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Dicarboxylic acylcarnitine biomarkers in peroxisome biogenesis disorders

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Abstract

The peroxisome is an essential eukaryotic organelle with diverse metabolic functions. Inherited peroxisomal disorders are associated with a wide spectrum of clinical outcomes and are broadly divided into two classes, those impacting peroxisome biogenesis (PBD) and those impacting specific peroxisomal factors. Prior studies have indicated a role for acylcarnitine testing in the diagnosis of some peroxisomal diseases through the detection of long chain dicarboxylic acylcarnitine abnormalities (C16-DC and C18-DC). However, there remains limited independent corroboration of these initial findings and acylcarnitine testing for peroxisomal diseases has not been widely adopted in clinical laboratories. To explore the utility of acylcarnitine testing in the diagnosis of peroxisomal disorders we applied a LC-MS/MS acylcarnitine method to study a heterogenous clinical sample set (n = 598) that included residual plasma specimens from nineteen patients with PBD caused by *PEX1* or *PEX6* deficiency, ranging in severity from lethal neonatal onset to mild late onset forms. Multiple dicarboxylic acylcarnitines were significantly elevated in PBD patients including medium to long chain (C8-DC to C18-DC) species as well as previously undescribed elevations of malonylcarnitine (C3-DC) and very long chain dicarboxylic acylcarnitines (C20-DC and C22-DC). The best performing plasma acylcarnitine biomarkers, C20-DC and C22-DC, were detected at elevated levels in 100% and 68% of PBD patients but were rarely elevated in patients that did not have a PBD. We extended our analysis to residual newborn screening blood spot cards and were able to detect dicarboxylic acylcarnitine

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MFW: Writing - Original Draft, Resources, Funding acquisition, Supervision. **BL:** Resources, Data Curation, Formal analysis. **RD:** Writing - Review & Editing, Resources. **SJ:** Resources, Data Curation**. PB:** Resources, Data Curation. **TW**: Resources. **MM:** Resources, Data Curation. **MJM:** Conceptualization, Formal analysis, Writing - Original Draft, Visualization, Project administration.

Conflict of Interest
The authors declare they have no conflicts of interest.

abnormalities in a newborn with a PBD caused by *PEX6* deficiency. Similar to prior studies, we failed to detect substantial dicarboxylic acylcarnitine abnormalities in blood spot cards from patients with x-linked adrenoleukodystrophy (x-ald) indicating that these biomarkers may have utility in quickly narrowing the differential diagnosis in patients with a positive newborn screen for x-ald. Overall, our study identifies widespread dicarboxylic acylcarnitine abnormalities in patients with PBD and highlights key acylcarnitine biomarkers for the detection of this class of inherited metabolic disease.

1. Introduction

Peroxisomes are single membrane-bound organelles, present in virtually all eukaryotic cells, and involved in diverse catabolic and biosynthetic metabolic processes critical for human development and health including oxidation of very-long-chain fatty acids (VLCFAs), as well as the turnover of polyamines, lysine, and glyoxylate [1–3]. Peroxisomes also synthesize plasmalogens (membrane ether-phospholipids that are major constituents of brain, kidney, lung, and skeletal muscle) and bile acids [1]. Peroxisomal enzymes, such as catalase, also provide balance in regulating cellular redox [4–6].

Peroxisomal disorders are divided into two main classes characterized by impaired organelle assembly (peroxisome biogenesis disorders, PBD) or single enzyme defects (SED) that affect the function of individual peroxisomal matrix enzymes directly responsible for peroxisomal metabolic activities [7]. All peroxisomal functions can be affected, at least to some extent, in patients with PBD. Nevertheless, many clinical features of PBDs overlap those of SEDs. D-bifunctional protein deficiency is a peroxisomal SED that is characterized by widespread clinical and biochemical abnormalities that can overlap with features of PBD [8, 9]. Numerous biochemical methods are available to detect and classify inherited peroxisomal diseases, including measurement of red blood cell plasmalogens, plasma very long chain monocarboxylic fatty acids, and plasma pipecolic acid [1, 10].

