Bloodspot Assay Using HPLC–Tandem Mass Spectrometry for Detection of Barth Syndrome

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BACKGROUND: Barth syndrome (BTHS) is a serious X-linked, metabolic, multisystem disorder characterized by cardiomyopathy, neutropenia, myopathy, and growth delay. Because early diagnosis and appropriate treatment are of key importance for the survival of affected boys, we developed a biochemical BTHS screening method based on analysis of the monolysocardiolipin:cardiolipin ratio in bloodspots.

METHODS: We performed chloroform/methanol extraction on quarter-inch punches of dried bloodspots on Guthrie cards from BTHS patients and controls. Extracts were dried (60 °C, N_2) and reconstituted in $CHCl_3$/methanol/H_2O [50:45:5 vol/vol/vol, 0.1% NH_3 (25%)]. HPLC–tandem mass spectrometry analysis was performed with a normal-phase HPLC column and multiple reaction monitoring transitions for monolysocardiolipin (MLCL) and cardiolipin (CL) with a total run time of 10 min. The ratio of MLCL and CL was used as screening parameter.

RESULTS: All BTHS patients (n = 31) had monolysocardiolipin:cardiolipin ratios >0.40 and all controls (n = 215) had monolysocardiolipin:cardiolipin ratios <0.23. Using a cutoff point of 0.30, a blind test of 206 samples (199 controls, 7 BTHS) had sensitivity and specificity of 100%. Bloodspots could be stored at 4 °C or room temperature for >1 year without affecting the test outcome. Three neonatal Guthrie cards of BTHS patients taken 3.6 to 5.8 years previously were correctly identified as positive for BTHS.

CONCLUSIONS: HPLC–tandem mass spectrometry analysis of dried bloodspots is an unambiguous screening test for BTHS with potential for rapid screening of neonates suspected of having BTHS, making remote and retrospective diagnosis accessible for a disease that is almost certainly underdiagnosed.

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Barth syndrome (BTHS)[4] is a life-threatening X-linked, metabolic, multisystem disorder (MIM 302060). It is characterized by cardiomyopathy, neutropenia, skeletal myopathy, and growth retardation (plus frequent striking catch-up growth during adolescence) (1–7). Other biochemical characteristics include 3-methylglutaconic, 3-methylglutaric, and 2-ethylhydracrylic aciduria (1, 2, 6, 8), low cholesterol (6, 7), and abnormal mitochondria (7, 9, 10). Mutations in the tafazzin gene (*TAZ*[5] previously known as G4.5) (11) cause BTHS. The gene product of the *TAZ* gene is a transacylase involved in the remodeling of cardiolipin (CL) (12–14), and its deficiency results in the most consistent biochemical abnormality: decreased CL levels and increased monolysocardiolipin (MLCL) content (15–17). CL is a stabilizing factor for the respiratory chain supercomplexes (18) and performs a key role in apoptosis (19, 20). The role of CL metabolism for the optimal function of cellular processes, and in particular, many proteins within mitochondria, has been reviewed recently (21–23). The unambiguous biochemical diagnosis of BTHS is made by the determination of the relative amounts and distribution of (monolyso-)cardiolipin species (17, 24–26) and confirmed by sequencing of the *TAZ* gene.

Although most children with BTHS manifest with typical characteristics, in practice the phenotypic spectrum is broad and heterogeneous, even within members of the same family (27). Because of this, and because patients visit a number of pediatric specialists,

including cardiologists, hematologists, neurologists, gastroenterologists, and geneticists, the diagnosis can easily be missed. Infants and children who die acutely from cardiomyopathy are often assumed to have viral myocarditis, especially as the associated neutropenia may be attributed to viral bone marrow suppression. Thorough diagnostic evaluation is not always performed, so misdiagnosis is likely and thus underestimation of the prevalence of BTHS. The retrospective analyses of stored bloodspot cards of patients who died of cardiomyopathy and/or infection with unresolved underlying causes may help to identify BTHS families. Such a study may also lead to awareness of the real prevalence of BTHS *(27)*. For these reasons, we consider the development of a rapid screening method to be a necessity.

