations, lower 0.25th and 0.5th percentile of distribution for the respective diseases, and empirically derived cutoffs for the respective disease

Disease	Average enzyme activity μmol/l/h ($n = 40{,}024$)	SD	Median	Lower 0.25th percentile	Lower 0.5th percentile	Cutoff used
Gaucher	17.7	9.8	15.7	3.26	3.85	3.50
Niemann Pick	9.2	4.6	8.3	1.94	2.3	2.00
Pompe	15.0	7.6	13.6	2.64	3.21	3.00
Fabry	11.0	8.0	9.0	2.09	2.45	2.50

ASM in 114 (0.29%), for GAA in 163 (0.41%), and for GLA in 224 (0.56%) samples, respectively. Twenty-one samples with questionable sample integrity (low activities for two or more enzymes) were excluded from further workup. In total, 663 (1.66%) samples were submitted for retesting in duplicates (Table 3).

After retesting, 17 (0.042%) samples with low GBA, 5 (0.012%) samples with low ASM, 64 (0.160%) with low GAA, and 34 (0.085%) with low GLA activity, respectively, in total 120, remained. These samples were considered highly suspicious of being affected with the respective disease and submitted to molecular diagnostics. The overall recall rate comprised 141 samples (0.352%) (Table 4).

Table 4 Summary of abnormal first and second screening results as well as resulting recall rates for the respective diseases

Disease	Abnormal first screening result	Percent	Abnormal after retesting	Percent recall
Gaucher (GBA)	141	0.352	17	0.042
Niemann Pick A/B (ASM)	114	0.285	5	0.012
Pompe (GAA)	163	0.407	64	0.160
Fabry (GLA)	224	0.560	34	0.085
Sample integrity questionable	21	0.052	21	0.052
Total	663	1.66	141	0.352

Table 5 Intronic sequence changes in patients suspected of Fabry disease. The intronic change c.-10C<T is known to be associated with elevated levels of Gb3 and lyso-Gb3 (Tanislav et al. 2011), though no symptoms of FD were seen in the patients reported by Tanislav et al.

Frequency	Male	Female	
1		x	c.370-81-77delCAGAA; c.640-16A>G (het); c.1000-22C>T (het)
1		x	c.-10C>T (het); c.640-981C>T (het); c.1000-22C>T (het)
2	x		c.-10C>T; c.370-81-77delCAGCC ; c.640-16A>G ; c.1000-22C>T
1		x	c.370-79G>C (het); c.640-16A>G (het); c.1000-22C>T (het)
2	X		c.-10C>T; c.370-81-77delCAGCC; c.640-16A>G; c.1000-22C>T

Molecular Genetics

From all samples where an abnormal second test result ($n = 120$) for GBA, ASM, GAA or GLA was obtained, we performed molecular genetic analysis for the respective disease.

Three newborns with Gaucher disease could be confirmed. They revealed the same genotype of c.1448 T>C (p.L483P)/c.1483 G>C (p.A495P) (Horowitz and Zimran 1994). The remaining seven abnormal MS/MS results revealed no pathological mutation. We detected seven carriers for Gaucher disease with the following mutations: 5 x c.1223 C>T (p.T408M) (Beutler et al. 1996), c.1226 A>G (p.N409S) (Tsuji et al. 1988), and c.475 C>T (p.R159W) (Horowitz et al. 1994).

In two of the five suspected sphingomyelinase deficiency cases, we detected the pathologic mutations c.108insCTGGC (p.L37fs)/c.108insCTGGC (p.L37fs) and c.880 C>A (p.Q294K)/c.1550 A>T (p.E517V) (Pavlů and Elleder 1997; Simonaro et al. 2002) identifying two Niemann-Pick A/B cases.

