Expanded Newborn Screening for Inborn Errors of Metabolism by Electrospray Ionization-Tandem Mass Spectrometry: Results, Outcome, and Implications

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ABSTRACT. *Objective.* **The aims of this study were to determine the impact of expanded newborn screening using tandem mass spectrometry (MS/MS) on the overall detection rate of inborn errors of metabolism in Germany and to assess the outcome for the patients that were diagnosed.**

Methods. **During the period of study, 250 000 neonates in a German population were investigated for 23 inborn errors of metabolism by electrospray ionization-MS/MS. The overall value of the screening program was estimated by 1) complete ascertainment of all positive tests; 2) definite assignment of all diagnoses including reconfirmation at 12 months; and 3) clinical follow-up of all detected patients in an overall interval of 42 months. The mean observation period was 13.5 months per child.**

Results. **In 106 newborns, confirmed inborn errors of metabolism were found. The disorders were classified as 50 classic forms and 56 variants. A total of 825 tests (0.33%) were false-positives. Seventy of the 106 newborns with confirmed disorders were judged to require treatment. Six children developed symptoms despite treatment. Three children had died. Among 9 children who became symptomatic before report of the results of screening, in 6 the diagnosis had been made in advance of the screening report. In evaluation of the screening program, 61 of the 106 identified children (58% of true-positives, or 1 of 4100 healthy newborns) were judged to have benefited from screening and treatment, because the diagnosis had not been made before screening. None of these infants had died and none developed psychomotor retardation or metabolic crisis during the follow-up period.**

Conclusions. **The screening by MS/MS for up to 23 additional disorders has approximately doubled the detection rate compared with that achieved by the conventional methods used in Germany. This strategy represents valuable preventive medicine by enabling diagnosis and treatment before the onset of symptoms.** *Pediatrics* **2003;111:1399–1406;** ***mass screening, metabolism, inborn errors, neonatal screening, outcome assessment, preventive medicine, spectrometry, mass, electrospray ionization.***

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ABBREVIATIONS. FAO, fatty acid oxidation; CI, confidence interval; MCAD, medium-chain acyl-CoA dehydrogenase; MS/MS, tandem mass spectrometry; PKU, phenylketonuria.

The application of electrospray ionization-tandem mass spectrometry (MS/MS) to newborn screening for inborn errors of metabolism offers the potential of substantially altering the natural history of these diseases by reducing morbidity and mortality. MS/MS permits efficient identification of groups of disorders with acceptable laboratory operating costs. The diseases detected include amino acidemias, fatty acid oxidation (FAO) disorders, and organic acidurias. The ability of MS/MS to screen for metabolic disorders was first demonstrated by Millington et al.[1] Chace and coworkers, and subsequently other groups, refined this method and applied it to newborn screening. Reports dealing with analytical variables, methodology, and descriptive characteristics have been published for phenylketonuria (PKU),[2–4] maple syrup urine disease,[5] homocystinuria,[6] medium-chain acyl-CoA dehydrogenase (MCAD) deficiency,[7–12] argininosuccinate lyase deficiency,[13] carnitine transporter defect,[14] carnitine palmitoyltransferase I deficiency,[15] carnitine palmitoyltransferase II deficiency,[16] and galactosemia.[17]

The use of MS/MS has permitted several neonatal screening laboratories throughout the world to expand the spectrum of disorders to include FAO disorders and organic acidurias and to increase considerably the number of amino acidemias detected. Initial reports on the experience of these expanded screening programs have focused on the frequency of the disorders detected, diagnostic specificity, cutoff criteria, logistics, and pitfalls.[18–22]

Despite emerging knowledge about the analytical aspects of MS/MS screening, only sparse data exist as to whether neonatal screening for such disorders and early institution of treatment in the presymptomatic phase effectively prevents mortality and morbidity. Most newborn screening programs have not reported data on outcome. In this report, we present data on 250 000 newborns who underwent expanded screening over a 42-month period by MS/MS for amino acidemias, FAO disorders, and organic acidurias. The work focuses on obtaining definitive diagnosis by detailed confirmation analysis, including enzymatic and mutational analyses, and clinical follow-up of all patients detected during

the period of study to assess the impact of newborn screening on short-term outcome. It is recognized that longer-term follow-up will be necessary to provide optimal assessment of impact on morbidity and mortality, but it appeared appropriate to provide a report of progress of potential usefulness to the many programs being initiated.

METHODS

Subjects

Specimens of dried blood spots were collected from infants born in Baden-Württemberg, Germany, between April 1998 and September 2001. The total number of infants tested was 250 000, and >98% were white. The recommended time of sampling was between the 3rd and 7th day of life. In our cohort, the median was the 5th day, with 0.88% of the samples being obtained before the 3rd, and 1.65% after the 7th day of life. Blood taken by heelstick was spotted on filter paper (Schleicher & Schuell 2992 up to 1999, Schleicher & Schuell 903 since 2000; Schleicher & Schuell, Dassel, Germany), dried, and sent by mail to the screening laboratory. Change of filter paper had no impact on results of MS/MS screening (data not shown). Blood specimens of preterm neonates were similarly assayed, but a second specimen from all preterm infants (<32 weeks of gestational age) was obtained and assayed after 14 days of life (~1% of all samples, ~2500 samples in total) following the current screening guidelines in Germany. The investigations of this study were added to the standard regional screening program already in place for hypothyroidism, congenital adrenal hyperplasia, PKU, galactosemia, and biotinidase deficiency, and were approved by the Ministry of Social Affairs of Baden-Württemberg, Germany. Parents were provided with information packages and signed informed consent was obtained. There were very few refusals based on the survey of 16 200 births. Medical staff and health professionals received detailed pamphlets about expanded screening.

