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ORIGINAL ARTICLE

Newborn screening for methylmalonic acidurias—Optimization by statistical parameter combination

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Summary With the introduction of tandem mass spectrometry, newborn screening for disorders of propionate metabolism became widely available. However, there is controversy whether population screening for these disorders should be performed. The most widely used primary metabolite C_3 itself has a poor specificity or lacks 100% sensitivity for milder forms and/or defects of cobalamin metabolism. Strategies to improve specificity have included the calculation of metabolite ratios (e.g. C_3/C_2) or second-tier strategies with analysis of methylmalonic acid or 2-methylcitric acid from the primary screening specimen. We report the results of a new statistical approach to identify parameter combinations that allow for 100% sensitivity as well as increased specificity. The promising results of this alternative approach will have to be substantiated on larger data sets.

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References to electronic databases: Propionic aciduria: OMIM #606054. Methylmalonyl-CoA mutase deficiency: OMIM #251000. Cobalamin disorders: CblA, OMIM #251100; CblB:, OMIM #251110; CblC/D, OMIM #277400/%277410; CblF, OMIM %277380.

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Abbreviations

Cbl	cobalamin
DBS	dried blood spot
MMA	methylmalonic aciduria
MS/MS	ESI tandem mass spectrometry
NBS	newborn screening
PA	propionic aciduria

Introduction

With the introduction of tandem mass spectrometry to newborn screening (NBS) programmes, screening for more than 20 monogenic disorders in one diagnostic run became possible (Schulze et al 2003; Wilcken et al 2003; Zytkovicz et al 2001). The organic acidurias that had previously been screened for only in certain very restricted areas (e.g. Quebec, Canada) by urine analysis (Sniderman et al 1999) could now realistically be included in general NBS programmes from dried blood. Among them the disorders of propionate metabolism show highest prevalence. They include propionic aciduria (PA, OMIM #606054) and methylmalonic acidurias (MMAs) including methylmalonyl-CoA mutase deficiency (OMIM #251000) and the cobalamin disorders (Cbl-A- (OMIM #251100), -B-(OMIM #251110), -C/D-(OMIM #277400/%277410) and -F (OMIM %277380) deficiencies). As a group these disorders are detected by elevation of propionylcarnitine (C_3) in dried blood spots (DBS). Further differential diagnosis is not possible by differences in C_3 concentration or by any additional parameter analysed in the routine NBS profile.

For several reasons, newborn screening for MMAs and PA is controversial and is handled differently in

various countries. Whereas in the USA the American College of Medical Genetics (ACMG) advocates that all propionic and methylmalonic acidurias be included as primary or secondary targets for newborn screening programmes (Watson et al 2006), doubts have been raised in the UK and Germany, and these targets were excluded from the national screening programmes. The major reason for these concerns is that most affected children will probably be diagnosed only after clinical manifestation has already occurred and that long-term outcome may not be improved by early detection and treatment (Leonard et al 2003). However, Walter (2003) has argued that even if no long-term benefit is proven, a definitive diagnosis in an affected newborn may be beneficial by avoiding unnecessary investigations and gives the opportunity for genetic counselling of families. Dionisi-Vici has stated that the benefit of early identification may be greatest for the subset of late-onset phenotypes, in which metabolic decompensation may be completely prevented (Dionisi-Vici et al 2006).

An important argument for excluding the disorders of propionate metabolism from neonatal screening programmes is the unfavourable balance between specificity and sensitivity of the primary parameter C_3. A high cut-off for C_3 will result in a significant rate of false negatives for disorders of cobalamin metabolism (Chace et al 2003; Wilcken et al 2003). On the other hand, with a lower cut-off a high rate of false-positive screening results may cause severe psychosocial problems within families (Hewlett and Waisbren 2006). In 2004, German recall rates of 11 screening laboratories for elevated C_3 alone ranged from 0.04 to 0.18 as compared to 0.08 to 0.69 for all other parameters of MS/MS screening taken together (DGNS 2004). As a consequence, disorders of propionate metabolism have been excluded from the German guideline for NBS starting from April 2005 (Bundesministerium für Gesundheit und Soziale Sicherung [1343 A] 2004).

To improve specificity, ratios of C_3 to various other acylcarnitine species (C_3/C_2, C_3/C_0 or C_3/C_{16}) have been used as secondary parameters (Chace et al 2003). To improve sensitivity, Wilcken replaced C_3 by the ratio C_3/C_2 as the primary parameter (Wilcken et al 2003).