Acylcarnitine profile analysis by flow injection mass spectrometry (FI-MS/MS) has also been reported to have utility in PBD diagnosis through the detection of long chain dicarboxylic acylcarnitine accumulation in plasma and urine [11, 12]. FI-MS/MS acylcarnitine profile analysis is widely used in biochemical genetics testing laboratories and newborn screening laboratories to detect inherited fatty acid oxidation disorders and organic acidemias [13]. Major limitations of this approach include the inability to resolve acylcarnitines of the same mass (e.g., isomeric compounds) and the potential for false positive detection of isobaric interferants. To overcome these limitations, liquid chromatography tandem mass spectrometry (LC-MS/MS) acylcarnitine assays have been developed that separate and uniquely identify isomeric acylcarnitines and remove interferants [14–16]. To date, these advanced methods have not been applied to the study of acylcarnitine biomarkers in PBD cohorts. In the following study we use a high resolution LC-MS/MS acylcarnitine method to confirm and expand the list of acylcarnitine abnormalities in patients with a PBD and we explore the clinical utility of these PBD biomarkers when applied to a large heterogenous clinical sample set.

2. Methods

2.1 Specimen collection

Heparinized plasma specimens used in this study were derived from residual patient samples submitted to the clinical biochemical genetics laboratory at the Indiana University School of Medicine or from residual study samples collected as part of H-44779, an IRB approved study at Baylor College of Medicine. Specimen identifiers for the Baylor College of Medicine cohort are consistent with the nomenclature used in their initial description [17] and can be identified in this study by their use of "Pex" in the name (for example Pex10–1). Blood spot specimens were derived from residual newborn screening materials at the Indiana State Newborn Screening Laboratory (ISNBS). Blood spot cards were collected between 24–48 hours of life, stored at −20 °C after the initial NBS analysis, and deidentified by the ISNBS prior to use in this project. Blood spot specimens used to establish reference intervals were randomly collected from residual specimens with no abnormalities detected by NBS (n = 50). This study was reviewed and approved by the Indiana University Institutional Review Board.

2.2 Sample preparation

For plasma acylcarnitine studies, patient specimens and associated calibrators and control specimens were prepared as previously described [16]. For blood spot acylcarnitine testing, two 3 mm punches were incubated with 125 μL of resuspension solution containing 6.3 μL of the same isotopic internal standard mixture used for plasma studies and 118.7 μL of 90% methanol. Blood spot resuspensions were incubated at room temperature for 20 minutes with periodic vortex mixing. Following incubation/mixing, both plasma and blood spot samples were centrifuged at 21,130 × g for 2 minutes on a bench top microcentrifuge. Clarified supernatant was transferred to a glass autosampler vial for LC-MS/MS analysis.

2.3 Acylcarnitine analysis

Acylcarnitine quantification was completed by LC-MS/MS as previously described [16]. Briefly, 5 μL of prepared specimen was subjected to chromatographic separation using an Acquity i-class UPLC system (Waters) equipped with a Scherzo SS-C18 column (100 mm × 3 mm, particle size = 3 μm; (Imtakt). Mass spectrometry analysis was completed using a Xevo TQS micro (Waters). Analytes were identified based on spectral and retention time matching. For each analyte, quantification was achieved by first calculating isotopic internal standard normalized response values and then comparing these values to a linear model generated by a six point calibration curve included in each batch. For long and very long chain dicarboxylic acylcarnitines (C18-DC to C22-DC), commercial calibration materials are not currently available. We therefore used hexadecanedioic acid mono-L-carnitine (C16-DC; Santa Cruz Biotech) as a surrogate calibrator for these analytes.

2.4 Other analytes

Methods used to generate pipecolic acid and hexacosanoic acid values for the Baylor College of Medicine sample set were previously reported [17]. For the remaining plasma specimens, pipecolic acid was quantified by LC-MS/MS essentially as described [18].

ISNBS blood spot C26:0-lysophosphatidylcholine (C26-LPC) values were determined by FI-MS/MS using Neobase™ 2 kit (Perkin Elmer). At ISNBS, the top 3% of specimen with the highest C26:0-LPC values are subjected to chromatographic separation in negative polarity mode by LC-MS/MS to mitigate possible interferences from isobaric compounds [19].