Acylcarnitine and Amino Acid Profiling

Acylcarnitines and amino acids were analyzed as butyl esters on an API 365 triple quadrupole tandem mass spectrometer (PE Sciex, Concord, Ontario, Canada) with an ion spray device as previously described with minor modifications.[4,23] One 3-mm (1/8-inch) diameter dot was punched from each 10-mm diameter dried blood spot specimen into a single well of a 96-well microtiter filter plate to which was added 100 μL of a methanol stock solution of internal deuterated standards (containing 0.76 μmol/L [$^2H_9$]carnitine, 0.19 μmol/L [$^2H_3$]acetylcarnitine, 0.04 μmol/L each of [$^2H_3$]propionylcarnitine, [$^2H_3$]butyrylcarnitine, [$^2H_9$]isovalerylcarnitine, [$^2H_3$]octanoylcarnitine, [$^2H_9$]myristoylcarnitine, 0.08 μmol/L [$^2H_3$]palmitoylcarnitine, 2.5 μmol/L each of [$^2H_4$]alanine, [$^2H_6$]valine, [$^2H_3$]leucine, [$^2H_3$]methionine, [$^2H_5$]phenylalanine, [$^{13}C_6$]tyrosine, [$^2H_3$]aspartate, [$^2H_3$]glutamate, [$^2H_2$]ornithine, [$^2H_4$,^{13}C]arginine, [$^2H_2$]citrulline, and 12.5 μmol/L [^{15}N,^{13}C]glycine). After 20 minutes, the samples were centrifuged and the eluate was evaporated to dryness, reconstituted in 60 μL of 3 N HCl/butanol, placed in sealed microtiter plates, and incubated at 65°C for 15 minutes. The resulting mixtures were again dried, and each residue was finally reconstituted in 100 μL solvent of acetonitrile/water/formic acid (50:50:0.025 by volume). A PE 200 autosampler transferred 25 μL of each into the collision cell at a solvent flow rate of 40 μL/min using a PE 200 high performance liquid chromatography pump. All acylcarnitines were measured by positive precursor ion scan of m/z 85 (scan range m/z: 200–500). Each acylcarnitine was quantified using the signal intensity ratio of the compound to its internal standard and related to concentrations using the slope derived from standard curves. When a specific stable isotope was not available, the following ratios were used for calculation: C2/C3-d3, C5:1/C5-d9, C5OH/C5-d9, C6/C5-d9, C8:1/C8-d3, C10:1/C8-d3, C10/C8-d3, C4-DC/C8-d3, C5-DC/C8-d3, C12/C14-d9, C14:1/C14-d9, C14OH/C14-d9, C16:1/C16-d3, C16:1OH/C16-d3, C16OH/C16-d3, C18:1/C16-d3, C18/C16-d3, and C18:1OH/C16-d3.

For amino acid profiling, different neutral loss scan functions were used as follows: neutral loss of m/z 102 for alanine, valine, leucine/isoleucine, methionine, phenylalanine, tyrosine, aspartate, glutamate, and special multiple reaction monitoring experiments for ornithine (m/z 189 → 70), arginine (231 → 70), argininosuccinic acid (459 → 70), glycine (132 → 76), citrulline (232 → 113), and homocitrulline (246 → 127). The specific isotope of each amino acid was used for quantification (except for argininosuccinic acid and homocitrulline, in which [$^2H_2$]citrulline was used as reference). The total time for 1 complete analysis of acylcarnitines and amino acids was 2.5 minutes per sample.

Decision Criteria, Interpretation, and Newborn Screening Protocol

Daily data management was divided into 2 main parts: 1) technical interpretation of acquired data, and 2) clinical interpretation and decision-making. For the first step, the technical interpretation, a decision limit (cutoff) for each analyte or analyte ratio was set on the 99.5th (0.05th) percentile, based on data collected and analyzed from 10 000 healthy neonates. Each analyte crossing the cutoff was flagged automatically. Samples in which 1 or more parameters were flagged underwent a repeated analysis from the same blood spot (13.8% of all specimens). A sample was classified as true-positive only if the first *and* second tests were positive. We decided to use this strategy to reduce the recall rate of samples close to the cutoff. If a distinct discrepancy between the first and second tests occurred, a third test was done and the mean of the 2 corresponding results was used. A distinct discrepancy was considered when the first test exceeded the cutoff by >30% and the second test was normal. In 5.8% of all specimens (ie, 42% of all repeated samples), 1 or more of the flagged parameters were confirmed. Data files of all the samples repeatedly flagged by 1 or more abnormal analyte were then transferred to the second step, the clinical interpretation and decision-making process, performed by an experienced metabolic disease specialist. He decided whether the analysis was abnormal (positive) or normal (negative) by using a rating system that included the magnitude of deviation from the cutoff limit and the overall profile of metabolites. As examples, in case of screening for maple syrup urine disease, the analysis has been judged as normal when only leucine/isoleucine were increased but valine was normal; or, in case of screening for MCAD deficiency, the analysis has been judged as normal when the octanoylcarnitine-to-acetylcarnitine ratio was normal, although octanoylcarnitine and decanoylcarnitine were increased. The rating system was optimized on the basis of measurements in patients with proven disorders, reports from the literature, analysis of diagnostic sensitivity and specificity in previous screening results, and the increasing experience of the investigator.

