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Mitochondrial respiratory chain disease discrimination by retrospective cohort analysis of blood metabolites

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Abstract

Diagnosing primary mitochondrial respiratory chain (RC) dysfunction has long relied on invasive tissue biopsies, since no blood-based biomarker has been shown to have sufficiently high sensitivity and specificity across the myriad of individual clinical presentations. We sought to determine whether cohort-level evaluation of commonly obtained blood analytes might reveal consistent patterns to discriminate a heterogeneous group of primary mitochondrial RC disease subjects both from control individuals and from subjects with pyruvate dehydrogenase deficiency.

METHODS—Following IRB approval, 62 biochemical analyte concentrations or ratios were retrospectively analyzed in three well-defined and intentionally heterogeneous subject cohorts reflective of clinical practice: [1] Primary mitochondrial disease (n=19); [2] Pyruvate dehydrogenase deficiency (n=4); and [3] Controls (n=27). Blood analyte categories included

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Author Contributions: M.J.F. designed the study. E.P. and C.C. consented subjects, reviewed medical records, obtained laboratory results, and maintained the database. C.C. and Z.Z. designed the study database. C.C., Z.Z., and R.X. performed all statistical analyses. N.S. identified additional subjects with pyruvate metabolism defects. M.B. provided laboratory control group subject data. M.Y. contributed to data analysis and interpretation. C.C., R.X., and M.J.F. wrote the manuscript. All authors approved of the final manuscript.

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comprehensive chemistry profile, creatine kinase, lipoprotein profile, lactate, pyruvate, and plasma amino acid profile. Non-parametric analyses were used to compare the median of each analyte level between cohorts.

RESULTS—Disease cohorts differed significantly in their median levels of triglycerides, lactate, pyruvate, and multiple individual plasma amino acids. Primary mitochondrial disease was significantly discriminated at the cohort level from pyruvate dehydrogenase deficiency by greater pyruvate and alanine elevation in pyruvate dehydrogenase deficiency, as well as significantly increased branched chain amino acid (BCAA) levels and increased ratios of individual BCAAs to glutamate in mitochondrial disease. In addition, significant elevation of median blood triglyceride level was seen in the primary mitochondrial disease cohort.

CONCLUSIONS—Blood metabolite profile analysis can discriminate heterogeneous cohorts of primary mitochondrial disease both from controls and from pyruvate dehydrogenase deficiency. Elevated BCAA levels, either absolutely or when considered relative to the level of glutamate, are common metabolic sequelae of primary mitochondrial RC disease. Prospective study is needed to validate observed plasma metabolite alterations as a potential biomarker of disease both in larger cohorts and at the individual subject level.

Keywords

primary human mitochondrial disease; pyruvate dehydrogenase deficiency; metabolites; branched chain amino acids

1. INTRODUCTION

Dysfunction of mitochondria, which support a myriad of cellular processes, causes diffuse cellular impairment or death. The minimal prevalence of mitochondrial respiratory chain (RC) disease is estimated at 1 in 5,000 worldwide [1, 2]. Primary mitochondrial diseases result either from mitochondrial DNA (mtDNA) mutations that conform to a maternal inheritance pattern or from nuclear gene mutations that can reflect any of the classic Mendelian inheritance patterns [3]. Mitochondrial DNA has 37 genes, including 13 that encode subunits of the energy-generating RC, while the remaining estimated 1,500 proteins necessary for mitochondrial activities are encoded by the nuclear genome [4, 5]. Clinical manifestations of mitochondrial disease are highly variable, both within a family, and often, in the same person over time. Multiple organ systems commonly are involved [1, 6], such that affected individuals may present at any age with a progressive range of neurologic problems including migraines, stroke, epilepsy, encephalopathy, and dementia, as well as vision loss, deafness, gastrointestinal dysmotility, muscle weakness, heart failure, arrhythmias, diabetes mellitus, and kidney or liver problems [1]. This inherent variation in the constellation of clinical presentations that may be seen in mitochondrial disease poses a substantial diagnostic challenge [4].

