Clinical Chemistry 47:11
1945–1955 (2001)

Molecular Diagnostics and Genetics

Tandem Mass Spectrometric Analysis for Amino, Organic, and Fatty Acid Disorders in Newborn Dried Blood Spots: A Two-Year Summary from the New England Newborn Screening Program

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Background: **Tandem mass spectrometry (MS/MS) is rapidly being adopted by newborn screening programs to screen dried blood spots for >20 markers of disease in a single assay. Limited information is available for setting the marker cutoffs and for the resulting positive predictive values.**

Methods: **We screened >160 000 newborns by MS/MS. The markers were extracted from blood spots into a methanol solution with deuterium-labeled internal standards and then were derivatized before analysis by MS/MS. Multiple reaction monitoring of each sample for the markers of interest was accomplished in ~1.9 min. Cutoffs for each marker were set at 6–13 SD above the population mean.**

Results: **We identified 22 babies with amino acid disorders (7 phenylketonuria, 11 hyperphenylalaninemia, 1 maple syrup urine disease, 1 hypermethioninemia, 1 arginosuccinate lyase deficiency, and 1 argininemia) and 20 infants with fatty and organic acid disorders (10 medium-chain acyl-CoA dehydrogenase deficiencies, 5 presumptive short-chain acyl-CoA dehydrogenase deficiencies, 2 propionic acidemias, 1 carnitine palmitoyltransferase II deficiency, 1 methylcrotonyl-CoA carboxylase deficiency, and 1 presumptive very-long chain acyl-CoA dehydrogenase deficiency). Approximately 0.3% of all newborns screened were flagged for either amino acid or acylcarnitine markers; approximately one-half of all the flagged infants were from the 5% of newborns who required neonatal intensive care or had birth weights <1500 g.**

Conclusions: **In screening for 23 metabolic disorders by MS/MS, an mean positive predictive value of 8% can be achieved when using cutoffs for individual markers determined empirically on newborns.**
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Amino acid and acylcarnitine analysis of newborn dried blood spots using tandem mass spectrometry (MS/MS)[5] is rapidly gaining worldwide support as the method of choice for the screening of metabolic disorders. Numerous reports in the literature describe the utility of MS/MS for screening for amino *(1–4)*, organic, and fatty acid disorders *(5–11)*, or screening for combinations of the

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Received April 27, 2001; accepted August 20, 2001.

[5] Nonstandard abbreviations: MS/MS, tandem mass spectrometry; NENSP, New England Newborn Screening Program; PKU, phenylketonuria; MSUD, maple syrup urine disease; HCU, homocystinuria; MCAD, medium-chain acyl-CoA dehydrogenase; C3, propionylcarnitine; C16, palmitoylcarnitine; C10, decanolycarnitine; NICU, neonatal intensive care unit; VLBW, very low birth weight; PPV, positive predictive value; HPhe, hyperphenylalaninemia; HMet, hypermethioninemia; HHH, hyperornithinemia, -ammonemia, -homocitrullinuria; ASS, arginosuccinate synthetase; ASL, arginosuccinate lyase; PA, propionic acidemia; MMA, methylmalonic acidemia; MCD, multiple carboxylase deficiency; IVA, isovaleric acidemia; 2-MBCD, 2-methylbutyryl-CoA dehydrogenase; C5, isovalerylcarnitine; HMG, 3-hydroxy-3-methylglutaryl-CoA lyase; MCC, 3-methylcrotonyl-CoA carboxylase; C5OH, 3-hydroxyisovalerylcarnitine; GA, glutaric acidemia; C5-DC, glutarylcarnitine; SCAD, short-chain acyl-CoA dehydrogenase; C4, butyrylcarnitine; VLCAD, very long-chain acyl-CoA dehydrogenase; C14:1, tetradecenoylcarnitine; C16OH, hydroxypalmitoylcarnitine; CPT II, carnitine palmitoyltransferase type II; and LCHAD, long-chain hydroxyacyl-CoA dehydrogenase.

latter two *(12–14)*. Early diagnosis and treatment can reduce the morbidity and mortality associated with these diseases.

Before February 1999, the New England Newborn Screening Program (NENSP) has routinely screened >80 000 Massachusetts newborns annually for 9 mandated disorders. The nine mandated disorders are biotinidase deficiency, congenital adrenal hyperplasia, hypothyroidism, hemoglobinopathies, toxoplasmosis, galactosemia, phenylketonuria (PKU), maple syrup urine disease (MSUD), and homocystinuria (HCU). The Massachusetts Department of Public Health authorized the expansion of the mandated disorders to include medium-chain acyl-CoA dehydrogenase (MCAD) deficiency. In addition, a pilot study was initiated to evaluate the incidence of, and to gather information on, the clinical benefit of screening for 19 additional metabolic disorders in the Massachusetts newborn population. The statewide study was implemented after Human Subjects Review of a protocol that included population-based "informed consent". The informed consent allows the parents of newborns to "opt in" or to "decline" testing for the pilot study. On February 1, 1999, the NENSP in the University of Massachusetts Medical School started using MS/MS to test for the 4 mandated metabolic disorders MCAD, PKU, MSUD, and HCU and for the 19 pilot disorders. The 19 pilot disorders include tyrosinemia, urea cycle defects, and several fatty acid and organic acid oxidation defects.

In addition to testing newborns in Massachusetts, the NENSP tests newborn specimens from Maine, New Hampshire, Vermont, and Rhode Island. Each state decides independently which disorders will be sought among its infants. Thus the number of infants described here varies according to the disorder. We present a review of our developing experience and results for the first 2 years of screening newborns by MS/MS. Portions of this report were presented as preliminary results at the APHL, Newborn Screening and Genetics Testing Symposium (Raleigh, NC, May 2001).

Materials and Methods

We used computers and data processing equipment from Compaq Digital Equipment Corporation. The MS/MS data were compiled by Neolynx software (Micromass, Inc).

Chemicals included HPLC-grade methanol and acetonitrile from Fisher Scientific. Anhydrous butanol and acetyl chloride were purchased from Aldrich Chemical Co. Butanolic-HCl was prepared by slowly adding 55 mL of acetyl chloride to 500 mL of butanol. The seven deuterium-labeled amino acid internal standards listed below were purchased from Cambridge Isotopes Labs. The five deuterium-labeled acylcarnitine internal standards listed below were purchased from HJ ten Brink.