In the case of a presumptive positive screening result (0.38% of all samples, false- and true-positives), a repeat dried blood spot specimen was obtained on recall from the infant, or alternatively, the infant was referred to a treatment center and hospitalization arranged, if avoidance of any further delay was felt to be essential for the patient's well-being. An additional urine sample for gas chromatography/mass spectrometry analysis was requested with the recall, if it was diagnostically relevant for the suspected disease, eg, an organic aciduria. Depending on the result of the recall, the screening result was classified as false-positive or true-positive (0.33% and 0.05% of all samples, respectively). For each true-positive, the metabolic specialist informed the responsible physician or midwife, and with physician approval direct contact was made with the parents and referral made to a medical center qualified to carry out definitive diagnosis and treatment. Decision criteria and confirmatory investigations are listed in Table 1.

Clinical outcomes were determined on the basis of the medical history from the hospitals, repeated surveillance, medical examinations, the medical records from the metabolic outpatient departments, and a detailed final examination on all patients detected by the expanded newborn screening program. The German surveillance program established for all children was performed in affected children at ages 4 to 6 weeks, 3 to 4 months, 6 to 7 months, 10 to 12 months, and 21 to 24 months by the primary care pediatricians as uniformly structured and predetermined investigation and reporting system on somatic and neurologic development including hearing, vision, and psychomotor development.

RESULTS AND DISCUSSION

Prospective Newborn Screening Results

Of 250 000 newborns studied, 106 were assigned with a confirmed inborn error of metabolism. The

TABLE 1. Decision Criteria and Confirmatory Analysis for Disorders Detected by Expanded Neonatal Screening With MS/MS

Disorder	Decision Criteria for "Positive" Testing in Neonatal Screening Amino Acids or Acylcarnitines		Confirmatory Analyses
	(Cutoff, μmol/L)	(Cutoff, Molar Ratio)	
PKU	Phe (>150)	Phe/Tyr (>1.7)	PKU: Phe >600 μM; non-*PKU-HPA:* Phe >150 and <600 μM
Tyrosinemia I	Tyr (>200) and positive succinylacetone test[28]		Succinylacetone in urine, increased α-fetoprotein in serum
Maple syrup urine disease	Leu(Ile) (>490) and Val (>390)		Plasma amino acids and presence of alloisoleucine; enzyme activity in fibroblasts; Classical: Leu 500–5000 μM, BCKD activity <2%; Variant forms: Leu 50–4000 μM, BCKD activity 2–40%
Citrullinemia	Cit (>65)	Orn/Cit (<1.5) Cit/Arg (>15)	Plasma amino acids; enzyme activity in fibroblasts; Classical: Cit ≫1000 μM; Variant forms: Cit >50 and <1200 μM, ammonia and arginine normal
Argininosuccinate lyase deficiency	Asa (>1)		Enzyme activity in fibroblasts; Asa in urine
Nonketotic hyperglycinemia	Gly (>2000)		Gly CSF/plasma ratio >0.06
Homocystinuria	Met (>65)	Met/Phe (>3) Met/Leu(Ile) (>1)	Enzyme activity in fibroblasts; mutational analysis; total homocysteine in plasma ≫20 μM
Long-chain 3-OH acyl-CoA dehydrogenase/mitochondrial trifunctional protein deficiency	C14OH (>0.12) or/and C16:1OH (>0.22) C16OH (>0.20) C18:1OH (>0.12) C18OH (>0.11)		Enzyme activity in fibroblasts/lymphocytes
Very long-chain acyl-CoA dehydrogenase deficiency	C14:1 (>0.43) or C14 (>0.76)		Enzyme activity in fibroblasts/lymphocytes
MCAD	C6 (>0.21) or/and C8 (>0.32) C10:1 (>0.28) C10 (>0.48)	C8/C2 (>0.02) C8/C10 (>1.6) C8/C12 (>1.6)	Enzyme activity in fibroblasts/lymphocytes; mutational analysis; phenylpropionate loading; hexanoyl and suberylglycine in urine; Classical: enzyme residual activity <5% or pathological phenylpropionate loading test or severe mutations; Variant forms: enzyme residual activity 5–35%; negative phenylpropionate loading test; mild mutations
Short-chain acyl-CoA dehydrogenase deficiency	C4 (>2)		Enzyme activity in fibroblasts/muscle; mutational analysis; ethylmalonic acid in urine; Classical: ethylmalonic acid in urine and development of clinical symptoms or proven disease causing mutations; Variant forms: ethylmalonic acid in urine and/or decreased enzyme activity, no severe mutations, no clinical symptoms
Multiple acyl-CoA dehydrogenase deficiency	Multiple elevations from C4 to C18		Enzyme activities in fibroblasts; lactic, glutaric, and ethylmalonic acid in urine
Carnitine transporter defect	C0 (<10) and AC (<5)		Carnitine uptake in fibroblasts; mutational analyses; decreased tubular carnitine reabsorption
Carnitine palmitoyltransferase I deficiency	C0 (>90) and C16 (<1.7) C18 (<0.6)	C0/(C16 + C18) (>70)	Enzyme activity in fibroblasts/lymphocytes
Carnitine palmitoyltransferase II/carnitine-acylcarnitine translocase deficiency	C0 (<10) and C16 (>10.6) C18 (>3.2)	C0/(C16 + C18) (<3)	Enzyme activity in fibroblasts/lymphocytes
Propionic aciduria	C3 (>6.8)	C3/C0 (>0.19) C3/C2 (>0.39)	Enzyme activity in fibroblasts; tiglylglycine, 3-hydroxypropionic acid, methylcitrate in urine; ammonia in plasma
Methylmalonic aciduria/cobalamine disorders	C3 (>6.8) or MMA (>1)	C3/C0 (>0.19) C3/C2 (>0.39)	Enzyme activities in fibroblasts; methylmalonic acid in urine; ammonia and homocysteine in plasma
Glutaric aciduria type I	Glut (>0.14)	Glut/C8 (>1.8) Glut/C16 (>0.06)	Enzyme activity in fibroblasts; glutaric and 3-hydroxyglutaric acid in urine
Isovaleric aciduria	C5 (>2)	C5/C2 (>0.06)	Enzyme activity in fibroblasts; 3-hydroxyisovaleric acid and isovalerylglycine in urine
HMG-CoA lyase deficiency	C5OH (>1)		Enzyme activity in fibroblasts; 3-OH-methylglutaric and 3-methylglutaconic acid in urine
Holocarboxylase synthetase deficiency	C5OH (>1)		Enzyme activity in fibroblasts; 3-OH-isovaleric, 3-hydroxypropionic acid, lactate, methylcitrate, and 3-methylcrotonylglycine in urine
3-Methylcrotonyl-CoA carboxylase deficiency	C5OH (>1)		Enzyme activity in fibroblasts; 3-OH-isovaleric acid and 3-methylcrotonylglycine in urine; Leu loading test; Classical: diminished enzyme activity or pathological Leu loading test; Variant forms: abnormal organic acids in urine but no increase of C5OH in the mother

