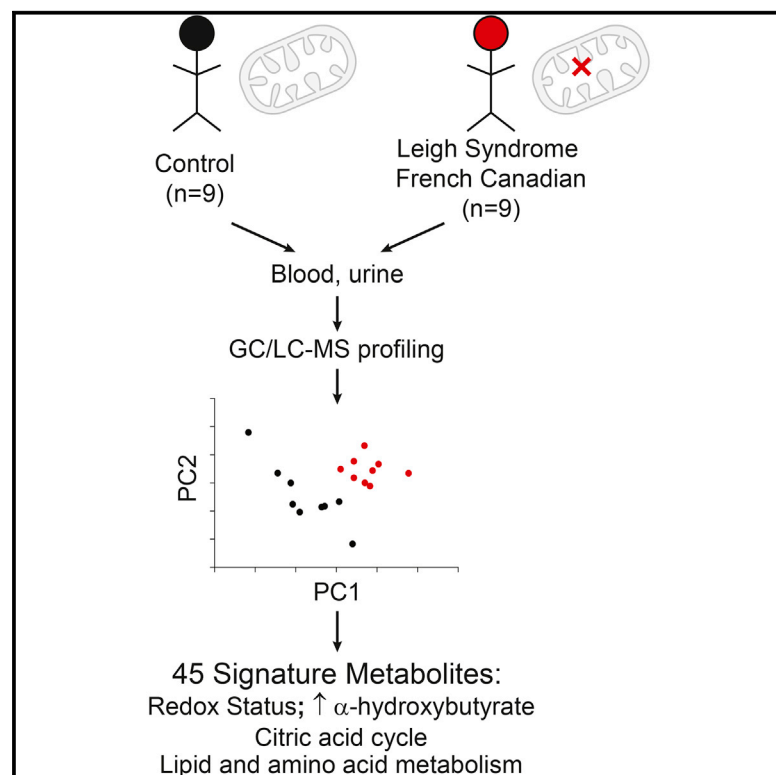


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A Metabolic Signature of Mitochondrial Dysfunction Revealed through a Monogenic Form of Leigh Syndrome

Graphical Abstract



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In Brief

In this prospective case-control study, Thompson Legault et al. applied comprehensive metabolic profiling in a genetically homogeneous form of Leigh syndrome to discover a robust signature of disrupted oxidative phosphorylation. The metabolic signature includes markers of mitochondrial disease, cardiometabolic risk, and disrupted NAD^+ as well as lipid and amino acid metabolism.

Highlights

- A metabolic signature is revealed in patients with a genetic mitochondrial disorder
- Profiling of 407 plasma/urine analytes identified 45 distinctive markers
- Markers reflect changes in cardiovascular risk as well as NAD^+ lipid and amine metabolism
- Markers also include metabolites linked to neurodegeneration



A Metabolic Signature of Mitochondrial Dysfunction Revealed through a Monogenic Form of Leigh Syndrome

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SUMMARY

A decline in mitochondrial respiration represents the root cause of a large number of inborn errors of metabolism. It is also associated with common age-associated diseases and the aging process. To gain insight into the systemic, biochemical consequences of respiratory chain dysfunction, we performed a case-control, prospective metabolic profiling study in a genetically homogenous cohort of patients with Leigh syndrome French Canadian variant, a mitochondrial respiratory chain disease due to loss-of-function mutations in *LRPPRC*. We discovered 45 plasma and urinary analytes discriminating patients from controls, including classic markers of mitochondrial metabolic dysfunction (lactate and acylcarnitines), as well as unexpected markers of cardiometabolic risk (insulin and adiponectin), amino acid catabolism linked to NADH status (α -hydroxybutyrate), and NAD⁺ biosynthesis (kynurenine and 3-hydroxyanthranilic acid). Our study identifies systemic, metabolic pathway derangements that can lie downstream of primary mitochondrial lesions, with implications for understanding how the organelle contributes to rare and common diseases.

INTRODUCTION

Mitochondrial dysfunction is increasingly being recognized as a hallmark of rare and common age-associated diseases. Among

inherited metabolic diseases, those affecting mitochondria are the most prevalent worldwide (1:5,000), frequently manifest in early childhood, and are associated with high morbidity and mortality (DiMauro, 2004; Torraco et al., 2009; Vafai and Mootha, 2013). These disorders may be caused by genetic lesions in either nuclear or mtDNA, which may disrupt numerous metabolic pathways housed in the mitochondria but most prominently the oxidative phosphorylation (OXPHOS) system (Debray et al., 2008; Munnich and Rustin, 2001). These disorders can impact virtually any organ system as a whole or in a tissue-specific manner. A subtle decline in OXPHOS is associated with skeletal muscle atrophy, type 2 diabetes, neurodegeneration, and the aging process itself (Vafai and Mootha, 2012), though the molecular basis and biochemical consequences are currently not known. There is growing interest in rare mitochondrial disorders, and studying them may shed insight into the role of the organelle to more common, age-associated disease.

