

New targets for monitoring and therapy in Barth syndrome

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Purpose: Barth syndrome (BTHS), an X-linked disorder caused by defects in *TAZ*, is the only known single-gene disorder of cardiolipin remodeling. We hypothesized that through analysis of affected individuals, we would gain a better understanding of the range of clinical features and identify targets for monitoring and therapy.

Methods: We conducted a multidisciplinary investigation involving 42 patients with BTHS, including echocardiograms, muscle strength testing, functional exercise capacity testing, physical activity assessments, cardiolipin analysis, 3-methylglutaconic acid analysis, and review of genotype data. We analyzed data points to provide a quantitative spectrum of disease characteristics and to identify relationships among phenotype, genotype, and relevant metabolites.

Results: Echocardiography revealed considerable variability in cardiac features. By contrast, almost all patients had significantly

reduced functional exercise capacity. Multivariate analysis revealed significant relationships between cardiolipin ratio and left ventricular mass and between cardiolipin ratio and functional exercise capacity. We additionally identified genotypes associated with a less severe metabolic and clinical profile.

Conclusion: We defined previously unrecognized metabolite/phenotype/genotype relationships, established targets for therapeutic monitoring, and validated avenues for clinical assessment. In addition to providing insight into BTHS, these studies also provide insight into the myriad of multifactorial disorders that converge on the cardiolipin pathway.

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Key Words: Barth syndrome; cardiolipin; cardiomyopathy; myopathy; 3-methylglutaconic acid

INTRODUCTION

Cardiolipin is one of the major phospholipids of the inner mitochondrial membrane and is involved in critical mitochondrial functions, including electron transport chain efficiency, apoptosis, mitochondrial fission and fusion, and mitochondrial interactions with other cellular mechanisms.^{1–3} Abnormalities in cardiolipin have been implicated in a wide range of common human diseases, including diabetes, heart failure, and Parkinson disease.^{4,5} However, Barth syndrome (BTHS) is the only known Mendelian disorder of cardiolipin remodeling.

BTHS (3-methylglutaconic aciduria type II, MIM 300394) is a rare X-linked disorder with an estimated prevalence of 1/300,000–400,000 live births and characterized by cardiomyopathy, neutropenia, growth abnormalities, and skeletal myopathy, among other features.⁶ The syndrome is caused by defects in tafazzin (*TAZ*, G4.5), an acyltransferase involved in the remodeling of the fatty acyl side chains of monolysocardiolipin.⁷ Tafazzin deficiency results in abnormal cardiolipin content and a reduction in mature tetralinoleoyl-cardiolipin.

Cardiac disease in BTHS typically presents early in infancy, most often as a dilated cardiomyopathy with heart failure and,

in many cases, left ventricular noncompaction.⁸ After infancy, the cardiomyopathy can have a waxing and waning course.⁸ Neutropenia can be persistent, intermittent, or cyclical, and it is responsive to granulocyte colony-stimulating factor administration.⁹ The most common reported complications associated with neutropenia of BTHS include mouth ulcers, bleeding gums, skin infections, and upper respiratory infections. Pneumonia, sepsis, and other severe infections also occur, but reportedly to a lesser degree.⁹

Skeletal myopathy in BTHS predominantly affects the proximal musculature and has a significant effect on exercise tolerance and quality of life. The myopathy often leads to developmental motor delay, with about 66% of affected children having a delay in sitting up and about 72% of affected children having a delay in walking.¹⁰ Additionally, 34% of individuals with BTHS reported the use of orthoses, walkers, or wheelchairs at some point in their lives.¹⁰ The exercise intolerance in patients with BTHS is thought to be due to both cardiac impairment and decreased skeletal muscle oxygen utilization.¹¹

The primary diagnostic metabolite measurement in BTHS is elevation of the ratio of monolysocardiolipin to cardiolipin

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(MLCL/CL ratio). This ratio has diagnostic sensitivity and specificity of 100% in blood spots.¹² The MLCL/CL ratio can also be measured in nucleated cells and tissues. Isolated measurements of CL are not sufficient for diagnosis because these have been occasionally reported in the normal range in known BTHS patients in the setting of an abnormal MLCL/CL ratio.¹³

Other biochemical abnormalities typically seen in BTHS include increased levels of 3-methylglutaconic acid (3MGC) in plasma and urine; however, normal urine levels of 3MGC have been reported in some patients.¹⁴ In one cohort of 28 affected individuals, all had elevated plasma and urine 3MGC levels.¹⁵ By contrast, in another BTHS cohort elevated 3MGC levels were found in only 8 of 16 patients.¹⁴

A diagnosis of BTHS can be molecularly confirmed by sequencing the *TAZ* gene; causative variants have been reported in every exon except for exon 5 and include missense variants, nonsense variants, splice variants, small in/dels, and large deletions.^{16,17} A genotype–phenotype correlation had not been uncovered until recently when Bowron *et al.*¹³ reported three genotypes occurring with normal tetralinoleoyl-cardiolipin content and possibly a milder phenotype. However, how these molecular variants lead to a milder cardiolipin abnormality is not clear. Additionally, controlled, multidisciplinary studies involving a large cohort of BTHS patients have not been undertaken to establish the relationships among phenotypes, genotypes, and metabolites.

We investigated relationships among the genotypes, specific metabolites, and cardiac and musculoskeletal phenotypes in 42 individuals with BTHS in order to gain insight into the pathophysiology of this disease. In doing so, we uncovered several genotypes with a “milder” biochemical phenotype and established metabolite and phenotype relationships. On the basis of our findings, we offer suggestions for long-term measures of clinical response to medical interventions.

In addition to establishing a better understanding of BTHS, these biochemical and phenotypic relationships have the potential to provide insight into more common multifactorial disorders that converge on the cardiolipin pathway, including idiopathic cardiomyopathy.