The existing analytical methods applied to lymphocytes, lymphoblasts, fibroblasts, thrombocytes, and muscle biopsies are based on sampling protocols that, for reasons of ethics and efficiency, are not suited as universal application for earliest detection, and hence ultimately for newborn screening. Because dried bloodspots are an easily accessible way of sampling in the practice of (newborn) screening, we investigated their usefulness for BTHS detection. Based on our experience with the analysis of MLCL/CL profiles in fibroblasts, lymphocytes, and other tissues, we found that bloodspots contain detectable amounts of MLCL and CL, which are sufficient to distinguish a BTHS patient from a healthy individual. In this report, we describe the validation of this method and its application as a screening tool.

Materials and Methods

PATIENT AND CONTROL SAMPLES
Control samples were collected from 215 men without BTHS (random extract of male hospital population) according to the institutional guidelines for sampling, including anonymization. Samples of proven BTHS patients (n = 31, age range 0–21 years) were collected according to institutional guidelines for sampling. All had characteristic symptoms and an identified disease-causative mutation of the *TAZ* gene. In cases for which samples were not anonymized, informed consent was obtained from the patient or guardian.

MATERIALS
Solvents: methanol, chloroform, and ammonia of analytical grade were purchased from Biosolve, LAB-SCAN, and Merck, respectively. H_2O was MilliQ® purified water. Tetramyristoylcardiolipin $[CL(14:0)_4]$ and dimyristoylphosphatidylcholine $[PC(14:0)_2]$ were purchased from Avanti Polar Lipids, and filter paper Whatman 903 was purchased from Drukkerij PAL.

SAMPLE PREPARATION
A punch (quarter-inch diameter) of a dried bloodspot on filter paper (Guthrie card) was transferred to a 4-mL tube, to which was added 1 mL methanol/chloroform (1:1, vol/vol). After the addition of 50 μL 8 μmol/L $CL(14:0)_4$ (0.4 nmol) and 100 μL 14.8 μmol/L $PC(14:0)_2$ (1.48 nmol) as internal standards, the sample was vortex-mixed and incubated for 15 min at room temperature in a sonicator bath (Branson 3510). The extraction fluid, with the filter paper removed, was transferred to a 4-mL glass tube and evaporated to dryness (60 °C, N_2). The residue was reconstituted in 150 μL mixture of $CHCl_3$/methanol/H_2O, 50:45:5 vol/vol/vol, 0.01% (vol/vol) 25% NH_3, transferred to a sample vial, and capped. We used 10 μL for HPLC–tandem mass spectrometry (HPLC-MS/MS) analysis.

HPLC-MS/MS
Chromatographic separation was achieved on a Surveyor modular HPLC System (Thermo Finnigan Corp.) consisting of cooled autosampler (T = 12 °C), low-flow quaternary MS pump, and analytical HPLC column (T = 25 °C): silica 2.1 by 250 mm, 5 μm (particle size) (Lichrospher; Merck). Samples were eluted at a flow rate of 300 μL/min and a linear gradient between solution A $[CH_3OH/H_2O$, 85:15 vol/vol, 0.1% NH_3 (25%)] and solution B $[CHCl_3/CH_3OH$, 97:3 vol/vol, 0.01% NH_3 (25%)]. For bloodspot analysis, we programmed the gradient as follows: from T = 0 min, 100% B toward T = 0.01 min, 100% A; T = 0.01–5 min, 100% A; T = 5–10 min, 100% B. We used a splitter between the HPLC column and the mass spectrometer and introduced 75 μL/min into the mass spectrometer.

