Cardiolipin metabolism in Barth syndrome

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Cardiolipin and monolysocardiolipin analysis in fibroblasts, lymphocytes and tissues using HPLC-mass spectrometry as a diagnostic test for Barth syndrome

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Abstract
Barth syndrome (BTHS) is an X-linked recessive disorder caused by mutations in the *tafazzin* gene and is clinically characterized by (cardio)myopathy, neutropenia and growth abnormalities. Biochemical abnormalities include decreased levels of the mitochondrial phospholipid cardiolipin, increased levels of monolysocardiolipin, and a lower degree of unsaturation of the (monolyso)cardiolipin acyl chains. Diagnostic testing for BTHS is routinely performed by *tafazzin* gene sequencing and recently a BTHS screening method in bloodspots has been developed, but both methods have important limitations. Since a validated confirmatory method is not yet available we set up and validated an HPLC-MS method for BTHS in cultured fibroblasts, lymphocytes and skeletal muscle based on cardiolipin, monolysocardiolipin and the monolysocardiolipin/cardiolipin ratio. In addition, we performed retrospective analysis of 121 muscle samples of patients with myopathy of which mitochondrial origin was presumed, and identified one patient with cardiolipin abnormalities similar to BTHS patients. Molecular analysis revealed a bona-fide mutation in the *tafazzin* gene. We conclude that (monolyso)cardiolipin analysis by HPLC-MS not only is a powerful tool to diagnose patients with clinical signs and symptoms of BTHS but should also be used in patients suffering from mitochondrial myopathies with unknown etiology.

Abbreviations
BTHS, Barth syndrome
CL, cardiolipin
MLCL, monolysocardiolipin
Introduction
Barth syndrome (BTHS; MIM 302060) is an X-linked recessive disorder, clinically characterized by cardiac and skeletal myopathy, neutropenia, and growth abnormalities [1-3]. BTHS is caused by mutations in the tafazzin (or TAZ) gene, which is located on chromosome Xq28 [4]. Alternative splicing of the TAZ mRNA results in various splice variants of which only two are thought to result in a catalytically active protein [5,6]. Based on sequence homology, the tafazzin proteins are believed to function as acyltransferases involved in the metabolism of phospholipids [7]. Recent studies have shown that the Drosophila melanogaster tafazzin functions in the transfer of linoleic acid acyl chains from phosphatidycholine to monolysocardiolipin (MLCL) [8]. As a result of the defective acyl chain remodeling by tafazzin, BTHS is biochemically characterized by lower levels of the phospholipid cardiolipin (CL) and reduced linoleic acid incorporation into CL, due to the defective acyl chain remodeling [9]. Later studies showed that TAZ deficient yeast cells [6], BTHS lymphoblastoid cells [10] and BTHS fibroblasts [11] also display an accumulation of MLCL, i.e. CL lacking one acyl chain, and have a lower degree of unsaturation of their acyl chains [6,10]. In mitochondria, CL has important functions with regard to the maintenance of the mitochondrial ultrastructure, the activity and supercomplex formation of the respiratory chain, and the execution of the mitochondrial apoptosome mediated apoptosis pathway [12,13]. Hence, deficiency of CL metabolism, as observed in BTHS cells, results in abnormally formed mitochondria [14,15], reduced respiratory chain activity, aberrant supercomplex formation [16-18], and an altered apoptotic response [19,20].
Molecular analysis of the TAZ gene is a powerful tool for the diagnosis of BTHS patients. However, if mutations are present in regulating sequences or result from DNA changes for instance in the promoter region, a false negative result can be obtained. Moreover, if CL analysis is omitted and replaced by molecular analysis, one may overlook other potential new defects in the CL biosynthetic pathway. In recent years, the characteristic CL abnormalities of BTHS (low CL levels and high MLCL levels) have been recognized as the biochemical parameter with the potential to be used in the diagnostic process of BTHS. CL analysis in BTHS has been reported in lymphoblasts [10], platelets [21] and fibroblasts [11], however, no validated methods have been published that describe the use of both CL and MLCL analysis for diagnostic purposes in different cells and/or tissues. We recently validated and reported a method in bloodspots, designed as a first screen for BTHS based on the MLCL/CL ratio [22]. Although this method proved to be very specific and sensitive, a positive screening result should be confirmed in a second specimen using a validated assay which investigates the whole CL spectrum. Therefore, we developed and validated a method for CL and MLCL analysis in fibroblasts, lymphocytes and skeletal muscle. We show that (ML)CL analysis followed by the calculation of the MLCL/CL ratio is an unambiguous test for the diagnosis of BTHS in these materials, and that additional confirmation can be obtained from inspection of the MLCL and CL mass spectra.
Materials and methods