MATERIALS AND METHODS

Study approval

This was an observational study open to any patient with a biochemically or molecularly confirmed diagnosis of BTHS who attended the Barth Syndrome International Conference in 2014. This study was approved under Johns Hopkins University IRB protocol “NA_00090474 Multidisciplinary Studies in Barth Syndrome.” Study enrollment took place in June 2014. Written informed consent was received from participants prior to inclusion in the study.

Participants

Participating individuals were males aged 24 months to 32 years 7 months who had molecular confirmation of BTHS ([Table 1](#)).

All blood samples were drawn at 3 to 4 hours of fasting after the last meal to ensure uniform dietary effects on the biochemical profiles. Patient *TAZ* genotype information was obtained through review of medical records. Age, weight, and height were measured and recorded for each participant by trained volunteers at the conference.

Statistical analysis

Linear regression models were used in the statistical data analysis program R to determine significant relationships between quantitative values.¹⁸

Quantitative data sets (cardiac parameters, 6-minute walk test (6MWT) distance, 3MGC, MLCL/CL, arginine, and other measures) were evaluated for a Gaussian distribution. Several values were above the distributions, including the following: participant 24 (left ventricular mass (LVM)), participant 23 (arginine), and participants 19, 23, and 24 (cardiolipin ratios). Regression analyses were calculated in two ways: including or excluding these strong outliers in the approximate Gaussian distribution. Inclusion or exclusion of outliers did not alter which relationships were statistically significant; therefore, all data were included in the analysis.

Modifiers included in each regression model were determined based on significantly related categories via Pearson correlations. Based on this Pearson correlation analysis, distance walked on the 6MWT was corrected for the modifier of height, and LVM, left ventricular internal diameter at end diastole (LVIDd), fractional shortening (FS), and left ventricular internal diameter at end systole (LVIDs) were corrected for height, weight, and age. Results from regression analyses were considered significant if the *P* value was less than or equal to 0.05.

Pearson *r*-correlations were determined for quantitative physical therapy data, including 6MWT distance versus physical activity questionnaire responses and 6MWT distance versus pre-/postexercise vital signs/Borg fatigue scoring. *R* correlations were considered very strong if greater than or equal to 0.7, strong if between 0.4 and 0.69, moderate if between 0.3 and 0.39, weak if between 0.2 and 0.29, and none/negligible if between 0.01 and 0.19.

Student's *t*-tests were used to determine the significance of manual and handheld dynamometry strength testing between cases and control values and fatigue measurements (as measured by the Borg scale) between younger subjects (ages 4–19) and older subjects (ages 20–32). Differences were considered significant if the *P* value was less than or equal to 0.05.

The 6MWT

The 6MWT is a well-established test that quantifies the distance an individual is able to walk over a total of 6 minutes on a flat surface, and this section of the study was open to participants older than age 3 years, which is the youngest age for which there are published normative values.^{19–21} The test was performed according to the American Thoracic Society Guidelines for the Six-Minute Walk Test, including a measuring wheel to measure distance walked, with the following exceptions secondary to

Table 1 Study participant characteristics

Study ID	Age at study (years)	Height (cm)	Weight (kg)	Distance walked in 6 minutes (m)	Cardiolipin ratio (normal <0.2)	Left ventricular size (cm)		LVIDd Z-score	Left ventricular systolic size (cm)	LVIDs Z-score	Left ventricular mass (g)	Left ventricular fractional shortening (%)	MGC (nmol/l) ^b	TAZ genotype	Comment
						diastolic size	size								
1	2	79.68 (-2.47)	10.8 (-1.77)	NA	34.0	2.88	-0.21	2.25	2.23	41	22.00	3,016.4	p.Trp194X	—	
2	4	88.39 (-3.07)	12.43 (-2.30)	NA	22.5	4.00	3.96	2.89	5.02	42	27.73	NA	c.778-24_778-7delinsA	—	
3	4	88.9 (-3.25)	12.43 (-2.60)	NA	17.3	3.44	1.54	2.44	2.60	60	29.06	1,048.5	p.His176tyr	—	
4	4	96.77 (-1.82)	12.75 (-2.66)	NA	3.9	4.82	7.11	4.07	10.89	68	15.55	592.9	c.583+5G>A	—	
5	5	119.48 (1.71)	24.22 (1.54)	NA	NA	3.79	0.05	2.52	0.47	121	33.46	NA	p.Gly140Arg	—	
6	6	103.48 (-2.3)	13.3 (-4.03)	NA	28.6	3.98	3.20	3.03	5.08	48	23.84	NA	p.Asp75His	—	
7	6	102.1 (-2.85)	17.04 (-1.77)	256.3	57.7	3.44	0.19	2.40	1.17	73	30.05	964.1	Ex1_Ex4 del	—	
8	6	118.3 (0.201)	21.34 (-0.021)	NA	19.7	NA	NA	NA	NA	NA	NA	NA	Unknown	Cardiac transplant	
9	7	112.4 (-1.33)	19.64 (-0.91)	313.9	NA	3.84	1.07	2.64	1.69	82	30.99	NA	p.Gly116Arg	—	
10	9	122.5 (-1.86)	18.64 (-3.52)	436.8	NA	4.46	3.45	3.26	4.52	110	27.05	NA	Ex1_Ex4 del	—	
11	9	121.3 (-2.69)	21.09 (-2.99)	411.5	18.4	NA	NA	NA	NA	NA	NA	NA	p.Ile54Asn	Cardiac transplant	
12	10	136 (-0.182)	24 (-1.70)	405.4	16.0	3.97	0.45	2.80	1.43	47	29.53	746.3	p.Gly197Arg	—	
13	10	133 (-1.00)	24 (-2.07)	457.2	NA	3.66	-0.63	2.40	-0.21	61	35.00	NA	Ex3_Ex5 del	—	
14	11	138.4 (-0.59)	31.18 (-0.66)	475.5	8.8	4.19	0.35	2.98	1.40	68	28.86	390.4	p.Gly197Arg	—	
15	11	139.5 (-0.43)	34.54 (-0.08)	404.2	NA	3.58	-2.04	2.47	-0.81	90	31.08	NA	p.Arg292Leufs*49	—	
16	11	121 (-3.32)	19.18 (-4.60)	304.8	40.9	5.25	6.43	4.04	8.03	144	23.06	NA	Ex1_Ex5 del	—	
17	11	131.5 (-2.06)	26.82 (-3.09)	527.3	2.7	3.98	0.21	2.95	1.81	78	25.83	705.6	p.Arg292Leufs*49	—	
18	11	132.5 (-1.91)	26.36 (-2.23)	336.5	4.8	3.75	-0.60	2.58	0.31	91	31.10	NA	p.Arg292Leufs*49	—	