Phe indicates phenylalanine; Tyr, tyrosine; Leu, leucine; Ile, isoleucine; Val, valine; BCKD, branched-chain α-ketoacid dehydrogenase complex; Cit, citrulline; Orn, ornithine; Arg, arginine; Asa, argininosuccinic acid; Gly, glycine; Met, methionine; C0, free carnitine; C2, acetyl; C3, propionyl; C4, butyryl; C5, isovaleryl; C6, hexanoyl; C5OH, 3-OH-isovaleryl or 2-methyl-3-OH-butyryl; C8, octanoyl; C10:1, decenoyl; C10, decanoyl; MMA, methylmalonyl; Glut, glutaryl; C12, dodecanoyl; C14:1, myristoleyl; C14, myristoyl; C14OH, 3-OH-myristoyl; C16, palmityl; C16:1OH, 3-OH-palmitoleyl; C16OH, 3-OH-palmityl; C18, stearoyl, C18:1OH, 3-OH-oleyl; C18OH, 3-OH-stearoylcarnitine; AC, total acylcarnitines (C3 to C18); HMG, 3-hydroxy-3-methylglutaryl.

overall prevalence of a metabolic disorder (classic forms and variants) identified on newborn screening was 1 in 2400 newborns (95% confidence interval (CI) 1:2900–1:2000). There were 65 newborns with amino acidemias (prevalence 1:3800, 95% CI 1:5100–1:3100); 24 with FAO disorders (1:10 400, 95% CI 1:17 400–1:7400); and 17 with organic acidurias (1:14 700, 95% CI 1:28 000–1:10 000). The 106 diseases could be further divided into 50 classic forms of disease and in 56 variant forms (Table 2). Most of the infants in the latter group required no treatment (eg, non-PKU-hyperphenylalaninemia, mild citrullinemia, and mild 3-methylcrotonyl-CoA carboxylase deficiency), but in some infants recommendation regarding treatment remains uncertain (eg, MCAD or short-chain acyl-CoA dehydrogenase deficiency); therefore, they were assigned to the "need treatment" group as a precaution.

In addition, 26 infants were judged positive on newborn screening and confirmed by recall, but a definite diagnosis remained questionable either because the diagnosis is difficult to achieve ($N = 17$) or because they were lost to follow-up ($N = 9$; Fig 1). The "diagnoses difficult to achieve" category includes short-chain acyl-CoA dehydrogenase deficiency in which butyrylcarnitine is increased, 3-methylcrotonyl-CoA carboxylase deficiency, holocarboxylase synthetase deficiency, and 3-hydroxy-3-methylglutaryl(HMG)-CoA lyase deficiency. In the latter 3, 3-hydroxy-isovalerylcarnitine is increased. None of the 17 children with suspected but unproven disorders displayed any clinical signs or symptoms of a metabolic disorder.