We performed mass spectrometry analyses on a TSQ Quantum AM (Thermo Finnigan Corp.) operated alternately in the negative and positive ion electrospray ionization (ESI) mode within runs. Standard mass resolution was 0.4 (Q3) in MS experiments and 0.3 (Q1) combined with 0.7 (Q3) in MS/MS mode. The source collision–induced dissociation was set at 10 V, spray voltage was 3600 V, and the capillary temperature was 300 °C. In the MS/MS experiments, argon was used as collision gas at a pressure of 0.5 mtorr; collision energy was 50 eV for the optimized transitions: m/z 582.9 → m/z 281.3; m/z 582.9 → m/z 255.2; m/z 619.9 → m/z 227.2; m/z 724.0 → m/z 279.2. In the positive mode, with collision energy set at 40 eV, we used a parent scan of m/z 184.1 (range m/z 650 to 850) to selectively detect PCs in the corresponding retention time window to check the efficiency of the bloodspot extraction. The analytical run time, from injection to injection, was 10 min.

MLCL/CL RATIO

As a parameter to distinguish between BTHS and controls, we used the ratio between (*a*) the chromatographic peak area for the multiple reaction monitoring (MRM) of m/z 582.9 → m/z 281.3 plus m/z 582.9 → m/z 255.2 as a representation of the abundance of MLCL in the bloodspot and (*b*) the chromatographic peak area for the MRM of m/z 724.0 → m/z 279.2 as a representation of the abundance of CL in the bloodspot. This ratio is referred to as the MLCL/CL ratio.

VALIDATION

Linearity. We tested linearity by measuring 2 linear series (A and B) of combinations of control and BTHS bloodspots (of the same control and of the same patient). By combining different amounts of control and BTHS bloodspots, we increased the MLCL content and concomitantly decreased the CL content. Each data point represents the average of 4 measurements of the same sample.

Precision. We established instrument variation by analyzing 10 consecutive injections of the same sample. As a measure of the overall variation in absolute response of the LC-MS/MS, we monitored the peak area of the CL internal standard $CL(14:0)_4$. Intraassay CV was determined by multiple analyses of bloodspots of each of 3 different BTHS patients and 3 controls within the same series. Interassay variation was tested by repeated analyses of bloodspots of 3 different BTHS patients and 3 controls on 5 different days over a period of 2 months. Recovery was extrapolated from data of consecutive extraction procedures of bloodspot punches. The limit of detection was expressed as the minimum amount of material of bloodspot necessary for an unambiguous screening result. We determined the cutoff values for attribution of BTHS vs control based on the results of controls (n = 215) and established BTHS patients (n = 31). We tested the influence of storage conditions by repeated analysis of bloodspots from the same controls and the same BTHS patient over a period of 1 year stored at room temperature or at 4 °C, non-BTHS bloodspots stored at 4 °C for >2 years (n = 10), and old Guthrie cards (3–6 years old) stored at room temperature.

As part of the validation, a blind test was performed. Bloodspots from 199 healthy male controls and 7 proven BTHS patients (3 with MLCL/CL ratios close to high normal) were anonymized and randomly assorted by an independent operator so as to simulate a test population. During the test, the tester did not know the number of BTHS patients included or in what order they would occur in the series. After designation of control or BTHS to the series of samples, the identities of the samples were disclosed and sensitivity and specificity were calculated.