LVIDs, left ventricular internal diameter at end systole; MGC, methylglutaconic acid.

^aZ-scores for height and weight are not available for individuals older than 240 months old. ^bNormal values for 3MGC at 0–2 years: 144 ± 58 (SD) nmol/l, at 2–13 years: 162 ± 68 (SD) nmol/l, and at older than 12 years 157 ± 66 (SD) nmol/l.

Table 1 Continued on next page

Table 1 Continued

Study ID	Age at study (years)	Height (cm)	Height (Z-score) ^a	Weight (kg)	Weight (Z score) ^a	Distance walked in 6 minutes (m)	Cardiolipin ratio (normal <0.2)	Left ventricular			LVIDd Z-score	Left ventricular systolic size (cm)	LVIDs Z-score	Left ventricular mass (g)	Left ventricular fractional shortening (%)	MGC (nmol/l) ^b	TAZ genotype	Comment
								ventricular diastolic size (cm)	ventricular size (cm)	ventricular mass (g)								
19	13	141.7 (-1.81)	44.54 (-0.099)	467.3	NA	3.88	NA	3.88	2.41	-1.76	134	37.94	NA	p.Gly197Arg	—			
20	14	134.25 (-3.40)	24.89 (-4.62)	332.8	17.6	3.79	14.3	3.79	2.22	-1.04	59	41.29	NA	Unknown	—			
21	15	144 (-2.74)	29.18 (-4.02)	490.7	14.3	NA	14.3	NA	NA	NA	NA	NA	NA	895.6	c.110-2A>G	Cardiac transplant		
22	15	163.4 (-0.57)	44.18 (-1.19)	367.0	27.1	NA	27.1	NA	NA	NA	NA	NA	NA	1,407.0	Unknown	—		
23	15	155 (-1.57)	34.64 (-2.82)	363.3	23.8	3.94	23.8	3.94	2.47	-0.98	110	37.38	700.5	c.110-2A>G	—			
24	15	143.5 (-3.00)	39.36 (-2.11)	386.5	17.0	4.11	17.0	4.11	2.66	-0.43	76	35.21	1,497.9	p.Gly161Arg	—			
25	16	163.6 (-1.38)	36.82 (-3.68)	345.3	21.8	4.38	21.8	4.38	2.95	0.53	106	32.70	790.5	p.Glu250X	—			
26	17	172.8 (-0.41)	41.27 (-3.66)	358.1	22.1	4.38	22.1	4.38	3.31	1.37	155	24.46	801.1	p.Trp79x	—			
27	18	165.4 (-1.44)	46.18 (-2.69)	425.2	24.2	4.56	24.2	4.56	3.51	1.82	124	23.13	415.3	p.gly80_Ser81ins12	—			
28	19	175.5 (-0.16)	58.36 (-1.19)	536.4	18.9	5.09	18.9	5.09	3.67	1.55	162	27.93	1,192.0	p.Ile209Asn	—			
29	20	147.29	29.5	NA	9.9	3.32	9.9	3.32	2.44	-2.63	38	26.54	NA	c.699+1G>T	Nonambulatory			
30	21	168.8	52.3	403.6	27.2	4.76	27.2	4.76	3.11	0.19	161	34.77	NA	p.Arg94cys	—			
31	21	165.8	65.4	405.4	15.3	5.50	15.3	5.50	3.91	2.06	130	28.84	555.9	p.Leu42x	—			
32	22	180.8	66.2	315.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	c.109+5G>A	Cardiac transplant			
33	22	173.3	72.0	350.5	NA	NA	NA	NA	NA	NA	NA	NA	NA	p.His69Gln	—			
34	23	181.2	75.2	441.4	23.6	4.31	23.6	4.31	2.54	-2.17	184	41.04	NA	p.Ile235Asnfs*76	—			
35	25	165	44.4	407.8	30.2	4.92	30.2	4.92	3.65	2.37	182	25.74	NA	p.Ile235Asnfs*76	—			
36	25	179.3	63.4	349.0	41.4	6.28	41.4	6.28	5.38	6.20	189	14.42	1,529.2	p.gly80_Ser81ins12	—			
37	26	181.1	92.2	339.9	36.5	5.26	36.5	5.26	3.63	-0.31	274	30.89	NA	c.110-2A>G	—			
38	27	178.7	72.2	484.6	20.0	6.13	20.0	6.13	4.63	6.31	201	24.45	2,507.8	p.Tyr51X	—			
39	28	183.1	60.5	379.2	22.3	4.66	22.3	4.66	3.21	0.04	184	31.13	541.2	c.238+2T>G	—			
40	31	159.8	64.1	300.5	51.4	5.92	51.4	5.92	2.85	5.11	180	17.27	2,259.0	p.Arg94Leu	—			
41	32	186.2	89.1	278.0	27.4	5.59	27.4	5.59	3.89	0.99	205	30.45	NA	p.Gly58AlafsX25	—			
42	33	168.5	64.2	301.8	54.1	4.63	54.1	4.63	3.15	-0.13	503	31.91	NA	p.Gln233X	—			

LVIDs, left ventricular internal diameter at end systole; MGC, methylglutaconic acid.

