Original Article

Impaired acylcarnitine profile in transfusion-dependent beta-thalassemia major patients in Bangladesh

Suprovath Kumar Sarker [a,i,1], Md Tarikul Islam [a,1], Golam Sarower Bhuyan [b], Nusrat Sultana [a],
Mst. Noorjahan Begum [a], Mohammad Al Mahmud-Un-Nabi [b], Md. Abdulla Al Noman Howladar [c],
Tashmim Farhana Dipta [d], A.K.M. Muraduzzaman [e], Syeda Kashfi Qadri [f], Tahmina Shirin [e], Salma Sadiya [g],
Manzoor Hussain [h], Waqar Ahmed Khan [g], Sharif Akhteruzzaman [i], Syed Saleheen Qadri [a],
Firdausi Qadri [a,j], Kaiissar Mannoor [a,*]

[a] Laboratory of Genetics and Genomics, Institute for Developing Science and Health Initiatives, Mohakhali, Dhaka 1212, Bangladesh
[b] Infectious Diseases Laboratory, Institute for Developing Science and Health Initiatives, Mohakhali, Dhaka 1212, Bangladesh
[c] Research and Development, Incepta Chemicals Ltd., Barabaria, Saturia, Dhankora, Manikganj, Dhaka 1810, Bangladesh
[d] Transfusion Medicine and Clinical Haematology, BIRDEM General Hospital and Ibrahim Medical College, Shahbag, Dhaka 1000, Bangladesh
[e] Department of Virology, Institute of Epidemiology, Disease Control and Research, Mohakhali, Dhaka 1212, Bangladesh
[f] Department of Paediatric Medicine, KK Women's and Children's Hospital, 100 Bukit Timah Road, Singapore 229899, Singapore
[g] Department of Biochemistry and Molecular Biology, Dhaka Shishu Hospital, Dhaka 1207, Bangladesh
[h] Department of Pediatric Medicine and Cardiology, Dhaka Shishu Hospital, Dhaka 1207, Bangladesh
[i] Department of Genetic Engineering & Biotechnology, University of Dhaka, Dhaka 1000, Bangladesh
[j] Department of Enteric and Respiratory Infectious Diseases, Infectious Diseases Division, International Centre for Diarrhoeal Disease Research, Bangladesh, Mohakhali, Dhaka 1212, Bangladesh

GRAPHICAL ABSTRACT
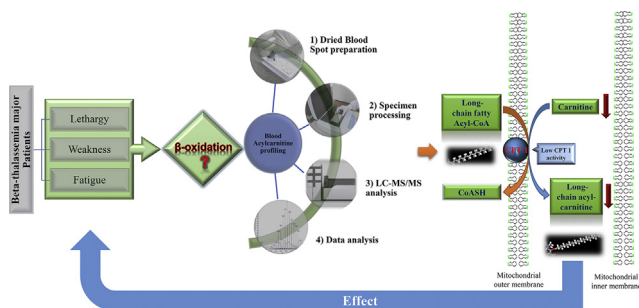
ARTICLE INFO

ABSTRACT

Patients with beta-thalassemia major (BTM) suffer from fatigue, poor physical fitness, muscle weakness, lethargy, and cardiac complications which are related to an energy crisis. Carnitine and acylcarnitine derivatives play important roles in fatty acid oxidation, and deregulation of carnitine and acylcarnitine metabolism may lead to an energy crisis. The present study aimed to investigate carnitine and acylcarnitine metabolites to gain an insight into the pathophysiology of BTM. Dried blood spots of 45 patients with BTM and 96 age-matched healthy controls were analyzed for free carnitine and 24 acylcarnitines by using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Although medium chain acylcarnitine levels were similar in the patients with BTM and healthy controls, free carnitine, short chain

https://doi.org/10.1016/j.jare.2018.04.002

Carnitine-acylcarnitine levels
Impairment in fatty acid oxidation
Carnitine Palmitoyltransferase-1 activity

acylcarnitines, long chain acylcarnitines, and total acylcarnitine levels were significantly lower in patients with BTM than in the healthy controls ($P < 0.05$). Moreover, an impaired fatty acid oxidation rate was observed in the patients with BTM, as manifested by decreased fatty acid oxidation indicator ratios, namely C2/C0 and (C2 + C3)/C0. Furthermore, an increase in the C0/(C16 + C18) ratio indicated reduced carnitine palmitoyltransferase-1 (CPT-1) activity in the patients with BTM compared with that in the healthy controls. Thus, a low level of free carnitine and acylcarnitines together with impaired CPT-1 activity contribute to energy crisis-related complications in the patients with BTM.

Introduction

Beta-thalassemia, an inherited hemoglobinopathy caused by beta-globin gene mutations and deregulation of the synthesis of the β-globin chain, is one of the most common autosomal recessive disorders worldwide. Approximately 65,000–70,000 babies are born each year with different types of thalassemia, most of whom are affected by beta-thalassemia major (BTM), the most severe form of the disorder [1]. In beta-thalassemia, pathophysiology caused by an imbalance in the ratio of alpha globin chains to beta globin chains, and the presence of excess alpha-globin chain in red blood cells (RBCs), results in oxidative damage to RBCs [2]. Consequently, patients with beta-thalassemia major suffer from hemolytic anemia, which triggers ineffective erythropoiesis through the action of erythropoietin, which is produced in excess by the kidneys [3]. These sequential phenomena lead to an expansion of bone marrow, ultimately causing bone deformities, hepatosplenomegaly, growth retardation, iron-induced dysfunctions of various organs, including the kidney and liver; and above all, muscular and cardiac complications [4]. The pathophysiology of these complications is associated with deregulation of metabolite levels in patients with BTM [5]. Therefore, metabolite profiling, in terms of free carnitine and acylcarnitines, amino acids, and other untargeted metabolites, might be a useful approach to manage BTM and to devise treatment strategies to ameliorate its complications.

Liquid chromatography tandem-mass spectrometry (LC-MS/MS) is a high-throughput technique that has shown analytical superiority for low molecular weight biomolecules or metabolites in biological specimens. For example, LC-MS/MS is now used as a dedicated high-throughput platform to screen and diagnose inborn errors of metabolism, such as aminoacidopathies, fatty acid oxidation disorders, and organic acidemia, in many countries worldwide [6]. An alteration in the metabolite profile of a biological specimen can provide valuable phenotypic information and mechanistic insights into the biochemistry of disease processes and their related pathophysiology. It is believed that multi-organ involvement in beta-thalassemia pathophysiology might be associated with an altered metabolite profile. However, very few studies have been conducted on metabolite profiling of BTM specimens [5,7,8].

L-Carnitine plays a significant role in fatty acid transportation across the mitochondrial membrane. In addition, L-carnitine participates in the fatty acid oxidation cycle by releasing Coenzyme-A (CoASH) molecules from shortened products of fatty acid oxidation [9]. Thus, L-carnitine and its acylcarnitine derivatives are important for energy production from fatty acids. Deregulation of carnitine and acylcarnitine levels inflicts damaging effects on organs that depend on fatty acid oxidation for energy, such as the heart and muscle [10]. Although previous studies reported data on free carnitine and total acylcarnitines, those studies did not focus on individual acylcarnitines [8,11]. The present study aimed to perform an LC-MS/MS-based analysis of dried blood spot (DBS) specimens of patients with BTM and age-matched healthy controls to gain an insight into the metabolite profiles of patients with BTM in terms of free carnitine and 24 individual fatty acylcarnitines.

Patients and methods

Ethical approval, study participants and specimen collection

Ethical approval for the present study was obtained from the Bangladesh Medical Research Council (BMRC) of National Ethics Review Committee (NERC), Dhaka, Bangladesh. The study enrolled 45 patients with transfusion-dependent BTM in the age range of 1–15 years. The patients were attending the Dhaka Shishu hospital for follow-up examination and chronic blood transfusions. All the patients continued to receive Desferoxamine (20–40 mg/kg/day) as an iron chelator. Patients with BTM with other comorbid conditions affecting the liver and kidney, and the respiratory, gastrointestinal, and cardiovascular systems, were excluded from the study. In addition to the patients with BTM, 96 age-matched healthy controls were included in the study. The healthy participants had no history of hemoglobinopathy or related disorders.

