methods

Monolysocardiolipin in cultured fibroblasts is a sensitive and specific marker for Barth Syndrome

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Abstract Barth Syndrome (BTHS) is an X-linked recessive disorder that results in abnormal metabolism of the mitochondrial phospholipid cardiolipin (CL). CLs are decreased and monolysocardiolipins (MLCLs), intermediates in CL metabolism, are increased in a variety of tissues. Measurement of decreased CL levels in skin fibroblasts has previously been proposed as a diagnostic test for BTHS. We investigated whether elevated MLCL is specific for BTHS and whether the MLCL-to-CL ratio is a more sensitive and specific marker for BTHS. We measured CLs and MLCLs in skin fibroblasts from 5 BTHS patients, 8 controls, and 14 patients with biochemical and clinical findings similar to those in BTHS (group D), using high performance liquid chromatography-mass spectrometry. Our results showed a clear decrease of CL in combination with a marked increase of MLCL in fibroblasts from BTHS patients when compared with controls. MLCL/CL ratios ranged from 0.03–0.12 in control fibroblasts and from 5.41–13.83 in BTHS fibroblasts. In group D, the MLCL/CL ratio range was 0.02–0.06. We therefore conclude that elevations of MLCLs are specific for BTHS and that the MLCL/CL ratio in fibroblasts is a better diagnostic marker than CL alone. We also report the finding of two novel mutations in the *TAZ* gene that cause BTHS.—van Werkhoven, M. A., D. R. Thorburn, A. K. Gedeon, and J. J. Pitt. **Monolysocardiolipin in cultured fibroblasts is a sensitive and specific marker for Barth Syndrome. *J. Lipid Res.* 2006.** 47: **2346–2351.**

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Barth Syndrome (BTHS, MIM 302060) is an X-linked recessive disorder which can be fatal in infants and children due to cardiac failure or sepsis. The clinical symptoms of this disease are variable in severity, but typically patients with BTHS have neutropenia, cardioskeletal myopathy, and short stature (1, 2). Biochemical parameters include low blood cholesterol and increased amounts of 3-methylglutaconic, 3-methylglutaric, and 2-ethylhydracrylic acids in urine (3), although urine organic acid analysis can sometimes be normal (4). BTHS patients have abnormal mitochondria in cardiac and skeletal muscle, and defects in the respiratory chain complexes I, III, and IV have been reported in muscle and fibroblasts (2, 5).

BTHS is caused by mutations in the tafazzin (*TAZ*) gene, which is located at Xq28 (6). Neuwald (7) showed that the predicted *TAZ* gene product has homology to acyltransferases involved in phospholipid metabolism and suggested that *TAZ* is involved in the remodeling of phospholipids. This proposal was substantiated by Vreken et al. (8), who found that the amount of the phospholipid cardiolipin (CL) was lowered in patients with BTHS even though the rate of biosynthesis of CL was normal when compared with control cells. Patients with BTHS have low CL in platelets, fibroblasts, and other tissues (9–12). Transformed yeast with a disrupted *TAZ* gene shows CL deficiency and elevations of monolysocardiolipins (MLCLs), a class of phosholipids related to CL (13). However, after transformation with normal human tafazzin, levels of both CL and MLCL normalized. This confirms the concept that *TAZ* is involved in the remodeling of CL. **Figure 1** shows the structures of CL and MLCL.

CL is almost exclusively found in the inner mitochondrial membrane and is made from phosphatidylglycerol. CL is required for maintaining the structure of some respiratory chain complexes and thus plays an important role in oxidative phosphorylation and in other mitochondrial functions (2, 5, 14–17). As shown in Fig. 1A, CL has

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Abbreviations: BTHS, Barth Syndrome; CL, cardiolipin; MLCL, monolysocardiolipin; m/z^-, mass-to-charge ratio, negative ion mode; *TAZ*, tafazzin.