^aZ-scores for height and weight are not available for individuals older than 240 months old. ^bNormal values for 3MGC at 0–2 years: 144 ± 58 (SD) nmol/l, at 2–13 years: 162 ± 68 (SD) nmol/l, and at older than 12 years: 157 ± 66 (SD) nmol/l.

space limitations at the study location: the walking course was 50 feet (~15 m) as opposed to 30 m (100 feet) and the walk was performed on a carpeted surface.^{22,23}

For subject safety, paramedics were present in the room where subjects completed the 6MWT. Prior to the test, subjects received a 10-minute seated rest break. After the rest period, the following baseline measurements were obtained utilizing a pulse oximeter: heart rate (HR) and SPO₂. Next, the subject stood and the tester assessed baseline dyspnea and fatigue utilizing the modified Borg scale²⁴ (**Supplementary Table S1** online). After completion of the test, dyspnea and fatigue were recorded utilizing the modified Borg scale and HR and SPO₂ were recorded utilizing the pulse oximeter. All participants in the 6MWT were independently ambulatory, and no participant required the use of adaptive mobility equipment that would limit his or her ability to smoothly make turns at the 15-m marks.

Muscle strength

Handheld dynamometry. Muscle strength was measured by a single physical therapist with expertise in muscle strength measurements using published, standardized methodology via a Microfet II handheld dynamometer (Hoggan Health Industries, Draper, UT). The Microfet handheld dynamometer has a sensitivity of 0.1 lb and a range of 0.8 to 150 lb. Force was read in pounds; the upper range was subjectively chosen at 90 pounds (~400 N) based on the actual force of the investigator. At the beginning of each day of testing, the dynamometer was calibrated with a 2.0-kg weight for two trials. Maximum isometric contraction values were measured in five different muscle groups by using the “make” technique for 5–6 seconds (the maximal force exerted by the subject while the examiner holds the dynamometer stationary with verbal instructions “push as hard as you can”).^{25,26} The order of muscle testing and the position of the subject and the dynamometer remained the same for each subject (**Supplementary Table S2** online). The muscle groups were tested bilaterally. The single best value obtained from either side was utilized for data analysis. The handheld dynamometry strength data are assessed in two ways: normalized to weight and raw to account for possible differences in body size. Results were compared with validated, published normal controls, as has been done previously in similarly published cohort studies.^{27–29}

Manual muscle testing. After completion of the muscle strength testing via handheld dynamometry, manual muscle testing (MMT) was completed in the following muscle groups: hip flexors, knee extensors, hip abductors, hip extensors, and knee flexors bilaterally. MMT was performed utilizing the techniques as reported previously^{25,30} and scored on a scale of 0–5 (**Supplementary Table S3** online).

Physical activity questionnaires

Prior to participation in the study, all subjects or their parents (depending on age) were asked to complete Physical Activity Questionnaires to gain knowledge of the general physical activity levels of the subjects during their daily typical routine.

Subjects/parents of subjects 6–14 years of age completed the Physical Activity Questionnaire for Children (PAQ-C). The PAQ-C is a well-validated, self-administered, 7-day recall instrument that was developed to assess general levels of physical activity for children 8 to 14 years of age.^{33,34} The PAQ-C provides a summary physical activity score derived from nine items, each scored on a 5-point scale. A PAQ-C activity summary score of 1 indicates low physical activity, whereas a score of 5 indicates high physical activity.^{31–33}

Subjects 15 years of age and older completed the International Physical Activity Questionnaire (IPAQ), Short Form, which is designed primarily for population surveillance of physical activity among adults (age 15–69 years). The IPAQ is a well-validated tool used to evaluate time spent being physically active, and the vigorousness of that activity, in the prior 7 days (**Supplementary Table S4** online).³⁶

Cardiac function

Transthoracic echocardiography was performed in 36 participants by a single sonographer using a Vivid E9 machine (General Electric Healthcare, Milwaukee, WI), with