Before sample collection, written informed consent was obtained from the guardians of both the patients with BTM and the healthy controls. Blood specimens (2 mL) were collected from overnight-fasted patients with BTM and the healthy controls using the standard venipuncture method. Blood collection from the patients with BTM was performed before their regular blood transfusion. Collected blood specimens were immediately spotted on Whatman™ 903 Generic Multipart filter paper (GE Healthcare, Westborough, MA, USA) (~75 μL per spot) to prepare DBS specimens. The rest of the blood was transferred to a BD vacutainer containing dipotassium EDTA (Becton Dickinson, Franklin Lakes, NJ, USA) for DNA isolation and subsequent genetic analysis.

Genotyping of study participants

Genomic DNA (gDNA) was extracted from the EDTA-treated blood using a QIAGEN flexigene® DNA kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The extracted DNA templates were amplified using polymerase chain reaction (PCR) with the forward primer 5′-GGCAGAGCCATCTATTGCTTAC-3′ and the reverse primer 5′-CAGGCCATCACTAAAGGCACC-3′, which together flanked a mutational hot-spot region of beta-globin gene (*HBB*) of the Bangladeshi population [12,13]. The following thermal cycling conditions were applied for PCR amplification: initial denaturation at 94 °C for 15 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 40 s and extension at 72 °C for 40 s; and a final extension at 72 °C for 5 min. The PCR composition was as follows: 4.0 μL of 10 × PCR buffer (with 1.5 mM MgCl2), 1.2 μL of MgCl2 (25 mM), 8.0 μL of Q-solution (Qiagen), 6.4 μL of dNTP mixture (2.5 mM), 0.8 μL of forward primer (10 mM) and 0.8 μL of reverse primer (10 mM), 0.4 μL of HotStart Taq DNA polymerase (Qiagen). Finally, a total reaction volume of 40.0 μL was made with nuclease-free water. PCR product purification was accomplished using a MinElute® PCR purification kit (Qiagen) following the manufacturer's instructions.

Using a BigDye® v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK), the column-purified PCR products were subjected to cycle sequencing to obtain the chain termination

products. Purification of the cycle sequencing products was performed using a BigDye® XTerminator™ purification kit (Applied Biosystems) following the manufacturer's instruction. Thereafter, the purified cycle sequencing PCR products were subjected to capillary sequencing in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and the data were obtained using data collection software version 3.1.0 (Applied Biosystems). The sequencing data were analyzed by alignment with the reference sequence (NC_000011.10; retrieved from the NCBI database) using the basic local alignment search tool (BLAST) to identify mutations in *HBB* among the study participants.

Method validation

Method validation using Shimadzu-8050 LC-MS/MS machine (Shimadzu Corporation, Kyoto, Japan) was performed using three different quality control DBS spots containing low, medium, and high levels of each acylcarnitine, provided with a NeoMass AAAC kit (Labsystems Diagnostics Oy, Vantaa, Finland). Method validation was performed in terms of measurement of inter-assay and intra-assay accuracy and precision, linearity, and recovery. The analytes were extracted from the quality control DBS spots and were analyzed for C0, C2, C3, C4, C5, C6, C8, C10, C12, C14, C16, and C18 for method performance.

Extraction and quantification of carnitine and acylcarnitines from DBS cards

A NeoMass AAAC kit (Labsystems Diagnostics Oy) was used to extract and quantify free carnitine and acylcarnitines from DBSs following the manufacturer's instructions. Lyophilized internal standards containing $^2H_9$-free carnitine (C0 IS), $^2H_3$-acetylcarnitine (C2 IS), $^2H_3$-propionylcarnitine (C3 IS), $^2H_3$-butyrylcarnitine (C4 IS), $^2H_9$-Isovalerylcarnitine (C5 IS), $^2H_3$-Glutarylcarnitine (C5DC IS), $^2H_3$-Hexanoylcarnitine (C6 IS), $^2H_3$-Octanoylcarnitine (C8 IS), $^2H_3$-Decanoylcarnitine (C10 IS), $^2H_3$-Lauroylcarnitine (C12 IS), $^2H_9$-Myristoylcarnitine (C14 IS), $^2H_3$-Palmitoylcarnitine (C16 IS), and $^2H_3$-Stearoylcarnitin (C18 IS) were reconstituted according to the guidelines provided with the kit. A working solution was prepared by diluting the reconstituted internal standards with extraction buffer at a 1:100 (v/v) ratio. From each DBS specimen, a 3.2-mm spot was punched out into the U-bottomed microplate well and 100 μL of extraction solution was added. The microtiter plate was covered with an adhesive sealer and incubated for 20 min at room temperature in a rotating shaker at 650 rpm. Thereafter, 70 μL of the supernatant was transferred into a V-bottom plate and covered with aluminum foil to minimize evaporation before placing it in the autosampler of a Shimadzu LCMS-8050 liquid chromatograph mass spectrometer (Shimadzu Corporation), equipped with an electrospray ionization source. Five microliters of the supernatant were injected using the autosampler and without chromatographic separation, the sample was subjected to flow injection analysis-electrospray ionization-tandem mass spectrometry (FIA-ESI-MS/MS).

Low, medium, and high-level DBS controls provided with the NeoMass AAAC kit (Labsystems Diagnostics Oy) were run in parallel with the samples following the same procedure to ensure the reliability of the resulting data.

Data collection and data processing

LC-MS/MS data were acquired in positive ion multiple reactions monitoring (MRM) mode using the Shimadzu LabSolutions (version 5.82 SP1) software (Shimadzu Corporation). The settings for the LC-MS/MS machine were as follows: isocratic elution of mobile phase: 200 μL/min; interface voltage: 4.5 kV; interface temperature: 250 °C; heat block temperature: 400 °C; dissolution line: 250 °C; nebulizing gas flow: 3.0 L/min; drying gas flow: 15.0 L/min; and collision gas (argon) pressure: 230 kPa. The settings for MRM data acquisition for quantification of free carnitine and acylcarnitines are shown in Table 1. The run time was 1.5 min and the data collection time was set to 0.9 min. The acquired data were processed using Shimadzu Neonatal Solution (Version: 2.20) and the concentration of each analyte was measured using the formula: Concentration of an analyte (nmol/mL) = the area of the analyte × the concentration of the internal standard/ the area of the internal standard. The processed results were exported as Excel files for further statistical analysis.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 7) software. Welch's unequal variances *t*-test was used to compare acylcarnitines and metabolic indicator ratios between the patients and the healthy controls. A *P*-value of less than 0.05 was considered statistically significant.

Results

Demography of study participants

Forty-five patients with chronic transfusion-dependent BTM and 96 non-BTM healthy controls were included in this study (Table 2). The average age of the BTM participants was 5.95 ± 3.67 years, whereas it was 6.99 ± 4.31 years for the age-matched controls. Patients with chronic transfusion-dependent BTM had an average transfusion interval of 28.23 ± 4.67 days.

Genotypic analysis of the patients with Beta-thalassemia major

Specimens from patients with beta-thalassemia major (BTM) were genotyped to determine the underlying beta-globin gene mutations involved in the disease process. The sequencing data demonstrated that 35 out of the 45 patients with BTM had homozygous beta-globin gene mutations and ten had compound heterozygous mutations (Table 3). Among the 35 homozygous mutations containing patients with BTM, 32 had IVS1_5G>C substitution. Two out of the remaining 3 samples had the homozygous c.92G>C mutation, whereas one sample had the homozygous IVS1_130G>C mutation. Each of the following compound heterozygous mutations combinations were found in two samples: c.47G>C plus IVS1_5G>C, c.27_28insG plus IVS1_5G>C, IVS1_5G>C plus c.126_129delCTTT and c.33C>A plus c.51delC. By contrast, compound heterozygous mutations c.92G>C plus IVS1_5G>C and IVS1_5G>C plus IVS1_130G>C were found in one sample each.

Validation of LC-MS/MS method performance

The details of the performance of method in terms of measurement of inter-assay precision, intra-assay precision, limit of detection (LOD), limit of quantitation (LOQ), linearity, and recovery using Shimadzu-8050 LC-MS/MS machine was determined according to the instructions provided with the NeoMass AAAC kit (Supplementary File 1). The inter-assay and intra-assay precision were measured by running three different controls (low, medium, and high controls) in fifteen replicates on five separate days. In case of inter-assay precision, the ranges of the coefficient of variation (CV) for the low, medium, and high level controls were 1.09–15.09%, 1.20–5.56%, and 2.20–6.08%, with mean values of 4.92 ± 3.71%, 2.73 ± 1.10%, and 4.13 ± 1.17%, respectively (Supplementary File 1: Table A). The inter-assay accuracy was reported

Table 1
The LC-MS/MS data acquisition set up for carnitine and acylcarnitines.

