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# Promotion of plasmalogen biosynthesis reverse lipid changes in a Barth Syndrome cell model

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ABSTRACT

In Barth syndrome (BTHS) mutations in tafazzin leads to changes in both the quantities and the molecular species of cardiolipin (CL), which are the hallmarks of BTHS. Contrary to the well-established alterations in CL associated with BTHS; recently a marked decrease in the plasmalogen levels in Barth specimens has been identified. To restore the plasmalogen levels, the present study reports the effect of promotion of plasmalogen biosynthesis on the lipidome of lymphoblasts derived from Barth patients as well as on cell viability, mitochondria biogenesis, and mitochondrial membrane potential. High resolution $^{31}$P NMR phospholipidomic analysis showed an increase in the levels of plasmenylethanolamine (the major plasmalogen in lymphoblasts), which reached values comparable to the control and a compensatory decrease in the levels of its diacyl-PE counterpart. Importantly, $^{31}$P NMR showed a significant increase in the levels of CL, while not altering the levels of monolysocardiolipin. Mass spectrometry measurements showed that the promotion of plasmalogen biosynthesis did not change the molecular species profile of targeted phospholipids. In addition, promotion of plasmalogen biosynthesis did not impact on cellular viability, although it significantly decrease mitochondria copy number and restored mitochondrial membrane potential. Overall, the results showed the efficacy of the promotion of plasmalogen biosynthesis on increasing the CL levels in a BTHS cell model and highlight the potential beneficial effect of a diet supplemented with plasmalogen precursors to BTHS patients.

## 1. Introduction

Barth Syndrome (BTHS) is an X-linked recessive disease caused by mutations in the gene that encodes tafazzin [1,2]. Tafazzin is a lysophospholipid-phospholipid transacylase involved in the last step of cardiolipin (CL) biosynthesis [3,4]. In BTHS loss-of-function of tafazzin leads to a lower content of CL as well as more heterogeneous molecular species of this lipid and accumulation of monolysocardiolipin (MLCL), which are considered the hallmark of BTHS [5–7]. In eukaryotes, CL is a mitochondria-specific lipid, which has been reported to play crucial roles in the structure and function of mitochondria [8]. Hence, it is not unexpected that a major focus of research in this disease has been to understand the molecular events resulting from changes in both the quantities and the molecular species of CL on mitochondrial structure and function.

In BTHS mitochondrial morphology is aberrant, presenting swollen and enlarged mitochondria with malformed cristae structures, which have lower surface area and appear round and disconnected from the peripheral membrane, instead of parallel cristae that often extend across the entire organelle observed in controls [9]. In addition, CL also plays a role in keeping the integrity of the inner mitochondrial membrane (IMM) and alterations that compromise it would result in dissipation of the proton gradient that drives ADP phosphorylation. Indeed, BTHS mitochondria exhibit increased proton leakage, decreased membrane potential, and a decreased respiratory coupling index [9–12]. Moreover, in Barth specimens, changes in CL lead to destabilization of mitochondrial super complexes (SC) [9,13,14]. Likewise, assembly of mitochondrial SC induces CL remodeling and, as a consequence, its stabilization [15]. Mitochondria are key players in cellular redox metabolism and disruption of mitochondrial SC is known to result in increased production of reactive oxygen species (ROS) [16,17]. Indeed, in several BTHS specimens elevated ROS production has been reported [9,12,18,19]. Cells from BTHS patients usually display increased mitochondria biogenesis as indicated by the rise in the AMP/ATP ratio and mitochondria copy number when compared to controls, which is proposed to occur for cellular compensation due to the

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impaired mitochondria [9,11].

While the alterations in CL associated with BTHS are well-established, currently there has been an increased awareness of more widespread changes in the lipid composition of cells depleted of tafazzin function. Of note, it is the recent identification of a decrease in the plasmalogen levels in organs obtained from tafazzin knockdown mice as well as blood cells derived from BTHS patients, which clearly indicate that plasmalogen loss is associated with BTHS [20,21].

Plasmalogens are members of a subclass of glycerophospholipids, which differ from their diacylglycerophospholipid counterpart by having a fatty alcohol linked *via* a vinyl-ether bond at the *sn*-1 position of the glycerol moiety rather than an esterified fatty acid. Plasmalogens comprise up to 20% of the total phospholipid mass in humans and are constituents of the plasma membrane as well as intracellular membranes (nucleus, endoplasmic reticulum, post-Golgi network, mitochondria) [22]. Plasmenylethanolamine (PE-Pls) is the predominant plasmalogen found in most of human cells, except in cardiac and skeletal muscles and mature spermatozoa where high proportions of both PE-Pls and plasmenylcholine are found [22]. Although the physiological role of plasmalogen remains elusive, several studies had shed light on some of their physical, chemical, and biological functions. For instance, it has been reported that the vinyl-ether bond at the *sn*-1 position of the glycerol moiety brings the aliphatic chains closer together in comparison to their diacyl counterparts [23,24]. This leads to more packed membranes with decreased fluidity and increased order, while more easily promoting the formation of non-bilayer phases. In addition, the vinyl-ether bond is oxidation-labile rendering plasmalogens the ability to scavenger ROS, which led to the proposition they could act as a protection against the oxidation of polyunsaturated fatty acids (PUFAs) and other oxidation prone lipids [25–27]. Moreover, plasmalogens are believed to act as reservoirs of second messengers as they are, usually, enriched with PUFAs at the *sn*-2 position of the glycerol moiety, which are biologically active lipid mediators released upon plasmalogen hydrolyses by PLA2 [22].

A decrease in the steady-state levels of plasmalogens is associated with an impaired biosynthesis or an enhanced degradation. Currently the mechanism of plasmalogen depletion in BTHS at the molecular level is not known. However, in several diseases characterized by depletion in plasmalogens, promotion of their synthesis has been shown as a successful strategy to alleviate/treat those conditions [22]. Furthermore, disruption of plasmalogen biosynthesis impaired mitochondrial dynamics, which could be rescued upon promotion of plasmalogen biosynthesis [28]. While restoring plasmalogen levels is relatively straightforward to accomplish, previous attempts to increase CL levels were not successful by direct administration of this lipid *in vivo* [29]. Hence, it was hypothesized that by promoting plasmalogen biosynthesis it would be possible to restore to normal the levels of this lipid in Barth specimens and, eventually, normalize the lipidome and mitochondria function. To do so lymphoblasts derived from BTHS patients were used and compared with controls. Cells were administered with plasmalogen precursors and the lipid profile was characterized by high resolution $^{31}$P NMR phospholipidomic analysis and mass spectrometry and compared with cells without the administration of plasmalogen precursors. The effect of the treatment on cell viability, mitochondria biogenesis, and mitochondria membrane potential were also evaluated.