Cells and tissues
For the validation of the CL analysis human skin fibroblasts were used of a healthy control and a BTHS patient (hemizygous for c.C153G which results in a premature stop codon (Tyr51X)[4]). Fibroblasts were cultured in HAM F-10 medium with L-glutamine and 25 mM HEPES, supplemented with 10% fetal calf serum (Invitrogen). Cells were grown to confluence in 162 cm$^2$ flasks, harvested by trypsinization, pelleted by centrifugation at 250 × $g$ for 5 minutes and washed twice with PBS and once with 0.9% NaCl. Pellets were stored at -80°C until analysis.

For the validation of the analysis in lymphocytes, a large batch of lymphocytes was isolated from a buffy coat derived from 500 mL whole blood (Sanquin). Patient and control lymphocytes were isolated from 5 mL EDTA-blood using Lymphoprep (Nycomed) according to the manufacturer guidelines. Anonymized muscle biopsies or muscle homogenates of patients with idiopathic (cardio)myopathy, of which a mitochondrial basis was presumed, were obtained from the tissue bank of the Nijmegen Center for Mitochondrial Disorders.

Phospholipid extraction
Fibroblasts and lymphocytes were resuspended in PBS. Muscle biopsies were homogenized in PBS using a potter homogenizer. All samples were sonicated for 20 seconds using a tip sonicator. The protein concentration was determined according to the Bradford protocol [23]. Phospholipids were extracted from fibroblast and lymphocyte homogenates using a single-phase extraction and muscle phospholipids were extracted using a two-phase extraction. In the single-phase extraction 3 mL of chloroform-methanol 1:1 (v/v) was added to a maximum of 300 µL of homogenized cells to prevent phase separation. After addition of CL internal standard (0.4 nmol of CL(14:0)$_4$ (Avanti Polar Lipids) dissolved in 50 µL chloroform), the mixture was shaken vigorously for 2 minutes and placed on ice for 15 minutes, followed by centrifugation at 1000 × $g$. The supernatant was transferred to another tube, and the protein pellet was extracted once again with 3 mL of chloroform-methanol 2:1 (v/v).

For the two-phase extraction, 3 mL of chloroform-methanol 2:1 (v/v) was added to 1 mL of muscle homogenate, which contained a maximum of 1 mg of protein. After addition of CL internal standard, the mixture was shaken vigorously for 2 minutes and placed on ice for 15 minutes, followed by centrifugation at 1000 × $g$. The lower, organic layer was transferred to another tube and the upper layer was reextracted with 3 mL of chloroform-methanol 2:1 (v/v). For both the single-phase and two-phase extraction, the organic layers were combined and evaporated under a stream of nitrogen at 45°C. The residue was dissolved in 150 µL of chloroform/methanol/water (50:45:5 v/v/v) containing 0.01% NH$_4$OH, and 10 µL of this solution was injected into the HPLC-MS system.