Only 825 tests (0.33%) were classified as false-positive, yielding an overall specificity of 99.67%. The specificity for amino acidemias and FAO disorders was 99.90% in each, and for organic acidurias it was 99.87% (Table 2).

Despite the low frequency of several disorders, the positive predictive value of the overall MS/MS

TABLE 2. Results of Expanded Newborn Screening in 250 000 Newborns: Frequency of Screened Disorders and Diagnostic Specificity, Sensitivity, and Positive Predictive Value of the Method

	Screening Positives			Prevalence of Disorder (With Variants)	False-Positive (Specificity, %)	False-Negatives (Sensitivity, %)	Positive Predictive Value, %
	Classic Form	Variants	Disorder Suspected				
Amino acidemias							
PKU	24	31	3	1:10,400 (1:4,500)	115 (99.95)	0 (100) PKU 4 (88.57) HPA	32.35
Tyrosinemia I	1	–	1	1:250,000	51 (99.98)	0 (100)	1.92
Maple syrup urine disease	1	1	–	1:250,000 (1:125,000)	23 (99.99)	0 (100)	8.00
Citrullinemia	1	5	–	1:250,000 (1:41,700)	12 (~100)	0 (100)	33.33
Argininosuccinate lyase	–	–	–	–	–	0	–
Nonketotic hyperglycinemia	1	–	–	1:250,000	39 (99.98)	0 (100)	2.50
Homocystinuria	–	–	–	–	8	0	–
FAO disorders							
Long-chain 3-OH acyl-CoA dehydrogenase/mitochondrial trifunctional protein	1	–	1	1:250,000	10 (~100)	0 (100)	9.09
Very long-chain acyl-CoA dehydrogenase	–	1	1	(1:250,000)	31 (99.99)	0 (100)	3.13
MCAD	12	4	–	1:20,800 (1:15,600)	46 (99.98)	0 (100)	25.81
Short-chain acyl-CoA dehydrogenase	–	3	4	(1:83,300)	23 (99.99)	0 (100)	11.54
Multiple acyl-CoA dehydrogenase	1	–	–	1:250,000	8 (~100)	0 (100)	11.11
Carnitine transporter defect	1	–	2	1:250,000	86 (99.97)	0 (100)	1.15
Carnitine palmitoyltransferase I	–	–	–	–	4	0 (100)	–
Carnitine palmitoyltransferase II/ carnitine-acylcarnitine translocase	1	–	–	1:250,000	47 (99.98)	0 (100)	2.08
Organic acidurias							
Propionic aciduria	1	–		1:250,000		0 (100)	
Methylmalonic aciduria	–	1	1	(1:250,000)	205 (99.92)	0 (100)	1.91
Cobalamin disorders	2	–		1:125,000		0 (100)	
Glutaric aciduria type I	3	–	–	1:83,300	59 (99.98)	0 (100)	4.84
Isovaleric aciduria	4	–	2	1:62,500	33 (99.99)	0 (100)	10.81
HMG-CoA Lyase	–	–		–		0 (100)	
Holocarboxylase synthetase	–	–	11	–	14 (99.99)	0 (100)	30.00
3-methylcrotonyl-CoA carboxylase	2	4		1:125,000 (1:41,700)		0 (100)	
Amino acidemias-total	28	37	4	1:8,900 (1:3,800)	258 (99.90)	4 (94.20)	20.12
FAO disorders-total	16	8	8	1:15,600 (1:10,400)	250 (99.90)	0 (100)	8.57
Organic acidurias-total	12	5	14	1:20,800 (1:14,700)	317 (99.87)	0 (100)	5.09
MS/MS screening-total	56	50	26	1:4,500 (1:2,400)	825 (99.67)	4 (96.36)	11.31

HMG indicates 3-hydroxy-3-methylglutaryl.