Definitive diagnosis of primary mitochondrial disease increasingly obliges identification of a pathogenic mutation in either the mitochondrial or nuclear genomes [7]. This can be a formidable undertaking, since more than 100 different genes have been associated with primary mitochondrial disease [4, 8] and upward of 1,000 other likely candidates remain based on current knowledge of mitochondria-localized proteins [9, 10]. Biopsy samples can be obtained from skin, muscle, or liver to determine not only the sequence and content of the mtDNA genome within each tissue, but also to assess for alterations in mitochondrial morphology and RC enzyme activity [2]. Such testing can differentiate biochemical-based mitochondrial dysfunction from other disorders [4], but approximately one third to one-half of patients with biochemically-confirmed mitochondrial RC disease have no readily identifiable pathogenic mutation in known mitochondrial disease genes even when newer

massively-parallel genomic sequencing is performed of either focused panels of candidate nuclear genes that include all currently known mitochondrial disease genes or of the whole exome [4, 11, 12]. Further, it is possible to have biochemical findings of altered RC function in other classes of genetic or environmental disease in which the causative defect is not a mutation in a gene linked to mitochondrial function [12].

Although previous studies have identified several individual blood analyte alterations that may raise the likelihood that a given patient has mitochondrial disease, none has sufficiently high sensitivity or specificity to be widely useful as a reliable biomarker. Elevated lactate and/or pyruvate levels commonly occur in mitochondrial disease, but may only be detected during an acute medical crisis or not at all [4]. Furthermore, lactate and pyruvate elevations can result from other diseases, nutritional deficiency, hypoxia, or most commonly, from poor specimen collection and handling techniques [4]. Elevated blood alanine can indicate pyruvate accumulation, a finding observed not only in mitochondrial RC disease, but also in other metabolic disorders such as pyruvate dehydrogenase (PDH) deficiency, pyruvate carboxylase (PC) deficiency, and urea cycle disorders [4]. The ratio of lactate to pyruvate is a well-established indicator of the cytoplasmic NADH/NAD⁺ redox ratio [4], which is typically, but not exclusively, elevated in mitochondrial RC disease and normal in pyruvate metabolism defects. Other plasma amino acids that have variably been reported to be elevated in primary mitochondrial disease include glycine, proline, sarcosine, and tyrosine [4]. Levels of glutathione and one of its precursor amino acids, citrulline, were recently found to be significantly decreased in the plasma of subjects with mitochondrial disease or organic acidemia, a finding attributed to ongoing oxidative stress [13]. Lipid alterations have also been noted to occur in some patients with mitochondrial disease [14]. In addition, several studies have suggested that altered creatine metabolites may be a hallmark of mitochondrial disease, as the creatine kinase (CK) reaction converts creatine to phosphocreatine, which donates a phosphate group to ADP to generate ATP [15]. Two studies identified elevated blood CK activity in some individuals with mitochondrial disease [14, 15]. Low phosphocreatine levels have also been reported to occur in both brain and muscle of RC disease patients [13]. Similarly, analysis of human myotubes treated with a mitochondrial complex I inhibitor, rotenone, identified elevated creatine and reduced phosphocreatine levels in the media [16]. FGF21 concentrations have also been shown to be a potentially reliable biomarker for RC diseases that involve muscle manifestations [17], although this has not yet been widely validated nor have clinical diagnostic assays for FGF21 been widely accessible.

Collectively, such studies are suggestive that alterations in a wide range of blood metabolites can and do occur in primary mitochondrial disease. However, most such alterations have been reported in small sample sizes because of the historic paucity of cases with a clearly-defined genetic etiology. In addition, many prior reports identified biochemical alterations in tissues, cells, or cell derivatives rather than analytes directly assayed in patients' blood. Given recent improvements in the ability to establish definitive genetic diagnoses in the setting of suspected mitochondrial disease, it is now feasible to systematically reevaluate whether any blood-based biochemical parameters are consistently altered across well-defined yet heterogeneous causes of primary mitochondrial RC disease. Such analyses may identify secondary biochemical alterations that commonly result from primary mitochondrial RC dysfunction, contribute to disease manifestations, and thereby, offer potential clinical benefit if therapeutically targeted. Comparison of the metabolic effects of primary RC deficiency and PDHc deficiency may be particularly informative to clarify two categories of mitochondrial dysfunction that impair cellular capacity to generate energy from nutrients, where only RC deficiency directly alters cellular redox balance and electron transport activity. To identify a common blood-based biochemical profile indicative of primary mitochondrial RC dysfunction, we report here results of a retrospective analysis

of 62 blood biochemical analyte concentrations or ratios performed in three well-defined cohorts of subjects with primary mitochondrial RC disease, pyruvate dehydrogenase complex (PDHc) deficiency, and controls having non-specific medical concerns but no clinical or laboratory evidence suggestive of metabolic disease.

