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Oxidized phosphatidylcholines suggest oxidative stress in patients with medium-chain acyl-CoA dehydrogenase deficiency

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ABSTRACT

Inborn errors of metabolism encompass a large group of diseases caused by enzyme deficiencies and are therefore amenable to metabolomics investigations. Medium chain acyl-CoA dehydrogenase deficiency (MCADD) is a defect in β-oxidation of fatty acids, and is one of the most well understood disorders. We report here the use of liquid chromatography–mass spectrometry (LC–MS) based untargeted metabolomics and targeted flow injection analysis–tandem mass spectrometry (FIA–TMS) that lead to discovery of novel compounds of oxidative stress. Dry blood spots of controls ($n=25$) and patient samples ($n=25$) were extracted by methanol/water (1/1, v/v) and these supernatants were analyzed by LC–MS method with detection by an Orbitrap Elite MS. Data were processed by XCMS and CAMERA followed by dimension reduction methods. Patients were clearly distinguished from controls in PCA. S-plot derived from OPLS-DA indicated that medium-chain acylcarnitines (octanoyl, decenoyl and decanoyl carnitines) as well as three phosphatidylcholines (PC(16:0,9:0(COOH))), PC(18:0,5:0(COOH)) and PC(16:0,8:0(COOH)) were important metabolites for differentiation between patients and healthy controls. In order to biologically validate these discriminatory molecules as indicators for oxidative stress, a second cohort of individuals were analyzed, including MCADD ($n=25$) and control ($n=250$) samples. These were measured by a modified newborn screening method using FIA–TMS (API 4000) in MRM mode. Calculated *p*-values for PC(16:0,9:0(COOH)), PC(18:0,5:0(COOH)) and PC(16:0,8:0(COOH)) were 1.927×10^{-14}, 2.391×10^{-15} and 3.354×10^{-15} respectively. These elevated oxidized phospholipids indeed show an increased presence of oxidative stress in MCADD patients as one of the pathophysiological mechanisms of the disease.

1. Introduction

Fatty acid oxidation disorders (FAODs) are metabolic diseases with a cumulative incidence of approximately 1:9300 (measured in 2010) based on newborn screening (NBS) in Australia, Germany and USA [1]. At least 15 different disorders of fatty acid metabolism are known today [2]. Medium chain acyl-CoA dehydrogenase deficiency (MCADD OMIM #201450) is one of the most

Abbreviations: MCADD, Medium chain acyl-CoA dehydrogenase deficiency; LC–MS, liquid chromatography–mass spectrometry; FIA–TMS, flow injection analysis–tandem mass spectrometry; PCA, principal component analysis; OPLS-DA, orthogonal partial least squares discriminant analysis; MRM, multi-raction-monitoring mode; FAODs, fatty acid oxidation disorders; NBS, newborn screening; MCAD, medium chain acyl-CoA dehydrogenase enzyme; C8, octanoylcarnitine; C6, hexanoylcarnitine; C10, decanoylcarnitine; C10:1, decenoylcarnitine; C2, acetylcarnitine; C12, dodecanoylcarnitine; PGPC, 1-O-hexadecanoyl-2-O-(9-carboxybutanoyl)-sn-glyceryl-3-phosphocholine; PAzPC, 1-O-hexadecanoyl-2-O-(9-carboxyoctanoyl)-sn-glyceryl-3-phosphocholine; FWHM, full width at half maximum; QC, quality control; CID, collision-induced dissociation; HCD, higher-energy collisional dissociation; FTMS, fourier transform mass spectrometry; LOESS, local regression; clr, centered logratio transformation; MSI, Metabolite Standards Initiative; ROS, reactive oxygen species; PUFAs, polyunsaturated fatty acids; PoxnoPC, 1-O-hexadecanoyl-2-O-(9-oxononanoyl)-sn-glyceryl-3-phosphocholine; PAPC, 1-O-hexadecanoyl-2-O-(5Z,8Z,11Z,14Z)-5,8,11,14-eicosatetraenoyl-sn-glyceryl-3-phosphocholine