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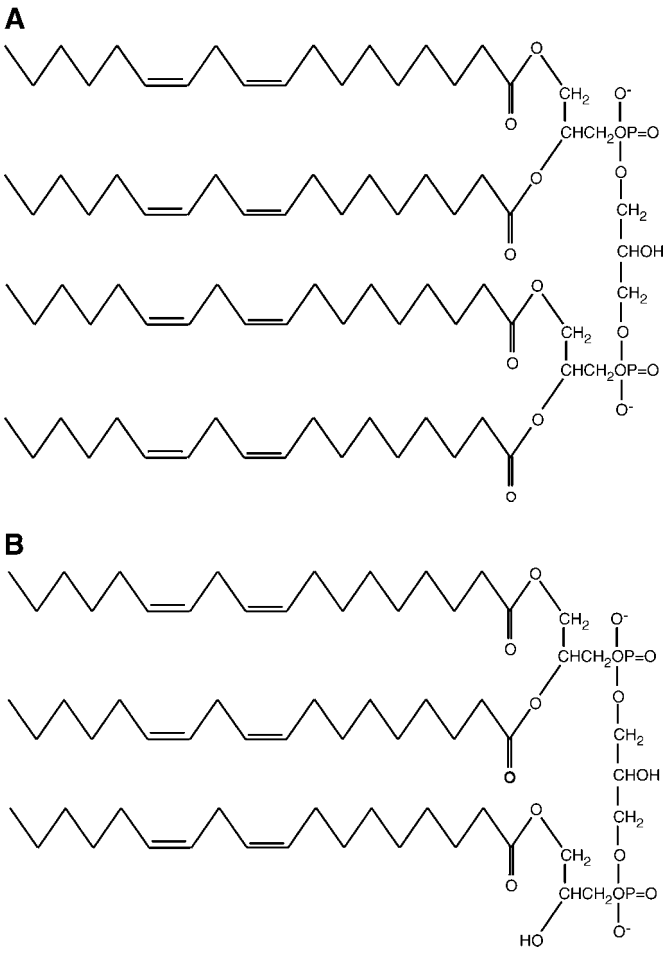

Fig. 1. Representative structures of (A) cardiolipin (CL) and (B) monolysocardiolipin (MLCL).

four fatty acyl side chains and two negatively charged phosphate groups. CL usually has C18 fatty acid side chains, and 80% of these are typically linoleic acid (C18:2) (18, 19). Incorporation of linoleic acid into CL and phosphatidylglycerol was reduced in BTHS patients (12). It is hypothesized that after synthesis of CL, the fatty acid side chains are remodeled by a deacylation-reacylation cycle to obtain different CL species and that this involves the formation of intermediate MLCLs (17, 20, 21).

Definitive diagnosis of BTHS by genetic testing is demanding, because most patients have different private mutations and because of the size of the *TAZ* gene. Previously, it has been reported by Valianpour et al. (12) that fibroblasts show low levels of CL and that this can be used as a diagnostic method for BTHS. Elevated levels of MLCLs were also found in muscle, heart, lymphocytes, and lymphoblasts from BTHS patients, and the MLCL level was found to be a good diagnostic marker in these tissues. However, MLCLs were not detected in fibroblasts, commonly used for diagnostic purposes (22). Also, it was not shown whether MLCL is indeed a specific marker for BTHS. We now describe the simultaneous measurement of CL and MLCL in fibroblasts and the use of the MLCL-to-CL ratio as a more sensitive diagnostic marker for BTHS than CL or MLCL levels alone. We also investigated cell lines from patients with clinical or biochemical symptoms similar to those of BTHS to see whether increased amounts of MLCL are unique to BTHS. Furthermore, we report two new mutations found in the *TAZ* gene that cause BTHS.

MATERIALS AND METHODS

In this study, fibroblast cell lines of five BTHS patients with typical clinical and biochemical features were used. Mutations in three of these patients have been previously described (6, 23–25), and two of the patients had novel pathogenic mutations. Genomic DNA preparation, amplification, and direct sequencing of the BTHS gene in those patients were as described previously (24).

Patient data are shown in **Table 1**. Control fibroblast cell lines were divided into two groups: in group C were controls (n = 8) known to have no defect in oxidative phosphorylation, and in group D (n = 14) were cell lines of patients with different deficiencies in oxidative phosphorylation. More detailed data about group D are listed in Table 1.