Recently, tremendous progress has been achieved in the genetic characterization of rare mitochondrial disorders, yet their management remains challenging because of the difficulty to track their progression and the absence of proven therapies (DiMauro and Mancuso, 2007; Orsucci et al., 2009; Pfeffer et al., 2013; Schapira, 2012). This is due in part to our lack of understanding of the metabolic consequences of OXPHOS defects beyond ATP and the commonly reported high blood lactate. In this regard, the recent emergence of metabolomics technologies offers a means to systematically measure thousands of low-molecular-weight compounds in order to provide a global view of alterations in metabolic pathways induced by a given perturbation, whether resulting from a gene mutation or disease onset. These methods have been applied to numerous diseases such as diabetes and cardiovascular disease (for reviews, see Roberts and Gerszten, 2013 and Shah et al., 2012). However, only few

Table 1. Clinical Characteristics of LSFC Patients and Matched Control Individuals

| | Patients | Controls |
|---------------------------------------|---------------------------------------|---------------------------|
| Gender (male:female) | 4:5 | 4:5 |
| Age, years | 22 (6–30) | 24 (5–32) |
| BMI in adults, kg/m ² | 23.5 (22.3–26.7) | 24.0 (21.6–29.2) |
| BMI for age in children, percentile | 72 (1–96) | 70 (2–92) |
| Serum glucose, mmol/l | 4.9 (4.2–5.5) | 4.7 (4.4–5.2) |
| Serum triglycerides, mmol/l | 1.32 (0.67–4.18); 3/9 | 1.12 (0.41–1.55); 1/9 |
| Serum total cholesterol, mmol/l | 4.74 (3.53–7.19); 3/9 | 4.39 (3.47–5.48); 1/9 |
| Serum LDL cholesterol, mmol/l | 2.76 (2.08–4.52); 3/8 ^a | 2.45 (1.59–3.45); 2/9 |
| Serum HDL cholesterol, mmol/l | 1.01 (0.76–1.49); 2/9 ^b | 1.46 (1.13–2.19) |
| Serum total cholesterol/ HDL ratio | 4.23 (3.13–7.33) ^b | 3.11 (2.05–3.96) |
| Serum AST, U/l | 23 (14–30) | 24 (12–91); 1/9 |
| Serum ALT, U/l | 21 (14–51); 1/9 | 18 (14–58); 2/9 |
| Serum GGT, U/l | 11 (8–86); 1/9 | 13 (6–37) |
| Plasma insulin, mU/l | 10.3 (3.8–26.2) ^b | 4.79 (3.2–11.9) |
| Plasma adiponectin, ng/ml | 4,200 (1,304– 10,410) ^b | 10,229 (3,580– 37,804) |

Data presented as median (min-max) and, where applicable, proportion of abnormal results when measured as part of the routine biochemical assay. Subjects' characteristics are described in more details in [Table S1](#).

^aData for LDL cholesterol were missing for one patient given that triglyceride level was too high.

^bp < 0.03 patients versus controls following permutation test (Platform 1).

studies have extended this method to mitochondrial disorders ([Clarke et al., 2013](#); [Leoni et al., 2012](#); [Shaham et al., 2010](#)), which pose significant challenges, owing to their genotypic and phenotypic heterogeneity combined with small patient populations.

To overcome these challenges, we performed metabolic profiling in a small but genetically defined cohort of patients with Leigh syndrome French Canadian variant (LSFC; MIM no. 220111). First described in 1993 ([Merante et al., 1993](#); [Morin et al., 1993](#)), LSFC is a recessive disorder prevalent in the north-eastern region of Quebec (~1/2,000 births; carrier rate 1/23) ([Merante et al., 1993](#); [Morin et al., 1993](#)). LSFC patients exhibit many hallmarks of mitochondrial disorders, including lactic acidosis and Leigh syndrome, a necrotizing encephalopathy ([Debray et al., 2011](#); [Finsterer, 2008](#); [Morin et al., 1993](#)). LSFC was the first human disorder whose underlying genetic locus was mapped via linkage disequilibrium (also known as genome-wide association study; [Lee et al., 2001](#)) and the underlying gene discovered via “integrative genomics” ([Mootha et al., 2003](#)). All known cases are due to missense (A354V) or deletion (C1277STOP) mutations in the nuclear leucine-rich pentatricopeptide-repeat-motif-containing (*LRPPRC*) gene, which encodes a mitochondrial localized RNA-binding protein. These mutations lead to a tissue-specific impact on mitochondrial OXPHOS complexes ([Gohil et al.,](#)

[2010](#); [Mourier et al., 2014](#); [Ruzzenente et al., 2012](#); [Sasarman et al., 2010, 2015](#); [Xu et al., 2004](#)). More recently, LSFC fibroblasts were found to display multiple alterations in mitochondrial function and increased susceptibility to nutrient-induced cytotoxicity, particularly saturated fat ([Burelle et al., 2015](#)).