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common fatty oxidation defects and it is inherited as an autosomal recessive trait. The medium chain acyl-CoA dehydrogenase (MCAD) enzyme (EC 1.3.99.3) metabolizes medium long chain fatty acids (carbon moiety length from 4 to 12) in a β-oxidation pathway which is localized in mitochondria and starts the mitochondrial β-oxidation pathway by introducing a double bond into a β-position of acyl-Coenzyme A (acyl-CoA). *ACADM* gene is located at 1p31.1 and one prevalent mutation c.985A > G accounts for up to 90% of mutant alleles identified so far. Up to now 36 mutations have been identified by newborn screening [2] and the frequency of mutation varies substantially between different populations with a particularly high incidence in north-central Europe: Denmark 1:8954 [3], England 1:10,000 [4], Netherlands 1:30,000 [5], Germany 1:133,000 [6], Greece 1:16,000 [7], and an overall incidence in USA 1:15,000 [8]. By contrast, with the exception of Greece, low incidence was generally found in southern Europe (1:300,000) [9] and no cases have been reported in Japan.

The clinical picture of the disease is highly variable ranging from severe-life threatening forms to asymptomatic patients identified retrospectively in families with positive newborn screening. The symptoms can appear at any age from newborn to adult, but the most common age of presentation is between 3 and 15 months. Symptoms in untreated patients cover hypoglycemic, acute encephalopathy initiated by common infectious diseases or fasting [10]. Up to 50% of the patients die during the first episode. Those, who survive, suffer from development delay, hypotonia and (cardio-)myopathy. The conditions can be effectively managed by preventing hypoglycemia. This is achieved by increasing feeding frequency with food containing a high starch content and a limited supply of medium chain triglyceride oils. Acute patients exhibit hyperamonemia and characteristic profile of elevated organic acids in urine – adipic, suberic, sebacic acids, hexanoic, octanoic acids [11] – and several other glycine conjugates – hexanoylglycine, 3-phenylpropionylglycine and suberylglycine [12]. This biochemical picture depends on the clinical status and can disappear during the period of normalcy.

The disease is screened in the majority of developed countries in the world. Nowadays, the common technique used in NBS is tandem mass spectrometry. This enables the rapid (typically 1 min/sample) measurement of dozens of diagnostically important metabolites with very high sensitivity and specificity [13]. The main diagnostic marker for MCADD is octanoylcarnitine [C8] and secondary markers include hexanoylcarnitine [C6], decanoylcarnitine [C10] and decenoylcarnitine (C10:1), respectively [14]. The screening of MCADD is based on determination of elevated levels of several acylcarnitines and their ratios with other acylcarnitines (e.g. acetylcarnitine (C2), dodecanoylcarnitine (C12) – C8/C2; C8/C12; C8/C6) in dried blood spots usually taken 1–3 days after birth [14].

In the area of inherited metabolic disorders untargeted metabolomics was firstly utilized by Siuzdak's group [15]. They used capillary reverse phase liquid chromatography with nonlinear alignment software (XCMS) data processing on samples from patients suffering from methylmalonic and propionic acidemia. Recently, this approach was already successfully applied on various defects in amino acid metabolism, organic acidurias and mitochondrial defects (including MCADD) [16]. The aim of this work was to investigate patho-physiological/-biochemical changes associated with MCADD using untargeted MS-based metabolomics.

2. Materials and methods

2.1. Chemicals

All solvents (acetonitrile, methanol and water) were of LC–MS quality purchased from Sigma-Aldrich (St. Louis, USA). Acetic acid and ammonium hydroxide were also purchased from Sigma-Aldrich. Standards of PGPC (1-O-hexadecanoyl-2-O-(9-carboxybutanoyl)-*sn*-glyceryl-3-phosphocholine) and PAzPC (1-O-hexadecanoyl-2-O-(9-carboxyoctanoyl)-*sn*-glyceryl-3-phosphocholine) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA).