All solutions were of analytical quality. Ethanol and chloroform were from Merck (Melbourne, Australia), and the analytical HPLC LiChrospher Si 60 column (2 × 250 mm and 5 μm particle size) and ammonia were from Merck (Darmstadt, Germany). Methanol was from VWR International (Poole, England). The $(C14:0)_4$-CL internal standard was purchased from Avanti Polar Lipids (Alabaster, AL), and Sigma (St. Louis, MO) supplied the $(C18:2)_4$-CL standard and the butylated hydroxytoluene (BHT). JRH (Lenex, KS) supplied us with cell culture medium, Oxoid (Hampshire, England) with phosphate-buffered saline (PBS), and Thermo (Melbourne, Australia) with trypsin.

Fibroblasts were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 18 mmol/l HEPES, 50 μmol/l uridine, and 1% penicillin/streptomycin and incubated at 37°C. For harvesting, one 175 cm^2 flask of cells (~5 × 10^6 cells) was trypsinized, and the cells were centrifuged for 5 min at 600 *g*. The pellet was washed once with 10 ml and once with 1 ml of PBS and then stored at −70°C.

Lipids were extracted as described by Valianpour et al. (22), except for minor modifications, as follows. Fibroblast pellets were resuspended in 1 ml of water and kept on ice. Samples were sonicated using a Branson B-30 sonifier with a microtip twice for 10 s at 30% duty cycle and power setting 3, after which 30 μl was saved for protein measurement. The bicinchoninic protein assay was used to measure the protein concentration (26). After extraction, the residue containing the lipids was dissolved in 200 μl of chloroform-methanol-water (50:45:5; v/v/v) + 0.01% butylated hydroxytoluene in ethanol.

Of this extract, 30 μl was introduced onto the HPLC column by a Waters 2777C autosampler. The column was maintained at room temperature. A Waters 1525 μ binary pump with built in degasser was used as the HPLC system. The CL and MLCL were separated from interfering compounds by a linear gradient using buffers A (chloroform + 0.0025% NH_3) and B [water-methanol (1:9; v/v) + 0.0025% NH_3], and the eluent was directly introduced into the mass spectrometer (Waters Micromass Quattro Micro API). The HPLC flow was set at 0.3 ml/min, and the gradient was as follows: 0–0.1 min, 0% B to 20% B; 0.1–5 min, 20% B to 100% B; 5–8 min, 100% B; 8–8.1 min, 100% B to 0% B; 8.1–15 min, 0% B. All the gradients were linear.

The mass spectrometer was used in the negative electrospray ionization mode, and double-charged CL and MLCL ions were measured. Four ions were monitored (**Table 2**). This was fol-

TABLE 1. Patient details

Patient	Diagnosis	Presenting Age	Mutation	3MGC	Biochemical/Clinical Findings
910158[a]	BTHS	4 Months	*TAZ* c.527-1 G>A	Yes	Short stature, neutropenia, dilated cardiomyopathy
910394[b]	BTHS	15 Months	*TAZ* c.868insT	Yes	Short stature, neutropenia, muscle weakness, dilated cardiomyopathy
0010379[c]	BTHS	Birth	*TAZ* c.919delC	?	Dilated cardiomyopathy, died at 6 weeks
910135[d]	BTHS	4 Months	*TAZ* c.655C>T (R123X)	Yes	FTT, muscle weakness, dilated cardiomyopathy
0030008[d]	BTHS	<12 Months	*TAZ* c.988-2 A>C	?	Cardiomyopathy
930132	COI liver specific	30 Months	[e]	Yes	Developmental regression, movement disorder, FTT
930160	COI	Birth	[e]	Yes	Mitochondrial cardiomyopathy
910590	COI liver specific	Birth	[e]	Yes	Mitochondrial cardiomyopathy, muscle weakness, elevated lactate
920052	COI, II	1 Month	[e]	Yes	Primary lactic acidosis, FTT
840136	RRF	6 Months	[e,f]	Yes	Mitochondrial encephalopathy, muscle weakness, FTT, cardiomyopathy
0010441	CO IV muscle specific	Birth	ND	Yes	Dilated cardiomyopathy, FTT
930227	RRF	Birth	ND	Yes	Mitochondrial encephalopathy
920421	COI	Birth	*NDUFS6*	No	LIMD, elevated lactate
980457	COI	6 Months	*MTND6*	?	Leigh Syndrome
0000382	COI	Birth	ND	No	LIMD, elevated lactate
950332	COI	6 Weeks	*MTND3*	No	Leigh Syndrome
990105	COI, IV	?	ND	No	Leigh Syndrome
0040012	COIV	6 Months	ND	No	Leigh Syndrome, FTT
950301	COIV	14 Months	*SURF1* hom exon 4 del	No	Leigh Syndrome