2.5 Statistical Analysis

Volcano plot p-values were calculated using a two-tailed homoscedastic t-test. The significance threshold was determined using a Bonferroni correction where alpha = 0.05 and the number of tests was equal to the number of analytes studied (n = 56). Z-scores were calculated by finding the standard deviation and mean value of median scaled $\log_2$-transformed patient values. Acylcarnitine reference intervals were determined through the study of residual specimens from unaffected individuals with the upper limit of normal set to the 99th percentile value and the lower limit of normal set to the 1st percentile value.

3. Results

3.1 Acylcarnitine abnormalities in peroxisomal disease

To identify plasma acylcarnitine biomarkers in peroxisomal disease we applied a LC-MS/MS method to quantify fifty seven acylcarnitines and carnitine metabolic precursors in unaffected individuals (n = 436) and in patients with PBD due to *PEX1* (n=18) or *PEX6* (n=1) deficiency or in a patient with the peroxisomal enzymatic defect, D-bifunctional protein deficiency (n=1; Table S1). The pool of unaffected individuals was curated to remove patients with a known inherited metabolic disease, a positive newborn screen, intravenous carnitine supplementation, renal failure, refeeding syndrome, or end-of-life sampling. The list of acylcarnitines quantified in this study is shown in Table S2 and includes all traditional acylcarnitine species covered by common flow injection methods such as hydroxylated species and monocarboxylic species ranging in chain length from C2 to C18. Owing to the use of LC-MS/MS, many non-traditional analytes were also studied including carnitine metabolic precursors (e.g., deoxycarnitine), resolved isobaric acylcarnitine species and long/very long chain dicarboxylic acylcarnitines.

As shown in Figure 1, widespread dicarboxylic acylcarnitine abnormalities were detected in patients with peroxisomal disease, with eight of the thirteen dicarboxylic acylcarnitines studied having significant elevations. Abnormalities included elevations of long chain dicarboxylic acylcarnitines hexadecanedioylcarnitine (C16-DC) and octadecanedioylcarnitine (C18-DC), consistent with prior reports [11]. Novel findings included the detection of significant malonylcarnitine (C3-DC) elevations and accumulation of very long chain dicarboxylic acylcarnitine species eicosanedioylcarnitine (C20-DC) and docosanedioylcarnitine (C22-DC). We failed to detect abnormal accumulation of short chain dicarboxylic acylcarnitines (C4-DC to C6-DC) or of any monocarboxylic acylcarnitines in the peroxisomal disease cohort.

3.2 Clinical utility

We next explored the clinical utility of dicarboxylic acylcarnitine biomarkers for the detection of peroxisomal disorders. For this comparison we included data from our full curated clinical sample set (n=598) representing a highly heterogenous group of clinical indications and genetic disorders that typify true clinical testing situations (Table S3). Acylcarnitine levels were converted to z-scored values allowing direct comparison of the patient distributions and relative biomarker strengths across analytes (Fig. 2).

The very long chain dicarboxylic acylcarnitines C20-DC and C22-DC achieved the highest z-score values and provided the best utility as individual biomarkers for peroxisomal disease. Using normal concentration cutoff values of C20-DC < 0.0060 μM and C22-DC < 0.0025 μM yielded a sensitivity of 100% and 68% for the detection of *PBD* (n=19), respectively, with a false positive rate of 0.35% (2/578) in both cases. Other dicarboxylic acylcarnitines C3-DC, C16-DC, C18:1-DC, and C18-DC were clearly elevated in the *PBD* cohort but were suboptimal biomarkers. For, example C3-DC elevations above the normal reference interval (<0.025 μM) provided a sensitivity of 84% for the detection of peroxisomal disease but had a false positive rate of 51/578 (8.82%).

Included in our cohort was a single individual with D-bifunctional protein deficiency, a peroxisomal enzyme defect that shares many clinical and biochemical abnormalities with PBD patients [20]. We identified mild dicarboxylic acylcarnitine abnormalities in this patient, accumulating in a pattern similar to that of our PBD cohort (Fig. 2).