2. METHODS

2.1. Human Subject Enrollment

The study was approved by the Institutional Review Boards of The Children's Hospital of Philadelphia and Arcadia University. Informed consent was obtained for all living subjects. A retrospective chart review was performed among subjects recruited from The Children's Hospital of Philadelphia as part of a larger study to investigate the metabolic consequences of mitochondrial disease (PI, M.J.F.). Only subjects on whom blood metabolite data were available were included in the present study. Data were obtained directly from clinical laboratory reports from all analyses available for each subject.

2.2. Clinical Cohort Assignment

Subjects were assigned to one of three clinical cohorts, as follows:

2.2.1 Primary mitochondrial disease—This cohort of 19 subjects (Table 1) included 15 females and 4 males (Table 1). Age at the time of phlebotomy ranged from 1 month to 53 years. Subjects were included in this cohort if their clinical histories were consistent with primary mitochondrial disease and they had either a known pathogenic mutation in a mtDNA or nuclear-encoded mitochondrial disease gene and/or tissue biochemistry studies consistent with significantly impaired respiratory chain enzyme activity (< 30 percent of control mean) (Supp File 1). Overall, 15 had definite genetic etiologies (11 subjects with confirmed pathogenic mtDNA mutations and 4 subjects with confirmed pathogenic nuclear gene mutations in *FBXL4*, *POLG*, *MPV17*, or *RRM2B*), two adult subjects (1041 and 1043) had severe multi-systemic disease, mitochondrial DNA mutations of uncertain pathogenicity, and a deceased first-degree relative who had severe complex I deficiency demonstrated on muscle biopsy, and 2 decedents (1097 and 1157) had severe muscle biopsy biochemical evidence of RC deficiency but no known genetic etiology. These latter two subjects were included as having “definite” RC disease based on meeting modified Walker criteria as follows: subject 1097 met one major (reduced RC complex IV activity at 8% of mean on muscle biopsy) and two minor criteria (increased metabolite indicators including lactic acidosis and hyperalaninemia, as well as widespread mitochondrial abnormalities on muscle electron microscopy); subject 1157 met two major criteria including having reduced RC complex I–III activity in muscle at 4% of control mean and autopsy-confirmed Leigh disease.

2.2.2 PDHc deficiency—This cohort of 4 subjects included 3 females and 1 male with enzyme-confirmed PDHc deficiency in a clinical diagnostic laboratory (Table 1), two of whom had confirmed *PDHA1* pathogenic mutations. Age at the time of phlebotomy ranged from 1 month to 3 years. Clinical characteristics are detailed in Supp File 1. Of note, subject 1273 was on the ketogenic diet at all phlebotomy timepoints evaluated. None of the other three subjects were treated on the ketogenic diet.

2.2.3 Controls—This cohort of 27 subjects included 15 females and 12 males who had no suggestive evidence of metabolic disease based on our review of all available clinical and metabolic data (Table 1). Age at the time of phlebotomy ranged from 1 month to 31 years. Each control subject was selected from Children's Hospital of Philadelphia Department of Pathology laboratory database of individuals who underwent medical evaluation during

which biochemical screening laboratory studies were obtained if official laboratory reports were available for review by the study team and their clinical presentation was not suggestive of a primary metabolic or mitochondrial disease following study team review of their medical records (Supp File 1). In addition, laboratory control ranges standardly calculated by the Children's Hospital of Philadelphia Department of Pathology are reported for each analyte (Supp File 2), although it was not possible to retrieve the individual data points used to generate the control ranges by the clinical diagnostic laboratory.

2.3 Database Organization

Analyte raw values were entered into a customized Access (Microsoft) database organized into five categories: (1) comprehensive chemistry profile, (2) creatine kinase, (3) lipoprotein profile, (4) blood lactate and pyruvate, and (5) plasma amino acid quantitative profile. A total of 62 discrete analytes or ratios were analyzed, including the following ratios: LDL:HDL cholesterol, alanine:lysine, alanine:(phenylalanine+tyrosine), alanine:glutamate, glycine:glutamate, valine:glutamate, leucine:glutamate, isoleucine:glutamate, and aspartate:glutamate. Standard collection methods were utilized to obtain proper pyruvate measurements, including immediate deproteinization in perchloric acid and on-site analysis in the Clinical Biochemical Laboratory of the Children's Hospital of Philadelphia. Data were included from all subjects for whom laboratory reports were available (Table 1). Given the retrospective nature of the study, no information was available as to intercurrent medications, medical conditions, or dietary modifications relative to the time of each blood draw. However, most of the phlebotomy draws on living patients seen in the outpatient clinic setting were performed approximately 3 to 4 hours after a meal, at the time immediately following outpatient evaluation in the Mitochondrial-Genetics Diagnostic Clinic at The Children's Hospital of Philadelphia. Fasting was avoided for all tests, including lipoprotein profiles, based on the confirmed or suspected mitochondrial disease status of subjects evaluated in this clinic. Since not all assays were performed and/or available for review on all subjects, subject numbers on whom data were analyzed differed by analyte category.