STATISTICAL METHODS

We analyzed data using statistical tools embedded in Microsoft Excel 2002 and SPSS 12.0.2. Subgroup distributions were analyzed by Kolmogorov-Smirnov (with Lilliefors significance correction) and Shapiro-Wilk tests for normality. For the comparison of the non-normally distributed groups, we used nonparametric 2-independent-samples tests; a P value <0.05 was considered significant.
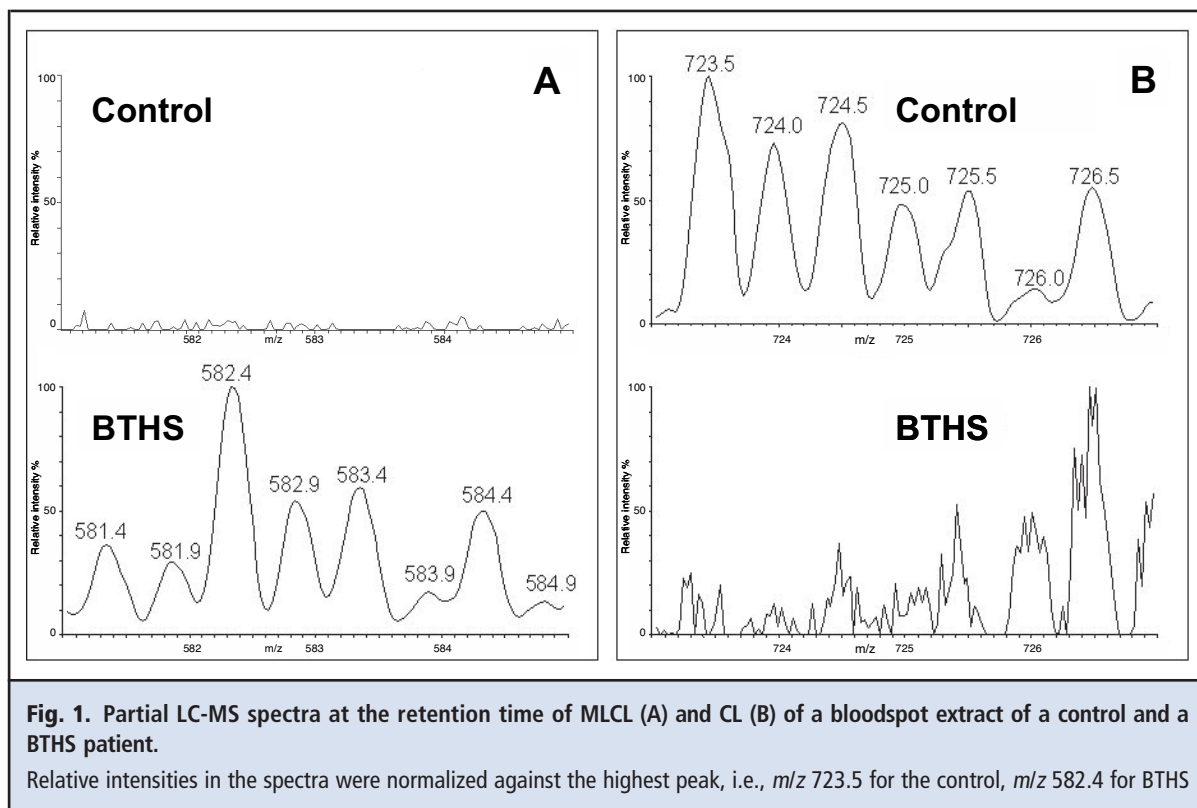
Results