We found nine Pompe cases with the following genotype: five cases with c.664G>A (V222M)/c.664G>A (p.V222M). All other genotypes occurred once, namely, c.-32-13T>G/c.-32-13T>G (Huie et al. 1994), c.664 G>A (p.V222M)/c.2174 G>A (p.R725Q), c.1216G>A (p.D406N)/c.1409A>C (p.N470T), and c.1552-3C>G/c.1552-3C>G (Kroos et al. 2006). Additionally, 25 carriers were detected: 4 x c.-32-13 T>G (Huie et al. 1994), c.307 T>G (p.C103G) (Hermans et al. 2004), 10 x c.664 G>A (p.V222M), c.763 C>T (p.Q255X), c.841 C>T (p.R281W), c.875 A>G (Castro-Gago et al. 1999), c.1437+1 G>A, c.1468 T>C (p.F490L), 2 x c.1552-3C>G (Kroos et al. 2006), c.1903 A>G (p.N635D), c.2237 G>T (p.W746L), and c.2482-2 A>G. Also, three cases remained uncertain: 2 x c.1048 G>A (p.V350M)/WT and c.-32-13 T>G (Huie et al. 1994)/c.-367-157 C>G as mutation analysis was inconclusive.

We have analyzed 34 filter cards being abnormal for the alpha-galactosidase enzyme activity usually found in Fabry disease in the MS/MS analysis. In three female cases we detected the already published (Eng et al. 1997) heterozygous mutation c.427 G>A (p.A143T) in the GLA gene. Twenty of these 34 have been without any detected mutation; two cases remain uncertain since we have detected the well-known mutation c.937G>T (p.D313Y) whose interpretation is conflicting. In seven cases we have been able to detect complex intronic sequence changes with combinations shown in Table 5.

In summary, the genetic analysis resulted in the detection of three Gaucher newborns, nine Pompe as well as two unclear Pompe cases, two Niemann-Pick A/B patients, and three confirmed as well as two unclear Fabry cases (Table 6). These numbers will result in frequencies ranging from of 1:13,341 for Gaucher, 1:4,447 at the maximum to at least 1:20,012 for Pompe, 1:13,341 for Fabry, and 1:20,012 to 1:40,024 for Niemann Pick A/B. The overall frequency for all four diseases is 1:2,354 at the best to 1:4,447 at the least assuming either all 17 detected cases or only the 9 cases with previously reported mutations.

The screening also identified seven heterozygous carriers for Gaucher disease (0.017%, 1:5,718) and 17 heterozygous carriers for Pompe disease (0.042%, 1:2354); however, due to our screening setup of genetically testing only those samples suspicious in biochemical analysis, we likely missed other heterozygotes. More probable frequencies for heterozygosity can be obtained by the Hardy-Weinberg principle utilizing the identified disease frequencies of the screening, resulting in a frequency of heterozygotes of 1.72% (1:58) for Gaucher disease, 1.40% to 1.00% (1:71 to 1:101) for Niemann-Pick A/B disease, and 2.95% to 1.40% (1:34 to 1:71) for Pompe disease.

Table 6 Summary of the frequency of confirmed LSD cases according to the newborn screening in the eastern area of Hungary as well as the carrier frequency, the unclear SNP cases in Fabry and Pompe disease, and range of measured enzyme activity in confirmed disease samples

	Total	Disease	Disease	Normal	Disease	SNP	Enzyme activity range
		Confirmed	Carrier	Wild type	Unclear		μmol/l
Gaucher	16	3	7	6			0.3–3.5
NP A/B	5	2	0	3			
Pompe	64	9	25	27	3		0.7–2.9
Fabry	34	3		20	1 male	9	0.8–1.6 confirmed
					1 female		1.7–2.1 unclear

Discussion

We report for the first time results of NBS for four lysosomal storage disorders based on the measurement of enzymatic activities in a multiplexed MS/MS assay in Europe. This assay originally was developed in 2004 for the screening of five lysosomal storage disorders (Li et al. 2004). Already 2 years later the NBS for Krabbe disease was started in New York State based on that method (Duffner et al. 2009). In order to prevent false negatives, the New York State model used a variable cutoff model. Samples with activities of less than 20% of the daily mean were retested in duplicates. Based on the average of three measurements, the sample was either submitted for DNA analysis (>8–12% of daily mean activity) or the newborn was referred to a treatment center for follow up diagnostics (<8% of daily mean activity). However, while this model may work for a single disease screening, we think that for multiplex screening for two or more diseases this model may be logistically challenging. In order to keep the recall rate low, we chose the classical NBS model with fixed cutoff values empirically set between the lower 0.25th and 0.5th percentile of distribution of measured enzyme activities. The cutoff values were a trade-off between higher recall rate and diagnosed patients. As we found affected cases with an initial screening result close to the cutoffs for Pompe and Gaucher diseases (Table 6), we think we were at the very limit and further reduction would have resulted in missed diagnoses. Recently, Han et al. (2011) reported a small-scale NBS for a Korean cohort, analyzing 211 normal newborns and 13 newborns with various LSDs to establish an NBS for LSDs in Korea; however, due to the small cohort no conclusion as to the prevalence of LSDs in Korea could be drawn.