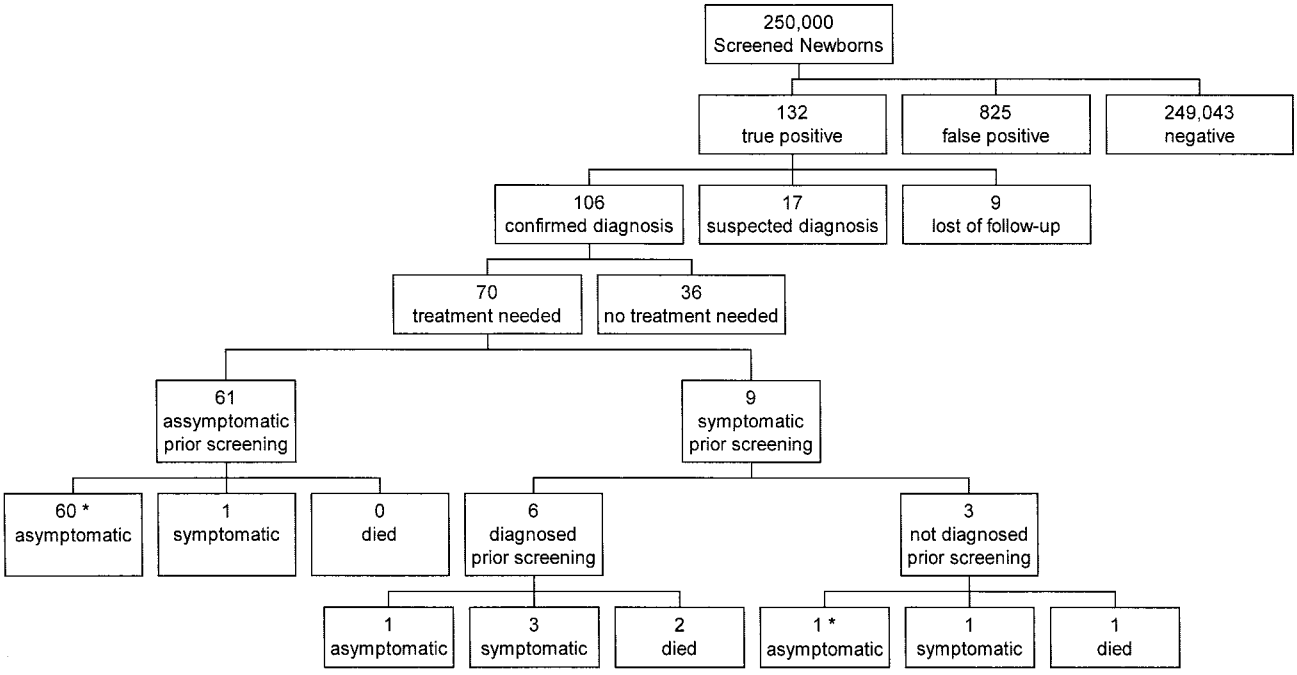

Fig 1. Flowchart of expanded newborn screening results. Asterisks denote children who were judged to have benefited from screening.

screening was found to be 11.31%, reflecting high diagnostic specificity of the method. The highest positive predictive value was obtained for amino acidemias (20.12%), followed by FAO disorders (8.57%) and organic acidurias (5.09%); the positive predictive value exceeds most of those known for conventional newborn screening, particularly for congenital endocrinopathies (for comparison, positive predictive values for conventional newborn screening programs in the United States and in Germany range from 0.5%–6.0% and 1.1%–9.5%, respectively)[24,25] (Table 2).

Diagnostic sensitivity of expanded screening for the different groups of disorders was found as follows: amino acidemias, 94.2% (4 patients with mild non-PKU-hyperphenylalaninemia were not identified by MS/MS screening but were detected by enzymatic PKU screening, which was conducted in parallel); and 100% for FAO disorders and organic acidurias. The overall sensitivity of MS/MS screening was 100% for classic forms of all disorders and 92.6% for variant forms. This estimate of sensitivity is based, to the best of our knowledge, on complete ascertainment of all affected infants. To date, monthly questionnaires to all pediatric hospitals and metabolic centers in Germany have not identified any child with an amino acidemia, FAO disorder, or organic aciduria missed by our newborn screening program.

Overall Outcome and Preventive Screening Effect

The results are shown in detail in Table 3. After a mean observation period of 13.5 months per child (range: 0.1–38 months), the clinical follow-up status of the 106 screen-positive newborns was determined. Ninety-seven infants (92%) were found asymptomatic. Subdivided into groups of disorders, this comprised 63 of 65 patients (97%) with amino acidemias, 22 of 24 patients (92%) with FAO disorders, and 12 of 17 patients (71%) with organic acidurias. Six children had symptoms of varying severity despite adequate treatment (1 maple syrup urine disease, 1 propionic aciduria, 2 cobalamin C/D deficiencies, 2 glutaric acidurias); in addition, 3 children had died (1 each of nonketotic hyperglycinemia, multiple acyl-CoA dehydrogenase deficiency, and mitochondrial trifunctional protein deficiency).

For the best estimate of the preventive value of the expanded screening program, we defined the benefit as prevention of mortality and morbidity in children with confirmed disorders who needed treatment, had not been diagnosed before screening, and remained asymptomatic during the observation period, with normal psychomotor development, no major disabilities, and no metabolic crises. In 70 of 106 diagnosed newborns, a disorder was established which required treatment. The remaining 36 patients were found to have milder disorders in which the necessity for treatment has not been established, such as non-PKU-hyperphenylalaninemia and variant forms of methylmalonic aciduria and 3-methylcrotonyl-CoA carboxylase deficiency. Nine newborns became symptomatic before availability of the results of screening. The diagnosis was actually established before receipt of the results of screening in 6 of the patients (3 amino acidemias, 1 FAO disorder, 2 organic acidurias). In the other 3 early symptomatic infants (1 each with amino acidemia, FAO disorder, and organic aciduria), the diagnosis was made by screening. Thus, the diagnostic efficiency of MS/MS screening was 94%. In 1 of these 3, symptomatology disappeared completely after the initiation of treatment (tyrosinemia type I).