Analyte	Analyte MRM transition (m/z)	Internal standard	ISTD MRM transition (m/z)	Target Dwell Time (msec)	Target Q1 Pre Bias (V)	Target Collision Energy (V)	Target Q3 Pre Bias (V)
C0	162.1 > 85	$^2H_9$ –C0	171.1 > 85	5	−12	−23	−15
C2	204.1 > 85	$^2H_3$ –C2	207.1 > 85	5	−10	−22	−14
C3	218.1 > 85	$^2H_3$ –C3	221.1 > 85	5	−11	−23	−15
C4	232.2 > 85	$^2H_3$ –C4	235.2 > 85	5	−12	−23	−15
C4OH	248.2 > 85	$^2H_3$ –C4	235.2 > 85	5	−12	−23	−15
C5	246.2 > 85	$^2H_9$ –C5	255.2 > 85	5	−13	−23	−15
C5OH	262.2 > 85	$^2H_9$ –C5	255.2 > 85	5	−13	−23	−15
C5DC	276.2 > 85	$^2H_3$ –C5DC	279.2 > 85	10	−13	−25	−16
C6	260.2 > 85	$^2H_3$ –C6	263.2 > 85	5	−10	−24	−15
C8	288.2 > 85	$^2H_3$ –C8	291.2 > 85	5	−11	−23	−15
C8:1	286.2 > 85	$^2H_3$ –C8	291.2 > 85	5	−11	−23	−15
C10	316.2 > 85	$^2H_3$ –C10	319.2 > 85	5	−12	−25	−15
C10:1	314.2 > 85	$^2H_3$ –C10	319.2 > 85	5	−12	−25	−15
C10:2	312.2 > 85	$^2H_3$ –C10	319.2 > 85	5	−12	−25	−15
C12	344.2 > 85	$^2H_3$ –C12	347.2 > 85	5	−13	−27	−14
C14	372.2 > 85	$^2H_9$ –C14	381.2 > 85	5	−14	−28	−15
C14:1	370.2 > 85	$^2H_9$ –C14	381.2 > 85	5	−14	−28	−15
C14:2	368.2 > 85	$^2H_9$ –C14	381.3 > 85	5	−14	−28	−15
C14OH	388.2 > 85	$^2H_9$ –C14	381.2 > 85	5	−14	−28	−15
C16	400.3 > 85	$^2H_3$ –C16	403.3 > 85	5	−15	−28	−15
C16:1	398.3 > 85	$^2H_3$ –C16	403.3 > 85	5	−15	−28	−15
C16:1OH	414.3 > 85	$^2H_3$ –C16	403.3 > 85	5	−15	−28	−15
C18	428.3 > 85	$^2H_3$ –C18	431.3 > 85	5	−16	−30	−15
C18:1	426.3 > 85	$^2H_3$ –C18	431.3 > 85	5	−16	−30	−15
C18:2	424.3 > 85	$^2H_3$ –C18	431.3 > 85	5	−16	−30	−15

Data in Table-1 represents LC-MS/MS data acquisition conditions. MRM transition represents: precursor ion > target ion. The analytes studied include C0 = Free carnitine; C2 = Acetylcarnitine; C3 = Propionylcarnitine; C4 = Butyrylcarnitine; C4OH = 3-Hydroxybutyrylcarnitine; C5 = Isovalerylcarnitine; C5OH = 3-OH isovalerylcarnitine; C5DC = Glutarylcarnitine; C6 = Hexanoylcarnitine; C8 = Octanoylcarnitine; C8.1 = Octenoylcarnitine; C10 = Decanoylcarnitine; C10.1 = Decenoylcarnitine; C10.2 = Decadienoylcarnitine; C12 = Lauroylcarnitine; C14 = Myristoylcarnitine; C14.1 = Myristoleylcarnitine; C14.2 = Tetradecadienoylcarnitine; C14OH = 3-Hydroxytetradecanoylcarnitine; C16 = Palmitoylcarnitine; C16.1 = Palmitoyleylcarnitine; C16.1OH = 3-Hydroxypalmitoleylcarnitine, C18 = Stearoylcarnitine; C18.1 = Oleylcarnitine; C18.2 = Octadecadienylcarnitine.

Table 2
Demographic information of study participants.

Parameters	BTM patients	Controls
Number of participants (Male/Female)	45 (24/21)	96 (46/50)
Age (years)	5.95 ± 3.67	6.99 ± 4.31
Transfusion interval (days)	28.23 ± 4.67	Not applicable

Table 3
Genotypic information of beta-globin gene in BTM patients.

Sl No.	Mutational status	Number of samples
1	IVS1_5G>C#	32
2	c.92G>C#	2
3	IVS1_130G>C#	1
4	c.47G>C* + IVS1_5G>C*	2
5	c.27_28insG* + IVS1_5G>C*	2
6	IVS1_5G>C* + c.126_129delCTTT*	2
7	c.33C>A* + c.51delC*	2
8	c.92G>C* + IVS1_5G>C*	1
9	IVS1_5G>C* + IVS1_130G>C*	1

Mutations of beta-globin gene that had been found in the beta-thalassmia major patients in the study are represented in the Table 3. # indicates homozygous mutation, whereas * indicates heterozygous mutation.

as a relative error and was within 20% of the target value for all the analytes except C8 in low level control. This might be due to a very low concentration of the analyte (0.14 nmol/mL) (Supplementary File 1: Table A). For the intra- assay precision, the ranges of the coefficient of variation (CV) for the low, medium, and high level controls were 2.95–15.93%, 3.14–7.77%, and 3.26–7.36%, with mean values of 9.70 ± 4.26%, 5.34 ± 1.44%, and 5.26 ± 1.18%, respectively (Supplementary File 1: Table B). The intra-assay

accuracy was within 20% of the target value for all the analytes except C8 and C10 in low level controls and such variations might occur due to very low levels of the analytes (C8 = 0.14 nmol/mL and C10 = 0.05 nmol/mL) (Supplementary File 1: Table B). As shown in Supplementary File 1: Table C, the LOD and LOQ values for each individual acylcarnitine were in the expected level for the Shimadzu-8050 LC-MS/MS machine. The average recovery rate for the acylcarnitines was 99.4% and the assay approach generated a linear correlation (R^2 < 0.99) for all acylcarnitine metabolites (Supplementary File 1: Table C). Overall, the data observed for the LC-MS/MS method performance showed that it was reliable to analyze carnitine and acylcarnitines of the study participants.

Deregulation of blood levels of free and total carnitine in patients with beta-thalassemia major

Evidence shows that patients with chronic transfusion-dependent BTM suffer from functional impairment of the liver and kidney because of iron overload [14–17]. This phenomenon leads to down-regulation of carnitine biosynthesis, which in turn, results in reduced availability of carnitine in blood circulation, tissues, and organs. As shown in Fig. 1 and Fig. 2, the free carnitine (C0) level was significantly lower ($P < 0.0001$) in the blood of the patients with BTM (31.59 ± 7.39 nmol/mL) than in the blood of the control group (42.93 ± 8.683 nmol/mL). Moreover, the total carnitine level was significantly lower in the blood of the patients with BTM (42.64 ± 10.79 nmol/mL) than in the blood of the control participants (64.25 ± 11.76 nmol/mL) ($P < 0.0001$).