The present study investigates the use of a different measure for discriminatory performance by a data mining procedure (Hu and Kibler 1996; Witten and Frank 2005), which included scoring and automatic preselection of new parameter combinations with the aim of substantially improving the sensitivity and specificity of NBS for disorders of propionate metabolism.

Table 1 Overview of acylcarnitines (and their symbols) used to develop discriminating analytes and analyte ratios

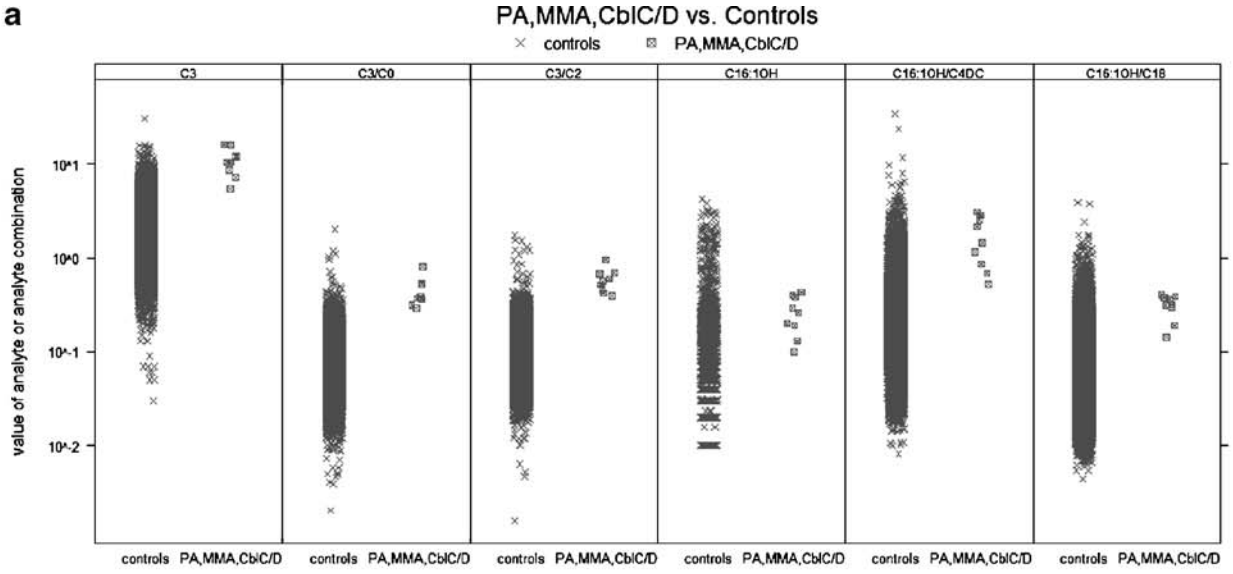
Main metabolite (same *m/z* in tandem-MS and relevant in the context of this paper)	Symbol
Free carnitine	C_0
Acetylcarnitine	C_2
Propionylcarnitine	C_3
Butyrylcarnitine	C_4
Isovalerylcarnitine	C_5
Hexanoylcarnitine	C_6
Octanoylcarnitine	C_8
Decanoylcarnitine	C_{10}
Dodecanoylcarnitine	C_{12}
Myristoylcarnitine	C_{14}
Hexadecanoylcarnitine	C_{16}
Octadecanoylcarnitine	C_{18}
Tiglylcarnitine	$C_{5:1}$
Octenoylcarnitine	$C_{8:1}$
Decenoylcarnitine	$C_{10:1}$
Myristoleylcarnitine	$C_{14:1}$
Hexadecenoylcarnitine	$C_{16:1}$
Octadecenoylcarnitine	$C_{18:1}$
Linoleoylcarnitine	$C_{18:2}$
Hydroxyisovalerylcarnitine	C_5OH
Hydroxytetradecadienoylcarnitine	$C_{14}OH$
Hydroxypalmitoylcarnitine	$C_{16}OH$
Hydroxystearoylcarnitine	$C_{18}OH$
Hydroxypalmitoleylcarnitine (cefotaxime metabolite)	$C_{16:1}OH$
Hydroxyoleylcarnitine	$C_{18:1}OH$
Methylmalonylcarnitine (succinylcarnitine)	C_4DC
Glutarylcarnitine	C_5DC
Methylglutarylcarnitine	C_6DC
3-Hydroxy-3-methylglutarylcarnitine	3HMG