HPLC mass spectrometry
The HPLC system consisted of a Surveyor quaternary gradient pump, a vacuum degasser, a column temperature controller and an autosampler (Thermo Electron Corporation, Waltham, MA). The column temperature was maintained at 25°C. The lipid extract was injected onto a LiChrospher 2×250 mm silica-60 column, 5 µm particle diameter (Merck, Darmstadt, Germany). The phospholipids were separated from
interfering compounds by a linear gradient between solution B (chloroform-methanol, 97:3, v/v) and solution A (methanol-water, 85:15, v/v). Solution A and B contained 1 mL and 0.1 mL of 25% (v/v) aqueous ammonia per liter of eluent, respectively. The gradient (0.3 ml/min) was as follows: 0–10 min: 20% A to 100% A; 10-12 min, 100% A; 12-12.1 min: 100% A to 0% A; and 12.1–17 min, equilibration with 0% A. All gradient steps were linear, and the total analysis time, including the equilibration, was 17 min. A splitter between the HPLC column and the mass spectrometer was used, and 75 µL/min eluent was introduced into the mass spectrometer. A TSQ Quantum AM (Thermo Electron Corporation) was used in the negative electrospray ionization mode. Nitrogen was used as nebulizing gas. The source collision-induced dissociation collision energy was set at 10 V. The spray voltage used was 3600 V, and the capillary temperature was 300°C. Mass spectra of CL and MLCL molecular species were obtained by continuous scanning from m/z 380 to m/z 1100 with a scan time of 2 s.

**Validation**

Linearity was analyzed in two separate experiments. In the first analysis we extracted phospholipids from increasing amounts of protein (0.25-2 mg of protein for fibroblasts and 0.1-1 mg for lymphocytes). The second analysis was based on extraction of phospholipids from equal amounts of protein but varying the phospholipid composition by mixing control and BTHS material in different proportions. Precision of the CL and MLCL analysis was assessed in several ways. The analytical or MS variation was determined by measuring CL and MLCL in the same phospholipid extract 21 times on the same day. The intra-assay variation was determined by CL and MLCL analysis in 10 identical samples, which were extracted separately but measured in the same analytical run. The inter-assay variation was determined by CL and MLCL analysis in one sample that was divided in five aliquots, which were extracted and measured in separate analytical runs. The limit of detection was expressed as the minimum amount of sample material necessary for an unambiguous result. This was determined by serial dilution of phospholipid extracts in chloroform/methanol/water (50:45:5 v/v/v) containing 0.01% NH₄OH. The discriminative power of the method was determined by the analysis of 7 healthy controls and 14 BTHS patients for the fibroblast validation. For the lymphocyte method we analyzed lymphocytes of 10 male and 8 female controls, 8 fathers of BTHS patients, 13 mothers of BTHS patients, and 20 BTHS patients.

**Data analysis**

Quantification of CL and MLCL levels was performed using the Xcalibur mass spectrometry quantification software. For quantification of CL and MLCL levels, the area under the curve of the HPLC profile corresponding to the complete mass spectra of CL (m/z 699-742) and MLCL (m/z 565-610) species was integrated, as well as that of the internal standard (m/z 619-622). The ratio of the CL or MLCL area divided by the area of the internal standard was used as a measure for CL or MLCL level, respectively. The ratio of MLCL over CL was used as an additional diagnostic marker, which is independent of the amount of material used for the phospholipid extraction. Data are represented as mean ± standard deviation. Statistical analyses were performed using the Mann Whitney U test.
Figure 1. Typical MLCL and CL spectra of control and Barth syndrome (BTHS) fibroblasts (A) and lymphocytes (B). For both control and BTHS fibroblasts, phospholipids were extracted from the equivalent of 1 mg of protein. For lymphocytes, phospholipids were extracted from the equivalent of 0.3 mg of protein of control and BTHS cells. In control cells, MLCL is hardly detectable, whereas in BTHS cells MLCL levels are markedly elevated. The acyl chain composition, both in number of carbon atoms and degree of unsaturation, is also altered in BTHS cells. As shown in the insets of the figures the most prominent peaks of the BTHS spectra are at a higher m/z indicating less double bonds, i.e. lower degree of unsaturation.
Results and discussion

Validation of the CL and MLCL analysis

In order to include MLCL as a diagnostic parameter we developed a new diagnostic HPLC-MS method suitable for the measurement of MLCL, which was unavailable until now. Figure 1 shows that we can detect both CL and MLCL in fibroblasts and lymphocytes of control and BTHS subjects using this method. As shown in figure 1, CL is decreased in the BTHS cells when compared to control cells, and MLCL levels are markedly elevated. Moreover, there is a lower degree of unsaturation of both CL and MLCL acyl chains in the BTHS cells (see inset of figure 1).