Next, we investigated whether genotypic variations among the patients with BTM had any influence on the decreasing trend of free carnitine and individual acylcarnitines. As shown in Table 4, irrespective of their different homozygous or compound heterozy-
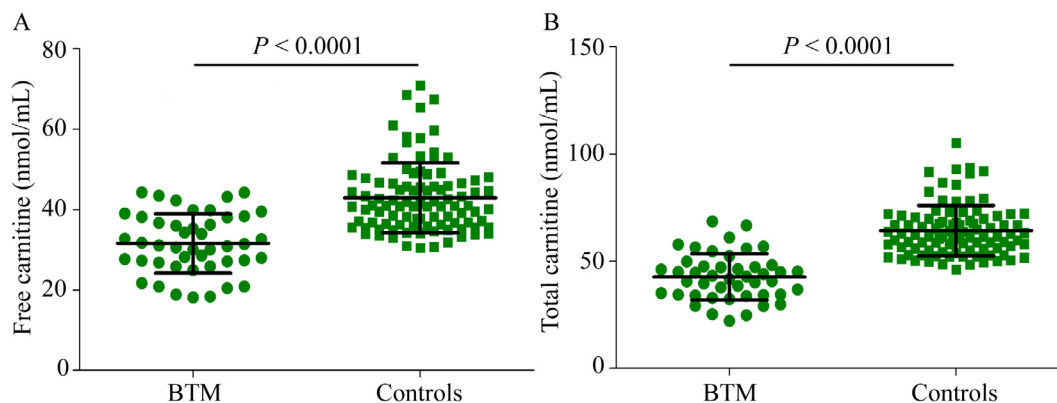
Fig. 1. Free and total carnitine levels in the BTM patients and control participants. The dried blood spot (DBS) specimens were analyzed using LC-MS/MS to determine free carnitine and total carnitine levels. The DBS results reflect levels of analytes in the blood of BTM patients (n = 45) and healthy controls (n = 96). (A) Free carnitine levels in the BTM patients (31.59 ± 7.39 nmol/mL) and healthy participants (42.93 ± 8.683 nmol/mL), (B) Total carnitine levels in the BTM patients (42.64 ± 10.79 nmol/mL) and healthy participants (64.25 ± 11.76 nmol/mL). A P-value of <0.05 was considered statistically significant.

gous mutation status, all the patients had lower levels of free carnitine and total carnitines compared with those in their healthy control counterparts.

Deregulation of the blood levels of acylcarnitines in patients with beta-thalassemia major

L-carnitine, a butyrate analog, is an essential cofactor that plays a crucial role in fatty acid transportation across the mitochondrial membrane, and provides a substrate for energy production through beta-oxidation [9]. Thus, a decrease in the L-carnitine level is very likely to affect fatty acid oxidation, and the resulting perturbation is expected to be reflected in the blood acylcarnitine profile.

In this study, the DBS specimens of patients with BTM and healthy controls were analyzed for free carnitine and 24 acylcarnitines using LC-MS/MS (Table-5, Figs. 2–5). The representative MRM chromatograms for short chain (SC) acylcarnitines, medium chain (MC) acylcarnitines, and long chain (LC) acylcarnitines in the blood of patients with BTM and healthy controls are presented in Figs. 2–4, respectively, and the representative MRM chromatograms for the internal standards are presented in Fig. 5.

The data presented in Table-5 demonstrate that the patients with BTM had significantly lower (P < 0.05) levels of SC-acylcarnitines including C2, C3, C4OH, C5, and C5OH than those of the control participants. However, the levels of SC-acylcarnitine derivatives C4 and C5DC were not statistically different from those of the control group. Altogether, the total concentration of SC-acylcarnitines in the patients with BTM was significantly lower than that of the control group (BTM: 9.51 ± 4.79 nmol/mL; control: 17.67 ± 3.80 nmol/mL; BTM vs. control: P < 0.0001).

Among the MC-acylcarnitine derivatives, the C6 and C10 levels were significantly higher (P < 0.05) in the patients with BTM than in the control group. However, other MC-acylcarnitine derivatives including C8, C8.1, C10.1, C10.2, and C12, did not differ significantly between the patients with BTM and the control participants (P > 0.05). The mean concentration of total MC-acylcarnitines did not differ significantly between the patients with BTM and the control participants (BTM: 0.43 ± 0.19 nmol/mL; control: 0.41 ± 0.16 nmol/mL; BTM vs. control: P < 0.4791) (Table 5).

The levels of most of the LC-acylcarnitine derivatives, including C14, C16, C16.1OH, C18, C18.1, and C18.2, were significantly reduced (P < 0.05) in the patients with BTM compared with those in the control participants (Table 5). However, the levels of LC-acylcarnitine derivatives C14.1 and C14.2 were significantly higher (P < 0.05) in the patients with BTM than in the controls, although

the C14OH and C16.1 levels remained at a similar level in the two groups. The mean concentration of all LC-acylcarnitines was significantly lower in the patients with BTM than in the control group (BTM: 1.162 ± 0.99 nmol/mL; control: 3.24 ± 0.86 nmol/mL; BTM vs. control: P < 0.0001).

Patients with BTM had lower fatty acid oxidation rate as well as downregulated CPT-1 activity

Next, we asked whether perturbation of the blood levels of free carnitine and different short chain, medium chain, and long chain acylcarnitines in the patients with BTM could define whether the fatty acid oxidation was impaired. Hence, the fatty acid oxidation rate was investigated to determine the underlying basis of the difference in acylcarnitine profiles between the patients with BTM and the control participants. First, the C2/C0 and (C2 + C3)/C0 ratios, which are known as indicator ratios for the fatty acid oxidation rate [18,19], were investigated for both the patients with BTM and the healthy controls (Table 6). The C2/C0 ratio was 0.26 ± 0.13 in the patients with BTM, whereas it was 0.37 ± 0.084 in the control group (P < 0.0001). A similar pattern was observed for the (C2 + C3)/C0 ratio, which was lower in the patients with BTM (0.29 ± 0.14) than in the control participants (0.40 ± 0.09) (P < 0.0001). Taken together, the results suggested that beta-oxidation was compromised in the patients with BTM.

The data demonstrated a lower fatty acid oxidation rate in the patients with BTM; therefore, the indicator ratio C0/(C16 + C18), which is known as an indicator of carnitine palmitoyltransferase-1 (CPT-1) activity, was further taken into consideration [20]. Patients with BTM are accompanied by conditions of systemic hypoxia and an increase in H_2O_2. Hypoxia and increased H_2O_2 could reduce *CPT-1* expression at the mRNA level and its enzyme activity [21]. As shown in Table 6, the C0/(C16 + C18) ratio was significantly higher in the patients with BTM (92.21 ± 52.02) than in the control group (25.80 ± 6.859) (P < 0.0001), implying lower CPT-1 activity in the patients with BTM. However, the indicator ratio for medium chain fatty acid metabolism (C8/C10) was not significantly different between the patients with BTM and the control participants (P > 0.05) [22].

Discussion

Diagnosis of beta-thalassemia can be performed using complete blood cell count (CBC), Hb electrophoresis and/or by genetic testing. Although these tests can confirm a diagnosis of
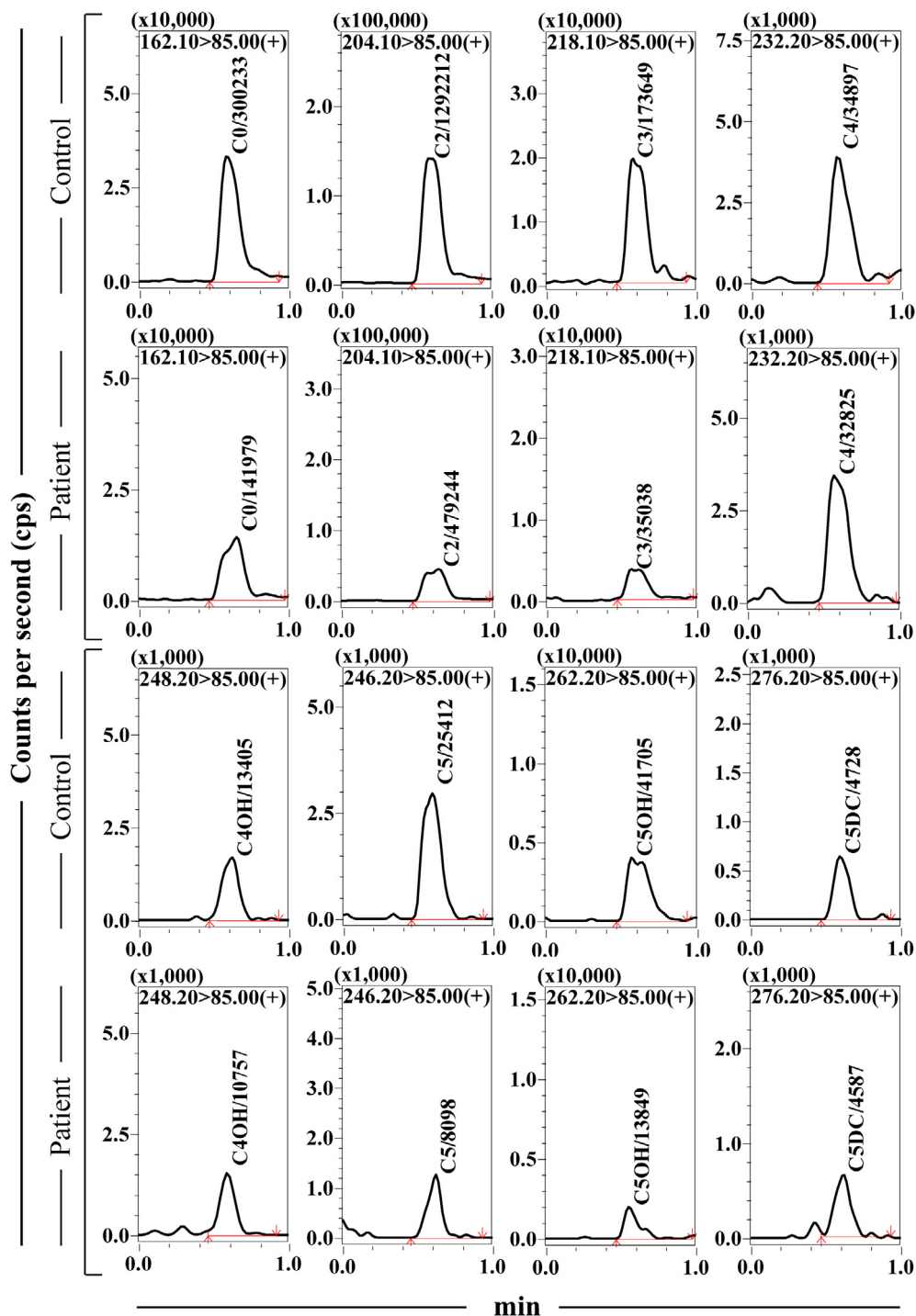
Fig. 2. LC-MS/MS-based representative MRM chromatograms for short chain acylcarnitines for BTM patients and healthy controls. The X-axis represents time after run in minute and Y-axis represent metabolites counted per second. The upper-left number inside the MRM chromatogram represents precursor ion followed by >sign and then the upper right number represents the target ion. Each metabolite detected has been shown vertically above the peak with a capital C followed by digit(s). The concentration of each short chain (SC) acylcarnitine is proportional to the peak area. The upper panel and lower middle panel indicate controls, whereas the upper middle panel and the bottom panel indicate patients.