2.4 Statistical Analyses

The significance of differences in analyte levels between clinical cohorts was evaluated using a non-parametric Mann-Whitney U test in SPSS v19. Some subjects had multiple blood draws for each metabolite, with potential variation in the time of day at which phlebotomy was performed, food intake, medication regimen, and medical stability versus intercurrent illness. For those subjects in whom replicate assays of a given analyte were performed, a single median value from all bloods draws per subject was used in the final analysis of each analyte. Metabolite control data ranges were obtained from The Children's Hospital of Philadelphia's Department of Pathology Clinical Pathology and Biochemical Laboratories.

3. RESULTS

3.1. Blood lactate and pyruvate were significantly more elevated in PDHc deficiency than in primary mitochondrial disease

Median blood lactate concentration in the PDHc deficiency cohort was significantly elevated relative to the control cohort ($p = 0.007$) (Fig 1a). A trend toward median blood lactate elevation was also seen in the mitochondrial disease cohort relative to the control cohort ($p = 0.063$) (Fig 1a). Median blood pyruvate concentration was significantly elevated in the PDHc deficiency cohort relative both to controls ($p = 0.006$) and to the mitochondrial disease cohort ($p = 0.046$). Interestingly, we noted that nearly all pyruvate values in the PDHc deficiency cohort fell at or above the upper limit of the reference range (Fig 1b).

Thus, whereas median pyruvate concentration did not exceed 0.25 mM/L in any subject within the mitochondrial disease cohort, the PDHc deficiency cohort had a median pyruvate concentration that exceeded 0.3 mM/L. No significant difference between clinical cohorts was seen in the median ratio of lactate to pyruvate (Supp File 2).

3.2. Plasma alanine and branched chain amino acid alterations significantly discriminated mitochondrial disease and PDHc deficiency cohorts

Several plasma amino acid concentrations showed statistically significant differences between cohorts (Table 2). Median levels of plasma alanine significantly differed from controls in both the mitochondrial disease cohort ($p = 0.006$) and in the PDHc deficiency cohort ($p = 0.044$) (Table 2a and Fig 2a). Interestingly, median plasma concentrations of all 3 branched chain amino acids (BCAA; leucine, isoleucine, and valine) were significantly different between cohorts, with the greatest and most significant difference in all BCAA median concentrations seen between the mitochondrial disease and PDHc deficiency cohorts (Figs 2b–d). Relative to median levels of individual BCAAs in controls, 13% to 22% increases were seen in the mitochondrial disease cohort (Table 2a) that reached marginal significance for leucine and isoleucine (Supp File 2), while decreases in the BCAA levels by 14% to 71% (Table 2a) were seen in the PDHc deficiency cohort that were statistically significant ($p < 0.05$) for both leucine and valine (Supp File 2). While one of the four PDHc subjects was on the ketogenic diet, plasma branched chain amino levels did not significantly differ in this subject relative to the other PDHs subjects (data not shown).

A relative increase in plasma concentrations of alanine and the BCAAs, as well as glycine, was particularly evident in the mitochondrial disease cohort when the individual ratio of each of these amino acids to glutamate was compared between the mitochondrial disease and control groups (Fig 3). While plasma median glutamate concentrations did not significantly differ between cohorts (Table 2a), we sought to evaluate each of these amino acid levels relative to glutamate since their formation depends on transamination of their respective α -ketoacids with glutamate. Such transamination occurs by an equilibrium reaction that is driven toward generation of the parent amino acid in the presence of an increased NADH:NAD⁺ redox ratio, as is typical of primary complex I-dependent RC dysfunction [18]. Indeed, the average ratio of each of these individual amino acid analytes relative to glutamate was consistently greater in the mitochondrial disease cohort than in controls (Fig 3). Notably, the ratio of alanine to glutamate showed the most substantial increase in the mitochondrial disease cohort relative to the control cohort. Of interest, all three BCAA:glutamate ratios significantly differed between cohorts, and were significantly lower in the PDHc deficiency cohort than in controls ($p < 0.05$) (Supp File 2). As expected, increases both in the ratio of alanine to lysine, and in the ratio of alanine to the sum of phenylalanine and tyrosine, as have commonly been recognized in primary mitochondrial disease [4], were also evident in the mitochondrial disease cohort relative to controls (Fig 3).