Most newborn blood specimens were obtained 1–3 days after birth. All specimens and controls tested by the NENSP were collected or prepared on S&S Grade 903 filter paper (Schleicher and Schuell).

sample preparation for ms/ms

A single 3.2-mm dried blood spot (equivalent to 3.1 μL of blood) was placed in a polypropylene microtiter plate (Corning). Methanolic internal standard solution (100 μL) was added either manually or with a Labsystems Multidrop Dispensor. The concentrations of the deuterium-labeled internal standards per liter of methanol were as follows: [$^2H_3$]-leucine ([$^2H_3$]-Leu), 10 μmol/L; [$^2H_3$]-phenylalanine ([$^2H_5$]-Phe), 5 μmol/L; [$^2H_3$]-methionine ([$^2H_3$]-Met), 5 μmol/L; [$^2H_4$]-tyrosine ([$^2H_4$]-Tyr), 5 μmol/L; [$^2H_6$]-ornithine ([$^2H_6$]-Orn), 5 μmol/L; [$^2H_2$]-citrulline ([$^2H_2$]-L-Cit), 2.5 μmol/L; [$^2H_4$]-[$^{13}C_1$]-arginine ([$^2H_4$]-[$^{13}C_1$]-L-Arg), 2.5 μmol/L; [$^2H_3$]-carnitine, 1.25 μmol/L; [$^2H_3$]-acetylcarnitine, 0.06 μmol/L; [$^2H_3$]-propionylcarnitine ([$^2H_3$]-C3), 0.06 μmol/L; [$^2H_3$]-octanoylcarnitine ([$^2H_3$]-C8), 0.06 μmol/L; [$^2H_3$]-palmitoylcarnitine ([$^2H_3$]-C16), 0.06 μmol/L. The microtiter plate was gently shaken during the 20-min extraction of the amino acid and acylcarnitine markers. The methanol extract was then manually transferred to a second polypropylene microtiter plate and dried by a hot air blower. Butanol-HCl (70 μL) was manually placed in each sample well and the microtiter plate covered with a thin Teflon sheet under a heavy weight and placed in 65 C° forced air oven for 15 min. After the plate was removed from the oven, the hot air blower removed the butanol-HCl. The butanol-derivatized samples were reconstituted with 100 μL of acetonitrile and water (80:20 by volume), and each plate was covered with aluminum foil. The samples were then ready for MS/MS analysis.

sample introduction and mass spectrometry

A series 1100 Hewlett Packard HPLC pump and a Model 215 Gilson autosampler equipped with a 20-μL loop were used for the solvent delivery and sample introduction into the MS/MS. The HPLC pump provided a flow of ~95-μL/min. The injector port was fitted with 1.6-mm (i.d.) Teflon tubing fitted with an Upchurch precolumn filter with a 10-μm stainless steel frit. A Micromass Quattro LC triple-quadrupole tandem mass spectrometer was used for the analysis. The samples (15 or 25 μL) were loaded into the sample loop. The injection-to-injection time was ~1.9 min. The acylcarnitine and amino acids were analyzed with multiple reaction monitoring. Precursor ion scans of the acylcarnitines were accomplished by focusing MS1 on the molecular mass (M+) of the butanol ester of the acylcarnitine, and MS3 was used to focus the "fragment" ion mass. The optimized cone voltage and collision energies are listed below in parentheses for the markers examined. The scan function for free carnitine (35 V, 17 eV) determined the ion abundance of the fragment ions at *m/z* 103, and the acylcarnitines (35–54 V, 25 eV) determined the ion abundance of the fragment ions at *m/z* 85.

Similarly, the neutral loss scan of *m/z* 102 was used for neutral amino acids (23V, 13–15 eV), loss of *m/z* 119 for Orn (17 V, 19 eV) and Cit (24 V, 20 eV), and loss of *m/z* 161 for Arg (27V, 27eV).

The markers were expressed as "concentration" if the marker concentration was calculated on the basis of its stable-isotope deuterium-labeled isomer (e.g., Phe/deuterium-Phe), or as a "ratio" if determined by comparison to a nonisomeric, deuterium-labeled compound [e.g., decanoylcarnitine/deuterium-C8 (C10/deuterium-C8)].

ANALYSIS FOR THE MCAD 985A→G MUTATION

Blood spots with C8 concentrations >0.5 μmol/L prompted an analysis for 985A→G. Two laboratories analyzed the DNA: one laboratory (Amino Acid Lab, Massachusetts General) used the method of Tanaka *(15)*, and the second (NENSP) used a Chelex procedure [the 3.2-mm blood spot was washed by shaking for 30 min in 300 μL of wash buffer (1× phosphate-buffered saline and 5 mL/L Triton X-100 by volume)]; after removal of the buffer, 120 μL of Chelex Solution (10% by volume) was added followed by a 30-min incubation at 100 °C. The ambient temperature samples were centrifuged, and the DNA was removed for amplification. The DNA amplification was performed according to a modified procedure of Gregersen et al. *(16)* that incorporated a hot-start PCR and isoschizomer of the restriction enzyme for more efficient digestion. Molecular analysis for MCAD mutations (by the laboratory of A.W.S.) in samples from individuals not homozygous for the 985A→G mutation were performed by direct DNA sequencing by the dideoxy-chain termination method in an automated Applied Biosystems DNA sequencer of amplified DNA from all 12 exons in both orientations.

RETEST AND FOLLOW-UP

Samples flagged for abnormal markers with the initial screen were retested. Repunching the dried blood spot and reanalyzing the sample confirmed the initial screening result. The mean of the results from the two tests was calculated, and if the mean was greater than the flag value, the results were reported to follow-up. This approach permitted the second test to confirm the flagged marker and confirm that the correct specimen has been tested. The policy for follow-up was if more than one specimen was out of range for a marker, a full metabolic workup was recommended. Generally, infants with mild or transient increases of markers that were without clinical signs were not worked up. Also, if the initial specimen had a very increased marker, then a full metabolic workup would be initiated. Confirmation for the disorders was as described or according to standard metabolic criteria *(17)*.

Results

The dried blood spots were generally taken 1–3 days after the infant's birth. The laboratory analysis of metabolic markers of disease began the day the specimens were received. The initial test results were available the next morning; retesting of flagged specimens was completed by 1200. Thus, final results were generally available within 28 h after receipt of the specimen.