The data obtained by the newborn screening in Szeged is representative of the ethnicities found in the entire Hungarian population. Of the country's population 95.5% are Hungarian and 2.0% are Gypsies (http://www.nepszamlalas2001. hu/eng/volumes/06/00/tabeng/2/load01_12_0.html), while in the Southern Great Plain county, where Szeged is located, 94.7% are Hungarian and 1.1% are Gypsies (http://www.nepszamlalas2001.hu/eng/volumes/06/00/tabeng/3/load01_9_0-7.html, data obtained from the Hungarian Population Census 2001). Though founder mutations for GM1-gangliosidosis in Spanish Gypsies (Santamaria et al. 2006) and novel mutations for Morquio disease type B in Bulgarian Gypsies (Sinigerska et al. 2006) have been reported, no literature on LSDs in Hungarian Gypsies is available. However, due to the similar distribution of ethnicities in the Szeged area and the whole of Hungary, we assume regional founder effects to be minimal.

As already mentioned, screening for Fabry disease yielded three females affected by a known causative mutation c.427 G>A (p.A143T) (Eng et al. 1997), though it is known to be associated with both the classic (Blaydon et al. 2001) and variant phenotype (Shabbeer et al. 2006) of Fabry disease. Since females are affected in a very heterogeneous manner, the biological significance of the mutation remains to be identified. In the other two patients, the uncertain mutation c.937G>T (p.D313Y) was identified and will have to be carefully analyzed concerning the pathological relevance since it has been described as disease-causing in older literature (Eng et al. 1993). More recent references interpret this mutation more in the sense of being an enzyme-modifying SNP with reduction of the enzyme activity up to 60% rather than a pathological mutation (Froissart et al. 2003). Additionally, combined intronic sequence changes associated with elevated Gb3 and lyso-Gb3 were detected, which may result in a variant phenotype of Fabry disease (Tanislav et al. 2011). Reported disease frequencies for Fabry are in the range of 1:1,250 for Fabry (Hwu et al. 2009) in Taiwan and 1:3,100 (Spada et al. 2006) in Italian males. Considering all certain Fabry cases we detected a frequency of 1:13,341. If we additionally consider the five cases with the intronic sequence change c.-10C<T described by Tanislav et al. (2011), we actually obtain a prevalence of 1:5,003, which is comparable to the Italian population (Spada et al. 2006) mentioned above. In summary, Fabry disease is more prevalent than original estimations of 1:40,000 suggested (Meikle et al. 1999).

Pompe disease was found at 1:35,000 in Taiwan (Chien et al. 2009). Our frequency for Pompe of 1:4,447 at best to 1:20,012 is higher compared to the Taiwanese population. In an anonymous Austrian pilot study for NBS of Pompe disease only 4 (0.039%) of 10,279 patients would have been recalled for retesting using a cutoff of 2 µmol/l/h (Dajnoki et al. 2008). Assuming all four patients were affected, a frequency of 1:2,570 can be derived. In our study, we found similar mean enzyme activity and standard deviation in whole sample cohort as was found in the Austrian study (Dajnoki et al. 2008). However, applying a cutoff value of 2 µmol/l/h would have missed three affected Pompe patients in our study. Activities of first screening results of these patients were in the range 0.7–2.6 µmol/l/h. Of all mutations identified only two were known, namely, c.-32-13T>G/c.-32-13T>G (Huie et al. 1994) and c.1552-3C>G/c.1552-3C>G (Kroos et al. 2006). The c.-32-13 T>G mutation is prevalent in 70% of the Caucasian patients and is associated with a juvenile or adult onset of Pompe disease. By aberrant splicing of exon 2 of the GAA gene, the residual enzyme activity is diminished to 5–10% compared to the wild type (Kroos et al. 2006). Likewise, c.1552-3C>G is associated with only 9% correctly spliced GAA, resulting in the loss of the coding sequence for the active site in wrongly processed GAA. Though only two of nine Pompe cases were associated with known juvenile and adult onset of Pompe disease, the screening also identified four carriers with the c.-32-13 T>G genotype (Huie et al. 1994) and two carriers with c.1552-3 C>G (Kroos et al. 2006) associated with juvenile or adult onset of Pompe disease. Two others of the 25 identified carriers displayed known disease-causing mutations, c.307 T>G (p.C103G) (Hermans et al. 1994) and c.875 A>G (Castro-Gago et al. 1999), respectively. The significance of the other spotted mutations remains ambiguous.