In sum, 61 (58%) of the 106 newborns first diagnosed by screening, needing treatment, and remaining asymptomatic can be assumed to have benefited from screening; these were 32 patients (49%) with

TABLE 3. Outcome of Expanded Newborn Screening by Means of MS/MS in 250 000 Newborns

	Confirmed Cases	Before Result of Screening		Age at Start of Treatment (D)	Current Clinical Status			Observation Period (Mo)
		Symptomatic	Diagnosis made prior		Asymptomatic	Symptomatic	Died	
Amino acidemias								
PKU	24	−	−			Not investigated*		
non-PKU-hyperphenylalaninemia	31	−	−			Not investigated*		
Tyrosinemia I	1	+	−	65	+	−	−	5
Maple syrup urine disease	1	+	+	10	−	(+)	−	38
MSUD-variant form	1	−	−	8	+	−	−	24
Citrullinemia	1	+	+	−	+	−	−	10
Citrullinemia-variant forms	5	0/5	0/5	−/−/−/−/12‡	5/5	−	−	14/6/9/3/6
Nonketotic hyperglycinemia	1	+	+	2	−	−	+	4 days
FAO disorders								
Long-chain 3-OH acyl-CoA dehydrogenase/ mitochondrial trifunctional protein	1	+	+	−	−	−	+	1 d
Very long-chain acyl-CoA dehydrogenase-variant form	1	−	−	185	+	−	−	35
MCAD	12	−	−	8.5 (7–90)*	12/12	−	−	17.0 (1–38)
MCAD-variant forms	4	−	−	65/39/18/12	4/4	−	−	16/27/21/19
Short-chain acyl-CoA dehydrogenase-variant forms	3	−	−	−/−/−	3/3	−	−	9/25/14
Multiple acyl-CoA dehydrogenase	1	+	−	6	−	−	+	3.5
Carnitine transporter defect	1	−	−	60	+	−	−	15
Carnitine palmitoyltransferase II	1	−	−	28	+	−	−	22
Organic acidurias								
Propionic aciduria	1	+	+	4	−	(+)	−	14
Methylmalonic aciduria-variant form	1	−	−	−	+	−	−	12
Cobalamin C/D deficiency	2	+/+	+/−	28/6	−	+/+	−	30/7
Glutaric aciduria type I	3	−	−	18/210/12	+	(+)/(+)	−	12/32/11
Isovaleric aciduria	4	−	−	10/7/11/11	4/4	−	−	31/19/14/12
3-Methylcrotonyl-CoA carboxylase	2	−	−	7/7	2/2	−	−	25/7
3-Methylcrotonyl-CoA carboxylase-variant forms	4	−	−	−	4/4	−	−	12/12/13/4
Amino acidemias-total	65	4/65	3/65	9.0 (2–65)§	8/10¶	1/10¶	1/10¶	7.5 (0.1–38)§
FAO disorders-total	24	2/24	1/24	11.0 (6–185)§	22/24	0/24	2/24	19.0 (1–38)§
Organic acidurias-total	17	3/17	2/17	10.5 (2–210)§	12/17	5/17	0/17	12.0 (4–32)§
MS/MS screening-total	106	9/106	6/106	10.0 (2–210)§	42/51¶	6/51¶	3/51¶	13.5 (0.1–38)§

MSUD indicates maple syrup urine disease.
* Not investigated in this study. A similar, overlapping cohort was recently evaluated by our group.[26]
‡ Four of these children were not treated; in the remaining 1, treatment was started at age 12 days.
§ Median (range).
¶ The PKU and non-PKU-HPA groups were excluded but should be considered asymptomatic.

amino acidemias, 22 (92%) with FAO disorders, and 7 (41%) with organic acidurias. It must be kept in mind that the natural course of some disorders diagnosed presymptomatically and qualified as to need treatment is not yet known. Therefore, it is still impossible to predict whether each individual child benefited from early detection, but the need for treatment in these disorders is generally accepted and the detection by screening the prerequisite for treatment. The estimated overall frequency of those newborns considered to benefit from screening was 1 in 4100 newborns in our population.

In addition to the direct screening effect of detecting affected newborns, a secondary effect was achieved in 3 cases. In the twin of the child with tyrosinemia type I, we were able to diagnose the disease after it had not been identified in the screen done by another screening laboratory. The diagnosis of carnitine palmitoyltransferase II deficiency in 1 girl led to the retrospective diagnosis of this disorder in a stored newborn filter card from a previous sibling who had died unexpectedly at 7 months of age. The diagnosis of multiple acyl-CoA dehydrogenase deficiency before the death of 1 infant led to prenatal diagnosis and the exclusion of this diagnosis in a subsequent pregnancy.