Among the 257 000 infants tested by MS/MS for PKU, MSUD, and HCU, 164 000 infants were also tested for Tyr, Orn, Cit, Arg, and acylcarnitine markers. Each microtiter plate analyzed had three dried blood spot controls, and one of these controls contained markers with concentrations near the flag value. The mean marker imprecision (CV) for this control was as follows: acylcarnitines (C3, C8, C16), 11%; amino acids Leu, Orn, Met, Phe, Cit, and Tyr, 9%; and Arg, 22%. In this report, we describe the infants with either out-of-range (flagged) amino acid or acylcarnitine concentrations.

Our approach to setting the flags for the various markers was as follows. The flags for Phe and Met were set after a comparison of the results generated by the MS/MS method with our HPLC and Guthrie Bacterial Inhibition Assay methods that were in use before installation of the MS/MS. We analyzed the blood from apparently healthy and diseased infants by the three methods and set the MS/MS flag close to the flags in use with the HPLC and Guthrie Bacterial Inhibition Assay methods. The MS/MS flag for Leu was set by a similar approach with the knowledge that the MS/MS method also quantifies the isomers of Leu. The flag for C8 was set after a review of the flags reported in the literature and a review of our results in an interlaboratory study of blood with added markers, including C8. The interlaboratory study included laboratories that had published their MS/MS experience. These interlaboratory results revealed that our values for C8 agreed with the other laboratories and the literature. For the remaining markers, the flags for Tyr, Orn, Cit, Arg, and the remaining acylcarnitines were set such that ~0.02% or less of a population of ~4000 randomly selected newborns would be flagged for each marker. Flags were set to balance the need to identify infants with these disorders with an acceptable degree of false positives. The number of infants flagged for each marker was routinely monitored and the flag value empirically adjusted upward if the number of infants flagged became problematic. The aggregate false-positive rate for all disorders was ~0.3%. For convenience and ease of comparison, this report will describe the flags as being set at several SD above the population mean of ~5800 newborns.

In the 2-year study, 633 infants were flagged for amino acid and acylcarnitine disorders. Of these, 392 infants were flagged only for amino acids, 232 infants were flagged only for acylcarnitines, and 9 infants were flagged for both amino acid and acylcarnitine markers.

AMINO ACID DISORDERS

In the screened population, there were 392 infants flagged only for increased amino acids. Of these, 132 had two or

Table 1. Amino acid disorders: reference values.

Marker components	Component mass	Associated disorders	Newborn population		Flag	
			Mean	SD	Value	SDs above mean
Phe/d-Phe	222/227	PKU (HPhe)	60[a]	13	139[a]	6
Phe/d-Phe/Tyr/d-Tyr[b,c]	222/227/238/242		0.7	0.3	1.5	
Leu/d-Leu	188/191	MSUD	145[a]	42	373[a]	5
Leu/d-Leu/Phe/d-Phe[b]	188/191/222/227		2.5	0.7	5	
Met/d-Met	206/209	HCU (HMet)	24[a]	7	67[a]	6
Met/d-Met/Phe/d-Phe[b]	206/209/222/227		0.4	0.1	1	
Tyr/d-Tyr	238/242	TYR[c]	90[a]	38	442[a]	9
Tyr/d-Tyr/Phe/d-Phe[b]	238/242/222/227		1.5	0.6	6	
Orn/d-Orn	189/195	HHH	52[a]	23	300[a]	11
Orn/d-Orn/Cit/d-Cit[b]	189/195/232/234		4.6	2.0	10	
Cit/d-Cit	232/234	ASS/ASL	14[a]	6	100[a]	13
Cit/d-Cit/Arg/d-Arg[b]	232/234/231/236		0.9	0.3	2	
Arg/d-Arg	231/236	ARG	19[a]	12	132[a]	9
Arg/d-Arg/Orn/d-Orn[b]	231/236/189/195		0.3	0.1	1	

[a] μmol/L concentration.
[b] Marker ratio.
[c] d-, deuterium-; TYR, tyrosinemia; ARG, argininemia.

more flagged amino acids and thus were classified as hyperalimented. The remaining 260 infants were flagged for only one amino acid, included 146 infants that were listed as being in the neonatal intensive care unit (NICU) or were of very low birth weight [(VLBW); <1500 g]. Table 1 lists the marker components and their respective masses that were used to measure each marker in the blood spot, the associated amino acid disorders, the mean and SD of the marker in a selected newborn population (n = 5800), and the current flag value for the marker. The screening results are summarized in Table 2, which lists the marker and the marker ratio, number of infants flagged, the number that were NICU/VLBW infants, the number of disorders identified, the positive predictive value (PPV), and the mean and range of the marker in the infants diagnosed with a disorder during follow-up.

Table 2. Amino acid disorders: screening results.

Marker and ratio	Flag	Number of infants flagged	Number of flagged infants that are NICU/VLBW (%)	Number of disorders identified	PPV, %	Marker mean of the disorder (range)
Phe[a]	139[b]	92	46 (50)	7 PKU, 11 HPhe	20	477 (267–606), 215 (167–282)[b]
Phe/Tyr[a]	1.5	64[c]	28 (44)	7 PKU, 11 HPhe	28	10.4 (5.5–15.6), 3.2 (1.6–6.8)
Leu[a]	373[b]	19	9 (47)	1 MSUD	5	458[b]
Leu/Phe[a]	5	8[c]	4 (50)	1 MSUD	12	11
Met[a]	67[b]	71	53 (75)	1 HMet	1	76[b]
Met/Phe[a]	1	32[c]	21 (66)	1 HMet	3	1.2
Tyr[d]	442[b]	42	21 (50)	0		
Tyr/Phe[d]	6	38[c]	18 (47)	0		
Orn[d]	300[b]	10	6 (60)	0		
Orn/Cit[e]	10	5[c]	5 (100)	0		
Cit[d,e]	100[b]	20	7 (35)	1 ASL	5	156[b]
Cit/Arg[e]	2	3[c]	1 (33)	1 ASL	33	7.3
Arg[d,e]	132[b]	6	4 (67)	1 ARG[f]	17	216[b]
Arg/Orn[e]	1	3[c]	2 (67)	1 ARG	33	4.5

[a] 257 000 infants tested.
[b] μmol/L concentration.
[c] Number of infants with marker and marker ratio flagged.
[d] 164 000 infants tested.
[e] Cit and Arg were initially assayed using deuterium-Orn as the internal standard and subsequently changed to deuterium-Cit and -Arg, respectively. The number of infants flagged is the sum of both analytical approaches. The table lists the current analytical approach.
[f] ARG, argininemia.