2.2. Samples

Dry blood spots from healthy newborns (control group, $n=25$) and patients suffering from MCADD (8 patients, $n=25$; from subsequent sampling) were chosen for the experiments. Four patients were compound heterozygotes and four were homozygous with mutation c.985A > G. In order to confirm the results and validate the chemical species (metabolites) identified in untargeted metabolomics experiment a second set of samples from 250 newborns was used for targeted metabolite analysis. All samples were collected in laboratory for inherited metabolic disorders (University Hospital Olomouc, CZ) within the pilot project of Czech newborn screening program. Written informed consent was obtained for all samples that were used in the analyses.

2.3. Untargeted metabolomics method

Two disks (3.2 mm) were dissected from dry blood spot and extracted in pure methanol (100 μL). After shaking (30 min, 25 °C) the sample was centrifuged (24,400*g*, 15 min, 4 °C). Supernatant (50 μL) was mixed with water (50 μL) and analyzed by the LC–MS untargeted metabolomics method adopted from Bajad et al. [17].

The stationary phase employed an aqueous normal phase separation system using amino-propyl stationary Luna NH_2 3 μm 100 Å, 150 × 2 mm^2 (Phenomenex, Torrance, USA). An Ultimate 3000 RS (Thermo Fisher Scientific, MA, USA) was used for liquid chromatography and binary gradient elution consisted of 20 mm ammonium acetate in water, pH 9.45 (mobile phase A) and acetonitrile (mobile phase B). The gradient elution with flow rate of 0.3 mL/min was as follows: $t=0.0$, 95% B; $t=15.0$, 30% B; $t=17.0$, 5% B; $t=23.0$, 5% B; $t=23.1$, 95% B; $t=28.0$ min 95% B. The injection volume was 10 μL.

An Orbitrap Elite (Thermo Fisher Scientific, MA, USA) operating in positive full scan mode (120,000 FWHM) within range of 70–1200 *m/z* was used for untargeted metabolomics experiments. Settings of the electrospray ionization were: heater temperature of 300 °C, sheath gas of 35 arb. units, auxiliary gas of 10 arb. units, capillary temperature of 350 °C and source voltage was +3.0 kV. A Thermo Tune Plus 2.7.0.1103 SP1 was used as instrument control software and data were acquired in profile mode using Thermo Excalibur 2.2 SP1.48 software (Thermo Fisher Scientific, MA, USA).

Quality control (QC) samples were prepared by pooling of all patient and control samples (10 μL). Blank sample was prepared by the same procedure without disks from dry blood spots. The order of healthy controls and MCADD patient's samples was randomized in the experiment batch. QC samples were analyzed and used as previously published [18]. Fragmentation spectra MS^n were acquired on an Orbitrap Elite using CID and HCD fragmentation method with detection via FTMS (resolution 60,000 FWHM) in both positive and negative mode as required. Settings for MS^2 and MS^3 experiments were as follows: act.Q. of 0.25, act. time of 10 ms (for MS^3 20 ms) and normalized collision energy of 35%. HCD fragmentation settings were as follows: act.Q. of 0.10 and normalized collision energy of 40%.

2.4. Metabolite target analysis for biological validation

In order to confirm new metabolic findings the samples from MCADD patients ($n=25$) and more healthy controls ($n=250$) were measured by FIA–TMS method routinely used for metabolite target analysis (MassChrom® Amino Acids and Acylcarnitines/Non Derivatised, Chromsystems, DE) with addition of specific transitions for the selected phospholipids (Supplementary data Table 1). Standard sample preparation obtains punching 3.2 mm dry blood spot disk of the filter card into a well plate, adding 100 μL of internal standard and agitation for 20 min. Injection volume on the LC–MS system is 10 μL. A liquid chromatography system Ultimate 3000 RS (Thermo Fisher Scientific, MA, USA) coupled with a triple quadrupole mass spectrometer API 4000 (AB Sciex, CA, USA) operating in MRM mode was used for metabolite targeted analysis.