HPLC-MS/MS

Based on our previously developed methods to analyze phospholipids and specifically CL and MLCL in several tissues and types of cells, the HPLC elution profile was adjusted for CL and MLCL determination in bloodspot extracts. The gradient separated CL and MLCL from other phospholipids, thus minimizing interference and suppression of signal of the analytes while maximizing signal-to-noise ratio (S/N) for both MLCL and CL, under similar conditions in a reduced runtime. LC-MS analysis of dried bloodspots showed tetralinoleonyl-cardiolipin $[CL(18:2)_4]$ as the most prominent CL species with highest S/N for the first isotope in the isotopic cluster of these $[M\text{-}2H]^{2-}$ ions, i.e., m/z 724.0. $MLCL(18:1)_2(16:0)$ was the most prominent MLCL species and the first isotope in the isotopic cluster of these $[M\text{-}2H]^{2-}$ ions, i.e., m/z 582.9 had the highest S/N (Fig. 1). The transition of m/z 724.0 $(CL(18:2)_4)$ → m/z 279.2 (C18:2) and the peak area of the summed transitions of m/z 582.9 $[MLCL(18:1)_2(16:0)]$ → m/z 281.3 (C18:1) and m/z 582.9 → m/z 255.2 (C16:0) resulted in the best S/N for these species in the LC-MS/MS analysis of dried bloodspots. The internal standard $CL(14:0)_4$ had the best S/N in dried bloodspot extracts for the transition m/z 619.9 → m/z 227.2 (C14:0). Reported data are based on the measurement of peak area of the chromatographic peaks of the optimized MRM transitions. In control bloodspots, the level of CL is relatively high and MLCL is low and often hardly detectable. BTHS bloodspots, however, showed a low abundance for CL and a clearly increased level for MLCL (Fig. 2). The presence of PC was measured by a parent scan of m/z 184.1. Only if the characteristic spectrum in the mass range m/z 650 to m/z 850 had a sufficient response, data were accepted for determination of the MLCL/CL ratio and used in the study.
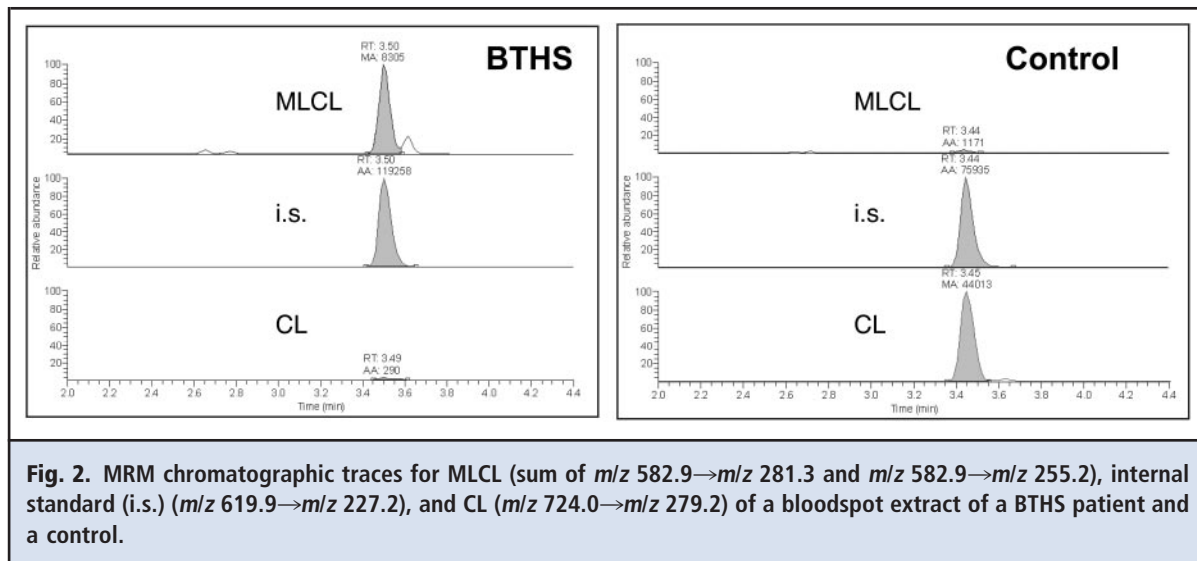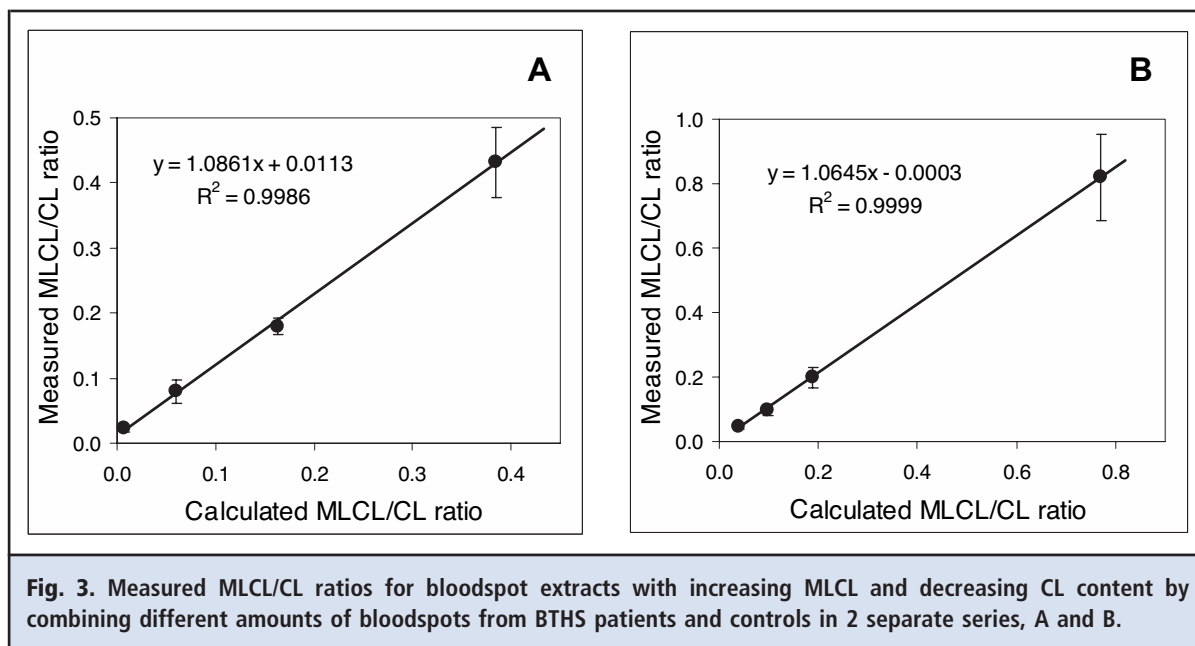
PRECISION