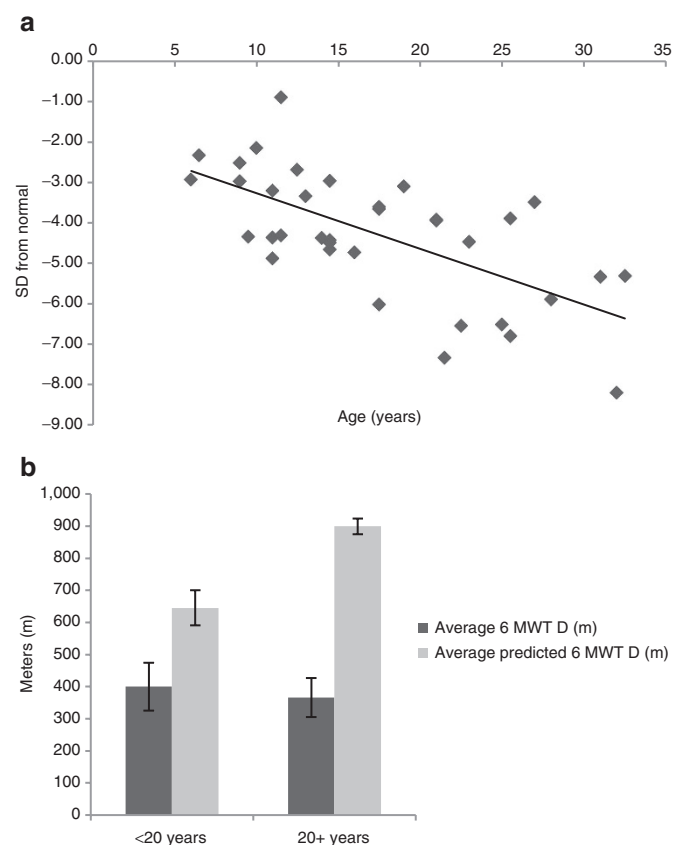


Figure 1 Performance on 6-minute walk test on a 15-m-long course. (a) As represented by SD from normal controls (refs. 19–23), only one individual performed within the normal range. (b) Average 6MWT distance (m) versus average predicted 6MWT distance (m) for subjects 4–19 years of age and for subjects 20–32 years of age. Average predicted values for subjects were calculated utilizing regression equations that take into account height and age.

measurements made offline by a single investigator who was blinded to the results of the other studies. The echocardiogram analysis included M-mode measurements of standard cardiac structure, size, and function parameters. LV mass was calculated using the method of Devereaux according to the American Society of Echocardiography guidelines.³⁵ Z-scores were calculated for all parameters.^{36–38}

Cardiolipin analysis

A punch (one-quarter-inch diameter) of a dried bloodspot on filter paper (Guthrie card) was transferred to a 2-ml tube, to which 1 ml methanol/chloroform (1:1, vol/vol) was added. After the addition of 5 µl of 10 µmol/l CL(14:0)₄, the sample was vortex-mixed and incubated for 15 minutes 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, N₂). The residue was reconstituted in 50 µl methanol, transferred to a sample vial, and capped. We used 10 µl for ultra -high -performance liquid chromatography–tandem mass spectrometry analysis. Chromatographic separation was achieved on a Thermo Scientific DionexUltiMate 3000 Rapid Separation system (Thermo Fisher Scientific, Waltham, MA) equipped with an analytical BEH C₈-column 2.1 by 50 mm and 1.7 µm particle size. Samples were eluted on a linear gradient between 0.1% NH₃ (25%) vol/vol and 2-propanol in 5 minutes. We performed mass spectrometry analyses on a Q Exactive plus (Thermo Fisher Scientific) operated in the negative ion electrospray ionization mode using parallel reaction monitoring for the following ions: CL(72:8): m/z 723.98>279.23, CL(14:0)₄; m/z 619.92>227.20, MLCL (52:2); and m/z 582.77>281.25 + m/z 582.77>255.23. The ratio between MLCL (52:2) and CL(72:8) was termed the MLCL/CL ratio.

3MGC analysis

Plasma 3MGC was measured in a random subset of plasma from 20 participants (in the remaining participants an insufficient volume of plasma was collected for this metabolite analysis). An aliquot of plasma was acidified in hydrochloric acid and combined with an isotopic internal standard of 3MGC. Samples were then extracted twice with ethylacetate, and the upper layer was removed and dried under a slow stream of nitrogen gas. Dried samples were derivatized with MTBSTFA (N-Methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide), combined with 100 µl of acetonitrile, and heated to 80 °C for 60 minutes. Gas chromatography mass spectrometry analyses were performed with monitoring for the characteristic ions m/z 315, 317, 318.

Plasma amino acid analysis

Plasma amino acids, including arginine, were measured on a random subset of plasma from 17 participants via ion exchange chromatography (in the remaining participants an insufficient volume of plasma was collected for this metabolite analysis). Samples were deproteinized with one-tenth of the sample volume of 35% sulfosalicylic acid. This was centrifuged for 3 minutes, and the supernatant was removed and used for further analysis. Quantification of amino acids was conducted by ion-exchange liquid chromatography with ninhydrin detection on a Biochrom 20 or 30 Amino Acid Analyzer (Biochrom US, Holliston, MA).

RESULTS

Patient cohort

The study group included 42 males ranging in age from 24 months to 32 years 7 months (average age 15 years 6 months ± 8 years (SD)) from 37 individual BTHS pedigrees. A mutation-confirmed diagnosis of BTHS was available for

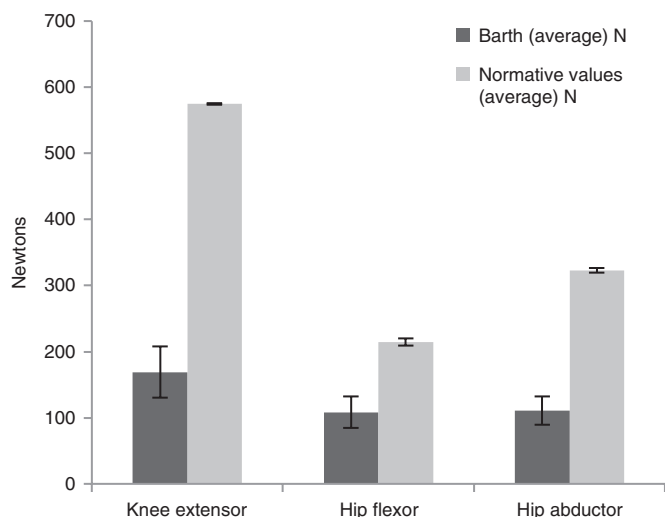


Figure 2 Average force of knee extensors, hip flexors, and hip abductors for subjects 20–32 (*n* = 13) years of age versus age-matched published normative values.²⁶ The average force is statistically significantly different from published normative values at *P* < 0.05.