2.5. Data processing

Data from untargeted metabolomics experiment were processed in R software [40] with *XCMS* [19–21], *CAMERA* [22] and *muma* [23] packages. Peak finding was performed by *XCMS* package using "matchedFilter" method and 1900 features were identified. Isotopes and adducts across the list of features were grouped by *CAMERA* package. First zero imputation was done by function of *XCMS* "fillpeaks" which integrates a noise in the same retention time of a missing peak. Features containing more than 30% of zeros were excluded from further processing. Final features were obtained after excluding isotopes and adducts.

Quality control-based robust LOESS (LOcal regrESSion) signal correction method was applied [18,24]. The curve was fitted through the QC points (based on LOESS) and smoothing factors were calculated for each feature. Smoothing factors with ratio of the maximum and the minimum of smoothing values higher than 10 were deleted from the data set. The feature values were divided by smoothing factors and used for further processing. Coefficients of variation were calculated and all features with a value higher than 30% were rejected from further processing, reducing the number of features to 273. Zero imputation was applied on the dataset. Zero values were replaced by two-thirds of minimal value per features within each sample group. Data were transformed by *centered logratio (clr) transformation* and mean centered, respectively [25].

For statistical evaluation both unsupervised (principal component analysis (PCA)) and supervised methods (orthogonal partial least squares discriminant analysis (OPLS-DA)) were applied. S-plot from OPLS-DA was used to elucidate features important for discrimination. This function was calculated using the *muma* package in R.

Spectra acquired in metabolite target analysis were evaluated in MultiQuant™ 2.1.1 (AB Sciex, CA, USA) and statistically processed in R. Data were referenced to the D3-octanoylcarnitine as an internal standard (1.36 μmol/L). Finally, the data were visualized by boxplots and scatter plots. Statistical significance (p-value) was calculated by non-parametric Wilcoxon Rank Sum test.

2.6. Confirmation of important discriminatory metabolite features

In order to identify features from the above process (S-plots from OPLS) three levels of identification reliability were applied [26] which follow the Metabolomics Standards Initiative MSI standards for metabolite identification [39]. MSI Level 1 – "confidently identified compound" – two orthogonal properties were used: *m/z*, fragmentation spectra and comparison of retention time with commercially available standards. Instead of simple fragmentation spectra approach "spectral trees" were applied (these are sets of MS^n data using different fragmentation techniques and collision energies (www.mzcloud.org)). MSI Level 2 – "putatively annotated compounds" – for identification the exact mass and comparison of fragmentation spectra with similar compounds found in databases (Metlin, HMDB, LipidMaps). MSI Level 3 – "putatively annotated compound classes" – based on specific peaks in fragmentation spectra, exact mass and the region of elution from chromatographic column [26]. The rest of the features, which do not belong to these three levels were marked as "unknowns". The determination of lipid acyls and the level of their saturation were elucidated in negative mode using CID fragmentation up to MS^3. PAzPC and PGPC standards were used for determination of general lipid retention time at this separation conditions.