The instrument variation for CL and MLCL in a control bloodspot extract was 13% and 44%, respectively.

Fig. 1. Partial LC-MS spectra at the retention time of MLCL (A) and CL (B) of a bloodspot extract of a control and a BTHS patient.

Relative intensities in the spectra were normalized against the highest peak, i.e., *m/z* 723.5 for the control, *m/z* 582.4 for BTHS

In a BTHS bloodspot extract, this variation was 47% and 41%, respectively. The CV in the measured intensity of the peak area of the internal standard in 200 consecutive analyses was 10%. The intraassay variation in the MLCL/CL ratio for control and BTHS bloodspots was 51% [ratio = 0.11 (0.05)] and 40%

[ratio = 11.3 (4.6)], respectively, as calculated for repeated complete analyses on 1 day in 1 series of HPLC-MS/MS measurements. The interassay variation in the MLCL/CL ratio for control (n = 3) and BTHS (n = 3) bloodspots was 47% and 46%, respectively, as calculated for the complete analysis on 5 different days over a period

Fig. 2. MRM chromatographic traces for MLCL (sum of *m/z* 582.9→*m/z* 281.3 and *m/z* 582.9→*m/z* 255.2), internal standard (i.s.) (*m/z* 619.9→*m/z* 227.2), and CL (*m/z* 724.0→*m/z* 279.2) of a bloodspot extract of a BTHS patient and a control.

Fig. 3. Measured MLCL/CL ratios for bloodspot extracts with increasing MLCL and decreasing CL content by combining different amounts of bloodspots from BTHS patients and controls in 2 separate series, A and B.

of 2 months. The high variance for these ratios can be attributed to the low levels of MLCL or CL in control and BTHS patients respectively, as explained in "Discussion."

LINEARITY

For the MLCL/CL ratio as a function of a series of mixes of BTHS and control dried bloodspot punches, the linearity of series A was characterized by $R^2 = 0.9986$, that of series B by $R^2 = 0.9999$ (Fig. 3).

RECOVERY

A repeated extraction step increased the extraction yield by approximately 15% for CL and 25% for MLCL, which indicated that recovery from a bloodspot with the procedure is <85% for CL and <75% for MLCL. Based on calculated estimates, the amount of $CL(18:2)_4$ in a bloodspot ranged between 0.2 and 2 nmol/punch for controls and between 0.05 and 0.8 nmol/punch for BTHS patients. As a limit of detection, we found that dilution of up to 4 times of the final volume for HPLC-MS/MS analysis did not affect the outcome of the screening result significantly for BTHS patients or controls. The results suggest that 25% of a quarter-inch punch is sufficient for screening.