High levels of long chain dicarboxylic acylcarnitines were noted in a subset of cases with a low clinical suspicion for a peroxisomal disease. These may serve as examples of possible false positive results that can occur when using C16-DC and C18-DC alone for the detection of PBD. Three notable cases (case1–3) had substantial long chain dicarboxylic acylcarnitine elevations (e.g, C16-DC = 0.24, 0.57, and 2.34 μM) but either normal or only mildly elevated levels of C3-DC, C20-DC , and C22-DC (Fig. 2 and Table S2). Case 1 was an adult with medium chain acyl-CoA dehydrogenase deficiency (MCAD, OMIM 201450) complicated by nonalcoholic fatty liver disease. Case 2 was an adolescent with neurofibromatosis type 1 (NF1, OMIM 162200) presenting with acute liver failure in the setting of acetaminophen toxicity. Case 3 was a six month old presenting with acute hepatitis, thought to be of viral etiology, leading to acute liver failure. None of the listed cases had clinical features concerning for a PBD. Long and very long chain dicarboxylic acylcarnitine elevations were also noted in one patient presenting early in life with carnitine palmitoyltransferase 2 deficiency (case 4, Fig.2; CPT2, OMIM 600649). However, in this patient, PBD could be ruled out by the detection of substantial long chain monocarboxylic acylcarnitine elevations (C16 to C18) that are a hallmark of CPT2 deficiency but not peroxisomal disease (Table S2). Patients with PBD can also be distinguished from patients with CPT2/VLCAD by calculating the ratios of dicarboxylic to monocarboxylic long chain acylcarnitines (Fig. S1).

3.3 Newborn blood spot analyses

Many states currently screen newborns for X-linked adrenoleukodystrophy (x-ald; OMIM 300100) by determining C26:0-lysophosphatidylcholine (C26:0-LPC) concentration in dried blood spot (DBS) using FI-MSMS with reflex to DBS LC-MS/MS analyses for C26:LPC or very long chain monocarboxylic fatty acids. Such screening programs, as a byproduct, will also detect individuals with peroxisomal biogenesis disorders. We were therefore interested in exploring the utility of DBS dicarboxylic acylcarnitine testing for narrowing the clinical differential in these situations.

Residual DBS specimens from the ISNBS were subjected to LC-MS/MS acylcarnitine analysis (Table1 and Table S4). Substantial dicarboxylic acylcarnitine abnormalities were detected in a DBS from a patient with *PEX6* deficiency (patient “NBS_1” in Table 1 and Table S4) but were otherwise undetectable in all unaffected specimens studied (n =50). Similarly three male patients with x-ald were found to have essentially normal dicarboxylic acylcarnitine levels consistent with prior reports regarding plasma C16-DC and C18-DC levels in x-ald patients [11].

4. Discussion

In the preceding report we describe the detection of widespread dicarboxylic acylcarnitine abnormalities in patients with inherited peroxisomal biogenesis disorders. However, the underlying mechanism of dicarboxylic acylcarnitine formation and accumulation remains unclear. Long chain dicarboxylic fatty acids can be introduced through diet and are also formed endogenously in the endoplasmic reticulum by omega oxidation of monocarboxylic fatty acids. Following conversion to acyl-CoA esters, long chain dicarboxylic fatty acids are catabolized primarily within the peroxisome via beta oxidation [21]. The peroxisomal membrane transporter ATP-binding cassette transporter 3 (ABCD3) plays a prominent role in transporting long chain dicarboxylic fatty acids into the peroxisome [22]. Importantly, the peroxisomal very long chain fatty acid transporter ABCD1 appears to have poor dicarboxylic fatty acid transporter activity [23], thus likely providing an important clue as to why dicarboxylic acylcarnitine accumulation is not observed in patients with *ABCD1* deficiency (i.e., x-ald).

When the peroxisome is defective the mitochondria may serve as an alternate, less efficient, site of dicarboxylic fatty acid metabolism. Unlike the peroxisome, long chain acyl-CoA transport into the mitochondria is mediated by the carnitine shuttle system and requires the transesterification of acyl-CoAs to acylcarnitines. Candidate carnitine acyltransferases involved in the formation of dicarboyxlic acylcarnitines include (i) mitochondrial localized carnitine palmitoyltransferase 1 (CPT1), (ii) peroxisomal localized carnitine octanoyltransferase (COT), (iii) and carnitine acetyltransferase (CAT) found in both the peroxisome and the mitochondria [24]. Further studies are needed to identify the enzymes involved in long chain dicarboxylic acylcarnitine metabolism and to determine the biological consequences of their formation.