PKU AND HYPERPHENYLALANINEMIA (HPhe)

The flag for Phe was set at 139 μmol/L, which is ~6 SD above our population mean. Of the 257 000 infants screened, 92 infants were flagged for Phe. Forty-six of the 92 infants were classified as NICU/VLBW infants. Seven infants were identified with PKU, and all seven were full-term, healthy babies (birth weights >2500 g). The mean Phe concentration in the PKU infants was 477 μmol/L (range, 267–606 μmol/L). The Phe/Tyr ratio for the initial and all repeat specimens for all PKU infants was >5. Eleven HPhe infants were also identified in the screened population. All 11 HPhe infants had birth weights >2500 g and 2 were NICU infants. The mean Phe concentration in the HPhe infants was 215 μmol/L (range,167–282 μmol/L); the Phe/Tyr ratio for the initial and all repeat specimens was >1.5.

Several infants with transient increases of Phe were observed. The Phe concentrations in these infants were 139–254 μmol/L, and their Phe/Tyr ratios were all <1.5. The overall prevalence of PKU and HPhe was ~1/14 000 (18 of 257 000). The PPV for PKU HPhe was 20% (18 of 92) if only Phe (>139 μmol/L) was used as the indicator of PKU HPhe. If the Phe concentration and the Phe/Tyr ratio were both considered, then the PPV increased to 28% (18 of 64).

MSUD

Because of the same *m/z* for Leu, isoleucine, and hydroxyproline, the Leu concentration included all three amino acids. The Leu flag of 373 μmol/L was set at 5 SD above the newborn population mean. MS/MS screening for MSUD in a population of 257 000 infants led to 19 flagged infants. Nine of the 19 infants were NICU/VLBW infants. The utility of the ratio, in this case Leu/Phe, was demonstrated; only 8 of the 19 infants flagged for Leu had a Leu/Phe ratio >5.

A single case of MSUD was identified. The initial specimen taken at 1 day of age had a Leu concentration of 458 μmol/L with a Leu/Phe ratio of 11. If only Leu was used as the flag for MSUD, then the PPV for MSUD was 5% (1 of 19). If both the flagged Leu and the flagged Leu/Phe ratio were used, then the PPV increased to 12% (1 of 8).

HCU AND HYPERMETHIONINEMIA (HMet)

The Met flag of 67 μmol/L was set at 6 SD above the population mean. There were 71 infants flagged for Met in the total population of 257 000 infants screened. Of the 71 infants flagged, 53 infants, or 75%, were NICU/VLBW. One full-term NICU infant with HMet was identified. If Met was used as the only indicator of HCU and HMet, the PPV was 1% (1 of 71). If both the flagged Met and the flagged Met/Phe ratio were used to calculate the PPV, then the PPV increased to 3% (1 of 32).

PILOT STUDY: AMINO ACID DISORDERS

Massachusetts infants (164 000) were also screened for four additional markers: Tyr, Orn, Cit, and Arg.

TYROSINEMIA

The Tyr flag was set at 442 μmol/L, which is 9 SD above the population mean. Of the 42 infants flagged for Tyr, 21 infants were NICU/VLBW. No cases of tyrosinemia were identified. The transient Tyr concentration range was 442–922 μmol/L, and the mean was 573 μmol/L. The Tyr/Phe ratio among the 42 infants was 4–20, and the mean was 9.2.

HYPERORNITHINEMIA, HYPERAMMONEMIA, HYPERHOMOCITRULLINURIA (HHH) SYNDROME

The analysis of the Orn marker for HHH syndrome also included asparagine because of their similar molecular weights. The Orn flag was set at 300 μmol/L, which is 11 SD above the newborn population mean. Only 10 infants were flagged for Orn out of a population of 164 000 that were screened. Six of the 10 were NICU/VLBW infants. The ratio used to support the increase of Orn was the Orn/Cit ratio; this was set at 10. Interestingly, only 5 of the 10 infants flagged for Orn also had a flagged Orn/Cit ratio. Three of five infants died, but none of these was reported to us as HHH syndrome; nor did we identify any confirmed cases of HHH syndrome.

ARGINOSUCCINATE SYNTHETASE (ASS) AND ARGINOSUCCINATE LYASE (ASL) DEFICIENCY

Cit was the marker used to identify ASS and ASL deficiency, and the Cit flag was set at 100 μmol/L, or 13 SD above the population mean. There were 20 infants flagged for increased Cit, and of these, 7 were NICU/VLBW infants. The ratio used to support the Cit marker was Cit/Arg, which was increased in only 3 of 20 infants flagged for Cit. One of those ratio increases was identified as ASL deficiency. The initial specimen, taken from the NICU infant at 2 days of age (birth weight, 1730 g) revealed a normal Cit value of 40 μmol/L, but a slightly increased (flagged) Cit/Arg ratio. The second specimen, taken at 14 days of age, was found to have a Cit concentration of 156 μmol/L and a very increased Cit/Arg ratio of 7.3. Confirmation of the disorder was by the presence of argininosuccinate in the urine and plasma and an absence of ASL activity in erythrocytes. The infant is well and without hyperammonemia.

If only Cit was used as the marker for ASS and ASL then the PPV was 5% (1 of 20). If both the flagged Cit and the flagged ratio Cit/Arg were considered, then the PPV was 33% (1 of 3).

ARGININEMIA

The marker for argininemia is Arg, and the flag was set at 132 μmol/L, which is ~9 SD above the population mean. There were six infants flagged for Arg, and four of them were NICU/VLBW infants. The ratio used to support the flagged Arg was Arg/Orn, which was increased in three of the six infants. A blood specimen taken from a female infant on the 3rd day of life who died in spite of hospital management of cerebral edema and hypertonia was

flagged for an Arg of 216 μmol/L and an Arg/Orn ratio 4.5-fold above the cutoff. This normal birth weight infant was diagnosed with argininemia. The disorder was confirmed by the increased Arg in the plasma and cerebral spinal fluid and by the absence of arginase activity in the erythrocytes.