beta-thalassemia, they cannot provide information about alterations of metabolites, which might play a role in disease pathology. Therefore, it is important to study metabolites in the patients with BTM to obtain valuable pathological information and a mechanistic insight into the biochemistry of the disease process. The phenotypic information and mechanistic basis underlying the pathological phenotype might help to devise adjunct medication and prognostic approaches with various management possibilities, and improve therapeutic approaches.

Different pathological stresses, including oxidative stress, cause damage to the organs and tissues in the patients with BTM [14], thus affecting the synthesis of biomolecules, which ultimately affects normal metabolic processes. Revealing the alterations of metabolic processes in the body fluids and tissues in the course

Table 4
Free and total carnitine level in levels in the blood of BTM patients with respect to their genotypic statuses.

Sl no	Genotypic status	Number of samples	Free carnitine (nmol/mL)	Total carnitine (nmol/mL)
1	IVS1_5G>C[#]	32	32.50 ± 7.34	42.77 ± 10.07
2	c.92G>C[#]	2	32.38 ± 6.16	54.13 ± 2.45
3	IVS1_130G>C[#]	1	28.62 ± 0.00	34.45 ± 0.00
4	c.47G>C[*] + IVS1_5G>C[*]	2	30.56 ± 16.55	45.71 ± 29.68
5	c.27_28insG[*] + IVS1_5G>C[*]	2	26.15 ± 1.62	35.71 ± 2.86
6	IVS1_5G>C[*] + c.126_129delCTTT[*]	2	34.06 ± 8.25	48.08 ± 18.38
7	c.33C>A[*] + c.51delC[*]	2	20.70 ± 0.27	32.08 ± 3.21
8	c.92G>C[*] + IVS1_5G>C[*]	1	32.78 ± 0.00	43.94 ± 0.00
9	IVS1_5G>C[*] + IVS1_130G>C[*]	1	32.67 ± 0.00	40.51 ± 0.00
10	Healthy controls	96	42.93 ± 8.683	64.25 ± 11.76

Table-4 represents the concentrations of free carnitine and total carnitine. Data presented as Mean ± SD; # indicates homozygous mutation, whereas [*] indicates heterozygous mutation.

of disease progression provides clues to predict disease pathology at the early stage and thus is useful to predict treatment outcome and survival of the affected patients. In addition, this strategy might help to develop symptom-specific medication. Iron overload and its associated organ damage are common phenomena in patients with BTM [14,17]. The kidney and liver, which are affected in patients with BTM, are the primary sites of carnitine biosynthesis [23]. Moreover, among many other clinical manifestations, the patients with BTM suffer from lethargy, fatigue, and weakness, indicating the presence of a chronic energy crisis state in these patients [8]. These clinical manifestations in patients with BTM might be associated with impaired carnitine and acylcarnitine levels, because these metabolites play an important role in fatty acid oxidation [24,25], and the study of these metabolites might reveal important information about disease pathology. Thus, the present study focused on the free carnitine and individual fatty acylcarnitine levels, and the effect of any changes in the levels of these metabolic parameters on fatty acid oxidation in the patients with BTM to gain insight into the pathophysiology of the disease.

The comparison of blood L-carnitine and total carnitines between the patients with BTM and the healthy controls revealed an altered carnitine profile in the disease group. The study demonstrated a lower level of L-carnitine in the patients with BTM compared with that in their control counterparts. This finding was consistent with previously published data [11,26]. Moreover, it has been reported that patients with BTM receiving L-carnitine supplementation showed improvement in maintaining RBC stability and prevention of RBC destruction, with the ultimate effect in improving RBC indices and blood transfusion intervals [27,28]. The lower level of L-carnitine in the patients with BTM could be explained by considering the affected organs, especially the involvement of compartmentalized organelles where L-carnitine is biosynthesized. The liver and kidney are the main organs of carnitine biosynthesis in the body, with a low level of carnitine also being biosynthesized in the brain [9]. Several studies have addressed compromised liver and kidney functions in patients with BTM, which could be attributed to their impaired carnitine biosynthesis [14,15,17]. Moreover, in vivo carnitine biosynthesis occurs from trimethyllysine which is produced in lysosomes from the degradation of proteins that undergo post-translational N-methylation to produce protein-6-N-trimethyllysine [23]. This step of trimethyllysine production can occur in the lysosomes of all organs and tissues. However, the lysosomes are highly susceptible to oxidative stress. Increased lysosomal damage and tissue injury have been reported in iron overload-related toxicity and oxidative stress [29–31], indicating that impaired in vivo carnitine biosynthesis in patients with BTM might be associated with lysosomal damage caused by iron overload.

L-carnitine plays an indispensable role in the transportation of long chain fatty acids across mitochondrial membranes for β-oxidation; hence, carnitine deficiency would affect fatty acid transportation [32]. The findings of the present study regarding fatty acid oxidation impairment in the patients with BTM were clearly reflected in the free carnitine and acylcarnitine profiles of the dried blood spots. The profile of acylcarnitine metabolites in the patients with BTM was compromised compared with that of the control group. In addition, it was evident that a low level of carnitine in the patients with BTM affected the levels of acylcarnitine derivatives, as manifested by lower levels of long chain and short chain acylcarnitines. Using an HPLC-based analysis, Tsagris et al. demonstrated depleted free carnitine and total acylcarnitine levels in patients with BTM [8], supporting the findings of the present study. However, unlike the aforesaid study, our LC-MS/MS-based study was much more sensitive and convenient to explain the impairment of long chain fatty acid oxidation because we could analyze not only free and total carnitines, but also individual long chain, medium chain and short chain acylcarnitine levels, which could help to explain the fatty acid oxidation-related disturbance.

Moreover, the findings suggested that the change in fatty acid oxidation was associated with CPT-1 activity. A significant decrease in CPT-1 activity in the patients with BTM compared with that in the healthy participants suggested deficient production of long chain fatty acylcarnitines, which in turn could be attributed to a decrease in the transport of long chain fatty acylcarnitine across the mitochondrial membrane. The decrease in CPT-1 activity could be accounted for by the low expression level of CPT-1 under hypoxic conditions [33,34], which is a common occurrence in patients with BTM. Moreover, H_2O_2 and H_2O_2-induced reactive oxygen species (ROS) also remain at an elevated level in the patients with BTM [35]. Higher H_2O_2 levels could cause a decrease in CPT-1 mRNA expression, and it has been demonstrated that L-carnitine supplementation in H_2O_2-exposed normal human hepatocyte cell line HL7702 could enhance CPT-1 expression by scavenging H_2O_2 and H_2O_2-induced ROS [21].