Linearity was tested by mixing control and BTHS fibroblasts in different proportions, keeping the combined amount of protein constant (1 mg). Because MLCL levels are low in controls and high in BTHS cells, whereas the reverse is true for CL, measurement of CL and MLCL in these mixtures yields a good indication of the linear response of the assay within a relatively constant matrix. Both CL and MLCL showed a good correlation between calculated and actually measured levels (figure 2A).

Figure 2. Linearity analysis of the CL (open circles) and MLCL (closed squares) (A, first panel). Control (high CL, low MLCL levels) and BTHS (low CL, high MLCL levels) fibroblast homogenates were combined in different proportions, all at a final protein content of 1 mg. By setting the lowest and highest values of CL and MLCL at 0% and 100% we established that there was a good correlation between the calculated and observed values (A, second panel). A second linearity test was performed by the CL and MLCL measurement in control and BTHS fibroblasts (B) and lymphocytes (C) by phospholipid extraction of increasing amounts of cell material. Data were normalized and expressed as arbitrary units (a.u.) in order to facilitate the interpretation of the linear analysis. Since the information concerning the signal intensity of (ML)CL (essentially the integrated (ML)CL signal divided by that of the internal standard) is lost by the normalization, the highest value is displayed as a boxed figure at the upper right of the graph.
Linearity of the CL and MLCL analysis was also determined by extracting phospholipids from increasing amounts of cells. To this end, CL and MLCL were extracted from control and BTHS fibroblasts (0.25-2 mg of protein) (figure 2B), and from control lymphocytes (0.1-1 mg of protein) (figure 2C). The analysis of CL was linear up to the maximum amount tested in control fibroblasts and BTHS fibroblasts. We found that in fibroblasts MLCL levels were linear with protein content, even though the MLCL content in control cells was close to the quantification limit.

The linearity of the CL analysis in lymphocytes was comparable to the linearity observed in fibroblasts (figure 2C). Unfortunately, BTHS lymphocytes were not available in sufficient amounts to analyze the MLCL linearity in these cells. Based on these experiments and the amount of material that is routinely available for diagnostic testing, we decided to use the equivalent of 1 mg of protein for all fibroblast analyses and 0.5 mg of protein for lymphocyte analyses.

To establish the precision of the CL and MLCL analysis, we determined the MS variation, the intra-assay variation and the inter-assay variation (figure 3). As shown in the box-and-whisker plots in figure 3A, the MS variation for CL and MLCL was between 5 and 10% in fibroblasts, except for MLCL in control fibroblasts (18%), which can be explained by the very low MLCL levels in control cells (see also figure 1A). The intra-assay variation of the CL and MLCL analysis in fibroblasts (figure 3A) was only marginally higher than the MS variation, indicating that the variation was mainly caused by the variation of the HPLC-MS system and that the contribution of the sample work-up, including the extraction, was limited. The inter-assay variation was high for all analytes (figure 3A). It is important to realize, however, that despite this high variation BTHS fibroblasts always showed a significant decrease in CL and increased levels of MLCL (both p<0.01). In a previous study we used the MLCL/CL ratio as a powerful determinant for the screening of BTHS in bloodspots [22]. As shown in figure 3A, this parameter was highly discriminative as demonstrated by the significant difference (p<0.01) between control and BTHS fibroblasts without any overlap between the two groups.