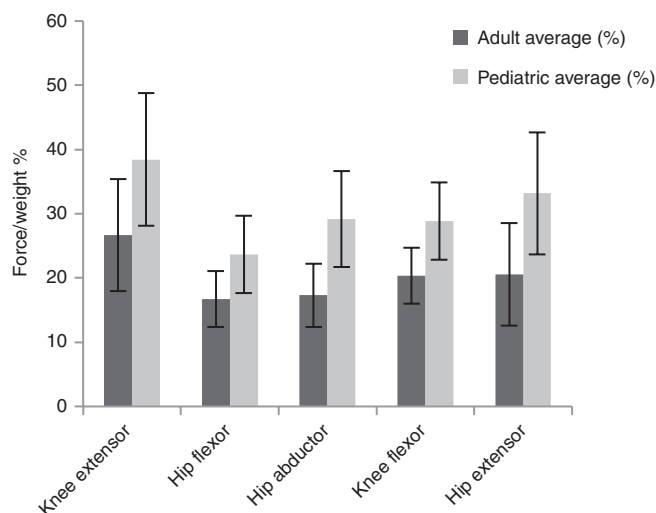


Figure 3 Comparison of average force/weight % of knee extensors, hip flexors, hip abductors, knee flexors, and hip extensors in adult (age 20 or older) versus pediatric subjects (age 6–19) with Barth syndrome.

39/42 participants (Table 1). Three patients with only parental report of a mutation-confirmed diagnosis had typical clinical features of BTHS (cardiomyopathy and neutropenia) as well as characteristic biochemical abnormalities, including increased plasma and urine levels of 3MGC. Baseline vital signs taken prior to clinical testing revealed that none of the participants had hypertension, all had heart rates in the normal range, and all had normal oxygen saturations.

The 6MWT

Functional exercise capacity (which requires cardiovascular exertion and muscular strength and endurance) was measured in 34/42 participants ages 6 years 8 months to 32 years 7 months via the 6MWT. Of the patients who did not participate in the 6MWT, one of eight was too young to participate (younger than 3 years), three of eight had noncardiac/nonskeletal muscle mobility-limiting disabilities, and four of eight had difficulty with compliance with the evaluation (all were younger than 6 years of age).

The 6MWT results were compared to those of controls described by Geiger *et al.*¹⁹ for individuals 3–18 years of age, and to adult controls described by Chetta *et al.*³⁹ Patients were compared to controls with a matched mid-height range, as opposed to age range, due to the significant growth delay characteristic of Barth patients and known effect of height on 6MWT results.⁴⁰ Subjects with BTHS had significantly reduced endurance, with Z-scores of -4.39 on 6MWT (range -0.9 to -8.18). Individuals in the oldest age cohort tended to have the worst performance during this evaluation (Figure 1). On average, the subjects aged 4–19 walked 62.3% of the value predicted on the 6MWT as seen by multiple regression accounting for age and height.⁴¹ Using the same kind of analysis, the subjects aged 20–32 years walked 40.63% of the value predicted on the 6MWT.^{39,40}

Dyspnea and fatigue and 6MWT distance

The difference between baseline fatigue between younger subjects (age 4–19) and older subjects (age 20–32) was statistically significant as measured by the Borg scale, with older subjects having more fatigue ($P < 0.05$, $P = 0.02$). For subjects aged 4–19, there was a moderate positive correlation between dyspnea and fatigue and the 6MWT distance (Supplementary Table S5 online). For subjects aged 20–32, there was a strong positive correlation between the difference of end fatigue and baseline fatigue and 6MWT distance ($r = 0.47$). Baseline HR, end HR, end HR-baseline HR, baseline SPO₂, final SPO₂ and final SPO₂-baseline SPO₂ for subjects aged 4–19 and 20–32 were also measured (Supplementary Table S5 online).

Strength values

Adults (subjects age 20–32 years). Strength measurements obtained via handheld dynamometry in participants 20–32 years of age were compared to published, normative values (such values are not available for matched younger age groups).

Individuals with BTHS had significantly lower knee extensor strength, hip flexor strength, and hip abductor strength (Figure 2). Subjects with BTHS demonstrate knee extensor strength that is 29.42% of normative values, hip flexor strength that is 48.21% of normative values, and hip abductor strength that is 34.28% of predicted values. These trends also hold true when normalized for weight (muscle force as a percentage of body weight) (Supplementary Figure S1 online).

As in prior publications, we found that strength scores obtained via MMT each correlated with a wide range of handheld dynamometry measurements³⁰ (Supplementary Tables S6–S9 online). For example, in adult BTHS subjects with a knee extensor manual measurement score of 5/5, handheld dynamometry measurements ranged from 93.86 to 236.65 N in individual patients. This finding has been attributed to the subjectivity of MMT, as well as the limited range of scoring possibilities in the MMT scale.³⁰ Therefore, we found the handheld dynamometry measurement to be a more useful assessment of participant muscle strength.

Comparison of adult versus pediatric strength measurements

The force/weight % generated by subjects 4–19 years of age was significantly more than the force/weight % generated by subjects 20–32 years of age for knee extensors ($P < 0.05$, $P = 0.001$), for knee flexors ($P < 0.05$, $P = 4.01 \times 10^{-5}$), for hip flexors ($P < 0.05$, $P = 0.001$), for hip extensors ($P < 0.05$, $P = 0.0003$), and for hip abductors ($P < 0.05$, $P = 3.60 \times 10^{-6}$) (Figure 3 and Supplementary Table S10 online).

In the entire cohort (adult and pediatric subjects) there was a weak positive correlation between knee extensor strength ($r = 0.25$), knee flexor strength ($r = 0.24$), and hip flexor strength (0.23) and distance walked on the 6MWT (Supplementary Table S11 online).