3. Results and discussion

Statistical evaluation by PCA showed clear separation between the control group and MCADD samples (Fig. 1A). This result was highly encouraging as PCA is an unsupervised learning method and thus any clusters that are evident are likely to be due to natural differences in the metabolomics profiles. The wider spread of the MCADD samples in PCA, compared to the controls, is caused by different levels of metabolic manifestation of MCADD and/or by date of subsequent sampling. As a complementary tool the supervised OPLS-DA was used (Fig. 1B) in order to discover which metabolites were highly diagnostic for group separation. The derived S-plot (see Supplemental data Fig. S1) is a visualization of covariance and correlation between features and the determined groups of samples. In order to select the most significant features the following parameters were used: $p1 \geq \pm 10$ and $p1(\text{corr}) \geq \pm 0.5$. Fourteen features meet the restrictive parameters of the S-plot and these are reported in Table 1. Four of the significant compounds from Table 1 were confidently identified to MSI Level 1: already known biomarkers of MCADD (C8, C10:1, C10) and one new compound 1-O-hexadecanoyl-2-O-(9-carboxyoctanoyl)-*sn*-glyceryl-3-phosphocholine (PAzPC). Two another phospholipids (1-O-octadecanoyl-2-O-(5-carboxybutanoyl)-*sn*-glyceryl-3-phosphocholine, PC(18:0;5:0(COOH)) and 1-O-hexadecanoyl-2-O-(8-carboxyheptanoyl)-*sn*-glyceryl-3-phosphocholine, PC(16:0;8:0(COOH))) were marked as putatively known compounds (identification reliability of MSI Level 2) based on a fragmentation similarity with PAzPC and other phosphatidylcholines present in external databases. For most of the lipid structures we have identified (identification reliability of MSI Levels 1 and 2), palmitic acid moiety at 255.2330 *m/z* was found. Another common fragment of phosphatidylcholines was 3-(hexadecanoyloxy)-2-hydroxypropyl 2-(dimethylaminoethyl) phosphate with *m/z* value of 480.3096. In case of PC(18:0;5:0(COOH)) fragments of stearic acid (283.2643 *m/z*) and 3-(octadecanoyloxy)-2-hydroxypropyl 2-(dimethylaminoethyl) phosphate (508.3398 *m/z*) were acquired. At MSI Level 3 four compounds were labelled as phosphatidyl cholines based on characteristic fragment of phosphocholine (184.0733 *m/z*) in positive mode. Confirmation of phosphocholine moiety was achieved by MS^3 fragmentation in positive mode. The other compounds in Table 1 were marked as "unknowns" (MSI Level 4). Chemical formulae and monoisotopic masses were calculated in QualBrowser software (Thermo Fisher Scientific, CA, USA). Regarding the structure of phosphocholine moiety the chemical formulae containing at least one nitrogen and one phosphorus atom were generated. The fragmentation spectral trees that we acquired are available as supplementary data (see Supplemental data Fig. S2).

Oxidized phosphocholines suggest the presence of oxidative stress in patients suffering from MCADD. In living organisms oxygen influences metabolism to produce free radicals (superoxide O_2^-, hydroxyl OH, alkoxyl RO nad peroxyl RO_2) and also non-radical species (hydrogen peroxide H_2O_2, peroxynitrite