Top panel shows the 5 BTHS patients, and bottom panel shows the 14 patients with different deficiencies in oxidative phosphorylation (group D). 3MGC, 3-methylglutaconic aciduria; CO, respiratory chain complex; FTT, failure to thrive; LIMD, lethal infantile mitochondrial disease; ND, not determined; RRF, ragged red fibers.

[a] GW in (6).
[b] IV-19 in (25) with same mutation found in cousin of OAT in (6).
[c] V.1 in (23) and MH in (24).
[d] Mutations not reported previously.
[e] *TAZ* gene sequenced but no mutation found.
[f] Reported in (29).

lowed by scans from mass-to-charge ratio, negative ion mode (m/z^-) 550 to 625 in 1 s at a cone voltage of 40 for MLCL species and from m/z^- 700 to 750 in 0.5 s at a cone voltage of 40 for CL species.

Chromatograms of the monitored ions were integrated in order to quantitate CL and MLCL species. CL and MLCL eluted at 3.8 and 3.9 min, respectively. Scanned mass spectra were also examined to confirm the presence of other CL and MLCL species (**Table 3**). Masslynx software and Quantify software (Waters) were used to measure the peak areas. Next, the surface area of the most abundant CL and the most elevated MLCL species was divided by the surface area of the internal standard (IS) peak, and this was expressed relative to protein concentration. *P* values were calculated for MLCL/CL, (MLCL/IS)/protein, and (CL/IS)/protein levels (BTHS patients versus control group C or D) using the one-tail distribution, equal variance Student *t*-test. Comparisons between control groups C and D used the two-tail distribution, equal variance Student *t*-test.

TABLE 2. Ions used to quantify CL and MLCL species

Ion	Dwell Time	Cone voltage
	s	
m/z^- 582.6 (C16:0)/$(C18:1)_2$-MLCL	0.2	30
m/z^- 595.6 $(C18:1)_3$-MLCL	0.1	30
m/z^- 619.7 $(C14:0)_4$-CL (Internal Standard)	0.1	40
m/z^- 725.8 $(C18:1)_2$ /$(C18:2)_2$ -CL	0.2	40

CL, cardiolipin; MLCL, monolysocardiolipin; m/z^-, mass-to-charge ratio, negative ion mode.

RESULTS

Figure 2A shows the spectra of the different CL species in a control and a BTHS patient. The levels of CL species clustered around m/z^- 712.8, 725.8, and 737.6 are substantially lower in the fibroblasts of the BTHS patient when compared with the control fibroblasts. The MLCL species were also monitored in the same samples, and Fig. 2B shows that the levels of MLCL species clustered around m/z^- 569.6, 582.6, 595.6, and 607.6 are significantly elevated in fibroblasts from the BTHS patient when compared with the control fibroblasts. The CL and MLCL species that showed the greatest difference between BTHS and control samples were m/z^- 725.8 and 582.6, respectively. These two species were therefore used to calculate the CL and MLCL levels

TABLE 3. Ions representing CL and MLCL species

m/z^-	Major Species
595.6	$(C18:1)_3$-MLCL
582.6	(C16:0)/$(C18:1)_2$-MLCL
581.6	(C16:1)/$(C18:1)_2$-MLCL
568.6	$(C16:0)_2$/(C18:1)-MLCL
569.6	$(C16:0)_2$/(C18:0)-MLCL
712.8	(C16:0)/(C18:1)/$(C18:2)_2$-CL
724.6	(C18:1)/$(C18:2)_3$-CL
725.8	$(C18:1)_2$/$(C18:2)_2$-CL
726.8	$(C18:1)_3$/(C18:2)-CL
727.8	$(C18:1)_4$-CL
737.6	(C18:2)/$(C18:1)_2$/(C20:4)-CL