In lymphocytes the coefficients of variation were only analyzed in control cells, because of the aforementioned lack of sufficient BTHS lymphocytes. The MS, intra-assay and inter-assay variation of CL in control lymphocytes were found to be around 20 to 25% (figure 3B). The large variation in MLCL levels was caused by the obvious near-absence of MLCL in control lymphocytes (see also figure 1B). The high variation in the lymphocyte method did not pose a diagnostic problem since the values of CL and MLCL in control and BTHS lymphocytes, and therefore also the MLCL/CL ratio, are in a completely different range, as will become apparent in figure 5.

As a limit of detection, we found that the HPLC-MS method could still be applied to as little as 31 µg of protein without affecting the outcome of the result for BTHS patients or controls (results not shown).

Control and BTHS patient analysis

To confirm the validity of the CL and MLCL analysis for the diagnosis of BTHS, we analyzed CL and MLCL in fibroblasts of 7 controls and 14 confirmed BTHS patients. As shown in figure 4A, there were large variations in CL and MLCL levels in fibroblasts of the individual controls and BTHS patients. On average, CL levels were significantly decreased in BTHS fibroblasts (figure 4A), but because of the large overlap this is
Figure 3. Precision of the CL and MLCL analysis in cultured fibroblasts (A), isolated lymphocytes (B) and skeletal muscle (C). The figure shows box-and-whisker plots of the CL and MLCL measurements and the calculated MLCL/CL ratio. The results in fibroblasts show that the coefficients of variation (CV%) are high. The discriminative power of the method, however, is good as shown by the significant (p<0.01) differences between control (CTRL) and BTHS fibroblasts in the levels of CL and MLCL and the MLCL/CL ratio (A). The variation coefficients of the CL and MLCL analysis in lymphocytes (B) and skeletal muscle (C) are comparable.

Figure 4. Box-and-whisker plot of the abundance of CL and MLCL (A) and the MLCL/CL ratio (B) in control (n=7) and BTHS (n=14) fibroblasts. CL levels are slightly decreased in BTHS cells, which is compensated by a high increase in MLCL levels (A). The MLCL/CL ratio is approximately 12.5 times higher in BTHS when compared to control fibroblasts (B). Data are means ± standard deviation. Statistical significance was calculated using the Mann Whitney U test.
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not suitable as the sole diagnostic parameter. MLCL on the other hand was markedly increased in BTHS fibroblasts and the values of controls and BTHS did not overlap (figure 4A). Using the MLCL/CL ratio we could easily distinguish between controls and BTHS patients without any overlapping values (figure 4B).

Figure 5 shows the CL and MLCL levels and the MLCL/CL ratio in lymphocytes of male and female controls, fathers of BTHS patients, mothers of BTHS patients and BTHS patients. As shown in figure 5, there was a large variation in CL levels (figure 5A), MLCL levels (figure 5B) and the MLCL/CL ratio (figure 5C). CL levels in BTHS lymphocytes were slightly but significantly lower than in the different control groups (p≤0.027). MLCL levels in BTHS lymphocytes were markedly increased, except for one patient who had MLCL levels in the control range. By calculating the MLCL/CL ratio, we found that both groups can clearly be distinguished and that the BTHS patient showing the control levels of MLCL was also positively identified as a BTHS patient, stressing the importance of this new parameter. Both mothers and fathers of BTHS patients were not statistically different from controls (male or female).

When the different control groups were combined, a highly significant increase in the MLCL/CL ratio was found with no overlap between the two groups (p=0.0001; figure 5D).