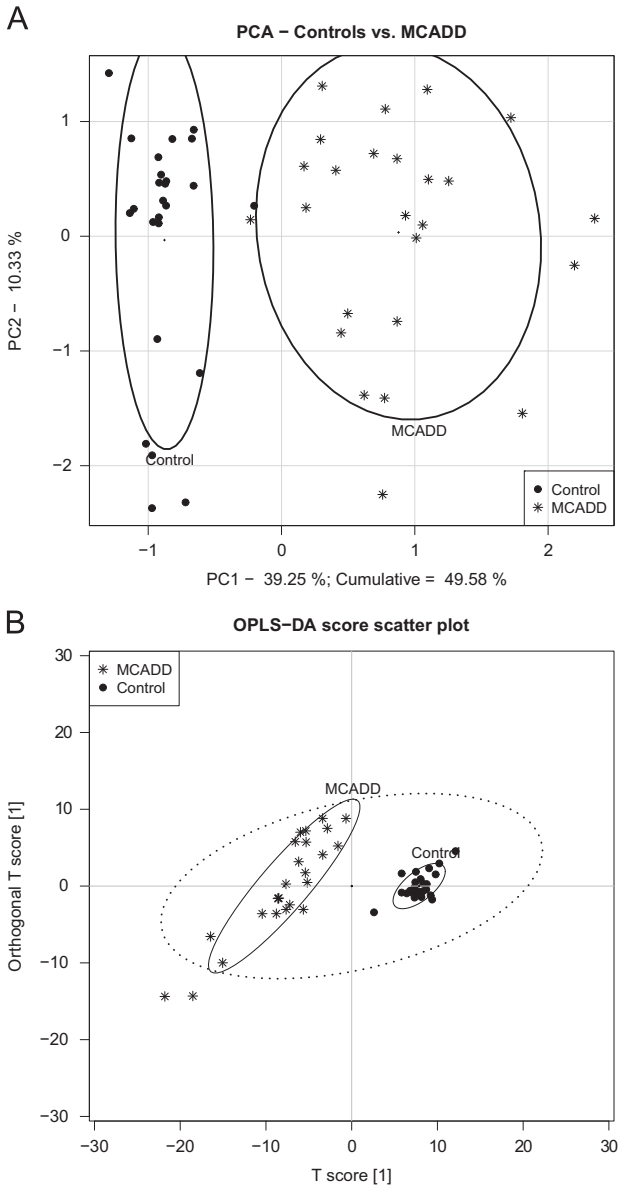

Fig. 1. Analysis from dried blood spots of MCADD and controls. (A) Two dimensional unsupervised PCA analysis, and (B) supervised orthogonal partial least squares-discriminant analysis (OPLS-DA), dashed line ellipses represent 95% quantile of all samples, full line ellipses represent 75% quantile of samples within the individual group.

ONOO–) [27]. Oxidative stress is defined as an imbalance between antioxidants and oxidants in favor of oxidants. Antioxidant defense is based on several enzymatic and non-enzymatic mechanisms [28]. Interaction of reactive oxygen species (ROS) with polyunsaturated fatty acids (PUFAs) is initiated by formation of carbon-centered radicals and/or hydroperoxides of PUFAs (peroxidation of PUFAs). This is one of the most well understood processes of oxidation [29]. Oxidations of these molecules can lead to appearance of short polar acyl chains in the structure (carbonyl and carboxyl groups at the end of their *sn*-2 chains). Some of the oxidized phospholipids were found to be stable and measurable (PAzPC, PoxnoPC (1-O-hexadecanoyl-2-O-(9-oxononanoyl)-*sn*-glyceryl-3-phosphocholine)) [30]. According the literature PAzPC is associated with oxidative stress [29,31].

Oxidative stress can cause severe damage to mitochondria. Reactive oxygen species (ROS) can decrease the mitochondria membrane potential, releasing cytochrome c, and subsequently causing the translocation of Bax to mitochondria and apoptosis-inducing factor to the nuclei [32]. Higher intracellular levels of ROS also affect the glucose homeostasis by impairment of the function of adipocytes [33] and they can be related to many other mitochondrial dysfunctions [34]. Accumulation of medium chain fatty acids as octanoic, decanoic, *cis*-4-decenoic acid as well as their glycine and L-carnitine derivates can cause oxidative stress in MCADD [35,36] as suggested by rat models. The origin of the PC(16:0;8:0(COOH)) can be derived from PAPC (1-O-hexadecanoyl-2-O-(5Z,8Z,11Z,14Z)-5,8,11,14-eicosatetraenoyl-sn-glyceryl-3-phosphocholine) as some of its oxidation products [37,38]. It is also supported by observation of decreased levels of PAPC in patients (see Supplemental data Fig. S3). Thus PAzPC can be considered to be a lineoic acid moiety oxidation product as has been previously reported [38], while PC(18:0,5:0(COOH)) can be considered as oxidation product of related lipids bearing unsaturated fatty acid at *sn*-2 position according to common oxidation mechanisms [37].