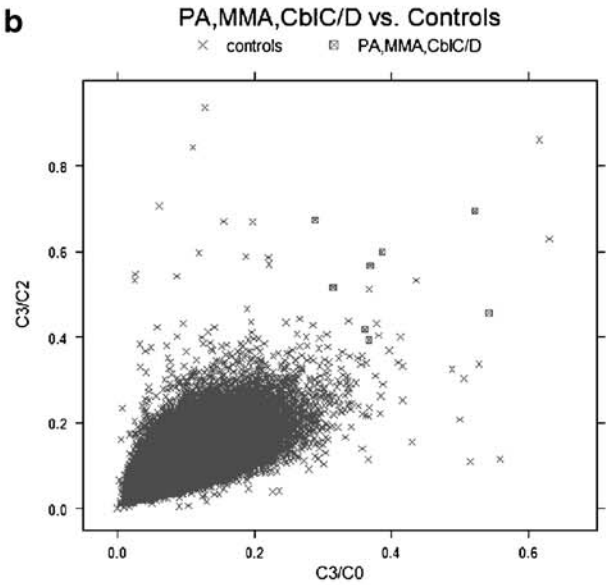
Methods

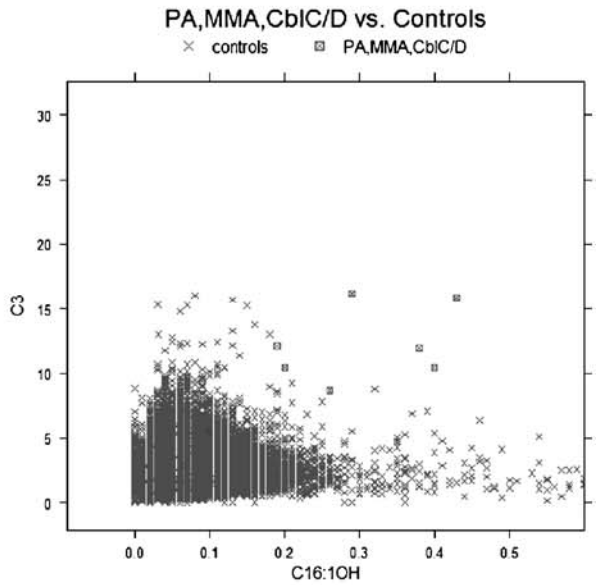
Data of all acylcarnitines measured in our laboratory's standard NBS programme were evaluated retrospectively (Table 1). Some of these data had to be blinded to the investigator in routine screening after the new German directive for newborn screening became operative in 2005.

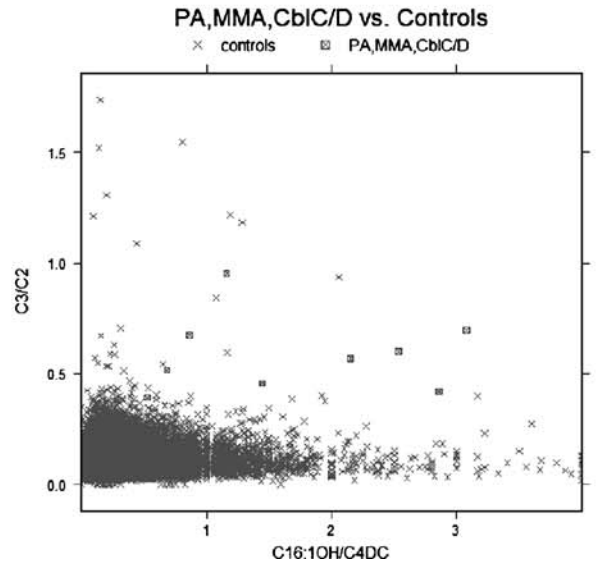
In a first step, all ratios of C_3 to other acylcarnitine concentrations were tested for their individual power

Fig. 1 Plots for the identification of informative parameters for the screening of disorders in propionate metabolism. (a) Strip plot of C_3 concentrations and other candidate analytes or analyte ratios which well discriminate true positives from controls as the base for further statistical analysis. Controls = false positives and normal control samples. (b) Scatter plot: comparison of the discriminative power: $C_{16:1}OH$ discriminates different controls versus C_3/C_2 and therefore can contribute effectively to reduction of the false-positive rates


a
PA,MMA,CblC/D vs. Controls
controls
PA,MMA,CblC/D
C3
C3/C0
C3/C2
C16:1OH
C16:1OH/C4DC
C16:1OH/C18
value of analyte or analyte combination
10^1
10^0
10^-1
10^-2
controls
PA,MMA,CblC/D


b
PA,MMA,CblC/D vs. Controls
controls
PA,MMA,CblC/D
C3/C2
C3/C0
0.0
0.2
0.4
0.6
0.8