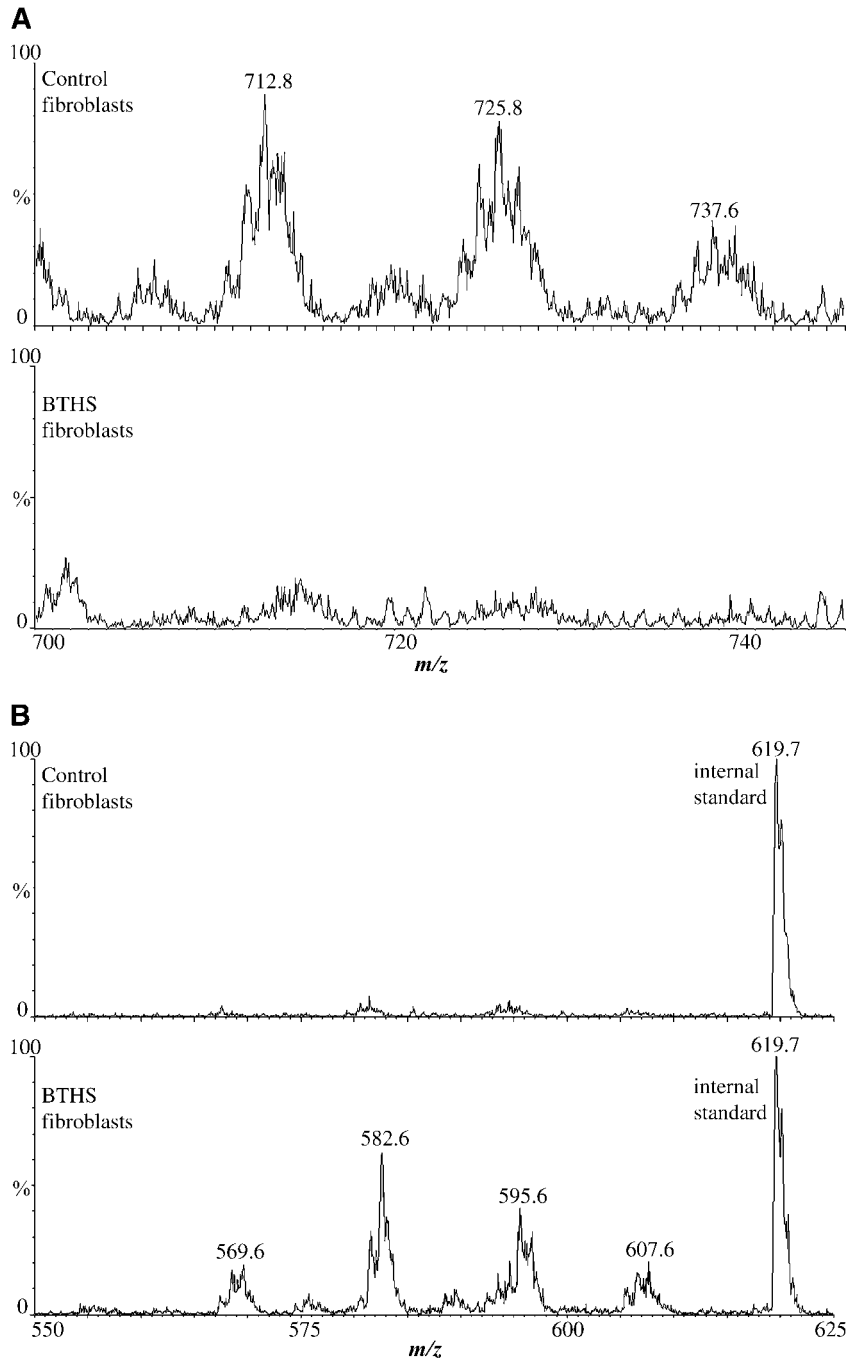

Fig. 2. A: Mass spectra of CL species in cultured control and Barth Syndrome (BTHS) fibroblasts. The spectra are displayed on the same scale. B: Mass spectra of MLCL species in cultured control and BTHS fibroblasts. The peak at mass-to-charge ratio, negative ion mode (m/z^-) 619.7 corresponds to the $(C14:0)_4$-CL internal standard.

and MLCL/CL ratios. The CL and MLCL data for a range of control and BTHS cell lines are shown in **Fig. 3A**. The mean CL (m/z^- 725.8) level is about four times lower in the BTHS fibroblast when compared with control fibroblasts. However the mean amount of MLCL (m/z^- 582.6) is 45 times higher in BTHS fibroblasts. Thus the mean MLCL/CL (m/z^- 582.6/725.8) ratio in the BTHS fibroblasts is 175 times higher when compared with that of control fibroblasts (0.03–0.12 in controls C versus 5.4–13.8 in BTHS).