We found the correlation between PAzPC and PC(16:0,8:0(COOH)) and PC(18:0,5:0(COOH)) was high and was $R=0.8929$ and $R=0.8096$, respectively, whilst the correlation of the phosphatidylcholines with octanoylcarnitine was below 0.54 (see Supplemental data Fig. S4). This suggests a common mechanism of formation in the patients. As previously mentioned PAzPC is related to oxidative stress [29,31], thus the correlation supports hypothesis that PC(16:0,8:0(COOH)) and PC(18:0,5:0(COOH)) can be also associated with oxidative stress in these patients. Nevertheless, in order to prove this hypothesis a prospective study has to be undertaken.

To confirm findings from untargeted metabolomics method we analyzed 25 MCADD samples and 250 control samples by FIA–TMS operating in MRM mode under specific transitions corresponding

Table 1
List of the most significant features identified by the S-plot. Restriction parameters ($p1=\pm 10$ and $p1(corr)=\pm 0.5$), levels of identification – see text for explanation; PC=phosphatidylcholines.

abs (*p*1) S-Plot	Positive (*m/z*)	Error (ppm)	Chemical formula	RT (s)	Name	Level of identification
+13.68	666.4354	2.0167	C33H65O10NP	514.1	PC(16:0;9:0(COOH))-PAzPC	1
+13.1	288.2172	1.0201	C15H30O4N	407.6	Octanoylcarnitine (C8)	1
−11.18	838.5666	1.7339	C39H85O15NP	511.1	Lipid (PC)	3
−10.93	625.5256	0.7066	C30H76O9NP	426.2	Lipid (PC)	3
−10.84	141.1136			411.8	Unknown	
−10.81	599.5092			427.2	Unknown	
+10.68	314.2327	0.2864	C17H32O4N	401.2	Decenoylcarnitine (C10:1)	1
−10.53	840.5834	3.7607	C39H87O15NP	510.9	Lipid (PC)	3
+10.48	638.4037	1.3941	C31H61O10NP	510.0	PC(18:0;5:0(COOH))	2
−10.46	623.5091	−0.7329	C30H74O9NP	426.1	Lipid (PC)	3
−10.4	385.2756			425.0	Unknown	
+10.21	260.1859	0.9685	C13H26O4N	417.4	Hexanoylcarnitine (C6)	1
+10.21	652.4194	1.5374	C32H63O10NP	515.2	PC(16:0;8:0(COOH))	2
+10.01	639.4089	6.5858	C38H58O5NP	510.3	Lipid (PC)	3

to the detection of the most significant phospholipids (see Supplemental data Table 1). Calculated *p*-values for PAzPC was 1.927×10^{-14}, for PC(16:0,8:0(COOH)) 3.354×10^{-15} and for PC (18:0,5:0(COOH)) was 2.391×10^{-15}. These markers are highly significant and thus corroborate that these 3 lipid species detected using metabolite target analysis were in full agreement with our original data from untargeted analyses.

4. Conclusions

In this study we first applied the approach of untargeted metabolomics on dry blood spots samples of 25 MCADD samples and 25 healthy controls. MCADD samples were clearly distinguished from controls by PCA. OPLS-DA was used to indicate which of the many 100s of metabolites we measured were statistically significant and we obtained 14 significant metabolite features. Among them, the currently known biomarkers of MCADD (C8, C10:1, C10) were confidently identified, and eight of the 14 significant features belong to the family of phosphatidylcholines. The presence of elevated levels of PAzPC (PC(16:0,9:0(COOH))) in the patients over controls, already associated with oxidative stress and two other phosphatidylcholines (PC(16:0,8:0(COOH)) and PC (18:0,5:0(COOH))) were also discovered to be significant features in the untargeted metabolomics method. These were confirmed by FIA–TMS in a second larger blood spot sample set (MCADD samples $n=25$, controls $n=250$; *p*-values $< 10^{-14}$). All these phosphatidylcholines correlated with disease mutually with $R > 0.8$ pointing to a common *mode* of creation. In conclusion using first untargeted metabolomics and then targeted metabolomics in a wider patient cohort we have point out that patients suffering from MCADD are experiencing oxidative stress.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2015.02.041.

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