If only Arg was used as the marker for argininemia, then the PPV for argininemia was 17% (1 of 6). The PPV increased to 33% (1 of 3) when both Arg and the Arg/Orn ratio were used as the indicators of argininemia.

FATTY AND ORGANIC ACID DISORDERS

A total of 164 000 Massachusetts newborns were tested for acylcarnitines. Maine requested that its newborn infants (20 000) also be screened only for the acylcarnitines associated with MCAD. Thus, the total number of infants screened for MCAD was 184 000.

The acylcarnitine markers were considered as primary or secondary markers. Primary markers were expected to be consistently expressed with their associated disorders, whereas the secondary markers added credibility or help with the differentiation. Table 3 lists 12 of the 20 acylcarnitines used to screen for the fatty and organic acid disorders. Eight additional secondary acylcarnitine markers used by our screening program are not shown. Table 3 lists the marker components and their mass, the disorders associated with the markers, whether the marker was primary or secondary, the mean and SD of the marker in a selected newborn population (n = 5800), and the flag value. The screening results in Table 4 list the marker, the number of infants flagged, the number of flagged infants that were NICU/VLBW infants, the number of disorders identified, the PPV, and the mean and range of the markers in the disorders identified at follow-up.

PROPIONIC ACIDEMIA (PA), METHYLMALONIC ACIDEMIA (MMA), AND MULTIPLE CARBOXYLASE DEFICIENCY (MCD)

C3 was the primary marker used to screen for PA, MMA, and MCD; the flag for C3 was set at 8 SD above the population mean. Methylmalonylcarnitine was a secondary marker for MMA. Only 36 infants were flagged for C3 in 164 000 newborns that were screened, and 8 of these were NICU/VLBW infants. Transient increases in C3 were frequently observed in infants whose ABO blood type differed from that of the mother. Repeat specimens from the 36 infants had normal amounts of C3 except for two infants. The C3 concentration in the repeat specimens from those two infants decreased in concentration but remained above the flag. The PA cases were confirmed with the finding of increased urinary tiglylglycine, 3-hydroxypropionate, and methylcitrate; the lymphocytes of both infants were without propionylcarboxylase activity. Both infants were asymptomatic at diagnosis, but one had several admissions for mild ketoacidosis, and yet no acute metabolic decompensation. The PPV for PA was ~6% (2 of 36).

ISOVALERIC ACIDEMIA (IVA) AND 2-METHYLBUTYRYL-CoA DEHYDROGENASE (2-MBCD) DEFICIENCY

Isovalerylcarnitine (C5) or its geometric isomer, 2-methylbutyrylcarnitine, is the primary marker for IVA and 2-MBCD deficiency *(9)*, and the flag was set at 11 SD above the population mean. Increased C5 requires additional testing, such as urinary organic acids and acylglycines to distinguish these two disorders. Although there were 35 infants flagged for C5 (28 of the infants were NICU/VLBW infants), we were not aware of any cases of IVA or 2-MBCD deficiency in the population screened.

Table 3. Acylcarnitine disorders: reference values.

				Newborn population		Flag	
Marker components	Component mass	Associated disorders	Markers[a]	Mean	SD	Value	SDs above mean
C3/d-C3[b]	274/277	PA/MMA/MCD	1/1/1	1.8[c]	0.8	8[c]	8
C3-2M-DC/d-C8	374/347	(MMA)	2	0.16	0.06	0.8	10
C5/d-C3	302/277	IVA/2-MBCD	1/1	0.22	0.09	1.2	11
C5OH/d-C8	318/347	BKT/HMG/MCC/MCD	2/1/1/2	0.14	0.05	0.8	12
C5:1/d-C3	300/277	(BKT)	1	0.02	0.01	0.08	6
C5-3M-DC/d-C16	402/459	(HMG)	2	0.02	0.01	0.12	11
C5-DC/d-C8	388/347	GA I, II	1/2	0.05	0.02	0.21	8
C4/d-C3	288/277	SCAD/IBCD	1/1	0.4	0.15	1.9	10
C8/d-C8	344/347	MCAD/GA II	1/2	0.11[c]	0.04	0.5[c]	11
C14:1/d-C16	426/459	VLCAD	1	0.12	0.06	0.9	12
C16/d-C16	456/459	CPT II/CATR	1	3.1[c]	1.2	12[c]	7
C16OH/d-C16	472/459	LCHAD	1	0.02	0.01	0.1	7

[a] 1, primary marker; 2, secondary marker.
[b] d-, deuterium-; C3-2M-DC, methylmalonylcarnitine; BKT, β-ketothiolase C5:1, tiglylcarnitine; C5-3M-DC, methylglutarylcarnitine; IBCD, isobutyryl-CoA dehydrogenase; CATR, carnitine-acylcarnitine translocase.
[c] μmol/L concentration.

Table 4. Acylcarnitine disorders: screening results.

Marker	Flag	Number of infants flagged[a]	Number of flagged infants that are NICU/VLBW (%)	Disorders identified	PPV, %	Marker mean of the disorder (range)
C3	8[b]	36	8 (22)	2 PA	6	15.2, (12–18.5)[b]
C3-2M-DC[c]	0.8	6	1 (17)			
C5	1.2	35	28 (80)			
C5OH	0.8	24	2 (8)	1 MCC	4	5.9
C5:1	0.08	7	5 (71)			
C5-3M-DC	0.12	21	15 (71)			
C5-DC	0.21	32	10 (31)			
C4	1.9	33	7 (21)	5 SCAD[d]	15	2.7, (2.4–3.0)
C8	0.5[b]	52[e]	25 (48)	10 MCAD	20	7.1, (1.8–22)[b]
C14:1	0.9	4	0 (0)	1 VLCAD[d]	25	4.8
C16	12[b]	2	1 (50)	1 CPT II	50	25.2[b]
C16OH	0.1	5	2 (40)			

[a] 164 000 infants screened unless otherwise noted.
[b] μmol/L concentration.
[c] C3-2M-DC, methylmalonylcarnitine; C5-3M-DC, methylglutarylcarnitine.
[d] Presumptive cases.
[e] 184 000 infants tested.