Even though the short chain and long chain acylcarnitine levels were lower in the patients with BTM than in the controls, the medium chain fatty acylcarnitine levels were similar between the two groups. The normal levels of medium chain fatty acylcarnitines in the patients with BTM could be attributed to their production in peroxisomes. The medium chain fatty acylcarnitines are produced in peroxisomes from very long chain fatty acids and from long chain dicarboxylic acids that are produced in the endoplasmic reticulum by ω-oxidation of long chain fatty acids [36].

Our study suggested that a reduced level of free carnitine and the associated anomaly in acylcarnitine levels in patients
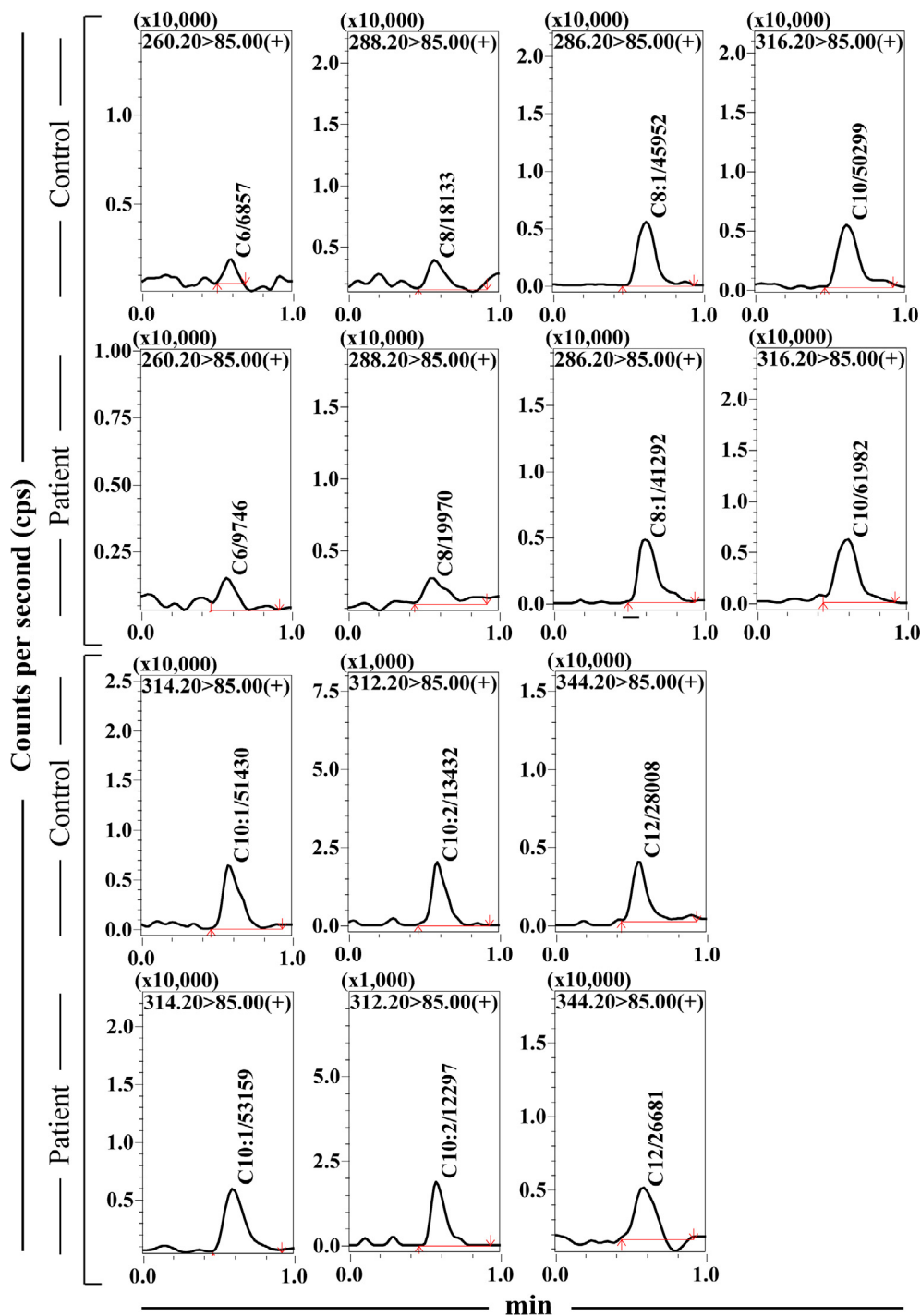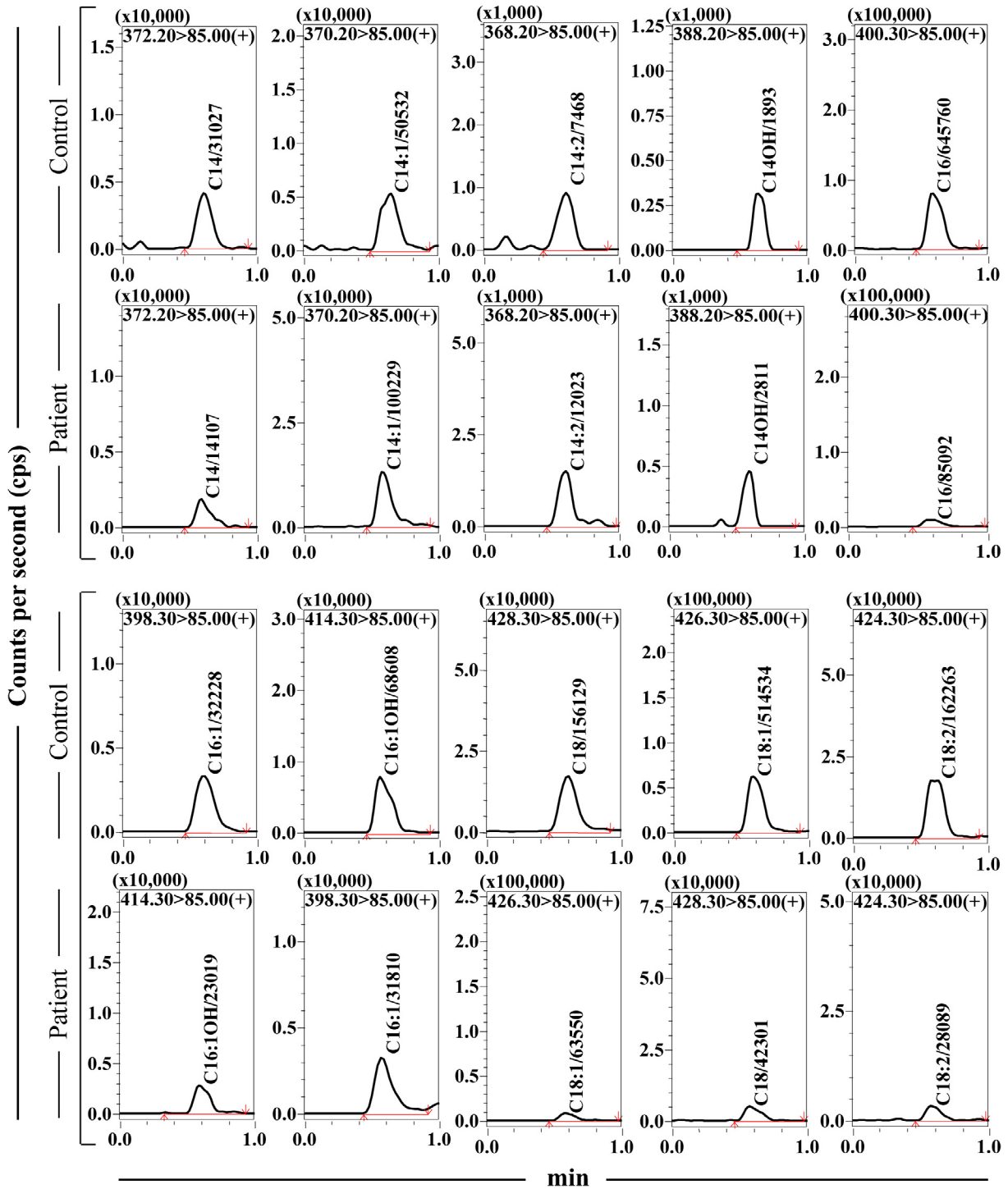
Fig. 3. LC-MS/MS-based representative MRM chromatograms for medium chain acylcarnitines for BTM patient and healthy control. The X-axis represents time after run in minute and Y-axis represent metabolites counted per second. The upper-left number inside the MRM chromatogram represents precursor ion followed by >sign and then the upper right number represents the target ion. Each metabolite detected has been shown vertically above the peak with a capital C followed by digit(s). The concentration of each medium chain (MC) acylcarnitine is proportional to the peak area. The upper panel and lower middle panel indicate controls, whereas the upper middle panel and the bottom panel indicate patients.

with BTM might impair fatty acid oxidation and thus the cells are forced to remain in a state of energy crisis. This energy crisis could explain the decrease in muscle mass, muscular weakness, and cardiac dysfunction in the patients with BTM [28,37]. L-carnitine supplementation has been shown to improve cardiac performance, physical fitness, and transfusion intervals in the patients with BTM [28]. Moreover, CPT-1 activity can be

modulated in the muscle and liver of young pigs using dietary L-carnitne supplementation [38]. This could explain why L-carnitine supplementation combined with chronic blood transfusion and chelation therapy helps to reduce energy crisis-related complications in the patients with BTM.