STORAGE

Repeated analysis of bloodspots of a control and a BTHS patient stored either at room temperature or at 4 °C did not show significantly different results over a period of 1 year. For quality control, we monitored the MLCL/CL ratio of control bloodspots (from a large series of bloodspots sampled on one occasion), which was typically 0.06 (0.04), n = 12. Guthrie cards of controls (n = 10) stored at 4 °C and room temperature without special precautions for approximately 2 years were screened as non-BTHS, and no false positives were observed. The analysis of bloodspots of 3 original neonatal Guthrie cards (3.6, 3.8, and 5.8 years old, stored at room temperature) of established BTHS patients (anonymized) were unambiguously identified as positive for BTHS. Screening results did not appear to be influenced by the age of the patient. Bloodspots of children (from 6 weeks of age), adolescents, and adults were analyzed with screening results in accordance with the diagnosis established by full MLCL/CL profile analysis in lymphocytes and/or molecular analysis of the *TAZ* gene.

SPECIFICITY AND SENSITIVITY

The measurement of bloodspots of 31 different BTHS patients and 215 controls showed that all BTHS patients had a MLCL/CL ratio >0.40 and all controls had a MLCL/CL ratio <0.23, without any overlap between the 2 groups. Statistically, the 2 groups were significantly different, $P < 0.0001$ (Fig. 4). Blind testing of a simulated test population derived from the existing BTHS patients and control samples (containing bloodspots from 7 BTHS patients and 199 controls) gave sensitivity and specificity of BTHS detection of 100% and 100%.
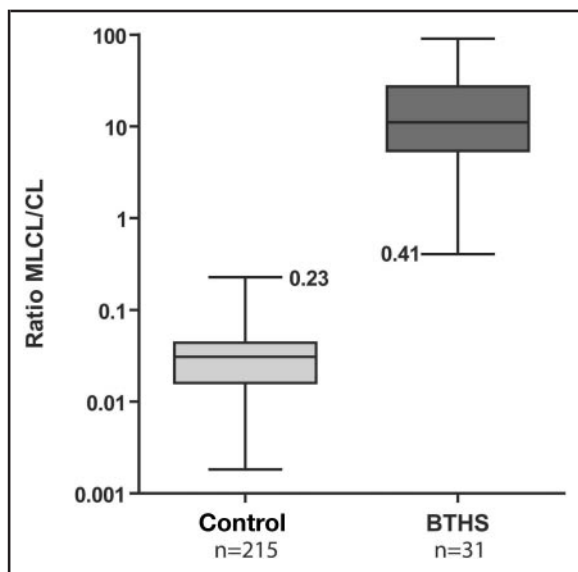
Fig. 4. Box-and-whisker plot of the MLCL/CL ratio of controls (n = 215) and BTHS patients (n = 31).
The cutoff value for BTHS vs control is set at MLCL/CL = 0.30

Discussion

Because the majority of material in dried bloodspots originates from erythrocytes, which do not contain mitochondria, the expected abundances for the biochemical markers of BTHS, CL and MLCL, were very low. Taking into account the complexity of bloodspots as a matrix, we chose HPLC-MS/MS with electrospray ionization as the analytical technique of choice for its combination of high selectivity and high sensitivity, properties that allowed for simple and rapid sample preparation combined with a relatively high throughput. After the first successful detection of MLCL and CL in bloodspots, it became clear that quantification of both analytes was difficult given their low levels and the inability to reliably introduce an internal standard in the dried bloodspot. Because BTHS patients have high levels of MLCL in combination with low levels of CL, and this is reversed in controls, we investigated whether combination of the inversely correlated parameters in a MLCL/CL ratio could differentiate between the two populations. Effectively, the MLCL/CL ratio became independent of the absolute amounts of MLCL and CL in the dried bloodspots. Data analysis substantiated this rationale, since the discriminating power of the calculated CL or MLCL concentration alone is insufficient to unambiguously differentiate BTHS from control. The concentration range of CL in BTHS overlaps with the encountered concentration range of CL in controls. The MLCL/CL ratio could, however, unambiguously distinguish BTHS from controls in all encountered cases.