β-KETOTHIOLASE, 3-HYDROXY-3-METHYLGLUTARYL-CoA LYASE (HMG), AND 3-METHYLCROTONYL-CoA CARBOXYLASE (MCC) DEFICIENCY AND MCD

3-Hydroxyisovalerylcarnitine (C5OH) or its isomers is a primary or secondary marker for these four disorders. Table 3 lists additional primary and secondary markers for these four disorders. The markers include tiglylcarnitine and 3-methylglutarylcarnitine. The C5OH flag was set at 0.8, or 12 SD above the population mean. A total of 52 infants were flagged for the four disorders, because either the primary or secondary markers shown in Table 4 were flagged. Of the infants flagged for C5OH, 22 were NICU/VLBW infants. One full-term infant's initial specimen taken 3 days after birth had a C5OH ratio of 5.9. A repeat specimen taken 4 days later revealed that the C5OH ratio had increased to 8.1. After referral to a metabolic specialist, the infant was diagnosed with MCC deficiency on the basis of the findings of increased urinary 3-hydroxyisovalerate and methylcrotonylglycine and the absence of MCC activity in the lymphocytes. At the completion of this report, the infant had been clinically well.

As mentioned earlier, nine infants were flagged for both amino acids and acylcarnitines. Four of these nine infants were on extracorporeal membrane oxygenator therapy. The infants on extracorporeal membrane oxygenator had very high Orn and 3-methylglutarylcarnitine, the secondary marker for HMG deficiency. It is not known whether the latter marker was actually 3-methylglutarylcarnitine or if it was an isomeric mass artifact associated with extracorporeal membrane oxygenator treatment. Three of the four infants died shortly after birth; it is not known whether the three infants died from any of the disorders listed here.

GLUTARIC ACIDEMIA I AND II (GA I AND II)

Glutarylcarnitine (C5-DC) is the primary marker for GA I and a secondary marker for GA II. The flag for C5-DC was set at 8 SD above the population mean. There were 32 infants flagged for C5-DC, and of these, 10 were NICU/VLBW infants. No cases of GA I or II were identified. But interestingly, of the 32 infants flagged for C5-DC, 15 were also flagged for at least one of the medium chain acylcarnitines [hexanoylcarnitine (C6), C8, decenoylcarnitine (C10:1), decanoylcarnitine (C10); see below]; 3 of these 15 were confirmed to have MCAD.

SHORT-CHAIN ACYL-CoA DEHYDROGENASE (SCAD) AND ISOBUTYRYL-CoA DEHYDROGENASE DEFICIENCY

Butyrylcarnitine (C4) and it geometric isomer, isobutyrylcarnitine, are the primary markers for SCAD and isobutyryl-CoA dehydrogenase deficiency *(18)*, respectively; the flag was set at 10 SD above the mean. The initial specimens of 33 infants were flagged for C4, and 7 were NICU/VLBW. Five infants were presumptively identified with the more common SCAD deficiency. Only one of the five infants had the marker C5 or its isomers flagged (data not shown). Four of the five infants had increased urinary ethylmalonate and methylsuccinate and/or increased plasma butyrylcarnitine. Fatty acid oxidation studies in two infants (only two were tested) were abnormal and consistent with SCAD deficiency. The urine of one infant did not have the increased urinary organic acids associated with SCAD deficiency but did have increased butyrylglycine. The infant was homozygous for 625G→A (personal communication, N. Gregersen, University of Aarhus, Aarhus, Denmark). The presumptive prevalence of the SCAD disorder is thus ~1 in 33 000.

MCAD DEFICIENCY AND GA II

C8 is the primary marker for MCAD with C6 and C10:1 as good secondary markers. C10 and C8 are good primary markers for GA II with C10 often more increased than C8 *(12)*. The C8 flag was set at 0.5 μmol/L, which is 11 SD above the population mean.

There were 52 infants flagged for C8, and 25 were NICU/VLBW infants. No infants with GA II were identified. In a population of 184 000 infants screened, 10 MCAD infants have been identified. Confirmation of nine of the MCAD disorders included positive urinary markers (hexanoylglycine and suberylglycine) consistent with MCAD deficiency and/or DNA analysis for the 985A→G mutation.

DNA analysis of the 10 infants identified with MCAD deficiency revealed that 4 were homozygous for the 985A→G mutation. Six of the MCAD-deficient infants had one copy of the 985A→G, and four of these underwent direct sequencing and were found to have the following sequence variants: 504A→C, 127G→A, 842G→C, and 698T→C. These sequence variants fall into the American College of Medical Genetics category as follows: "Sequence variation is previously unreported and is of the type which may or may not be causative of the disorder". Additional studies are necessary to establish whether these mutations contribute to the disorder.

To assess the prevalence of 985A→G in infants with low flagged concentrations of C8, the following study was performed. Seventeen infants with borderline C8 concentrations (0.5–0.7 μmol/L) were tested for the 985A→G mutation. The results revealed that eight infants had one copy of the 985A→G mutation and nine were without the mutation. These results, as compared with our local prevalence of ~1:120 for the 985A→G mutation, indicate why further study is needed to understand the relationship between initial increases of C8 and MCAD activity.

The C8 concentrations in the initial and repeat specimens for the 10 MCAD deficiency infants are shown in Fig. 1. The mean initial C8 concentration in the four infants homozygous for the 985A→G mutation was 13.8 μmol/L with a range of 9–22 μmol/L. The initial specimen from infants with a single copy of the 985A→G mutation had a mean C8 concentration of 2.6 μmol/L with a range of 1.9–3.2 μmol/L. A substantial decrease in the C8 concentration with time after birth is shown for the MCAD infants homozygous for the 985A→G mutation. Some MCAD infants with a single copy of the 985A→G mutation had C8 concentrations that decreased to values close to but above the C8 flag. All MCAD infants are clinically well; currently, most are on carnitine supplementation. Only one has been admitted for vomiting, at which time blood glucose was 3.3 mmol/L. The PPV for MCAD deficiency with the use of C8 was ~19% (10 of 52). The overall prevalence of MCAD deficiency was ~1 in 18 000.