We recruited all known LSFC patients and, using a prospective, case-control study design, we performed metabolic profiling in plasma and urine using two different targeted mass spectrometric (MS)-based methods. We report a metabolic signature consisting of 45 compounds or ratios that distinguish LSFC patients from controls. The metabolites provide insight into the metabolic pathway derangements that ensue from a root mitochondrial lesion and provide a valuable resource for understanding the biochemical consequences and basis of mitochondrial dysfunction.

RESULTS

We performed metabolic profiling of all known living patients with LSFC. Eight of the nine patients are homozygotes for the A354V founder mutation, and one patient is compound heterozygous for this mutation and a premature stop ([Mootha et al., 2003](#)). Our study design is described in [Experimental Procedures](#) and depicted in [Figure S1](#). Patients were prospectively matched with controls for gender, age, BMI, physical activity level, and nutritional status. Metabolic measurements encompassed standard clinical biochemical parameters and multiple metabolite classes in plasma and urine. These analyses generated two independent data sets, hereafter referred to as “platforms,” which were processed through a statistical workflow for quality control filtering, missing data imputation, principal component analysis (PCA), and permutation testing. Significance threshold was set at a false discovery rate of 10%, corresponding to a p value < 0.03 for Platform 1 and <0.023 for Platform 2. Characteristics of LSFC patients and controls are summarized in [Table 1](#) and described in more detail in [Table S1](#). As expected given the intended matching, there were no statistical differences between patients and controls for age, BMI, or physical activity (paired t test; $\alpha = 0.05$).

Clinical Biochemical Assays in LSFC Patients versus Controls

Standard biochemical and hormonal assays, measured as part of Platform 1, showed that LSFC patients had significantly increased total cholesterol/HDL ratio and insulin levels, as well as reduced levels of HDL cholesterol and adiponectin. A trend for a significant increase in triglyceride levels was also observed (p = 0.039; [Tables 1](#) and [S2](#)), whereas serum glucose was similar to controls (p = 0.320). Collectively, these changes would suggest an increased cardiometabolic risk ([Leiter et al., 2011](#)) in LSFC patients.

Global Metabolic Profiles in LSFC versus Controls

PCA was first applied to the post-quality control, imputed data set from each platform, namely 137 analytes including standard biochemistry and hormones for Platform 1 and 156 for Platform 2, with an overlap of 37 metabolites between the two platforms. As shown in [Figure 1](#), the first PC from each platform

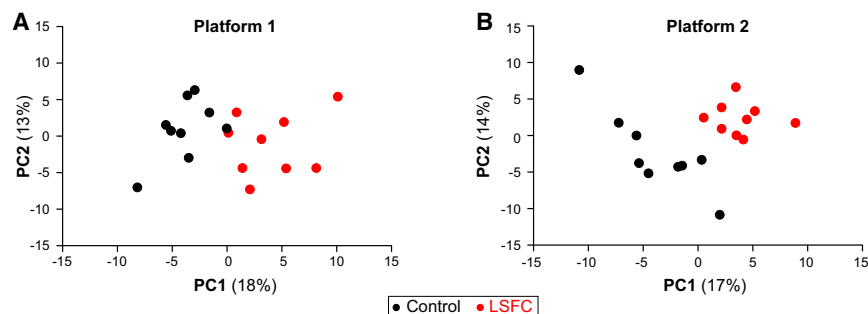


Figure 1. Principal-Component Analysis Discriminates LSFC Patients and Controls

For each panel, the post-quality control, imputed data set was used to perform the analysis.

(A) Platform 1; PC1 and PC2 account for 18% and 13% of variation, respectively.

(B) Platform 2; PC1 and PC2 account for 17% and 14% of variation, respectively.

Loading scores are reported in Table S3; see also Figure S1 for an overview of analytes submitted to the PCA and Table S2 for raw data sets.

fully discriminated patients from controls, indicating a distinct metabolic profile in LSFC patients. In each data set, the top two PCs accounted for 31% of the total variation (see Table S3 for loading scores). Metabolites contributing the most to the separation included lactate, β -hydroxybutyrate, acylcarnitines of various chain lengths, and α -hydroxybutyrate; to the best of our knowledge, the latter has not been previously reported in plasma from patients with an inherited mitochondrial disorder.