It should be noted that the MLCL/CL ratio is based on the ratio of the chromatographic peak areas of different MRMs of different components with different response factors. Therefore the actual value of this ratio is not the molar ratio of the MLCL and CL species in the bloodspot sample; it is a practical parameter that expresses the combined effect of two separate relevant indicators. Internal standards were added to monitor retention time and system response. The detection of the spectrum of PC was used as a qualitative check for the extraction of the bloodspot. As PC originates from the bloodspot, it is a better parameter than the added internal standard to check the extraction efficiency.

The high inter- and intraassay variation found for the MLCL/CL ratio (both about 50%) can be explained by the fact that in a bloodspot measurement either MLCL (in controls) or CL levels (in BTHS) are close to the detection limit. As a result, the contribution of a random amount of (electronic) noise contributes to the integrated area of the low-abundance component and introduces a relatively large variation in the calculated MLCL/CL ratio. The intra- and interassay variation for the MLCL/CL ratio is lower when both MLCL and CL are relatively high, as encountered in a few cases within the group of BTHS patients where there was a substantial increase in MLCL but a mild decrease in CL (interassay CV values 15%). Despite the (explainable) high variation of the MLCL/CL ratio, the screening result has proven to be very robust, as demonstrated by the 100% sensitivity and specificity score in the blind test of 205 samples including 7 BTHS patients. Therefore, we concluded that the precision of the ratio does describe fluctuations but is not relevant to evaluate the screening potential of our method—it is too dependent on individual bloodspot features.

We also investigated whether the BTHS bloodspot assay has the potential to be used as a neonatal screening tool. Because all the validation tests were performed with established BTHS patients, none of whom were neonates, the ultimate test was the measurement of bloodspots of original Guthrie cards from a neonatal screening program. Anonymized neonatal Guthrie cards were obtained from 3 known BTHS patients; in all 3 cases, BTHS was unambiguously the outcome of the test. Together with our data with respect to storage, this retrospective analysis demonstrates the potential of the assay for retrospective analysis of bloodspots of patients with a reasonable chance of missed diagnosis for BTHS. Such a study of children who died of unexplained cardiomyopathy and/or infection might substantiate the hypothesis that the prevalence of BTHS is underestimated. Current methods for BTHS diagnos-

tics require lymphocytes or fibroblasts, materials not easily obtained and that cannot be transported without special (expensive) precautions. A bloodspot, however, has proven a very suitable and stable matrix that can be stored and easily transported, thereby allowing samples to be sent by mail from anywhere in the world, at very low cost.

Although the assay described here is likely to be too time-consuming for incorporation in existing neonatal screening programs, it has potential as a quick screening method of neonates with any likelihood for BTHS. Early diagnosis and appropriate treatment, which is essentially supportive in nature, are of key importance for the survival of boys affected with BTHS. Usually, cardiomyopathy calls for the most attention but, because of possible neutropenia, prompt hematological evaluation and treatment of symptoms are required during fever or infections (28). Because BTHS children require a lower intake of calories to maintain a normal growth rate, calcium and other minor nutrients have to be managed carefully (28). It is estimated that alert management of the individual symptoms of BTHS patients can increase life expectancy into adulthood up to 90%; if diagnosis is missed at an early stage, only 30% will survive childhood (28). We hope the development of this screening assay will result in an increase in early identification of new BTHS patients so that the appropriate treatment can be initiated as soon as possible. In our daily practice, we already use the assay for quick screening of patients with a suspicion of BTHS. Positive screening results are always confirmed by analysis of complete MLCL/CL profiles in lymphocytes or fibroblasts with (if consistent with BTHS) subsequent molecular analysis of the *TAZ* gene.

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