Figure 5. Abundance of CL (A) and MLCL (B) and the MLCL/CL ratio (C) in lymphocytes of male controls (n=10), female controls (n=8), fathers to BTHS patients (n=8), mothers to BTHS patients (n=13) and BTHS patients (n=20). CL is decreased in BTHS lymphocytes when compared to the different control groups and MLCL is markedly elevated, with only one BTHS patient in control range for MLCL. The MLCL/CL ratio is higher in BTHS patient lymphocytes when compared to the control groups without exception, making this an excellent marker for diagnosis. This is also shown in the box-and-whisker plot (D) showing that there is no overlap in the MLCL/CL ratio between controls and the BTHS lymphocytes. Statistical significance was calculated using the Mann Whitney U test.
Retrospective BTHS testing of muscle samples of unknown mitochondrial myopathies

Because it is thought that BTHS is underdiagnosed [24], we aimed to perform a retrospective analysis of muscle samples of patients with mitochondrial myopathies of unknown origin. Typically, muscle CL is very rich in tetrailinoleoyl-CL as reported previously [25] and as shown in figure 6A, CL can be easily measured in control muscle. As little as 5 mg of wet weight tissue was sufficient for successful analysis (not shown). We applied a limited validation protocol to the (ML)CL analysis in skeletal muscle and showed that the variation of the method is comparable to the fibroblast and lymphocyte analysis (figure 3C). Besides the variation in the MS analysis as observed for the fibroblast and lymphocyte method, the muscle measurement is particularly susceptible to variations in the biopsy for instance with regard to connective tissue content, and efficiency of homogenization. To overcome these issues, we used the MLCL/CL ratio as the diagnostic parameter for BTHS in muscle.

Using the validated assay, we performed a retrospective analysis of 121 patients with myopathy of unknown origin, of which a mitochondrial basis was presumed. The samples were derived from the repository of the Nijmegen Center for Mitochondrial Disorders and in most (but not all) patients respiratory chain deficiency was confirmed by enzymatic analysis, without subsequent confirmation at the molecular level. In the screen we identified one patient with markedly elevated MLCL/CL ratio, indicative for BTHS (figure 6B). As shown in figure 6C, the (ML)CL mass spectrum of the patient is completely different from the spectrum of the typical control muscle, including a decrease in CL, increased MLCL levels and a shift towards more saturated CL and MLCL species. Sequencing of the TAZ gene identified a disease causing mutation, which confirmed the BTHS diagnosis (manuscript in preparation).

**Figure 6.** (A) MLCL and CL spectrum of a typical control muscle sample. Muscle CL is typically very rich in linoleic acid, which is observed as the high peak at m/z 723. MLCL species are hardly present in control muscle. (B) MLCL/CL ratio of muscle samples of 121 patients with idiopathic mitochondrial myopathy. The highest value for controls in this screen is 0.18, whereas one patient has a MLCL/CL ratio of 3.31, indicative for BTHS. A confirmed BTHS patient was included in the analysis and showed a ratio of 0.96. (C) MLCL and CL spectrum of muscle of the BTHS patient shown in figure 6B. CL levels are very low and MLCL levels are markedly elevated.
To summarize, in this study we developed and validated an HPLC-MS method for the diagnosis of BTHS in lymphocytes and in cultured fibroblasts, as these materials are widely used for diagnostic testing. The MLCL/CL ratio was successfully identified as a reliable marker for BTHS. Although the calculation of the MLCL/CL ratio is sufficient to establish the BTHS diagnosis, the detailed analysis of the HPLC-MS spectra poses the opportunity to collect additional evidence to support the BTHS diagnosis. Especially in cases when the MLCL/CL ratio is close to the normal range (which is almost never the case), it is useful to have an additional parameter namely the degree of unsaturation of the CL and MLCL species, which is lowered in BTHS cells when compared to controls (insets of figure 1). This method complements the recently developed method for the screening of BTHS in bloodspots [22] as it can (and should) be used to confirm a positive screening result. Finally, we validated the (ML)CL method for skeletal muscle and used this method for a retrospective screen for BTHS in a group of 121 patients with mitochondrial myopathy of unknown origin. The identification of a previously undiagnosed patient in this group suggests that BTHS is underdiagnosed and therefore we advocate low threshold screening for patients suffering from mitochondrial disease with unknown origin, since BTHS might be the underlying disorder.

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