It is shown that the promotion of plasmalogen biosynthesis could restore the low levels of PE-Pls in this BTHS cell model to levels comparable to the control. In addition, the data showed a compensatory effect of the diacyl counterpart lipid upon plasmalogen precursors administration; that is, a decrease in the steady-state levels of diacyl-PE. Noteworthy, promotion of plasmalogen biosynthesis led to a significant increase in the levels of CL in BTHS lymphoblasts, while no changes were observed in the MLCL levels. Promotion of plasmalogen biosynthesis did not impact cell viability, although it significantly decrease mitochondria copy number and restored mitochondrial membrane potential to levels comparable to controls. Overall, the results highlight the efficacy of the promotion of plasmalogen biosynthesis on increasing the CL levels in a BTHS cell model and suggest that a diet supplemented with plasmalogen precursors could be beneficial for BTHS patients.

## 2. Materials and methods

### 2.1. Materials

Sodium dodecyl sulfate (SDS) was from Bioshop (Canada). 2-ethanesulfonic acid (MES), ethanolamine, CsOH, butylated hydroxytoluene (BHT), ethylenedinitrilo-tetraacetic acid (EDTA), bovine serum albumin (BSA), carbonyl cyanide p-(trifluoromethoxy)phenyl-hydrazone (FCCP), methanol (HPLC grade), and chloroform (HPLC grade) were from Sigma-Aldrich (St. Louis, MO). $D_2O$ (99.9 atom %D) was from Cambridge Isotope Laboratories (Tewksbury, MA). Roswell Park Memorial Institute (RPMI) 1640 Medium was from Gibco (Gaithersburg, MD). Fetal bovine serum (FBS) was from Wisent (Canada). 1-O-hexadecyl-*sn*-glycerol (HG) was from Avanti Polar Lipids (Alabaster, AL). All reagents were used as received.

### 2.2. Cell culture and plasmalogen precursor administration

Lymphoblast cell lines were established by Epstein–Barr virus transformation of the leukocytes isolated from the whole blood of two BTHS patients and their gender- and age-matched control subjects using Ficoll–Hypaque gradients as previously described [11]. BTHS and control lymphoblasts were cultured in RPMI 1640 medium in the presence of 10% FBS, penicillin (50 IU/mL), and streptomycin (50 μg/mL) at 37 °C in a humidified 5% $CO_2$ atmosphere. Cells were seeded at a density of *ca.* $2 \times 10^5$ cells/mL and passaged every 2–3 d. Administration of plasmalogen precursors was conducted by adding 30 μM of HG and 5 μM of ethanolamine (this component was added to avoid it becoming rate limiting for the synthesis of PE-Pls) 20 h before harvesting the cells [30]. Other two concentrations (2.5 fold above and below the one mentioned) of HG and ethanolamine were tested, but data is only presented for the above concentrations as they yielded the better recovery of PE-Pls and CL. HG was dissolved in ethanol and the final ethanol concentration in the cell suspension was 0.25% (v/v). Administration of vehicle to control and Barth lymphoblasts did not change significantly the phospholipids as measured by $^{31}$P NMR in either case (data not shown). Cells were harvested by centrifugation at 1000 ×*g* for 5 min at RT, the cell pellets were washed twice with PBS buffer, snap frozen in liquid $N_2$, and stored in a – 80 °C freezer until used.

### 2.3. Human lymphoblast lipid extraction for high resolution $^{31}$P NMR

Lipid extraction from lymphoblast cells for high resolution $^{31}$P NMR was conducted as described in Kimura and collaborators [21]. Briefly, a Cs-EDTA buffer was prepared by titration of a 200 mM EDTA solution with 50 wt% aqueous CsOH until pH 6.0 was reached, addition of 50 μM BHT, readjustment of the pH to 6.0, and degassing by argon bubbling [31]. Lymphoblast cell pellets were thawed on ice, resuspended in cold Cs-EDTA buffer (1.2 mL of buffer to *ca.* 600 μL cell pellet), and homogenized by striking the cell suspension 30 times with a Teflon pestle in a tight-fit glass vessel. Lipids from the homogenized cell suspension were, then, extracted by the Folch method using a chloroform:methanol (2,1, vol/vol) solvent mixture containing 250 μM BHT [32]. After removal of the top aqueous phase, the organic phase was washed twice with cold Cs-EDTA buffer. The organic phase was, then, collected, dried under a $N_2$ flux, and vacuum-dried for at least 2 h to form a lipid film. For high resolution $^{31}$P NMR measurements, lipid films were hydrated with 600 μL of degassed 10% (wt/v) SDS buffer (50 mM MES, 50 μM BHT, pH 6.0) containing 10% (v/v) $D_2O$.

### 2.4. High-resolution $^{31}P$ NMR

The phospholipidomic analysis of the lipids extracted from the lymphoblasts was conducted by measuring the high-resolution $^{31}P$ NMR spectra of the SDS solubilized lipids as described by Kimura et al. [21]. Briefly, samples were placed in 5-mm-diameter NMR tubes and their spectra were acquired on a Bruker AVANCE-III 700 MHz spectrometer ($^{31}P$ frequency, 283.4 MHz) equipped with a QNP cryoprobe at 25 °C. Spectra were acquired with an 80° excitation pulse on $^{31}P$ nuclei and inverse-gated broadband $^{1}H$ decoupling with the WALTZ-16 sequence at a 3.8 kHz decoupling frequency. Spectra were acquired over 12 ppm (3.4 kHz) bandwidth, 2.4 s acquisition time, 1.0 s recycle delay, and 2048 scans. The chemical shift scale was referenced to an external 85% (wt/v) phosphoric acid standard set to 0 ppm.