To see whether increased amounts of MLCL are specific for BTHS, we measured MLCL/CL ratios in 14 patients who had clinical and biochemical features overlapping those of BTHS but who had, in fact, different abnormalities (control group D). This group included patients with elevated levels of 3-methylglutaconic acid, dilated

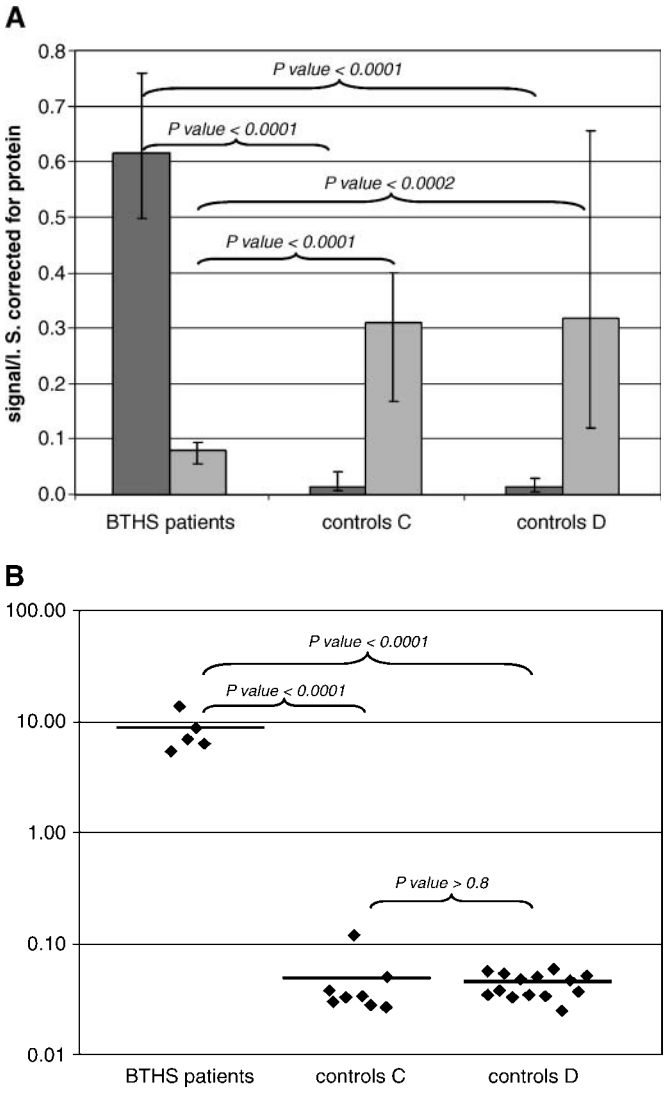

Fig. 3. A: A semiquantitive representation of the levels of CL (m/z^- 725.8) and MLCL (m/z^- 582.6) in the BTHS group and the different control groups. The bars represent the mean ± range. Dark-gray bars represent MLCL/internal standard (MLCL/IS)/protein, and light-gray bars represent (CL/IS)/protein. B: MLCL/CL ratios for the individual patients in the different groups. The horizontal bar represents the mean. Note that the y-axis is in log-scale.

cardiomyopathy, failure to thrive, muscle weakness, and respiratory chain enzyme defects. The MLCL/CL ratios in control group D ranged from 0.02–0.06 and did not differ significantly from control group C (see Fig. 3B). The 95% confidence intervals for MLCL/CL ratios in group C, group D, and the BTHS patients were 0.00–0.13, 0.02–0.06, and 1.54–14.94, respectively, and *P* values calculated for comparison of the different groups are depicted in Fig. 3A, B.