3.3. Analysis of comprehensive chemistry profiles and CK activity showed few significant alterations in the mitochondrial disease cohort

Few significant cohort differences were identified among the analytes assayed on the comprehensive chemistry panel (Supp File 2). Serum bicarbonate median levels in the PDHc deficiency cohort (median = 18.25 mg/dL) were significantly decreased relative both to controls (median = 24 mg/dL, $p = 0.024$) and to the mitochondrial disease cohort (median = 25, $p = 0.046$). Serum chloride values were significantly increased in the PDHc deficiency group (median = 107 mmol/L) relative both to controls (median chloride = 104 mmol/L, $p = 0.046$) and to the mitochondrial disease cohort (median = 102 mmol/L, $p = 0.006$). Serum glucose was increased in the PDHc deficient cohort relative only to the mitochondrial disease cohort ($p = 0.026$). No statistically significant differences were observed in the

median levels between the 3 cohorts of the remaining analytes measured on the comprehensive chemistry panel, which included sodium, potassium, calcium, anion gap, urea nitrogen, creatinine, total bilirubin, albumin, total protein, alkaline phosphatase, alanine aminotransferase, and aspartate aminotransferase (Supp File 2). Unlike prior studies reporting increased CK activity in mitochondrial RC disease [14–16], CK activity median values fell within the reference for 9 of 10 mitochondrial disease subjects on whom data was available, where the only exception was elevated CK activity in subject 1093 who had *MPV17* mutations causing mitochondrial DNA depletion (Supp File 2).

3.4. Plasma triglycerides were significantly increased in primary mitochondrial disease

The only lipoprotein profile analyte that was significantly altered in the mitochondrial disease cohort was plasma triglyceride level (Fig 4a), which was increased by 89.8% relative to controls ($p = 0.02$) (Fig 4b). No significant differences were seen between the mitochondrial disease and control cohorts in total cholesterol, HDL cholesterol, LDL cholesterol, or in the LDL:HDL ratio (Fig 4a; Supp File 2). No lipoprotein data was available for analysis on subjects from the PDHc deficiency cohort.

4. DISCUSSION

To investigate whether a common metabolic response to primary mitochondrial RC disease is discernible regardless of the notorious variability in disease etiology and intercurrent factors, we performed a retrospective analysis at the global cohort level, rather than at the level of individual subjects, of blood metabolite profiles from intentionally heterogeneous subjects having clear biochemical and/or genetic causes of mitochondrial disease. Metabolite data were compared to laboratory-based controls, as well as to a PDHc deficiency cohort in effort to quantitatively distinguish these clinically overlapping inborn errors of metabolism, and potentially to identify a common metabolic response to primary RC dysfunction. Indeed, cohort-level analyses of 62 blood analyte concentrations and ratios did allow discrimination of primary mitochondrial RC dysfunction and PDHc deficiency cohorts, both from one another and from controls.

Just as mitochondrial disease is known to be inconsistently characterized by elevations in blood lactate, pyruvate, and lactate to pyruvate ratio, we did not find these analytes to be the most discriminating set of metabolic alterations at the mitochondrial disease cohort level (Fig 1). Rather, the PDHc deficiency cohort had significantly greater increases in absolute concentrations of blood pyruvate (Fig 1) and alanine (Fig 2) than the mitochondrial disease cohort. Considered together with the significantly lower serum bicarbonate level and trend toward higher anion gap (Supp File 2), these metabolite alterations were suggestive that a more pronounced and consistent metabolic acidemia occurs in PDHc deficiency than in primary mitochondrial disease. However, these differences may also be reflective of either more acute metabolic compromise of the particular PDHc subjects analyzed and/or to potentially desired effects of the ketogenic diet, which is a common treatment for PDHc deficiency, although only one of the four PDHc subjects studied was treated on the ketogenic diet. In addition, given that PDHc is a more biochemically and homogeneous condition than is primary RC disease, it is possible that combined analysis of this heterogeneous disorder masked variability among distinct subgroups of RC disease. Interestingly, whereas in no subject did individual pyruvate concentration exceed 0.25 mM in the mitochondrial disease cohort, the median pyruvate concentration exceeded 0.3 mM in the PDHc deficiency cohort (Fig 1b). Thus, the degrees of lactate, pyruvate, and alanine elevations were neither consistent nor unique indicators of mitochondrial RC disease. However, substantial elevation of these analytes may actually be a more consistent finding of PDHc deficiency.