### 2.5. Human lymphoblast lipid extraction and LC-MS analysis

Lipid extractions were performed as previously described [33]. Briefly, lymphoblast cell pellets were resuspended in 1 mL of cold PBS. An aliquot of the mixture was taken to measure protein concentration using a Coomassie (Bradford) Protein Assay Kit (Thermo Scientific, Waltham, MA) following the manufacturer's instructions. Lipids were extracted after adding in 1 mL of methanol and 2 mL of chloroform into the remaining material in PBS using a glass Dounce homogenizer. The sample was transferred into a 2 dram glass vial and was centrifuged at 4 °C, 500 $\times g$ for 15 min to separate the aqueous and organic phases. The organic phase was transferred into a 1 dram glass vial and the solvent was removed by rotary evaporation. The dried lipids were re-suspended in chloroform, using volumes calculated to normalize samples based on protein concentration. Lipids were analyzed by LC-MS using Agilent Infinity 1260 HPLC / Agilent 6530 Jet Stream ESI-QTof-MS system using methods that were previously described [33]. The lipid extracts for each condition were combined and aliquoted for analysis.

For analysis of diacyl PE species, Mobile phase A consisted of 95% water and 5% methanol, Mobile phase B consisted of 60% isopropanol, 35% methanol and 5% water in negative ionization mode. The separation was performed using a Gemini C18 column with a C18 guard cartridge, and 0.1% (w/v) ammonium hydroxide was added to the mobile phases. The analysis of samples was performed using 5 μL injection volume and began with 5 min of 0% B at 0.1 mL/min, then the flow rate was increased to 0.5 mL/min and a mobile phase gradient began with 0% B to 100% B over 60 min, maintained at 100% at B for 7 min and switched to 0% B for 8 min. Data analysis was performed using Agilent MassHunter Qualitative Analysis Software where corresponding *m/z*'s for each ion were extracted and peak areas for each ion were manually integrated.

For analysis of MLCL and CL species, Mobile phase A used consist of 95% water and 5% methanol, Mobile phase B consisted of 60% isopropanol, 35% methanol and 5% water in negative ionization mode. The separation was performed using a Luna C5 column with a C5 guard cartridge, and 0.1% (v/v) ammonium acetate was added to the mobile phases. The analysis of samples was performed using 5 μL injection volume and began with 5 min of 0% B at 0.1 mL/min, then the flow rate was increased to 0.5 mL/min and a mobile phase gradient began with 0% B to 100% B over 40 min, maintained at 100% at B for 7 min and switched to 0% B for 8 min. Chromatographic alignments and data analysis were carried out using Agilent MassHunter Profinder Software due to the low abundances of these species.

### 2.6. Cellular ATP content

Cell viability was estimated by measuring the ATP content of the lymphoblasts using the ATPlite (Perkin-Elmer, Waltham, MA) assay system, which is based on the production of light (luminescence) caused by the reaction of ATP with added luciferase and d-luciferin. Briefly, 50 μL of lysis buffer were added to 100 μL of a lymphoblast (at a cell density of *ca.* $10^6$/mL) cell suspension per well in a 96-well microplate and incubated for 5 min at RT in a rocking shaker. Following, 50 μL of luciferase/d-luciferin solution were added to each well and incubated for 5 min at RT in a rocking shaker. The plate was dark adapted for 10 min and the luminescence was measured in a Synergy 4 microplate reader (BioTek, Winooski, VT). An ATP standard solution was used to build a calibration curve and the number of cells was quantified in a Countless cell counter from Invitrogen (Carlsbad, CA).

### 2.7. Mitochondria copy number

The mitochondria copy number in the lymphoblast cells were measured by quantifying the ratio of mitochondrial to nuclear DNA as previously described [34]. Briefly, the genomic DNA of lymphoblasts was extracted using NucleoSpin Tissue kit from Takara Bio (Japan) and had their concentration and purity quantified using a nanodrop. In a real-time PCR 96-well microplate (BioRad – Hercules, CA) the real time PCR master mix (TB green premix Ex TaqII, Tli RNAseH Plus; Takara Bio – Japan) was added, followed by a set of four optimized PCR primer pairs (one per well) targeting two nuclear and two mitochondrial genes from Takara Bio (Japan) and 10 ng of genomic DNA. Finally, real-time PCR was performed in a CFX96 qPCR from BioRad (Hercules, CA) and the ratio between mitochondrial and nuclear DNA was determined from the differences in Ct values for mitochondrial and nuclear DNA.

### 2.8. Mitochondria membrane potential

The mitochondria membrane potential was measured as previously described [11]. Briefly, *ca.* $10^6$ cells were incubated in 1.5 mL culture medium with 7.5 μM JC-1 (a mitochondrial membrane potential probe) from EMD Millipore (Burlington, MA) for 10 min at 37 °C in a humidified 5% $CO_2$ atmosphere. Stained cells were washed three times with PBS and suspended in 1 mL PBS containing 5% (g/100 mL) BSA. Following, stained cells were filtered with a Falcon round-bottom tube with a cell strainer and immediately analyzed by flow cytometry in a BD LSR Fortessa™ flow cytometer and data were analyzed by FlowJo (v. 10.6.1) software. The aggregate/monomer JC-1 emission ratio (red/green fluorescence ratio) was used as an estimate of mitochondrial membrane potential. As a reference, uncoupled mitochondria (cells pretreated with 1 μM FCCP) were used. Data is presented as the apparent mitochondrial membrane potential ($\Delta\Psi^{App}$ (%) = $[(F^{Red}{:}F^{Green} - F^{Red}_{FCCP}{:}F^{Green}_{FCCP}) / F^{Red}{:}F^{Green}] \times 100$).

### 2.9. Statistical analysis

Statistical analysis was done by performing one-way ANOVA test on data set and statistical significance was assumed for p-values $< 0.05$.

## 3. Results

### 3.1. Effect of the promotion of plasmalogen biosynthesis on the phospholipidome of BTHS lymphoblasts

To reverse the low levels of plasmalogens found in Barth specimens, a strategy based on the promotion of their biosynthesis was used. The effects of the promotion of plasmalogen biosynthesis were studied using lymphoblasts derived from BTHS patients and compared with controls. The cells from Barth patients have all the characteristics of cells from patients with this Syndrome, including the decrease in the levels of plasmalogens [9,11,21]. Plasmalogen biosynthesis begins in peroxisomes and is finalized in the endoplasmic reticulum (ER) where mature plasmalogen is formed and prepared to be transported to plasma and organelle membranes [22]. 1-O-alkylglycerols are plasmalogen precursors that enter the biosynthesis pathway at the ER; *i.e.*, downstream of peroxisomal steps [22]. Administration of alkylglycerols *in vitro* and