One of the more unexpected findings from his study was the detection of malonylcarnitine (C3-DC) accumulation in PBD patients. We were able to detect this previously unrecognized

PBD abnormality due to our method's chromatographic resolution of C3-DC from isobaric acylcarnitine species (e.g., C4:OH) that interfere with malonylcarnitine detection when using common flow injection methods. The mechanism of malonylcarnitine accumulation is unclear but its presence suggests there may be an underlying accumulation of its precursor malonyl-CoA. If so, this could be related to a reduction of malonyl-CoA decarboxylase activity, a key enzyme in malonyl-CoA processing that is found in both the cytoplasm and the peroxisome [25]. Malonyl-CoA is a potent inhibitor of carnitine palmitoyl transferase I (CPT1), a mitochondrial enzyme that facilitates acyl-carnitine formation; malonyl-CoA therefore acts as a critical signaling molecule mediating the switch between fatty acid synthesis and oxidation [26]. Given the important intracellular role of malonyl-CoA homeostasis, additional work is needed to clarify the mechanism of malonylcarnitine elevations and its downstream consequences in patients with PBD.

It should be noted that our study did not test the levels of very long chain monocarboxylic acylcarnitines (C22, C24 and C26). Accumulation of this class of acylcarnitine can also occur in patients with PBD or x-ald but prior reports indicate that very long chain monocarboxylic acylcarnitine abnormalities are unreliable biomarkers that occur sporadically and at relatively low levels in patients with peroxisomal disorders [3, 11, 12, 27]. This class of compounds also present chromatographic challenges; including them in our study would have required substantial modifications to our LC-MS/MS method to achieve timely elution of these strongly hydrophobic compounds. For these reasons we chose to focus on dicarboxylic acylcarnitine abnormalities in peroxisomal disorders.

An additional limitation of this study involves our use of a surrogate calibrator for some analytes. Most relevant to the focus of this manuscript, pure reference material was not commercially available for C18:1-DC, C18-DC, C20-DC, and C22-DC acylcarnitine species. We therefore calibrated the concentration of these analytes to the most chemically similar compound for which calibration material was available (C16-DC). Such surrogate calibration approaches are routinely used in acylcarnitine studies, where pure standards are not available for many analytes of interest [13]. These methods can generate precise and reproducible semiquantitative values but suffer from suboptimal quantitative accuracy. Future synthesis of additional long and very long chain dicarboxylic acylcarnitine reference materials will improve our ability to provide absolute quantification values for these analytes.

Our results demonstrate that patients with PBD have a characteristic pattern of widespread dicarboxylic acylcarnitine abnormalities. However, care should be taken to avoid false positives when reviewing acylcarnitine data. We identified three patients with substantial accumulation of long chain dicarboxylic acylcarnitines (C16-DC, C18:1-DC and C18-DC) that could not be attributed to an inherited peroxisomal defect. Instead, liver dysfunction was the common feature in all three cases thus potentially indicating an important novel biomarker association. Malonylcarnitine, frequently elevated in our PBD cohort, can also be elevated in patients with malonyl CoA decarboxylase deficiency (OMIM 248360; not represented in our sample set) or in patients with renal dysfunction (in our experience). In summary, we would advocate for the use of the full acylcarnitine profile and careful

clinical correlation when relying on acylcarntine data to determine the risk for a peroxisomal disease.