Physical activity

Subjects age 6–14. In total, 16/17 subjects between ages 6 and 14 who participated in the physical therapy portion of the study completed the PAQ-C. Of the 16 PAQ-C questionnaires that were completed, two were removed from analysis secondary to legibility issues. The average PAQ-C Activity score calculated from the questionnaires was 2.41 ± 0.73 (SD). This indicates that, on average, the subjects assessed at ages 6–14 demonstrate a low to moderate level of physical activity. There was a strong positive correlation between PAQ-C Activity score and distance walked on the 6MWT ($r = 0.64$), supporting the impact that exercise tolerance has on quality of life.

Subjects aged 15–32. In total, 17 subjects 15–32 years of age completed the IPAQ. One questionnaire was removed from analysis secondary to subjects participating in atypical physical activity for 2 weeks prior to the conference. From the completed IPAQ forms, the following were calculated: total metabolic equivalent of task-minutes per week, the average amount of time

spent sitting per day, and a categorical classification regarding activity level (**Supplementary Table S12** online). On the 16 questionnaires utilized in the analysis, five subjects indicated a low physical activity level, four reported a moderate physical activity level, and seven reported a high physical activity level. There was a strong positive correlation between days per week spent walking and 6MWT distance ($r = 0.53$). There was a weak, negative correlation between average amount of time sitting per day and distance walked on 6MWT ($r = -0.28$).

Cardiac function

Transthoracic echocardiography was performed for 36/42 participants. Of the 6 patients who did not receive echocardiograms, 4 had cardiac transplants and 2 refused echocardiograms. Measurements were taken of LVIDd and LVIDs, left ventricular FS, and left ventricular posterior wall thickness at end-diastole (**Table 1**).

Of the 29 individuals who completed the 6MWT and underwent echocardiogram, left ventricular size and function, including LVIDd, LVIDs, and FS, were not related to the performance on the 6MWT as analyzed via a generalized linear regression model in the statistical analysis program R.¹⁸ Lack of correlation between cardiac function and 6MWT performance has previously been reported in other studies of cardiac disease.⁴²

However, it should be noted that the seven participants who underwent echocardiograms but did not complete the 6MWT were significantly younger than those who underwent echocardiograms and completed the 6MWT (6.6 years \pm 6.2 (SD) vs. 15.61 years \pm 8.77 (SD) years (P value 0.013)) and had a significantly lower fractional shortening (25.34% \pm 5.6 (SD) vs. 29.8% \pm 6.18 (SD) (P value 0.010)). Cardiac status was not the apparent reason for nonparticipation in these seven nonparticipants (see above).

Cardiolipin analysis

MLCL/CL ratios were measured in 34/42 patients. The remaining 8 patients refused blood draws or we were unable to obtain blood after two vein-puncture attempts. The average MLCL/CL ratio was 23.5 \pm 13 (SD) (range, 2.67–54.05), with normal controls of <0.23 .

Data were analyzed using a generalized linear regression model in the statistical analysis program R.¹⁸ When correcting for height, MLCL/CL was inversely correlated with the distance walked on the 6MWT, with a P value of 0.00014.

In terms of MLCL/CL relationship to cardiac parameters, when we corrected for height, age, and weight, increasing MLCL/CL was correlated with increasing LVM, with a P value of 0.0374 (**Supplementary Figure S2** online). There was no correlation between cardiolipin ratio and LVIDd, LVIDs, or FS.

Arginine and 3MGC measurements

Average arginine in the subset of 17 participants was 58.2 $\mu\text{mol/l}$ \pm 38 (SD). Normal values for this amino acid are 69.8 $\mu\text{mol/l}$ \pm 36 (SD).¹⁵ Average plasma 3MGC in the random

subset of 20 participants was 1127.8 nmol/l \pm 724 (SD). Normal values for this metabolite are 162 \pm 68 (SD) nmol/l.¹⁵

LVM, LVIDd, LVIDs, FS, and 6MWT distance were not significantly related to either 3MGC or arginine via regression analysis.

Genotype

A TAZ mutation-confirmed diagnosis of BTHS was available for 39/42 of the participants. Of 39 patients, 13 have a missense mutation, 6 have a nonsense mutation, 8 have a splicing mutation, 6 have a small out-of-frame insertion or deletion, 2 have a small in-frame insertion, and 4 have a large deletion encompassing several exons. This is similar to the overall breakdown of mutation types in the general BTHS population.¹⁶

Of the patients with missense mutations, a total of 11 individual alleles accounted for 13 patients. Of the patients with nonsense mutations, a total of six individual alleles accounted for six patients. Of the patients with splicing mutations, a total of six individual alleles accounted for eight patients. Of the patients with out-of-frame in/dels, a total of three individual alleles accounted for six patients. Of the patients with in-frame insertions, one allele accounted for two patients. Of the patients with large deletions, a total of four individual alleles accounted for four patients. Several notable genotype/cardiolipin relationships were uncovered among these.

Two alleles accounted for the three patients with the lowest MLCL/CL ratios. Two siblings (study IDs 17 and 18) had 5 bp duplication at the end of exon 11, causing an addition of 49 amino acids. They had MLCL/CL ratios of 4.76 and 2.67. The third patient (study ID 4) had a single-base-pair variant in the +5 position of exon 7, which is thought to lead to a splicing abnormality. He had a MLCL/CL ratio of 3.92.

Interestingly, the two patients (study IDs 7 and 16) with large deletions (exons 1–4 and exons 1–5) who had MLCL/CL measurements had significantly higher MLCL/CL ratios than the three patients with nonsense mutations early in the gene, all of which are in exon 2 (average ratios 49.2 vs. 19.1, $P = 0.021$). This implies that these nonsense mutations may not represent “true biochemical nulls” and that there may, in fact, be a mechanism allowing for some residual activity. Whether this is due to an alternative start site or another mechanism for read-through is not clear at this time. Alternatively, the possibility that proximal upstream modifying genes are disrupted in the individuals with these large 5' deletions has not been excluded. This requires further analysis.