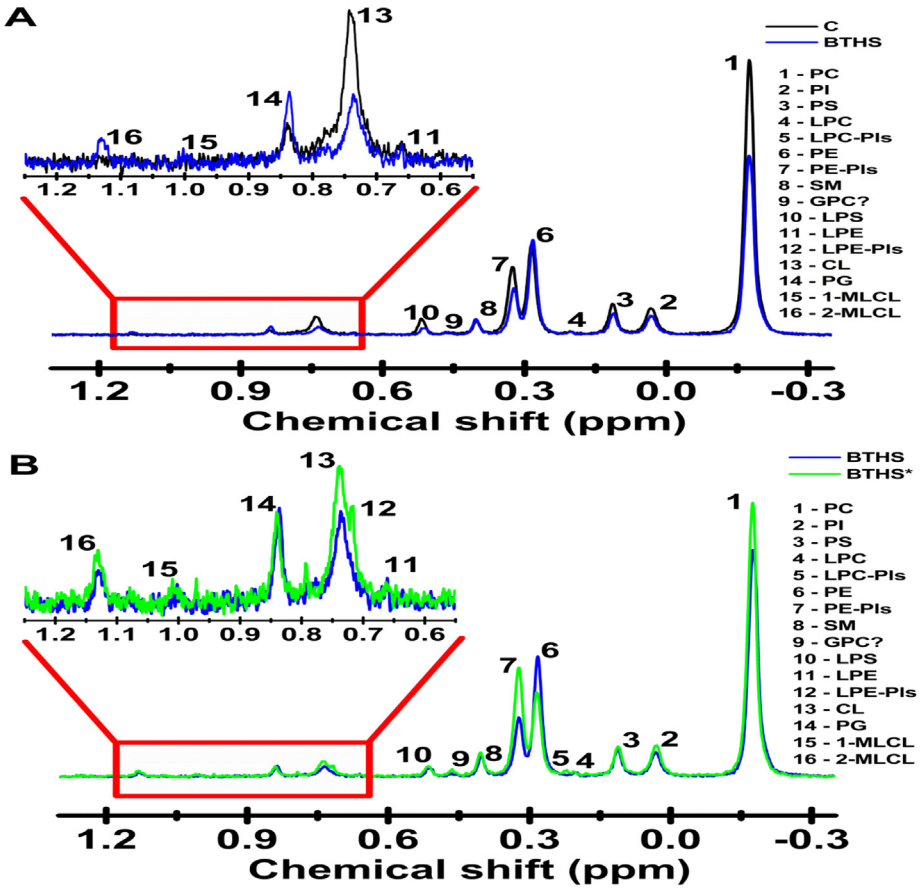


**Fig. 1.** High resolution $^{31}$P NMR spectra of lymphoblasts phospholipids. (A) High resolution $^{31}$P NMR spectra of phospholipids extracted from control, C, and Barth, BTHS, lymphoblasts are overlayed for comparison. (B) High resolution $^{31}$P NMR spectra of phospholipids extracted from Barth lymphoblasts, BTHS, and Barth lymphoblasts administered with plasmalogen precursors, BTHS*, are overlayed for comparison. The insert shows a magnification of the spectral region highlighted in red. Spectra are representative of 4 independent measurements. Peaks are numbered and the legend presents the assignment of each peak to a phospholipid class (or subclass). PC: diacyl-phosphatidylcholine (+plasmanyl-phosphatidylcholine), PI: phosphatidylinositol, PS: phosphatidylserine, LPC: lyso-phosphatidylcholine, LPC-Pls: lyso-plasmenyl-phosphocholine, PE diacyl-phosphatidylethanolamine (+plasmanyl-phosphatidylethanolamine), PE-Pls: plasmenyl-phosphoethanolamine, SM: sphingomyelin, GPC: glycerophosphocholine, LPS: lysophosphoserine, LPE: lyso-phosphoethanolamine, LPE-Pls: lyso-plasmenylphosphoethanolamine, CL: cardiolipin, PG: phosphatidylglycerol, 1-MLCL: 1-monolyso-cardiolipin, and 2-MLCL: 2-monolysocardiolipin.

*in vivo* has shown to be a successful strategy to increase plasmalogen levels [28,35–39]. Hence, BTHS and control lymphoblasts were administered with an alkylglycerol, namely 1-O-hexadecylglycerol (HG), aiming to promote plasmalogen biosynthesis. In addition to HG, ethanolamine was co-administrated to avoid it being a rate limiting factor as the major plasmalogen in lymphoblasts is plasmenylethanolamine (PE-Pls) [21,30].

To evaluate the effects of promotion of plasmalogen biosynthesis on the phospholipidome of the lymphoblasts, high resolution $^{31}$P NMR spectra were measured on their lipid extracts. This methodology presents high selectivity as well as ease of performance and analysis for lipid extracts. Sharp NMR peaks are obtained by solubilizing the lipids in detergent micelles (SDS in the present study), which leads to unique chemical shift values of the phosphate group for the different phospholipid classes and subclasses avoiding the need of separation of components of the mixture prior to measurement. Fig. 1A presents the overlapped high resolution $^{31}$P NMR spectra of BTHS and control lymphoblast lipid extracts. These spectra are characterized by the presence of 12–14 peaks, which are ascribed to different phospholipid classes and subclasses. Kimura and collaborators have previously assigned most of these peaks based on comparison with spectra of phospholipid standards, literature data, and the use of different detergents [20,21]. Table 1 summarizes the relative abundance of the individual phospholipids identified in lymphoblasts of BTHS and control individuals. The results showed differences in the content of 6 phospholipid species; those were PE, PE-Pls, SM, CL, PG, and MLCL. In BTHS lymphoblasts the content of PE, SM, PG, and MLCL were significantly increased, while that of PE-Pls and CL were decreased, in agreement with the previous report [21]. On average PE, SM, and PG increased their individual content by 44, 42, and 100%, respectively; and PE-Pls and CL decreased their individual content by 22 and 57%, respectively (Fig. 2 and Table 1). MLCL was not detected in control lymphoblasts by high resolution $^{31}$P NMR.