Inclusion of PBD biomarkers in acylcarnitine testing has the potential to improve clinical outcomes for patients with PBD in a number of important ways. Acylcarnitine testing is one of the most commonly ordered biochemical genetics tests and is considered a part of a complete workup for inherited metabolic disorders. Given the prevalence of laboratories offering acylcarnitine profile analysis, this testing, and especially STAT testing, is likely more accessible to many patient populations compared to other genetic tests for PBD. Furthermore, the routine use of acylcarnitine testing increases the odds of detecting mild cases where a PBD was not suspected or low on the differential diagnosis. To be clear, dicarboxylic acylcarnitine elevations alone should not be considered definitive diagnostic biomarkers for peroxisomal disease and we would still strongly advocate for additional confirmatory testing by alternate molecular and biochemical methodologies whenever an acylcarnitine result is abnormal for PBD. Finally, blood spot acylcarnitine testing offers an orthogonal second-tier test that can be implemented by NBS laboratories to follow up on cases with elevated C26:0-lysophosphatidylcholine (C26:0-LPC). In conclusion, our study shows that widespread dicarboxylic acylcarnitine abnormalities are a consistent finding in PBD patients that can provide sensitive and specific detection of peroxisomal disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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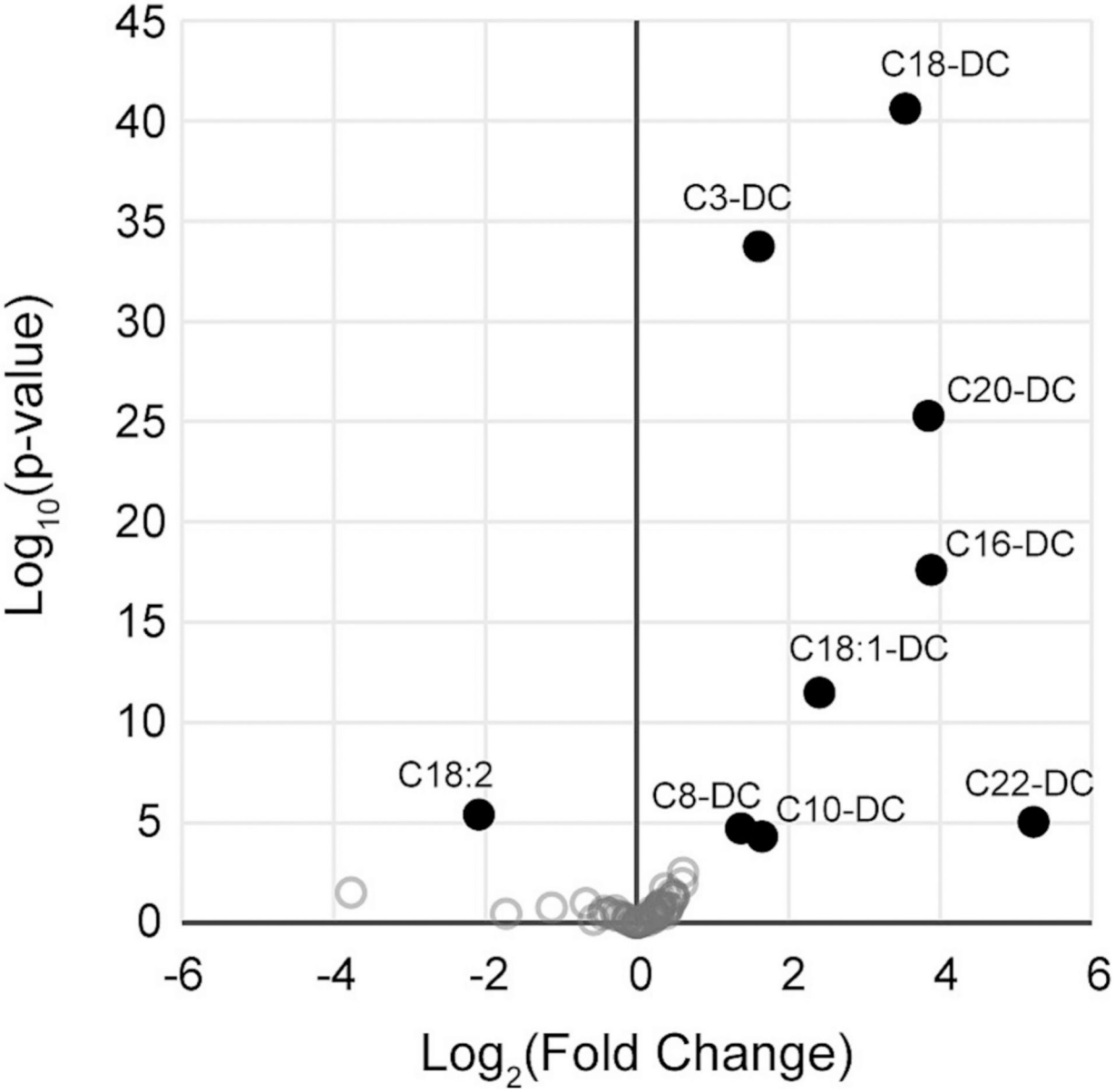

Fig. 1.