PA,MMA,CblC/D vs. Controls
controls
PA,MMA,CblC/D
C3
C16:1OH
0
5
10
15
20
25
30
0.0
0.1
0.2
0.3
0.4
0.5


PA,MMA,CblC/D vs. Controls
controls
PA,MMA,CblC/D
C3/C2
C16:1OH/C4DC
0.0
0.5
1.0
1.5
1
2
3

to discriminate 9 truly affected newborns from healthy controls. The most promising candidates were then combined into diagnostic criteria that allowed for 100% sensitivity and improved specificity.

Population studied

The data for the analysis was extracted from the neonatal screening data obtained from May 2004 to October 2007 at the Heidelberg newborn screening centre. Recommended time for blood sampling was between 48 and 72 hours of life. The screening centre analysed about 350 000 samples from South German neonates and about 32 000 samples from Qatari neonates. C_3 screening for German neonates ended in 2005 but was continued for the Qatari samples.

In this period, 7 true positive patients were detected in regular NBS: 3 PA (one of them because of a positive family history), 4 with MMAs (2 with methylmalonyl-CoA mutase deficiency, and 2 with Cbl C/D deficiencies). After discontinuation of C_3 screening in Germany, one patient with MMA (Cbl-C deficiency) was diagnosed because of suggestive clinical symptoms at the age of 3 months and one with PA because in error her complete tandem-MS profile was evaluated and the reporting physician decided not to withhold this information. The data from these two patients' initial newborn screening tests are included in this study. Gestational ages at birth in weeks were 40 (2, MMA), 39 (1, Cbl C/D), 38 (2, PA and Cbl C/D), 37 (1, PA), 36 (1, PA), 35 (1, PA) and 34 (1, Cbl C/D).

To our knowledge, no patient with PA, MMA or Cbl deficiency was missed in the screened population.

Analytical methods

Acylcarnitine and amino acid profiling

Acylcarnitines and amino acids were analysed as butyl esters using a Micromass triple-quadrupole tandem mass spectrometer (Micromass /Waters, Eschborn, Germany) with an ion spray device as previously described with minor modifications (Schulze et al 2003).

Definition of cut-offs: The cut-off for 'abnormal C_3' was set at the minimum C_3 concentration of 9 true-positive cases with enzymatically proven diagnoses, i.e. 4.9 μmol/L, corresponding to the 98.5th centile of all measurements. C_3 as the only marker metabolite would therefore result in a false-positive rate of 1.5%. The 'control group' comprises data of 121 newborns showing elevated concentrations in the primary marker C_3 and the data of randomly chosen presumably healthy children (n=1472) with C_3 <4.9 μmol/L.

For the calculation with C_3/C_2 as the primary marker, the cut-off for C_3/C_2 was set to 0.27, corresponding to the 99.8th centile of all samples.

Statistical methods

All analytes and the computed analyte ratios were scored by computing the chi-squared value as a measure for discriminatory performance. The scores were computed with the data mining framework weka (Witten and Frank 2005). The algorithm first discretizes the inspected analyte or analyte ratio by a method described in Fayyad and Irani (1992). This method aims at minimizing the number of intervals, while simultaneously minimizing the information loss of the discretization with respect to the class variable (in this case the diagnosis 'PA, MMA, Cbl defect' versus 'controls').

In the second step the chi- squared score is computed on the discrete variable. This is done analogously to the chi-squared test for independence, where the covariation of two variables is analysed. High chi-squared values indicate good discriminatory performance.

We then used visual inspection of strip plots and boxplots of the metabolites and metabolite ratios to verify the usability of the chi-squared measure for identification of discriminatory analyte ratios for our study data. We manually selected those that best separated true positives from false positives and further investigated them for their ability to increase specificity in diagnostic criteria.

Combinations of informative parameters identified were tested for their influence on specificity with a reference data set (n=212 508) comprising all samples analysed between May 2004 to June 2006 and random samples from the period July 2006 to October 2007. This reference data set includes the 1601 samples used for the identification of new markers (nine results from patients with confirmed diagnoses and 1593 samples of controls).