**Table 1**
Effect of promotion of plasmalogen biosynthesis on the phospholipidome of Barth lymphoblasts. Phospholipids, their $^{31}$P NMR chemical shifts, and individual contents (mol%, relative to total phospholipid) are presented. Values are presented for control (C) and Barth (BTHS) lymphoblasts as well as Barth (BTHS*) lymphoblasts that have been administered with plasmalogen precursors. Data were generated from the simulation of the high resolution $^{31}$P NMR spectra presented in Fig. 1 and are presented as mean ± S.D. (n = 4).

| Lipid | Chemical shift (ppm) | C | BTHS | BTHS* |
|---|---|---|---|---|
| PC[a] | −0.174 | 54 ± 2 | 52 ± 2 | 49.1 ± 0.5[c,d] |
| PI | 0.033 | 5.2 ± 0.3 | 4.6 ± 0.8 | 6.0 ± 0.5[d] |
| PS | 0.114 | 4.8 ± 0.2 | 4.3 ± 0.4 | 4.8 ± 0.4 |
| LPC | 0.204 | 0.3 ± 0.2 | 0.04 ± 0.03 | 0.3 ± 0.1[d] |
| LPC-Pls | 0.220 | n/d[b] | n/d[b] | 0.15 ± 0.02 |
| PE[a] | 0.284 | 16 ± 1 | 23 ± 1[c] | 13.1 ± 0.8[c,d] |
| PE-Pls | 0.324 | 11.5 ± 0.6 | 9.0 ± 0.5[c] | 17.9 ± 0.7[c,d] |
| SM | 0.404 | 1.9 ± 0.5 | 2.7 ± 0.3[c] | 2.6 ± 0.1[c] |
| GPC? | 0.466 | n/d[b] | 0.3 ± 0.2 | 0.4 ± 0.3 |
| LPS | 0.513 | 1.4 ± 0.8 | 0.5 ± 0.4 | 0.9 ± 0.6 |
| LPE | 0.662 | 0.3 ± 0.2 | 0.13 ± 0.06 | 0.07 ± 0.01 |
| LPE-Pls | 0.721 | n/d[b] | n/d[b] | 0.6 ± 0.3 |
| CL | 0.736 | 2.1 ± 0.2 | 0.9 ± 0.3[c] | 1.44 ± 0.09[c,d] |
| PG | 0.838 | 0.8 ± 0.2 | 1.6 ± 0.3[c] | 1.20 ± 0.06[c,d] |
| 1-MLCL | 1.009 | n/d[b] | 0.09 ± 0.04 | 0.08 ± 0.01 |
| 2-MLCL | 1.131 | n/d[b] | 0.5 ± 0.2 | 0.7 ± 0.2 |

[a] These phospholipids comprise the sum of both their diacyl and plasmanyl molecular species.
[b] These lipid species were not detected (n/d).
[c] Statistical significant in comparison to C ($p < 0.05$).
[d] Statistical significant in comparison to BTHS ($p < 0.05$).

Upon administering lymphoblasts with plasmalogen precursors notable changes were observed in the high resolution $^{31}$P NMR spectra of

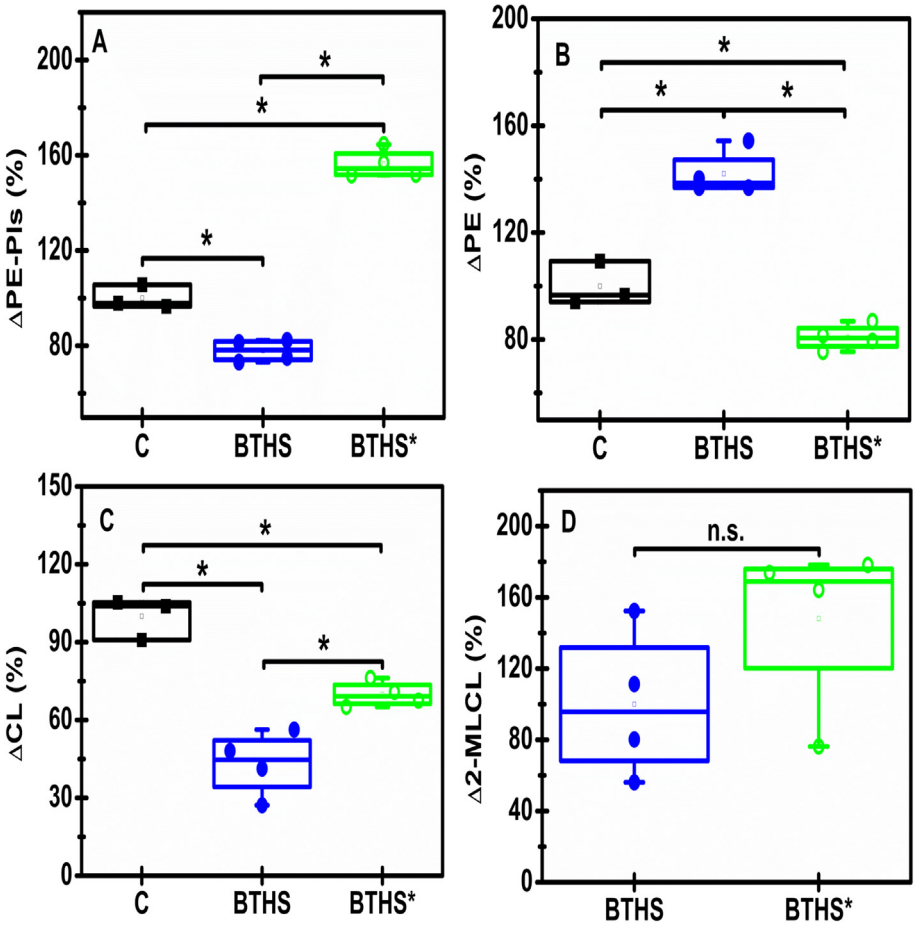


**Fig. 2.** Effect of promotion of plasmalogen biosynthesis on the individual content of selected phospholipids of Barth lymphoblasts. Data was generated from the normalization of their individual content presented in Table 1 to their individual abundance in controls, C, and are presented for (A) PE-Pls, (B) diacyl-PE (+ plasmanyl-PE), (C) CL, and (D) 2-MLCL. Data is presented as a box plot (n = 3 for C and 4 for BTHS and BTHS*; *p < 0.05). Note that since there was no MLCL measured in controls, in (D) data was normalized for the values in Barth lymphoblasts, BTHS,. Data is only presented for 2-MLCL as this was the isomer with higher content, although a similar trend was observed for 1-MLCL (not shown). BTHS* are the values for Barth lymphoblasts that were administered with plasmalogen precursors. Values reflect data obtained by high resolution $^{31}$P NMR measurements.