In contrast to these traditionally considered but poorly sensitive and specific biomarkers of RC disease, the mitochondrial RC disease cohort in our study was uniquely characterized by an analyte alteration profile consisting of significantly increased absolute alanine concentration, increased alanine:glutamate ratio (Fig 2a), and consistently increased ratio relative to glutamate of all amino acids formed by the NADH/NAD⁺ driven transamination equilibrium reactions (Fig 3). Thus, plasma amino acids that are specifically formed by transamination with glutamate are uniquely increased relative to plasma glutamate in human mitochondrial RC disease, similar to what we have previously observed in both *C. elegans* and mouse models of primary mitochondrial RC dysfunction [18, 19]. The PDHc deficiency cohort, by comparison, actually showed a significant reduction relative to controls in BCAA median concentrations, particularly involving leucine and valine (Fig 2b–d), as well as a reduction in the ratio of individual BCAA to glutamate levels when compared to that of the mitochondrial disease cohort (Supp File 2). Thus, analysis of absolute concentrations of blood alanine and pyruvate, together with the ratios of both alanine to glutamate and each BCAA to glutamate, appear superior to conventional analysis of only blood lactate concentration and lactate to pyruvate ratio in their collectively ability to accurately discriminate primary mitochondrial RC disease from PDHc deficiency at the overall cohort level. Thus, while PDHc is a mitochondrial matrix localized enzyme that impairs the generation of energy from cellular nutrients, it does so without impairing cellular redox balance and has distinct metabolic effects from that of primary RC disease that disrupts mitochondrial oxidative phosphorylation capacity, ATP production, and cellular redox balance. It is important to recognize that this present study was neither designed nor powered to assess the sensitivity or specificity of this ratio at the individual patient level, although these data are suggestive that these may be important parameters to prospectively assess in a larger cohort of mitochondrial disease patients and matched controls.

Alterations in several blood analytes, including citrulline and creatinine, which have variably been reported in prior studies of mitochondrial disease subjects were not replicated here. Most notably, we saw no significant decrease in plasma citrulline, despite prior reports that attributed such reduction in mitochondrial disease to increased oxidative stress [13]. However, it is not possible to determine in this retrospective study whether normal citrulline concentrations in the mitochondrial disease cohort might reflect subject use of antioxidants in “mitochondrial cocktails.” Similarly, no increases were seen at the cohort level in either median levels of blood creatinine or CK activity, as have been suggested in cell models of mitochondrial dysfunction and in blood from mitochondrial disease subjects [14–16]. It remains possible that our negative findings in creatine metabolism are reflective of the specific clinical phenotypes prevalent in the mitochondrial disease cohort studied (Supp File 1). Further, it remains important to obtain a screening CK level in individuals presenting with muscle or cardiac evidence of suspected mitochondrial disease to assess for other disease classes having similar clinical presentations.

Lipid alterations in mitochondrial disease are intriguing. Altered lipid metabolism has previously been observed in mitochondrial disease [14], with histologic tissue studies in patient liver or skeletal muscle specimens commonly disclosing non-specific lipid accumulation within mitochondria. Here, we observed significantly increased plasma triglyceride median concentration in the mitochondrial disease cohort relative to controls (Fig 4). While clearly not characteristic of every individual subject, this significant group difference suggests that plasma triglyceride elevation may be a common metabolic sequela of primary mitochondrial RC dysfunction. It is possible that altered plasma triglyceride levels may relate to a secondary impairment in mitochondrial fatty acid oxidation that is caused by altered NADH/NAD⁺ redox poise in primary RC dysfunction, and/or by alterations in central transcriptional factors such as in the PPAR family that influence lipid metabolism [20], which will require further confirmation by prospective study.