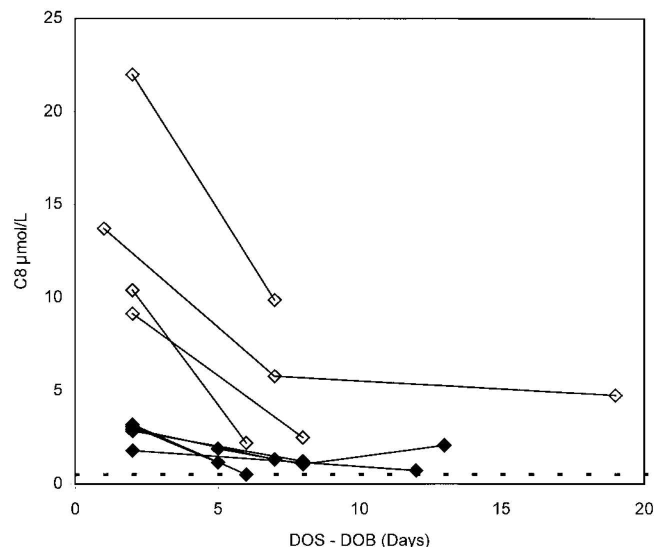

Fig. 1. Date of sample minus date of birth (*DOS − DOB*) in days plotted against the C8 concentration for the 10 infants with MCAD deficiency. ◇, infants with two copies of the 985A→G mutation; ◆, infants with one copy of the 985A→G or the 985A→G/variant disorder; - - - - denotes the current C8 flag of 0.5 μmol/L.

VERY LONG-CHAIN ACYL-COA DEHYDROGENASE (VLCAD) DEFICIENCY

Tetradecenoylcarnitine (C14:1) is the primary marker used to identify VLCAD, and the flag of 0.9 was set at 12 SD above the population mean. There were four infants flagged for C14:1. One normal birth weight infant with very increased C14:1, tetradecadienoylcarnitine, and hydroxypalmitoylcarnitine (C16OH) was presumed to have VLCAD deficiency on the basis of increased C14:1 and tetradecadienoylcarnitine. The original specimen from the infant, taken at 2 days of age, had a C14:1/d-C16 ratio of 4.8, which decreased to 1.2 in the second specimen taken at 7 days after birth. Additional testing such as fatty acid β-oxidation studies or assay for VLCAD activity in fibroblasts is needed for confirmation. However, the infant is clinically well, and the parents have, therefore, resisted further testing.

CARNITINE PALMITOYLTRANSFERASE TYPE II (CPT II) AND CARNITINE-ACYLCARNITINE TRANSLOCASE DEFICIENCY

The primary marker for CPT II and carnitine-acylcarnitine translocase deficiency is C16, and the flag of 12 μmol/L was set at 7 SD above the population mean. Two infants in the screened population of 164 000 were flagged for C16. One normal birth weight infant in the NICU had very increased C16 and long-chain hydroxyacylcarnitines. The specimen taken 3 days after birth had a C16 concentration of 25.2 μmol/L, and the following marker ratios were flagged: oleylcarnitine at 9.5, C16OH at 0.3, hydroxyoleylcarnitine at 0.2. The infant had presented acutely as a newborn with dysmorphic features and severe brain, liver, and kidney disease, and died at 3 days of age.

Muscle and skin biopsies confirmed the diagnosis of CPT II deficiency *(19)*.

LONG-CHAIN HYDROXYACYL-CoA DEHYDROGENASE (LCHAD) DEFICIENCY

The primary marker for LCHAD is C16OH, and the flag was set at 7 SD above the population mean. The differential diagnosis for long-chain hydroxyacylcarnitine markers would include the trifunctional protein deficiency. Five infants were flagged for C16OH; two were NICU/VLBW infants. Two of the five infants flagged for C16OH were the cases of CPT II and VLCAD described above. No cases of LCHAD deficiency were observed.

Discussion

The NICU/VLBW population poses a challenge to screening laboratories because the marker profiles in this population differ from those of normal birth weight infants. In our total screened population, the NICU/VLBW constituted 56% of the infants flagged for one amino acid and 40% of the infants flagged for the acylcarnitines. Thus, ~50% of the infants flagged for either a single amino acid or acylcarnitines were NICU/VLBW infants, although the NICU/VLBW population constituted only 5% of the total population screened. An example of the marker differences in our subpopulations is the substantially lower C16 concentration (mean, 1.5 μmol/L; SD, 0.9) observed in infants <1500 g. Sweetman et al. *(20)* described several differences in marker mean values in full-term infants, premature infants, and premature infants (usually VLBW) on total parenteral nutrition. The age of the infant is another factor because the reference interval for some markers can change over the first few weeks of life. There is a need to establish age- and weight-adjusted flags for these subpopulations. The overrepresentation of flagged infants among NICU and VLBW infants clearly demonstrates a need to establish a different set of screening criteria for these infants. If subpopulation flags were in use, our false-positive rate would be greatly reduced. The false-positive rate for the full-term infants would remain the same because the current flags are probably appropriate for this group.

Among the 42 cases of metabolic disorders identified, 9 were NICU/VLBW infants. Five of these were amino acid disorders (two HPhe, one each of HMet, ASL, and argininemia), and four were acylcarnitine disorders (two SCAD and one each of CPT II and MCAD). Thus, 21% of the disorders identified were NICU/VLBW infants. Our data suggest that there is a fivefold greater disease prevalence in this subpopulation than in the general newborn population. To identify disorders in this subpopulation, it is our policy that specimens from NICU infants should be submitted to the screening laboratory every 2 weeks as well as a specimen at discharge from the NICU.

To summarize screening for the remaining amino acid disorders, we conclude that transient tyrosinemia continues to be one of the problematic areas of detection and differentiation. We observed that the high concentrations of Tyr persisted for up to 7 weeks. Transient tyrosinemia reportedly can exist for nearly 3 months *(21)*. The urea cycle disorders present additional challenges. For example, regarding argininemia, we were successful in identifying one case prospectively by a flagged Arg concentration of 216 μmol/L in the specimen taken at 3 days of age. Unrelated to our prospective screening, another 3-day specimen that had been in storage for 3 years also had an increased Arg concentration concordant with the diagnosis of argininemia that had been made clinically. Unlike these two examples of argininemia, the one infant with ASL deficiency was not identified by the first specimen, but did have a substantial flagged Cit increase in the second specimen taken 14 days after birth. We remain uncertain whether other cases of ASL can be identified in the early neonatal period.