their lipid extracts (Figs. 1B and S1). A summary of the relative abundance of the individual phospholipids identified in BTHS lymphoblasts that were administered with plasmalogen precursors is presented in Table 1. The phospholipid species that were significantly affected by administering plasmalogen precursors to BTHS lymphoblasts were PE, PE-Pls, CL, and PG. In addition, two lysophospholipids were identified in BTHS lymphoblasts that were administered with plasmalogen precursors, namely, LPC-Pls and LPE-Pls (Fig. 1B and Table 1). When compared with BTHS lymphoblasts, administering plasmalogen precursors led, on average, to 43 and 25% decrease in the individual content of PE and PG, respectively; and a 100 and 60% increase in the individual content of PE-Pls and CL, respectively (Fig. 2 and Table 1). Interesting, in control lymphoblasts administered with plasmalogen precursors, a similar trend in the relative change of these lipid species as well the presence of LPC-Pls and LPE-Pls were observed, corroborating the finding in BTHS lymphoblasts administered with plasmalogen precursors (Figs. S1–2 and Table S1). It is important to mention though that none of these changes restored their individual content found in control lymphoblasts (Figs. 2 and S2 and Tables 1 and S1). In addition, as MLCL accumulation is a hallmark of BTHS it is important to mention that administration of plasmalogen precursors to BTHS lymphoblasts did not change the levels of this lipid (Fig. 2D). To deepen the understanding of the observed phospholipid changes upon promoting plasmalogen biosynthesis in these lymphoblasts liquid chromatography-mass spectrometry (LC-MS) was conducted targeting three phospholipids; namely, PE, CL, and MLCL (Figs. 3 and S4). In control lymphoblasts the major PE molecular species bears 36:1 acyl chains, which is also the prevalent molecular species in BTHS lymphoblasts (Fig. 3A). The major change in the PE molecular species profile in BTHS lymphoblasts when compared to control was an overall increase in longer polyunsaturated acyl chains (36:4, 38:3 and 38:4) (Fig. 3A) whereas shorter less unsaturated acyl chains (34:1 and 36:2) were affected modestly. CL molecular species that were detected mainly showed depletions in BHTS as compared to control lymphoblasts (72:6, 72:7, 72:8 and 72:9, Fig. 3B), except for 72:0 and 72:2 molecules species which were less affected. In control lymphoblasts two MLCL species were detected, albeit their low abundances (Fig. 3C). 54:5 MLCL was detected at similar levels in control and BHTS; and 54:6 showed a slight accumulation (Fig. 3C).

Upon promotion of plasmalogen biosynthesis, LC-MS data showed that the majority of the PE molecular species detected were depleted in BTHS lymphoblasts (BTHS *vs* BTHS*, Fig. 3A), except for longer polyunsaturated acyl chains (36:4, 38:3 and 38:4). The results also showed that the CL molecular species profile is not significantly changed upon the administration of plasmalogen precursors (BTHS and BTHS*), except for C72:0 CL which showed a modest accumulation in BTHS* when compared to BTHS (Fig. 3B). The promotion of plasmalogen biosynthesis did not change the MLCL molecular species profile when comparing BTHS lymphoblasts with or without administered plasmalogen precursors (Fig. 3C). In control lymphoblasts administered with plasmalogen precursors, the majority of the PE and MLCL species detected were depleted; whereas there was an accumulation of the majority of the CL species detected in comparison to controls, supporting the findings obtained by $^{31}$P NMR (Fig. S4).

### *3.2. Effect of the promotion of plasmalogen biosynthesis on cell viability and mitochondrial properties of BTHS lymphoblasts*

Overall, these results indicate the success of the strategy to restore the low levels of PE-Pls in BTHS lymphoblasts. Of note, was the observation that this strategy also led to a significant increase in the levels of CL. However, to substantiate these findings having the perspective of

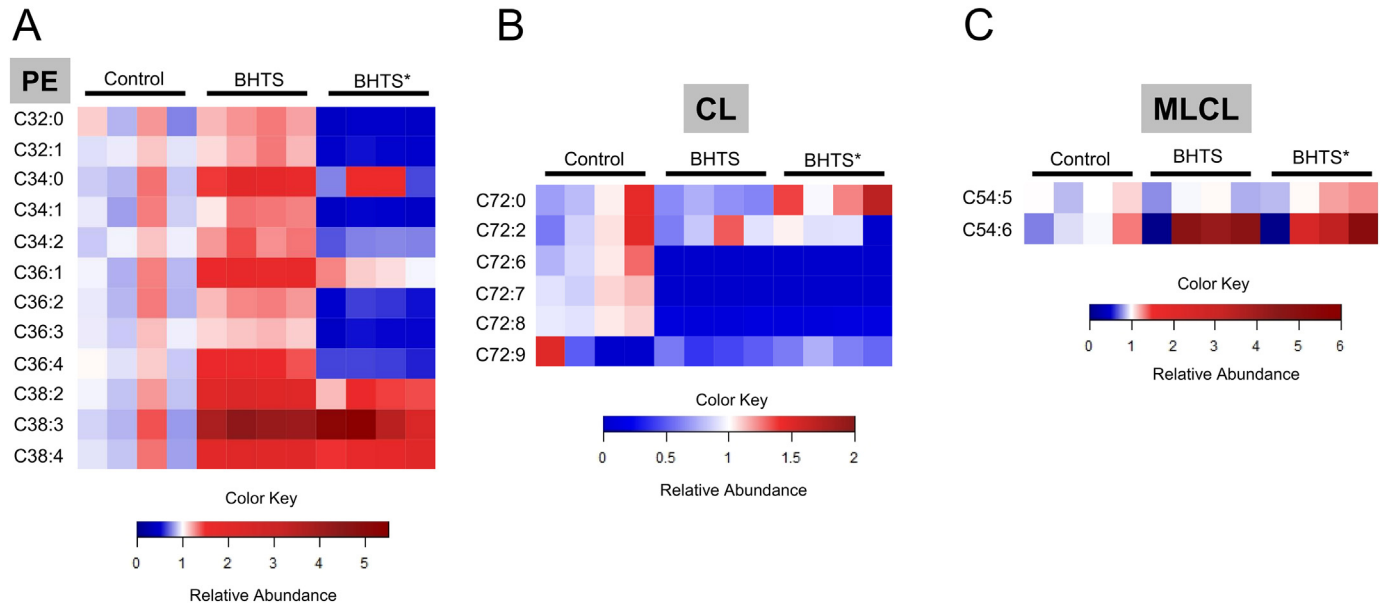


**Fig. 3.** Effect of promotion of plasmalogen biosynthesis on the molecular species profile of targeted phospholipids of Barth lymphoblasts. Heatmap representation of relative abundance of different molecular species of (A) PE, (B) CL, and (C) MLCL are shown. Relative abundances were calculated by dividing the raw abundance of each lipid molecular species by the average abundance of the same lipid species in all samples. Red coloring indicates high levels while blue indicates low levels. Species that were not detected were assigned zero (0) abundance. Each sample in the heatmaps displays the values of 4 independent measurements. Values reflect data obtained by mass spectrometry measurements.