DISCUSSION

We conducted an extensive multidisciplinary investigation in a large cohort of patients with BTHS with the goal of gaining a greater understanding of the pathophysiology of this disease. Through this study we defined previously unrecognized metabolite/phenotype relationships and individual metabolite/phenotype/genotype relationships, established potential targets for therapeutic monitoring, and validated new avenues for clinical assessment.

There is a lack of reliable measures of outcome in BTHS because the characteristic waxing and waning nature of the cardiac function in BTHS make this a difficult target for measuring success of clinical interventions. Additionally, 3MGC, an excreted metabolite characteristic of BTHS, is very variable over time in any single individual and does not correlate with cardiac status (unpublished observations). In fact, it is well recognized that some individuals with significant disease have had normal measurements of urinary 3MGC.¹⁴ Our study further confirms this lack of a direct relationship between 3MGC and quantifiable clinical parameters.

Our studies show that the cardiolipin ratio has at least an association with, and possibly an impact on, several important aspects of clinical status, including functional exercise capacity and LV mass. Therefore, the MLCL/CL ratio has the potential to represent a viable biochemical target for long-term therapeutic monitoring. Likewise, 6MWT and LV mass have the potential to serve as independent clinical markers for therapeutic monitoring.

The association of increasing LVM and abnormal tetralinoleoyl-cardiolipin was previously reported in a rat model of spontaneous heart failure in which investigators uncovered a statistically significant inverse relationship between LVM and tetralinoleoyl-cardiolipin content in interfibrillar and subsarcolemmal cardiac mitochondria not associated with the aging process.⁵ The authors further studied several human hearts affected with idiopathic dilated cardiomyopathy and uncovered both a reduction in tetralinoleoyl-cardiolipin and an increase in minor cardiolipin species.⁵ Thus, abnormalities in cardiolipin content appear to be an intrinsic abnormality in some cases of human heart failure and, even more significantly, seem to be directly related to cardiac mass in the mouse model. Although the mechanisms for abnormal cardiolipin accumulation in idiopathic forms of heart failure are not clear, our findings provide evidence for a common downstream biochemical mechanism for both common and rare forms of cardiac failure.

A quantitative correlation between increasing muscle dysfunction and abnormal cardiolipin species has not been reported prior to this study. However, chronic use and disuse of muscles in a rat model was shown to affect expression of cardiolipin synthetic enzymes.⁴³ More research is warranted to determine whether cardiolipin abnormalities are a mechanism of skeletal dysfunction in other conditions, although this seems to be a plausible hypothesis.

In term of BTHS progression, there are several indicators of the progression of skeletal muscle disease with age, including worsening functional exercise capacity, decreased strength as measured by force/weight % of knee and hip musculature, and increased reported fatigue. Possibly, progression of muscle disease is due solely to an intrinsic characteristic of the primary pathology of BTHS or is also compounded by other mechanisms of cardiolipin dysfunction secondary to chronic muscle disuse.

As previously described by Bowron *et al.*,¹³ some BTHS patients have less severe cardiolipin abnormalities. This may

occur in conjunction with a relatively modified phenotype in some cases.¹³ They described this milder cardiolipin ratio and phenotype in three genotypes: c.170G>T (p.Arg57Leu), c.118A>G (p.Asn40Asp), and c.553A>G (p.Met185Val). Here, we additionally describe two genotypes with a milder cardiolipin ratio: c.583+5G>A and c.873_874dupCCTGG (p.Arg292LeufsX49). The two individuals with the c.873_874dupCCTGG have no history of neutropenia, and, although they had significant cardiomyopathy as infants, they now have normal cardiac function. One of these siblings was the only participant to perform in the normal range on the 6MWT. By contrast, the individual with the c.583+5G>A variant had severe cardiac disease necessitating cardiac transplantation but did not have “typical” BTHS facies and had better than average skeletal muscle mass bulk and physical evaluation.

From a practical clinical standpoint, we recommend that the 6MWT be administered at regular intervals during the assessment of individuals with BTHS, similar to the regularity with which echocardiograms are performed. This study revealed the 6MWT performance to be independent of cardiac function and correlated with measures of quality of life, including daily activity, and reported dyspnea and fatigue. The 6MWT distance is potentially susceptible to differences in testing conditions (in this case a 15-m walk track was used as opposed to a 30-m walk track).⁴⁴ Therefore, when using 6MWT to monitor clinical status, practitioners should be careful to maintain consistent test conditions.

Additionally, we found that muscle weakness may become more pronounced with age in BTHS, and sensitive quantification of this feature is probably an important measure of disease progression. There was great variability in handheld dynamometry scores in individuals who scored 5/5 in manual muscle testing of knee extensor strength. This suggests that MMT is not sensitive enough to detect and quantify important areas of muscle weakness in BTHS, such as in the knee extensors, and we recommend quantitative handheld dynamometry measures for the most accurate clinical assessment.

One drawback of this study is that it represents a single time-point observation in individual patients; therefore, we cannot definitively conclude that the MLCL/CL ratio will relate to clinical status over the lifetime of a patient or to the nature of the stability of this measurement over a patient's lifetime. Additionally, due to the single time-point observations, we cannot comment regarding the effectiveness of medical intervention (e.g., inotropic agents) on cardiac status. However, this type of long-term observation could potentially take years to complete, and the cumulative data presented above strongly suggest more abnormal parameters with age. To solve this question, long-term natural history studies are ongoing in our group. Studies relating the degree of cardiolipin abnormalities to cardiac or skeletal muscle phenotypes have also not been performed in animal models but could represent another interesting avenue for further research.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/gim>

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DISCLOSURE

The authors declare no conflict of interest.

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