Results

Figure 1a and b shows examples of strip plots and scatterplots. Table 2 shows the results of the chi-squared test for the most promising parameters and some other parameters performing less well. The chi-squared scores allowed us to reject many analytes and analyte ratios with low chi-squared values, which in the visual inspection also did not perform well. The parameters and ratios C_3, C_3/C_0, C_3/C_2 and C_3/C_{16},

Table 2 The 10 highest results of chi-squared measures for analytes or ratios discriminating the group of true positives from controls

Parameter or ratio	Chi squared
C_3/C_2	1122
$C_{16:1}OH/C_{18}$	920
$C_{16:1}OH/C_4DC$	746
$C_{16:1}OH/C_{16}$	659
$C_{16:1}OH/C_{18:1}$	646
C_3/C_0	601
$C_3/C_{18:1}$	542
$C_{16:1}OH$	511
C_3	373
$C_{16:1}OH/C_2$	32

already used in practice, were confirmed with high chi-squared values but, to our surprise, the calculation for $C_{16:1}OH$ and ratios of $C_{16:1}OH$ and other acylcarnitines also suggested a high discriminatory power (Fig. 1). $C_{16:1}OH$ discriminates different controls from C_3/C_2 and therefore can contribute effectively to reduction of the false-positive rates.

Owing to the low numbers of diagnoses of PA and MMAs, the best chi-squared scores did not always reflect the impression from the visual inspection. Nevertheless, the method proved effective for automatically preselecting promising analytes or analyte ratios.

Combinations of the parameters with the highest associative power in the chi-squared analysis were tested on the extended data set of 212 508 samples for their ability to improve specificity. The combination '$C_3/C_2 > 0.27$ (99.8th centile) *AND* $C_3/C_0 > 0.24$ (99.7th centile) *AND* $C_{16:1}OH/C_4DC > 0.45$ (91.1st centile)' provided 100% sensitivity while reducing the false-positive rate to 0.012%. (Table 3).

Table 3 Reduction of false positive rate by application of different metabolite combinations with C_3 and C_3/C_2 as the primary parameter for screening

Criteria	TP	Specificity
C_3>4.9 μmol/L[a]	**9**	**98.465**
C_3>4.9 and C_3/C_0>0.24 and $C_{16:1}OH/C_2$>0.005	9	99.923
C_3>4.9 and C_3/C_0>0.24[b]	9	99.888
C_3>4.9 and C_3/C_2>0.27[4]	9	99.903
C_3>4.9 and C_3/C_2>0.27 and C_3/C_0>0.24	9	99.954
C_3>4.9 and $C_{16:1}OH/C_2$>0.005[c]	9	99.961
C_3>4.9 and C_3/C_2>0.27 and $C_{16:1}OH/C_2$>0.005	9	99.983
C_3>4.9 and $C_{16:1}OH/C_4DC$>0.45	9	99.779
C_3/C_0>0.24 and C_3/C_2>0.27 and $C_{16:1}OH/C_2$>0.005	**9**	**99.985**
C_3/C_2>0.27[d]	**9**	**99.764**
C_3/C_2>0.27 and C_3/C_0>0.24	9	99.923
C_3/C_2>0.27 and $C_{16:1}OH/C_4DC$>0.45[e]	9	99.956
C_3/C_2>0.27 and C_3>4.9 and $C_{16:1}OH/C_{18}$>0.1	9	99.979
C_3/C_2>0.27 and C_3/C_0>0.24 and $C_{16:1}OH/C_4DC$>0.45	**9**	**99.988**

TP, true positive.
[a] C_3>4.9 μmol/L=98.5th centile.
[b] C_3/C_0>0.24=99.74th centile.
[c] $C_{16:1}OH/C_2$>0.005=94th centile.
[4] C_3/C_2>0.27=99.76th centile.
[5] $C_{16:1}OH/C_4DC$>0.45=91.10st quantile.

Discussion

C_3 is one of the most problematic parameters to be analysed in MS/MS newborn screening. It is used as the primary metabolite for detection of disorders of propionate metabolism, but there is poor specificity when 100% sensitivity for all disorders, including the cobalamin deficiencies, is aimed at. As a consequence, second-tier strategies analysing methylmalonic acid, 3-hydroxypropionic acid and 2-methylcitric acid from the initial DBS have been developed (La Marca et al 2007; Matern et al 2007), but not all NBS laboratories may be able to adopt this approach. In the paper by Matern there is no calculation of the real costs that are involved in the second-tier testing from the initial DBS in comparison, for example, with a second DBS. To the best of our knowledge there is also no available published detailed information on the method used. Thus necessary resources in the laboratory cannot be estimated.