developing a strategy to alleviate/treat BTHS patients, it's important to evaluate the effect of the promotion of plasmalogen biosynthesis on cell viability. In addition, since BTHS mitochondria are dysfunctional it is also relevant to evaluate the effect of administering the cells plasmalogen precursors on mitochondria biogenesis and functional properties. To evaluate the effect of administering the lymphoblasts plasmalogen precursors on cellular viability the measurement of cellular ATP was used as an estimation of cell proliferation and cytotoxicity. Fig. S3 compares the total cellular ATP content of control and BTHS lymphoblasts with and without the administration of plasmalogen precursors. BTHS lymphoblasts had *ca.* 2.3-fold higher total cellular ATP than controls, which is in agreement with the known cellular compensatory effects of BTHS lymphoblasts to circumvent the disrupted mitochondria; *i.e.*, higher glycolysis and mitochondria content [9,11,18]. Plasmalogen precursors' administration didn't change significantly the cellular ATP content of BTHS lymphoblasts, indicating that the treatment did not impact to a large extent on cell proliferation and cytotoxicity (Fig. S3). No changes were also observed in the cellular ATP content of control lymphoblasts that were administered plasmalogen precursors, corroborating the indication that administering lymphoblasts with plasmalogen precursors did not impact on cell proliferation and/or cytotoxicity (Fig. S3). One of the strategies BTHS cells use to compensate their mitochondrial dysfunction is to increase mitochondria biogenesis; *i.e.*, its mitochondrial copy number [9]. To evaluate the effect of the promotion of plasmalogen biosynthesis on the mitochondrial copy number we measured the ratio of mitochondrial to nuclear DNA in the lymphoblasts, which is an accurate way to quantitate the mitochondria copy number (Figs. 4A and S5A). The results showed that BTHS lymphoblasts had on average 3-fold more mitochondria than control lymphoblasts, in agreement with the literature [9,11]. Interestingly, upon administering plasmalogen precursors to BTHS lymphoblasts there was a small but significant decrease in the mitochondria copy number, which now had, on average, 1.8-fold more mitochondria than control lymphoblasts, a 40% decrease from BTHS lymphoblasts (Fig. 4A). However, it should be mentioned that contrary to BTHS lymphoblasts, in control lymphoblasts administering plasmalogen precursors led to an increase in the mitochondria copy number, which was, on average, 1.8-fold higher than control lymphoblasts (Fig. S5A).

The measure of mitochondrial membrane potential is an indicator of respiratory chain maturation [40]. It has been shown that in Barth specimens mitochondrial membrane potential is lower than in control cells, which is a consequence of a lower content and more heterogeneous CL molecular species decreasing the stability of respiratory complexes and SC [11]. To evaluate the effect of the promotion of plasmalogen biosynthesis on the mitochondrial membrane potential we measured the aggregate/monomer (red/green) JC-1 fluorescence ratio as an estimation of mitochondrial membrane potential (Figs. 4B and S5B). BTHS lymphoblasts showed, on average, a 34% lower apparent mitochondrial membrane potential (Fig. 4B). Interestingly, upon promotion of plasmalogen biosynthesis the mitochondrial membrane potential was fully restored to levels compared to control lymphoblasts (Fig. 4B). This result was also corroborated by a small, but significant, 13% average increase in the apparent mitochondrial membrane potential of control lymphoblasts that were administered plasmalogen precursors (Fig. S5B).

## 4. Discussion

Currently, there is no specific treatment for BTHS. Contrary to the well-established alterations in CL content and molecular species in BTHS, the recent identification of a marked decrease in another class of lipids, namely plasmalogens, in BTHS has opened a new possibility, up to now not considered, as a potential strategy to alleviate/treat this condition, which is through the promotion of plasmalogen biosynthesis [20,21]. It has been shown in other systems that restoring plasmalogen levels is beneficial at the cellular and mitochondrial levels, in restoring biological function [22,28]. Noteworthy, in several diseases characterized by depletion in plasmalogens the use of plasmalogen replacement therapy (*via* the promotion of their synthesis) has been shown as a successful strategy to alleviate/treat those conditions [22]. Hence, the present work describes the effect of the promotion of plasmalogen biosynthesis on the phospholipidome of BTHS lymphoblasts, which is a simple cell model that bears all the characteristics of this disease. In addition, an evaluation of the administration of the plasmalogen precursors on cell viability, mitochondria biogenesis, and mitochondrial membrane potential were evaluated. It is shown that

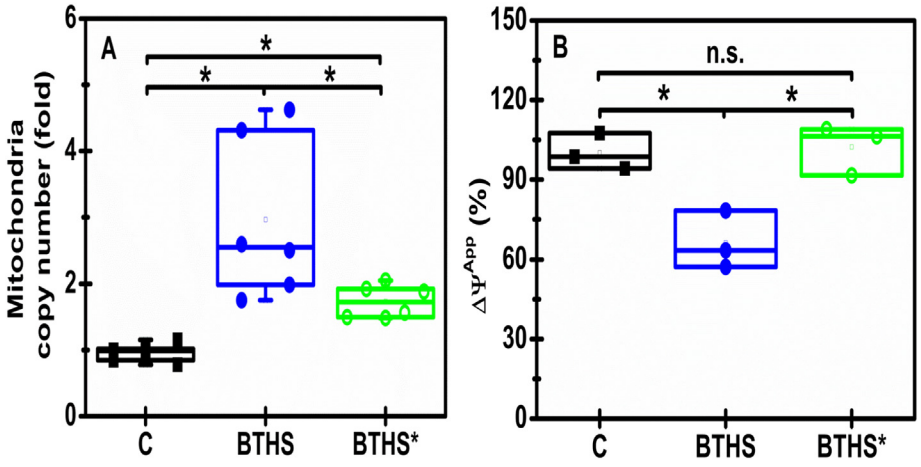


**Fig. 4.** Effect of promotion of plasmalogen biosynthesis on mitochondrial properties of Barth lymphoblasts. (A) Mitochondria copy number and (B) Apparent mitochondrial membrane potential. Data were normalized for values of control lymphoblasts, C. Data are presented as a box plot (n = 6 and 3 in A and B, respectively; *p < 0.05) for Barth lymphoblasts without administration (BTHS) and with administration (BTHS*) of plasmalogen precursors.

administering BTHS lymphoblasts with plasmalogen precursors led to an increase in the low levels of PE-Pls (the main plasmalogen in these cells) with a counterbalancing decrease in its diacyl-PE content. Of note is the significant increase in the content of CL, while not altering the content of its hydrolysis product MLCL. Moreover, the treatment did not impact on cellular viability, although it significantly decrease mitochondria copy number and restored mitochondrial membrane potential.