The retrospective nature of this study underscores consideration of several important limitations. Relevant analyte data was not collected or available from all study subjects and controls. Thus, no conclusion regarding lipoprotein alterations in PDHc deficiency can be drawn as no data was available in this cohort. Further, plasma lipoprotein levels may be affected by age, gender, diet, or familial hypertriglyceridemia, a relatively common condition that was not assessed in this study. Multiple testing can be a concern since over 60 biochemical parameters were analyzed in this study, although cohort differences among most of the major analyte alterations discussed reached a relatively stringent level of statistical significance ($p < 0.02$). Unfortunately, the potentially influential contributory role of intercurrent illness, medications, nutritional status, and prandial state on plasma metabolites could not be assessed. Therefore, validation of findings suggested by this retrospective cohort level analysis will require prospective study to control for the potential influence of relevant factors such as concurrent medications, diet, prandial status prior to phlebotomy, body mass index, general health status, parental analyte data, and family history. Finally, it is important to recognize that even if these metabolite alterations are prospectively confirmed in RC disease subjects, their clinical utility will likely be as a screening test to support further diagnostic testing in patients with suspected RC disease or potentially to monitor either disease progression or treatment effects. However, the presence or absence of such biomarkers alone are unlikely to permit definitive diagnosis or exclusion of mitochondrial disease, and may not be specific to primary versus secondary causes of RC disease.

In summary, we report here results from retrospective blood analyte analysis that identified a common profile of metabolite alterations across a heterogeneous cohort of primary mitochondrial disease and distinguished mitochondrial disease both from controls and a PDHc deficiency cohort. These findings offer novel insight into blood analyte alterations that may be common in human mitochondrial RC disease patients, and are supported by similar amino acid and lipid alterations that we have previously seen in animal models of primary RC disease [18, 19]. If validated in prospective clinical studies both in larger cohorts and at the individual subject level, recognition of a common metabolite profile may aid in the preliminary discrimination of mitochondrial RC disease from pyruvate metabolism defects. A blood-based biochemical analyte profile might also offer a non-invasive means to objectively monitor disease severity and therapeutic response. Finally, identification of secondary blood metabolite alterations that commonly occur in the setting of primary mitochondrial disease and that may themselves contribute to clinical disease manifestations, such as triglyceride elevation and increased BCAA to glutamate ratios, may potentially offer novel therapeutic targets in primary mitochondrial RC disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

RC respiratory chain

PDH	pyruvate dehydrogenase
BCAA	branched chain amino acids
CK	creatine kinase
PC	pyruvate carboxylase deficiency
PDHc	pyruvate dehydrogenase complex deficiency

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HIGHLIGHTS

- We report retrospective cohort-level analysis of blood analytes in human RC disease
- Branched chain amino acids (BCAA) are commonly increased in human RC disease
- PDH deficiency has lower BCAA & higher alanine and pyruvate levels than RC disease
- Blood metabolite profiles discriminate common sequelae of heterogeneous RC diseases

Figure 1. Blood lactate (A) and pyruvate (B) median concentrations in mitochondrial disease, PDHc deficiency, and control cohorts

Box plots depict results of analyses from median of all replicate values for each analyte per subject in each clinical cohort. The length of the box represents 25th to 75th percentile inter-quartile range, interior horizontal line represents median, and vertical lines issuing from the box extend to minimum and maximum values of the analysis variable. Values of upper and lower reference range bounds are indicated with horizontal black lines. Outlier values are indicated by an open circle and subject identifier, with detailed subject information per Supp File 1. N indicates number of subjects on whom laboratory data was available from each cohort. Statistical significance is indicated by #, $p < 0.10$; *, $p < 0.05$; and **, $p < 0.01$ as determined by non-parametric Mann-Whitney U test.

Figure 2. Plasma alanine and branched chain amino (BCAA) median concentrations in mitochondrial disease, PDHc deficiency, and control cohorts. (A) Alanine Median plasma alanine was significantly elevated in both the mitochondrial disease ($p = 0.006$) and the PDHc deficiency cohort ($p = 0.044$) relative to controls. **(B) Valine.** Median plasma valine levels were within the reference range for all cohorts, except for two outliers in the mitochondrial disease cohort (Subject 1007 had *FBXL4*-based severe complex I–III deficiency with multi-systemic disease including chronic lactic acidosis and Subject 1097 died in infancy of Leigh syndrome with severe complex IV deficiency). However, valine was significantly increased in the mitochondrial disease cohort relative to PDHc deficiency cohort ($p = 0.002$), and was significantly decreased in the PDHc deficiency cohort relative to controls ($p = 0.019$). **(C) Leucine.** Median plasma leucine levels were elevated in the mitochondrial disease cohort above the upper limit of the reference range and relative to controls ($p = 0.067$), but significantly decreased in the PDHc deficiency cohort relative to both the mitochondrial disease cohort ($p = 0.002$) and controls ($p = 0.044$). **(D) Isoleucine.** Median plasma isoleucine levels were within the reference range for all cohorts, with marginally significant elevation in the mitochondrial disease cohort relative to controls ($p = 0.056$) and significant reduction in the PDHc deficiency cohort relative to the mitochondrial disease cohort ($p = 0.047$). Throughout all panels, box plot component interpretation is same as indicated in Fig 1 legend. Statistical significance is indicated by #, $p < 0.10$; *, $p < 0.05$; and **, $p < 0.01$ as determined by Mann-Whitney U test.