Follow-up studies of the presumptive positive cases of the fatty acid and organic acid disorders often begin with the analysis of urinary organic acids and acylglycines and/or plasma acylcarnitines *(7, 8)*. Timing of these follow-up tests can be critical for many disorders because the abnormal markers may not be informative if the infant is not metabolically decompensated *(8)*. In addition, a decrease in the acylcarnitines is expected when regular feedings are established. In vitro testing of the fibroblasts or leukocytes of the infant can be used to confirm the disorder *(22)*. Recent studies have described DNA testing for confirmation of some of the fatty and organic acid disorders *(15, 16, 23, 24)*. For many disorders, there are no tests for the alleles. Our working definition of a disorder is one that has positive screening results followed by test results that are consistent with the disorder, i.e., analysis for urine and/or blood markers, DNA, and in vitro testing.

SCAD deficiency was thought to be a rare disorder, but five presumed cases of SCAD deficiency have been identified in 164 000 Massachusetts newborn infants. Workup for the disorder included urine analysis for ethylmalonic acid, although the urinary markers for this disorder may not be increased *(8)*. Additional follow-up testing can include DNA testing for associated mutations. Gregersen et al. *(24)* reported that two polymorphic variants of SCAD are 625G→A and 511C→T, but these mutations are frequent in the general population. Both mutations contribute susceptibility to ethylmalonic aciduria but do not lead to clinically relevant SCAD deficiency *(24)*. It has been postulated that other genetic, cellular, and environmental factors can affect the SCAD enzyme, leading to the onset of clinical symptoms *(25)*.

The laboratory identified 10 MCAD-deficient infants. There does appear to be an association between the amount of the primary marker, C8, and the number of copies of the 985A→G mutation. The mean C8 value was 13.8 μmol/L in four of the infants homozygous for the 985A→G mutation, whereas MCAD-deficient infants with a single copy of the 985A→G had a mean C8 value

only one-fifth as much. As shown in Fig. 1, caution must be followed in interpreting the repeat specimens because the repeat specimen may not reveal the very high C8 concentration that was seen in the earlier specimens. The results demonstrate the need for immediate retesting of infants for urinary or blood markers to confirm or support the presence of the disorder. This is especially true when DNA testing is not readily available for confirming the initial screen. Bonafe et al. *(8)* reported that urinary acylglycine markers for MCAD were always pathologic and independent of clinical status, but the urinary organic acids can be mildly increased or unaltered. Thus, if retesting is delayed or if the individual is not catabolically stressed, some of the urinary markers may no longer be increased enough to help clarify the diagnosis. It is very important to confirm the MCAD disorder and inform the family because the mortality rate can be as high as 60% for the first clinical episode of hypoglycemia between the ages of 15 and 26 months *(26)*.

The common mutation for the MCAD mutation is 985A→G. We observed that this mutation accounted for 70% of the MCAD alleles (14 of 20) in the 10 cases. This value agrees with the previous a report *(27)* that 72% of the alleles in nine MCAD cases were 985A→G. However, others have reported *(15, 28–31)* that 88.9% of the alleles in 172 patients with MCAD were 985A→G. These 172 patients were diagnosed by enzymatic confirmation, with increased medium-chain acylcarnitines in the blood or urine, urinary acylglycines, or urinary excretion of phenylpropionylglycine after a phenylpropionic acid-loading test. Because the 172 cases were likely diagnosed on the basis of clinical presentation and would not have included milder cases detected by routine screening, the higher prevalence of 985A→G in the latter group is not surprising. On the other hand, it is also possible that the 172 cases were identified in a population with a higher prevalence for the 985A→G mutation.

The prevalence of 985A→G in our mixed population is estimated to be ~1 in 120. If we were detecting all carriers of this mutation, then we would have expected to identify >1500 infants with increased C8 values. Because only 52 infants were flagged for C8, we are not detecting the 985A→G carriers. This result is supported by the observation that only 8 of 17 infants with slightly increased C8 values had the 985A→G mutation. It remains to be seen whether these 17 infants may have a mild form of MCAD deficiency.

Our results reveal that the secondary markers for MCAD can be variable. The marker, C6, was always flagged in all 10 MCAD infants, but C10 varied from normal to very increased. One MCAD-deficient infant (985A→G/127G→A) had the highest C10 ratio (2.11) observed in the entire population screened. The C10 ratio was higher than the C8 concentration. This infant also failed to express the urinary markers usually observed with MCAD. A second MCAD infant (985A→G/504A→C) did not express a C10 ratio (0.21) high enough to be flagged. We observed two MCAD-deficient infants with C8 concentrations of 3.1 and 1.8 μmol/L but with C8/C10 ratios of only 1.8 and 0.9, respectively (data not shown). Our results reveal that the C8/C10 ratio was increased in the infants homozygous for the 985A→G mutation but not for the MCAD infants with one copy of 985A→G. Our experience indicates that C10 is not a reliable secondary marker for MCAD and that it may or may not be substantially expressed in infants with MCAD deficiency that are identified by our prospective screening program.

The six heterozygous cases identified by screening and DNA testing may not have the "classic" MCAD deficiency. These infants are part of an ongoing study, and additional workup of enzymatic and/or biochemical studies is needed to better understand the degree to which they may have this disorder. The heterogeneous nature of the MCAD deficiency is suggested by the data presented here and by a recent report *(32)* of mild MCAD cases identified by prospective screening.

To summarize our results over the 2-year period, the PPV for all amino acid disorders was 8% (22 of 260). If one uses the flagged amino acid and its flagged ratio, then the PPV increases to 14% (22 of 153). The PPV for all the acylcarnitine disorders identified was 9% (20 of 233). The PPV reported here is better than the reported value for some other disorders detected by other methods. For example, in a review of detection methods not including MS/MS, Kwon and Farrell *(33)* describe the PPV for biotinidase deficiency (6%), galactosemia (0.5%), congenital hypothyroidism (2%), and congenital adrenal hyperplasia (0.5%). Measurement of false-negative rates and of the expanded screening outcomes of detected cases *(34)* is beyond the scope of the current report, but we look forward to using the database we are developing to answer these important questions. To date, we have not been informed of any clinical cases in screening-negative infants but are reluctant to use this early experience as a basis for accurate estimates of "false-negative" rates.

We are indebted to Amii Rush, Timothy Foley, Denise Rojas, and Jacalyn Gerstel-Thompson for technical support during the course of this study. The assistance of Rosemarie Collard is also appreciated.

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