The frequency for Gaucher of 1:13,341 appears high when compared to the prevalence of, e.g., 1.13:100,000 in the Czech Republic (Poupetova et al. 2010), leading to the conclusion that M. Gaucher is highly underdiagnosed. In all three cases we were able to detect a compound heterozygosity for L483P, a mutation predisposing for a severe course of Gaucher disease, where early detection is of high importance (Montfort et al. 2004). Furthermore, seven carriers for Gaucher disease were identified, the mutations c.1226 A>G (p.N409S) (Tsuji et al. 1988) and c.1223 C>T (p.T408M) (Beutler et al. 1996) occurring once and five times, respectively, and both linked to the type I of Gaucher disease (Amaral et al. 2000; Miocić et al. 2005; Nichols et al. 2009). In addition, both c.1223 C>T (p.T408M) (Beutler et al. 1996) and c.475 C>T (p.R159W) (Horowitz and Zimran 1994) have been proven to be connected with Parkinson's disease (Hodanová et al. 1999; Amaral et al. 2000; Mitsui et al. 2009). Overall, clinically relevant phenotypes in Gaucher patients as well as carriers have been identified.

We found a very small recall rate for Niemann-Pick A/B disease, although the abnormal rate after the first screening was comparable to the other diseases; the three of the five remaining potentially affected cases turned out to be genetically normal. We conclude that the incidence of Niemann-Pick A/B is significantly less in our population compared to the other three diseases screened. However, one of the two identified NP A/B cases carried mutations previously described (c.880 C>A (p.Q294K)/c.1550 A>T (p.E517V) (Pavlů and Elleder 1997; Simonaro et al. 2002)), while the other (c.108insCTGGC (p.L37fs)/c.108insCTGGC (p.L37fs)) is a novel mutation whose clinical relevance remains uncertain, resulting in a frequency of at least 1:40,024 and 1:20,012 at the maximum.

A limitation of the study is the detection of altogether eight patients with novel mutations in the respective genes associated with low enzyme activities. As a result of the anonymized study design, follow-up and clinical evaluation of the patients was not possible, hence not resulting in clear-cut cases. Another drawback is the detection of uncertain mutations that are not necessarily connected to the full-blown picture of the respective LSD raising ethical issues of detecting hereditary disease at an early age when manifestation of the disease is still questionable. However, we believe the benefit of early disease detection and thereby early treatment, especially in LSDs where enzyme replacement therapy is available is of most importance.

Although the assay requires precise sample preparation and cleanup procedures, it proves to be robust and suitable for large-scale screening. An experienced operator can perform the cleanup of 4,500 samples, the daily workload of an average size screening laboratory, within 4–6 h. Recent introduction of online HPLC sample cleanup technology would save the time-consuming cleanup procedure as well as consumables but requires longer analysis time (LaMarca et al. 2009) or more sophisticated equipment (Kasper et al. 2010). However, using online HPLC cleanup on MS/MS, the instrument very likely can be used only for LSD screening and is blocked for other analysis. We think that in resource-limited screening laboratories with second tier testing methods and selective screening assays running on MS/MS, the manual assay will be the first choice.

The overall recall rate of 0.352% and the overall frequency of 1:2,354 at the best and 1:4,447 considering only previously described mutations for all four diseases in this study is comparable to the expanded NBS programs for acylcarnitines and amino acids (Schulze et al. 2003). Thus, we consider NBS for multiple LSDs economically justifiable.

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Conflict of Interest

No conflict of interest to declare.

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