Figure 3. Ratio of cohort means for amino acids relative to glutamate, as well as ratio of alanine relative to either lysine or sum of phenylalanine and tyrosine, in mitochondrial disease and control cohorts

Bars convey the mean cohort ratio values for each individual amino acid ratio. A consistently higher mean ratio was observed for each of the respective amino acids to glutamate, as well as for the alanine-dependent ratios, in the mitochondrial disease cohort relative to controls.

A.

Lipid Profile (mg/dL)	Percent change relative to control
Triglycerides	↑ 89.85 *
Cholesterol	↑ 34.8
LDL Cholesterol	↑ 19.18
HDL Cholesterol	↓ 0.09
LDL:HDL Ratio	↑ 0.54

B.

Figure 4. Plasma triglyceride elevation in mitochondrial disease cohorts. (A) Percent change in median blood lipoprotein analytes by cohort

Plasma triglyceride median levels were significantly increased by 89% in the mitochondrial disease relative to control cohort ($p = 0.02$). Total and LDL cholesterol levels showed a non-significant increase in the mitochondrial disease cohort relative to controls. Numbers indicate percent change between cohort medians for each analyte relative to controls. Up and down arrows convey increase or decrease, respectively, relative to controls. *, $p < 0.05$ as determined by Mann-Whitney U test. **(B) Plasma triglyceride boxplots by cohort.**

Median plasma triglyceride elevation exceeded the upper limit of the control range in the mitochondrial disease cohort. Box plot component interpretation is same as indicated in Fig 1 legend. *, $p < 0.05$ as determined by Mann-Whitney U test.

Table 1

Cohort enrollment overview

The total number of subjects is detailed for each of three clinical cohorts studied, along with the number of subjects for whom data was available for each blood analyte panel. Complete subject details are provided in Supp File 1.

Cohort	Number in Cohort	Gender Distribution	Age ranges at evaluation	Subject Number			
				Lipid Profile	Plasma Amino Acids	Chemistry Panel	Creatine Kinase Lactate and Pyruvate
Mitochondrial Disease	19	4 M 15 F	1 month - 35 years	10	17	17	10 17
PDHc Deficiency	4	1 M 3 F	1 month - 3 years	0	3	3	2 3
Control	27	12 M 15 F	1 month - 31 years	6	14	3	0 12

Table 2
Plasma amino acid alterations in mitochondrial disease and PDHc deficiency cohorts

Numbers indicate the percent change in each median analyte concentration relative to controls.

Cohort		
	Mitochondrial Disease	PDHc Deficiency
Amino Acid (mg/dL)	Median percent change relative to control	Median percent change relative to control
alanine	161.81 **	258.49 *
leucine	22.4 #	39.34 *
isoleucine	13.06 #	14.27
valine	21.48	70.92 *
glycine	32.49	52.93 #
ornithine	18.79	3.79
histidine	17.82 *	11.04 #
phenylalanine	1.48	10.13
B-alanine	1.07	NA
aspartic acid	2.38	10.97 *
1-methyl-histidine	17.6 **	11.5 *
a-amino-n-butyric acid	6.27	0.74
arginine	10.67	23.65 *
citrulline	5.68	7.52 #
cystine	6.52 #	3.92
ethanolamine	32.12	NA
glutamic acid	26.94	46.00 *
glutamine	29.1	7.45
lysine	40.13 **	28.76 #
methionine	0.45	4.73
proline	7.72	40.06
taurine	2.03	10.06 #
tyrosine	22.94	6.18
hydroxy-proline	31.88	11.96 *
asparagine	19.67 **	29.16 *
serine	1.69	25.01
threonine	12.44	26.32 *

Arrows indicates direction of percent change (increase or decrease) relative to control cohort, where significance is conveyed by #, $p < 0.10$, *, $p < 0.05$, ** $p < 0.01$ based on non-parametric Mann-Whitney U test.