Acylcarnitine abnormalities in peroxisomal disease. The volcano plot compares average acylcarnitine levels in patients with PBD (n=19) or D-bifunctional protein deficiency (n=1) to average levels an unaffected reference population (n= 436). In total, 56 acylcarnitines or carnitine metabolic intermediates were studied. All statistically significant abnormalities are labeled and indicated by filled black circles.

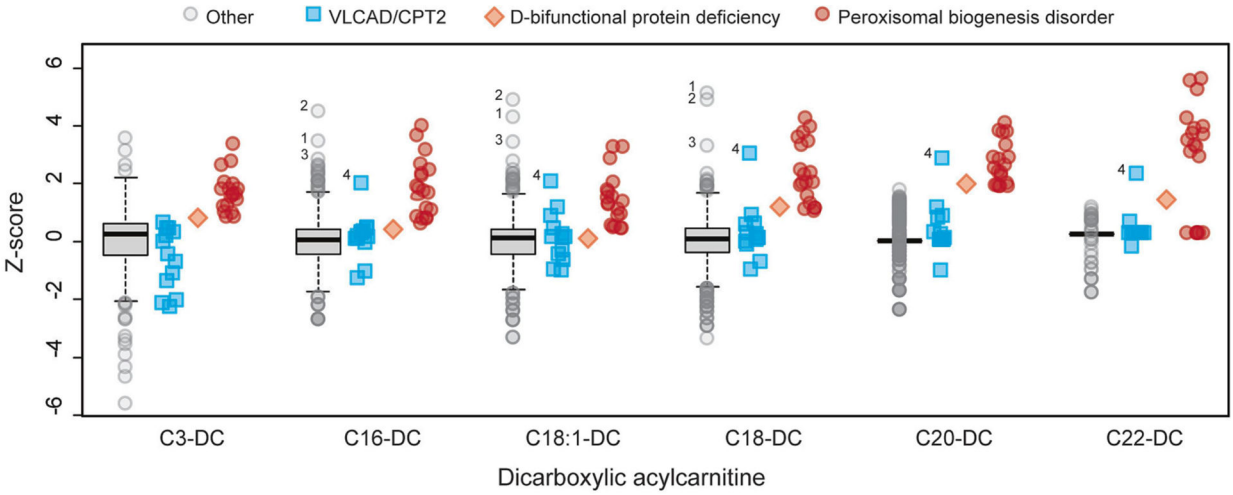

Fig. 2.

Dicarboxylic acylcarnitine abnormalities across a large heterogenous clinical sample set (n=598). Relevant disease states are shown in color. The “Other” category (grey circles) includes both unaffected individuals as well as individuals with inherited metabolic diseases that are not currently associated with dicarboxylic acylcarnitine or long chain monocarboxylic acylcarnitine abnormalities. Patient case numbers are listed for interesting outliers. For more information see Tables S2 and S3.

Table 1:

Blood spot dicarboxylic acylcarnitine biomarker levels in patients with peroxisomal disorders (see Table S4 for more details)

Patient	Diagnosis	C3-DC	C16-DC	C18:1-DC	C18-DC	C20-DC	C22-DC
NBS_1	PBD	**0.049 (H)**	**0.252 (H)**	**0.041 (H)**	**0.061 (H)**	**0.005 (H)**	N.D.
NBS_2	X-ALD	0.007	0.009	0.004	0.004	N.D.	N.D.
NBS_3	X-ALD	0.020	0.096	0.025	0.016	N.D.	N.D.
NBS_4	X-ALD	0.020	0.153	**0.044 (H)**	0.016	N.D.	N.D.

Reference Interval (0 to 0.022) (0 to 0.193) (0 to 0.039) (0 to 0.016) (0 to 0.001) (0 to 0.001)

N.D. = not detected