In the present study, blood specimens from the patients with BTM were collected a couple of hours before blood transfusion. It
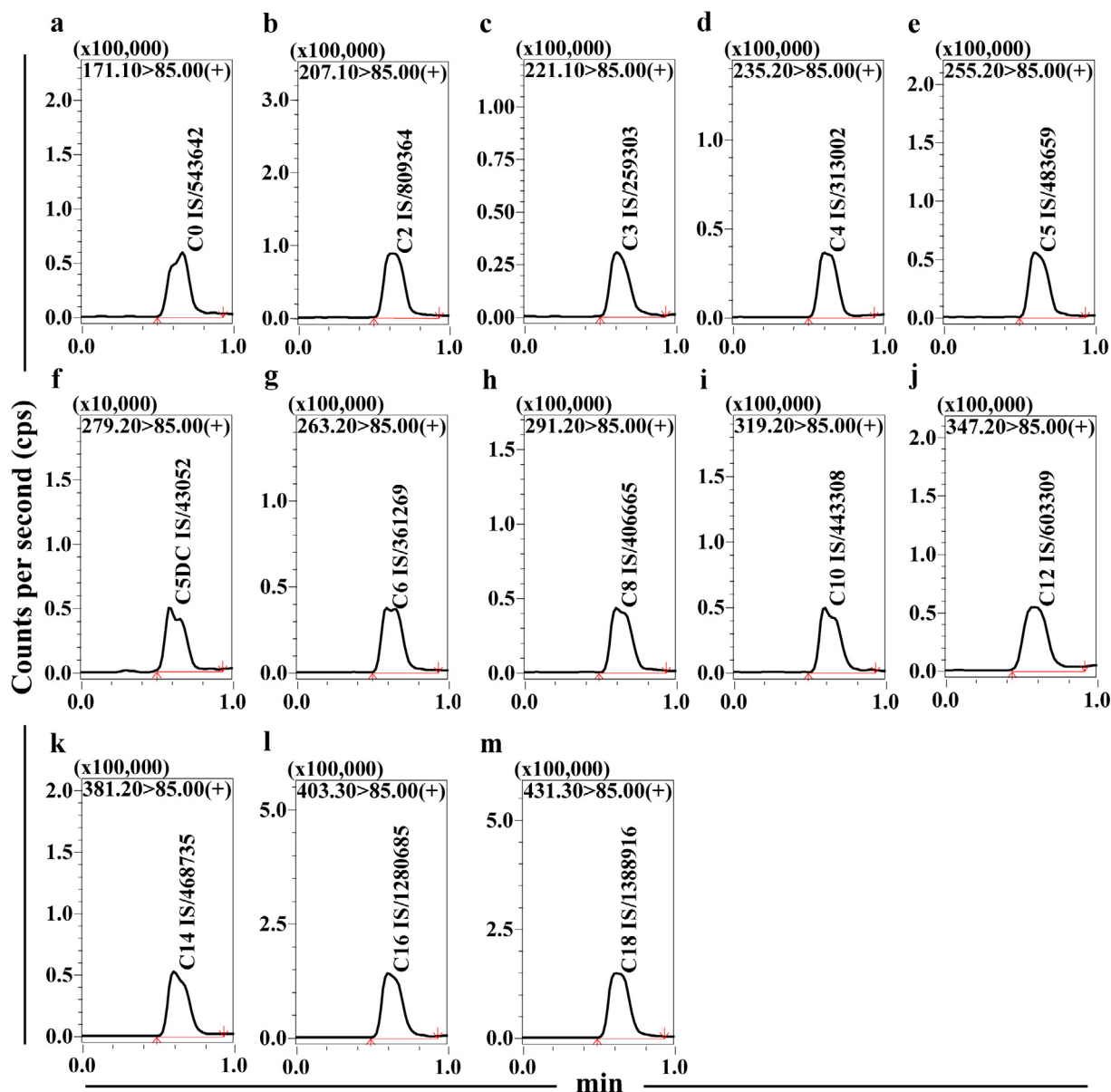
Fig. 4. LC-MS/MS-based representative MRM chromatograms for long chain acylcarnitines for BTM patient and healthy control. The X-axis represents time after run in minute and Y-axis represent metabolites counted per second. The upper-left number inside the MRM chromatogram represents precursor ion followed by >sign and then the upper right number represents the target ion. Each metabolite detected has been shown vertically above the peak with a capital C followed by digit(s). The concentration of each long chain (LC) acylcarnitine is proportional to the peak area. The upper panel and lower middle panel indicate controls, whereas the upper middle panel and the bottom panel indicate patients.

has been observed that a portion of thalassemic erythroblasts escapes early destruction to complete their maturation into reticulocytes, which in turn are released from the bone marrow into the peripheral circulation [39]. Thus, RBCs of two different origins, namely donor-origin RBCs and the patients' deformed RBCs that exit the bone marrow as a result of ineffective erythropoiesis

are expected to circulate in patients with transfusion-dependent BTM. Therefore, to strongly support the findings that there were significantly reduced levels of carnitine and acylcarnitines in the blood of patients with BTM, it was important to consider the quality of the transfused blood. However, in the Dhaka Shishu Hospital, where blood transfusion was performed, it was a common practice

Fig. 5. LC-MS/MS generated representative multiple reaction monitoring (MRM) chromatograms for internal standards. The X-axis represents time after run in minute and Y-axis represent metabolites counted per second. The upper-left number inside the MRM chromatogram represents precursor ion followed by >sign and then the upper right number represents the target ion. Each metabolite detected has been shown vertically above the peak with a capital C followed by digit(s) and then IS for internal standard. The concentration of each metabolite is proportional to the peak area. The internal standards are presented in the chromatogram as (a) $^2H_9$-free carnitine (C0 IS), (b) $^2H_3$-acetylcarnitine (C2 IS), (c) $^2H_3$-propionylcarnitine (C3 IS), (d) $^2H_3$-butyrylcarnitine (C4 IS), (e) $^2H_9$-Isovalerylcarnitine (C5 IS), (f) $^2H_3$-Glutarylcarnitine (C5DC IS), (g) $^2H_3$-Hexanoylcarnitine (C6 IS), (h) $^2H_3$-Octanoylcarnitine (C8 IS), (i) $^2H_3$-Decanoylcarnitine (C10 IS), (j) $^2H_3$-Lauroylcarnitine (C12 IS), (k) $^2H_9$-Myristoylcarnitine (C14 IS), (l) $^2H_3$-Palmitoylcarnitine (C16 IS), and (m) $^2H_3$-Stearoylcarnitin (C18 IS).

that the parents or guardians of patients would bring the donors with them and the blood collection, processing and transfusion into the patients with BTM were done on the same day, thereby ensuring transfusion of good quality blood to patients with BTM. Thus, blood storage-related decreases in carnitine and acylcarnitines could be excluded. The overall decrease in carnitine and acyl-carnitines observed in this study could be attributed to secondary carnitine deficiency caused by a decrease in the biosynthesis of these molecules as a result of anemia-induced oxidative stress and/or iron overload-induced toxicity on the patient's liver and kidneys where carnitine is produced [14,15,17].