Amino Acidemias

MS/MS screening for amino acidemias revealed the highest frequency of affected newborns (1:3800), the highest positive predictive value (20.12%), and good clinical outcome in all but 2 patients (Tables 2 and 3). Twenty-four patients with PKU and 31 with non-PKU-hyperphenylalaninemia made up the majority of these amino acidemias. The diagnostic specificity of MS/MS screening of 99.95%, which was somewhat higher than observed with enzymatic determination of phenylalanine (99.67%),[26] was attributed to the determination of tyrosine as well as phenylalanine and the calculation of the phenylalanine/tyrosine ratio.[3,4]

FAO Disorders

FAO disorders represent a group of inborn errors of mitochondrial β-oxidation and inborn errors of the carnitine cycle which were not detected by conventional screening methods. The number and favorable outcome of these newborns provides the strongest argument for improved outcomes with expanded screening by MS/MS. There was a high frequency of newborns affected with FAO disorders (1:10 400), a high positive predictive value (8.57%), and good clinical outcome in all but 2 patients (Tables 2 and 3). MCAD deficiency, the diagnosis in 16 newborns (1:15 600), represented the majority of the FAO disorders with a very high specificity (99.98%), a high positive predictive value (25.81%), and a favorable outcome that followed simply the avoidance of fasting and supplementation with carnitine. In 4 of the 16 patients, a mild form of MCAD deficiency was diagnosed.[12] In view of the lack of long-term data on the need for treatment and the relatively simple treatment, we currently recommend treatment and follow-up of all patients with mild MCAD deficiency.

Organic Acidurias

MS/MS screening for organic acidurias revealed the lowest frequency of affected newborns (1:14 700), a lower positive predictive value (5.09%), and a good clinical outcome in 12 of 17 patients. The best results were achieved by screening for isovaleric aciduria (Tables 2 and 3). In 11 asymptomatic children, increased 3-OH-isovalerylcarnitine (or 2-methyl-3-OH-butyrylcarnitine) persisted but could not be attributed to an underlying disorder. These children represent the main proportion of unconfirmed but suspected disorders.

Secondary Abnormalities of Acylcarnitine and/or Amino Acid Profiles

Secondary reasons for distinct but nonspecific abnormalities in acylcarnitine and/or amino acid profiles were found in 108 patients. Extreme immaturity of preterm neonates accounted for 56% of this group, severe congestive heart failure 8%, total parenteral nutrition 8%, galactosemia 3%, and other severe nonmetabolic life-threatening neonatal illnesses 26%. The screening guidelines in Germany mandate a second specimen at 14 days of life from all preterm infants <32 weeks of gestational age. This led to the detection of carnitine deficiency in a substantial number of preterm infants if their free carnitine was related to the reference values of healthy term neonates. These decrease of carnitine levels in some preterm infants should be considered normal, although not necessarily physiologic.[27]

CONCLUSIONS

Electrospray ionization-MS/MS is increasingly being advocated for expanded newborn screening programs because it is targeted toward a broad range of amino acidemias, FAO disorders, and organic acidurias. Our study has revealed this approach to be a reliable method for the early diagnosis of these disorders. Carefully adjusted tracking, confirmatory diagnosis, and follow-up by metabolic specialists is essential to the success of such a program.

Diagnostic specificity was favorable. In this study, the false-positive rate for PKU screening with MS/MS was 0.05%, distinctly lower than the 0.23% with the enzymatic phenylalanine determination.[26] An especially important feature is the overall number of recalls. Expanding newborn screening by MS/MS to >20 disorders (overall prevalence 1:2400) resulted in 0.33% false-positives, which is similar to the recall rate obtained in the former screening for PKU alone. These results are impressive in view of the recent review of the magnitude and negative consequences of false-positive results in conventional newborn screening programs in both Germany and the United States.[24]

The combined frequency of newborns for whom expanded newborn screening was of value amounted to 1 in 4100 healthy newborns in our population. Besides a proven preventive effect of early detection/treatment in the classic disorders, disclosure of the disorder might also be helpful in several variant forms despite the lack of necessity for

treatment. This might be of relevance to non-PKU-hyperphenylalaninemia, in which it might lead to the prevention of maternal PKU in affected females. The retrospective or prospective detection of affected siblings or other family members of positively tested newborns is another positive effect of screening.

The detection of FAO disorders, particularly MCAD deficiency, represents a major advantage for expanded screening of a white population.

Although a cost analysis is not feasible without factoring in a defined testing volume, the cost per procedure by MS/MS was estimated at 7.5 US dollars per test. The observed frequency of 1 in 4100 newborns yielded a cost for detection of 1 affected infant at 30 750 US dollars. The increased costs would be quickly offset by the reduction of expenses for hospitalization and medications, ~30 000 to 40 000 US dollars per year, or 0.5 to 1.5 million US dollars per life for a patient with an inborn error of metabolism not diagnosed and treated in a timely fashion.

The detection of rare and unfamiliar metabolic disorders, the need for immediate follow-up, complicated logistics, timing of confirmatory testing, specialized treatment, and the need to avoid unnecessary anxiety in families necessitate a well-organized network closely linking the screening laboratory with an experienced metabolic unit. The results of expanded screening by MS/MS indicate that these expanded screening programs will become the standard of care. Long-term follow-up will provide the data necessary for their evaluation and modification.

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This article is dedicated in memory of Horst Bickel for his commitment to the introduction and continuing improvement of newborn screening. He was born in Hamburg, Germany, on June 28, 1918. In 1964, he started the first European Screening and Treatment Program for PKU with the Guthrie test. He died on December 1, 2000.

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