We also measured MLCL and CL levels in BTHS and control lymphoblasts (three BTHS patients and two controls) and found that the mean amount of MLCL (m/z^- 582.6) is 115 times higher in BTHS lymphoblasts when compared with control lymphoblasts, and the ratio of MLCL/CL (m/z^- 582.6/725.8) was 815 times higher (data not shown). We also report the finding of two novel mutations that cause BTHS. Patient 910135 had a novel c.655 C>T mutation, predicting a stop codon at codon 123. Patient 0030008 had a novel c.988-2 A>C mutation, predicting a splicing abnormality (see Table 1).

DISCUSSION

Molecular diagnosis of BTHS by sequence analysis of the *TAZ* gene can be time consuming because of its size (10 kb, 11 exons) and the lack of common *TAZ* mutations. Also, relatively few laboratories around the world offer molecular diagnosis of BTHS samples. Measuring 3-methylglutaconic acid is useful, but elevations of 3-methylglutaconic acid are not specific for BTHS (see Table 1). The finding of decreased activities of respiratory chain enzymes can provide supporting evidence of BTHS, but this is also not very specific. Therefore, a rapid and reliable biochemical test is needed so that appropriate clinical care may be administered prior to confirmatory gene analysis. Valianpour et al. (12) described measurement of CL levels in fibroblasts as a diagnostic assay for BTHS in patients, using high performance liquid chromatography-mass spectrometry, a technique that is available at many biochemical genetics laboratories around the world.

MLCLs were previously reported (22) to be elevated in heart, skeletal muscle, and cultured lymphoblasts of BTHS patients but were found only at very low levels in cultured fibroblasts. We investigated whether elevations of MLCLs were detectable in cultured fibroblasts for two reasons. First, fibroblasts are used more widely than Epstein-Barr virus-transformed lymphoblasts in most diagnostic centers. Second, for diagnosing inborn errors of metabolism, it is usually more sensitive to measure an increased amount of a metabolite that is normally present at very low levels than to measure a partial decrease in amount of a metabolite present in healthy individuals.

Our data clearly show that MLCL levels are significantly increased in fibroblasts of BTHS patients and that measurement of MLCL and CL levels in fibroblasts is a better diagnostic tool than measuring just CL levels. Like Valianpour et al., we found increased amounts of MLCL in BTHS lymphoblasts (115 times higher versus control lymphoblasts), but in addition, we found increased MLCLs in BTHS fibroblasts (45 times higher versus control fibroblasts). The m/z^- 582.6 species was the most elevated MLCL species in fibroblasts, but in lymphoblasts, the m/z^- 595.6 and the m/z^- 582.6 MLCL species were equally elevated (data not shown).

To be able to measure an increase in MLCLs and a decrease of CLs *in the same sample* and to calculate the ratio of MLCL to CL adds further diagnostic value to this test, because the MLCL/CL ratios are substantially more increased in BTHS patient fibroblasts when compared with control fibroblasts (see Fig. 3B). Use of the MLCL/CL ratio also minimizes some of the variability associated with extraction, culturing conditions, etc. The reason that we

were able to measure MLCL in BTHS fibroblasts when Valianpour et al. could not probably relates to the amount of sample analyzed. Valianpour et al. apparently introduced only about 20% of the sample volume that we used onto the HPLC column and analyzed only 10% of the eluant in the mass spectrometer (12, 22), whereas we analyzed all the eluant.

In previous reports, it was unclear whether increased amounts of MLCLs are specific for BTHS (22). Here we show that elevated MLCL levels are indeed highly specific for BTHS patients, because fibroblasts of controls with similar clinical or biochemical features but with a different genetic disorder (control group D) have MLCL levels comparable to those in fibroblasts of control patients known to have no defect in energy metabolism (group C). Analysis of fibroblast MLCL and CL is a relatively simple diagnostic test that can be applied to a readily available patient sample and that could potentially be used to test proposed therapies in vitro. This also makes it suitable for investigation of patients with clinical or biochemical features that overlap BTHS and either a low or high degree of suspicion of mutations in the *TAZ* gene. Examples include patients with cardiomyopathy with or without 3-methylglutaconic aciduria, with left ventricular noncompaction (27), or with a recently described autosomal recessive syndrome with features in common with BTHS (28).

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