The total cellular level of PE glycerophospholipids tends to remain constant. It has been previously shown that to keep the overall PE content constant, a decrease in PE-Pls content is compensated with an increase in the content of its diacyl-PE counterpart; likewise, an increase in PE-Pls content is compensated with a decrease in the content of its diacyl-PE counterpart [41–43]. The total cellular content of PE lipids derived from the Kennedy pathway are controlled by the levels of CDP-ethanolamine, through the action of CTP:phosphoethanolamine cytidyltransferase. CDP-ethanolamine is coupled to alkylacylglycerol or diacylglycerol to yield PE-Pls and diacyl-PE, respectively [43]. Hence, if the level of CDP-ethanolamine is kept constant, the relative amounts of PE-Pls and diacyl-PE will adjust according to the relative levels of alkylacylglycerol and diacylglycerol, respectively. The studies reported here are in very good agreement with previous findings showing that the total PE content of lymphoblasts are held constant at *ca.* 30 mol% [21]. In addition, the results suggested that the low PE-Pls:diacyl-PE ratio found in BTHS lymphoblasts are due to a decreased alkylacylglycerol:diacylglycerol ratio. This is corroborated by the present studies where by increasing the alkylacylglycerol:diacylglycerol ratio by administering lymphoblasts with plasmalogen precursors led to an increase in the levels of PE-Pls with a concomitant decrease in the levels of diacyl-PE.

Plasmalogens are usually enriched with PUFAs at the *sn*-2 position of the glycerol moiety [22]. PUFAs are biologically active mediators of signaling processes, which cannot be synthesized *de novo* in higher mammals and need to be acquired through the diet; *i.e.*, they are "*essential fatty acids*". Thus, their levels are tightly regulated. It has been shown that in conditions where plasmalogen levels are decreased, the increase in diacyl-PE is favored for those molecular species enriched in PUFAs, which seems to be a way to maintain the total PUFAs levels in these lipids [43,44]. The results presented here are in very good agreement with the literature, showing that in BTHS lymphoblasts the counterbalanced increase in diacyl-PE due to the decreased PE-Pls levels are preferential for those diacyl-PE molecular species enriched in PUFAs. Interestingly, the promotion of plasmalogen biosynthesis, although it increased the total cellular levels of PE-Pls with a compensatory decrease in the total cellular levels of its diacyl-PE counterpart, it did not change the profile of the diacyl-PE molecular species, which continued to be enriched in PUFAs.

The hallmark of BTHS is the alterations that happen in CL content and molecular species due to the lack of functional tafazzin [5,7,45]. Previous attempts to increase CL levels were not successful by direct administration of this lipid *in vivo* [29]. Interestingly, the results presented here showed that by promoting plasmalogen biosynthesis in addition to increasing plasmalogen levels, it also leads to an increase in CL levels. This result suggests that increased plasmalogen levels might regulate CL biosynthesis and/or degradation pathways. The understanding of the molecular mechanisms that triggered CL content increase upon promotion of plasmalogen biosynthesis is beyond the scope of the present work. However, it seems reasonable to propose that the regulation might be happening in either one or a combination of the following biochemical reactions that control CL levels; those are: (i) CL hydrolysis by a phospholipase (iPLA2β, iPLA2γ, cPLA2, and/or sPLA2), (ii) MLCL acylation by ALCAT1 and/or MLCLAT1, (iii) protection of CL oxidation, and (iv) increased CL synthesis [46–48]. The results presented here suggest that (i) and (ii) are less likely than (iii) and (iv) if those are considered as single rather a combination of reactions affected by increased plasmalogen levels. The reason is that in (i) and (ii) by themselves a decrease in the steady-state levels of MLCL would be expected upon promotion of plasmalogen biosynthesis, which was not observed. Future studies will shed some light on how promotion of plasmalogen biosynthesis leads to an increase in CL content.

In BTHS the alterations in CL as a consequence of the lack of tafazzin function lead to dysfunctional mitochondria [9,11,12,18]. The results presented here show that upon promotion of plasmalogen biosynthesis mitochondria copy number was decreased and mitochondrial membrane potential was fully restored to control levels. It is known that in BTHS cellular compensation occurs due to the dysfunctional mitochondria, one of which is the increase in mitochondria copy number as a consequence of a less robust electron transport chain activity [9]. The decreased in the mitochondria copy number upon promoting plasmalogen biosynthesis suggested that the treatment led to a more robust electron transport chain activity. Indeed, the finding that the mitochondrial membrane potential was fully restored upon promotion of plasmalogen biosynthesis corroborated this indication. It is known that plasmalogen imparts different physical properties to the membrane when compared to their diacyl counterparts [23]. Thus, it is conceivable that the increase in plasmalogen content had led to a more packed membrane, which would, in turn, make the membrane less "leaky", increasing the mitochondria membrane potential. Likewise, the increase in CL levels could have led to a more efficient electron transport chain activity.

## 5. Conclusions

To sum up, the present study showed that it is possible to restore the

low levels of plasmalogen in a BTHS cell model upon promotion of its biosynthesis. Importantly, the administration of plasmalogen precursors did not impact on the cell viability, while it increased to some extent the CL levels as well as restored mitochondrial properties. Until the association of plasmalogen loss with BTHS such strategy could not have been considered and the results presented here suggest that a diet supplemented with plasmalogen precursors could be beneficial for BTHS patients.

## Declaration of competing interest

The authors declare that they have no conflicts of interest with the content of this article.

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## Author contributions

J.C.B. Jr. performed cell work, cellular ATP content quantification, mitochondria copy number quantification, and high resolution $^{31}$P NMR measurements. D.L. performed mass spectrometry measurements under the supervision of G.E.A.-G. J.C.B. Jr. and R.M.E. designed the experiments, analyzed the data, and wrote the manuscript.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbalip.2020.158677.

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