In the method published by La Marca and colleagues describing quantification of methylmalonic, succinic and 3-hydroxypropionic acid at least some additional analytical time and effort (including column separation) has to be expended. That would probably exceed the capacity in smaller NBS centres where only one MS/MS device is available.

Various empirically identified combinations of parameters have been evaluated for their potential to reduce the rate of false-positive results. We applied a statistical approach to identify predictive parameter combinations 'hidden' within the set of analytes assessed in the routine NBS run. In our programme the sensitivity of $C_3 > 4.9$ μmol/L (98.5th centile) showed 100% sensitivity, and to the best of our knowledge there have not been any false negatives during the period when all newborns were screened for this parameter in our laboratory. We therefore first used C_3 in combination with other parameters to improve specificity. The combination '$C_3/C_0 > 0.24$ *AND* $C_3/C_2 > 0.27$ *AND* $C_{16:1}OH/C_2 > 0.005$' improved specificity to 99.985% (vs. 98.465% with C_3 alone).

However, in other programmes patients with CblC deficiency were not detected even with lower cut-offs for C_3 (Wilcken et al 2003). In the Australian newborn screening programme the primary metabolite used is

C_3/C_2 (Wilcken et al 2003). Therefore we also tested C_3/C_2 as the primary parameter on our data. This ratio alone already proved to have a much higher specificity than C_3,with 100% sensitivity even for our CblC patients. The combination of '$C_3/C_2 > 0.27$ *AND* $C_3/C_0 > 0.24$ *AND* $C_{16:1}OH/C_4DC > 0.45$' further increased specificity to 99.99%.

This new combination was found by arithmetic derivations only and without any specific biochemical hypothesis behind it and it has not yet been tested with a larger set of true-positive samples. The fact that a higher ratio of $C_{16:1}OH/C_4DC$ contributes to increased specificity of our parameter combination warrants a biochemical explanation.

A decrease of C_4DC caused by low succinylcarnitine behind the metabolic block could be responsible. Methylmalonylcarnitine, which also contributes to C_4DC in MS/MS acylcarnitine profiling, is never elevated in NBS samples of patients with MMAs or PA.

No effect of propionic acid or one of its metabolites on long-chain fatty acid oxidation causing elevation of $C_{16:1}OH$ has been shown so far. In 5 of our patients the $C_{16:1}OH$ concentration was slightly elevated (data not shown). $C_{16:1}OH$ elevation may be caused by a metabolite of cefotaxime, an antibiotic commonly used in sick neonates, but only one of our patients was treated with that drug and at the time of blood sampling her $C_{16:1}OH$ concentration was within the normal range. So we still do not have an explanation for the discriminating power of the $C_{16:1}OH/C_4DC$ ratio, but it is certainly not an artefact due to cefotaxime treatment.

According to the scarce literature available, levels of $C_{16:1}OH$ seem not to be influenced significantly by gestational age (Meyburg et al 2002) or those of $C_{16:1}OH$ and C_4DC by the age at sampling (Cavedon et al 2005) and our patients' data confirm this.

Other ratios, e.g. C_3/C_{16} as proposed by Chace (Chace et al 2001), were not able to further improve specificity in our data set (data not shown).

Conclusion

Statistical methods may help in improving the performance of newborn screening for disorders of propionate metabolism by reducing the number of false-positive samples while maintaining a high sensitivity.

The optimized combination of parameters found within our set of nine true positives certainly will have to be validated prospectively in larger series before application in daily practice can be recommended.

For these studies the quantiles of the respective parameters rather than absolute concentrations have to be used as cut-off levels because absolute quantitative data from MS/MS devices still differ substantially between laboratories. The sensitivity and specificity of our approach could be compared to those of the second tier strategy with analysis of methylmalonic acid, 3-hydroxypropionic acid and 2-methylcitric acid from the initial DBS (La Marca et al 2007; Matern et al 2007). In Germany this could be done only within a research project owing to very restrictive screening guidelines. Applications for EC approval and funding are under consideration.

Certainly a revision of the German panel of screening disorders should re-include disorders of propionic acid metabolism screened for by one of the available methods with improved specificity.

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