A low-carnitine diet consumed by young adult women has been reported to affect the carnitine and acylcarnitine levels by vitamin B6 depletion and repletion [40]. Thus a limitation of this study is that we did not investigate the Vitamin B6 status of the healthy controls and the patients with BTM, indicating that further studies involving Vitamin B6 levels are required to clarify the matter. Another limitation of this study was that we used only three different concentrations (low, medium, and high) of acylcarnitine controls instead of four or more because the NeoMass AAAC kit contained only three different concentrations of each analyte to check the linearity of the method performance. Even though only three levels of each analyte were used for linearity calculation, the obtained linearity values for all acylcarnitines were satisfactory.

Table 5
The acylcarnitine profiles in beta-thalassemia major patients and control participants.

Carnitine derivatives	BTM (N = 45)			Control (N = 96)			P- value (BTM vs. Control)
	Mean ± SD	95% CI	% CV	Mean ± SD	95% CI	% CV	
C2	8.282 ± 4.34	6.97–9.585	52.38%	15.52 ± 3.55	14.80–16.24	22.90%	<0.0001
C3	0.76 ± 0.46	0.62–0.89	60.15%	1.41 ± 0.39	1.33–1.49	27.56%	<0.0001
C4	0.15 ± 0.12	0.11–0.18	80.85%	0.13 ± 0.05	0.12–0.14	40.81%	0.3465
C4OH	0.06 ± 0.05	0.04–0.08	92.66%	0.09 ± 0.05	0.08–0.10	49.88%	0.0055
C5	0.10 ± 0.04	0.08–0.11	43.00%	0.14 ± 0.05	0.13–0.15	34.87%	<0.0001
C5OH	0.09 ± 0.03	0.08–0.10	35.31%	0.30 ± 0.09	0.28–0.32	29.01%	<0.0001
C5DC	0.06 ± 0.03	0.05–0.07	54.83%	0.07 ± 0.03	0.06–0.08	40.09%	0.1783
TSCC	9.51 ± 4.79	8.07–10.95	50.41%	17.67 ± 3.80	16.90–18.44	21.51%	<0.0001
C6	0.03 ± 0.02	0.02–0.04	77.16%	0.02 ± 0.02	0.01–0.024	98.80%	0.0130
C8	0.024 ± 0.03	0.01–0.03	121.33%	0.02 ± 0.03	0.01–0.02	186.06%	0.1445
C8.1	0.14 ± 0.08	0.12–0.16	54.13%	0.17 ± 0.09	0.15–0.19	55.58%	0.1012
C10	0.10 ± 0.07	0.08–0.12	67.38%	0.07 ± 0.04	0.06–0.08	59.24%	0.0136
C10.1	0.07 ± 0.04	0.6–0.08	50.67%	0.07 ± 0.04	0.06–0.08	54.62%	0.8793
C10.2	0.015 ± 0.007	0.012–0.046	49.51%	0.0175 ± 0.009	0.015–0.019	53.73%	0.0527
C12	0.05 ± 0.03	0.04–0.06	59.29%	0.05 ± 0.02	0.04–0.05	38.77%	0.4909
TMCC	0.43 ± 0.19	0.38–0.49	43.37%	0.41 ± 0.16	0.38–0.44	39.67%	0.4791
C14	0.07 ± 0.06	0.05–0.08	89.37%	0.09 ± 0.04	0.08–0.095	45.41%	0.0347
C14.1	0.12 ± 0.06	0.10 – 0.13	53.65%	0.07 ± 0.03	0.06 – 0.08	44.67%	<0.0001
C14.2	0.04 ± 0.03	0.03 – 0.05	62.84%	0.03 ± 0.02	0.02 – 0.034	52.96%	0.0290
C14OH	0.012 ± 0.008	0.009 – 0.015	71.69%	0.010 ± 0.007	0.009 – 0.012	69.60%	0.1596
C16	0.36 ± 0.35	0.25 – 0.46	100.52%	1.28 ± 0.40	1.20 – 1.36	31.80%	<0.0001
C16.1	0.06 ± 0.03	0.05–0.07	58.54%	0.06 ± 0.02	0.06 – 0.064	29.5%	0.5876
C16.1OH	0.012 ± 0.012	0.008 – 0.016	98.65%	0.032 ± 0.014	0.028 – 0.034	44.17%	<0.0001
C18	0.16 ± 0.15	0.11–0.21	94.20%	0.49 ± 0.16	0.45 – 0.51	33.03%	<0.0001
C18.1	0.26 ± 0.28	0.17 – 0.34	109.43%	0.83 ± 0.24	0.78 – 0.88	28.75%	<0.0001
C18.2	0.06 ± 0.06	0.04 – 0.08	101.76%	0.33 ± 0.10	0.30 – 0.35	30.92%	<0.0001
TLCC	1.162 ± 0.99	0.86–1.46	85.34%	3.24 ± 0.86	3.06–3.42	26.64%	<0.0001

The data presented here in Table 5 were derived from analysis of DBS specimens by using LC-MS/MS. The short chain acylcarnitinine or their derivatives that were analyzed in the study included C2, C3, C4, C4OH, C5, C5OH and C5DC (upper panel), whereas C6, C8, C8.1, C10, C10.1, C10.2, and C12 were analyzed as the medium chain acylcarnitinines or their derivatives (middle panel). On the other hand, the long chain acylcarnitines or their derivatives that were analyzed included C14, C14.1, C14.2, C14OH, C16, C16.1, C16.1OH, C18, C18.1 and C18.2 (bottom panel). C2 = Acetylcarnitine; C3 = Propionylcarnitine; C4 = Butyrylcarnitine; C4OH = 3-Hydroxybutyrylcarnitine; C5 = Isovaleryl-carnitine; C5OH = 3-OH isovalerylcarnitine; C5DC = Glutarylcarnitine; TSCC = Total short chain Acylcarnitine; C6 = Hexanoylcarnitine; C8 = Octanoylcarnitine; C8.1 = Octenoylcarnitine; C10 = Decanoylcarnitine; C10.1 = Decenoylcarnitine; C10.2 = Decadienoylcarnitine; C12 = Lauroylcarnitine; TMCC = Total medium chain acylcarnitine; C14 = Myristoylcarnitine; C14.1 = Myristoleylcarnitine; C14.2 = Tetradecadienoylcarnitine; C14OH = 3-Hydroxytetradecanoylcarnitine; C16 = Palmitoylcarnitine; C16.1 = Palmitoyleylcarnitine; C16.1OH = 3-Hydroxypalmitoleylcarnitine, C18 = Stearoylcarnitine; C18.1 = Oleylcarnitine; C18.2 = Octadecadienylcarnitine; TLCC = Total long chain acylcarnitine; BTM = Beta-thalassemia major; 95% CI = 95% confidence interval; CV = Coefficient of variation; P < 0.05 was considered significant.

Table 6
Metabolic indicator ratios in BTM patients and control participants.

Indicator ratio (IR)	BTM (N = 45)			Control (N = 96)			P-value (BTM vs. Control)
	IR values	95% CI	%CV	IR values	95% CI	%CV	
C2/C0	0.26 ± 0.13	0.23–0.31	49.49%	0.37 ± 0.084	0.35–0.38	22.87%	<0.0001
(C2 + C3)/C0	0.29 ± 0.14	0.25–0.33	47.74%	0.40 ± 0.09	0.38–0.42	21.52%	<0.0001
C0/(C16 + C18)	92.21 ± 52.02	76.58–107.8	56.41%	25.80 ± 6.859	24.41–27.19	26.59%	<0.0001
C8/C10	0.26 ± 0.32	0.17–0.36	122.02%	0.21 ± 0.41	0.13–0.29	189.57%	0.4451

Data presented in Table-6 illustrate metabolic indicator ratios for fatty acid oxidation rate [C2/C0 and (C2 + C3)/C0)], Carnitine Palmitoyltransferase-1 (CPT-1) activity [C0/(C16 + C18)], and medium chain metabolism (C8/C10). The values are presented as Mean ± SD. Statistical analysis was performed using t-test with welch's correction. 95% CI = 95% confidence interval; CV = Coefficient of variation; P < 0.05 was considered significant.

Conclusions

In summary, patients with chronic transfusion-dependent BTM have lower levels of free carnitine, as well as long chain and short chain acylcarnitines. Their fatty acid oxidation becomes impaired not only because of lower carnitine/acylcarnitine levels, but also via impaired CPT-1 activity. Further studies are needed to exploit this valuable metabolic information to devise better treatment and management strategies for the disease.

Conflict of interest

The authors have declared no conflict of interest.

Acknowledgements

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jare.2018.04.002.

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