The two data sets were then independently subjected to permutation analyses, and statistical significance was reached between patients and controls for 24 analytes/ratios out of 137 in Platform 1 ($p < 0.03$) and for 31 analytes out of 156 in Platform 2 ($p < 0.023$), with an overlap of nine significant metabolites between both platforms (Table S4). All analytes are depicted as volcano plots in Figure S2 and listed in Table S2. Combining the results of both platforms, 46 single variables (representing 45 unique compounds/ratios) out of the 256 single variables submitted to the permutation test were statistically different between LSFC patients and controls, as depicted in Figure 2. Of note, the compound heterozygote was similar to the other LSFC patients (A354V homozygotes).

Concurring with the multivariate PCA, the most striking differences were observed for (1) plasma β -hydroxybutyrate and lactate, as well as the β -hydroxybutyrate/acetoacetate ratio; (2) acylcarnitines, and more specifically myristoylcarnitine and palmitoylcarnitine (C14 and C16, respectively); and (3) α -hydroxybutyrate (Figure 2). Altogether, the changes in metabolite levels can be organized into a small handful of metabolic pathways. First, these include elevated NADH/NAD⁺ ratio (increased β -hydroxybutyrate, lactate, and β -hydroxybutyrate/acetoacetate ratio) and closely related alterations in citric acid cycle (CAC) reactions, including those catalyzing entry or removal of carbons from this cycle (increased isocitrate, malate, and propionate, albeit decreased succinate and methylmalonate). Then, elevated C2, C6, C12, C14, C14:1, C16, C18:1, and C18:2 acylcarnitine but decreased C9 and C10:2 species reflect disrupted fatty acid oxidation, whereas impaired biosynthesis is suggested by increased vaccenic acid (C18:1n-7), dihomo- γ -linolenic acid (C20:3n-6), and phosphoethaloamine. Of particular interest, there were also unexpected changes in plasma metabolites relevant to amino acid catabolism, further substantiating the complexity of metabolic perturbations linked to NAD⁺ metabolism. These include (1) an increase in α -hydroxybutyrate, a metabolite arising from the reduction of α -keto-

butyrate formed from methionine and/or threonine, and (2) various intermediates of the tryptophan degradation pathways involved in NAD⁺ synthesis (kynurenine and 3-hydroxyanthranilic acid) or product of gut bacterial metabolism (indoxyl sulfate; Zhu et al., 2011). Finally, LSFC patients displayed increased plasma levels of creatine and decreased levels of the bile acid glycocholate, observations that were also made in patients affected by various OXPHOS defects (Pajares et al., 2013; Shaham et al., 2010) and with mutations in mitochondrial pantothenate kinase 2 (PANK2) (the rate-limiting enzyme in mitochondrial coenzyme A biosynthesis; Leoni et al., 2012), respectively.

Quantitative Analyses of Metabolic Indices of NADH/NAD⁺

Because both PCA and permutation tests revealed that a perturbed redox status was a prominent feature of the distinctive metabolic signature in LSFC patients, an isotope dilution GC-MS method was developed in order to quantify with precision lactate and β -hydroxybutyrate as well as their corresponding oxidized counterparts, namely pyruvate and acetoacetate, respectively. In addition, we included α -hydroxybutyrate, a finding issued from the metabolic profiling on Platform 2 but that was not assessed on Platform 1, because we suspected that its elevation was also linked to the increased NADH/NAD⁺ ratio. Hence, it was measured along with its oxidized counterpart, namely α -ketobutyrate. The results of these analyses are consistent with the initial metabolic profiling data. In fact, the plasma concentration of lactate, pyruvate, β -hydroxybutyrate, and α -hydroxybutyrate, as well as the lactate/pyruvate and β -hydroxybutyrate/acetoacetate ratios, were all significantly higher in LSFC patients (Figure 3). Importantly, this analysis revealed that the α -hydroxybutyrate/ α -ketobutyrate ratio was also significantly increased. Collectively, these quantitative measurements further support the findings of an elevated plasma level of α -hydroxybutyrate in LSFC patients, revealing an additional metabolic perturbation linked to the altered redox status in these patients.

DISCUSSION

In this prospective study, we applied a comprehensive targeted metabolomics approach to a genetically homogenous cohort of patients with an inherited mitochondrial respiratory chain disorder. Despite a small sample size, the current case-control study design enabled the identification of a distinct metabolic

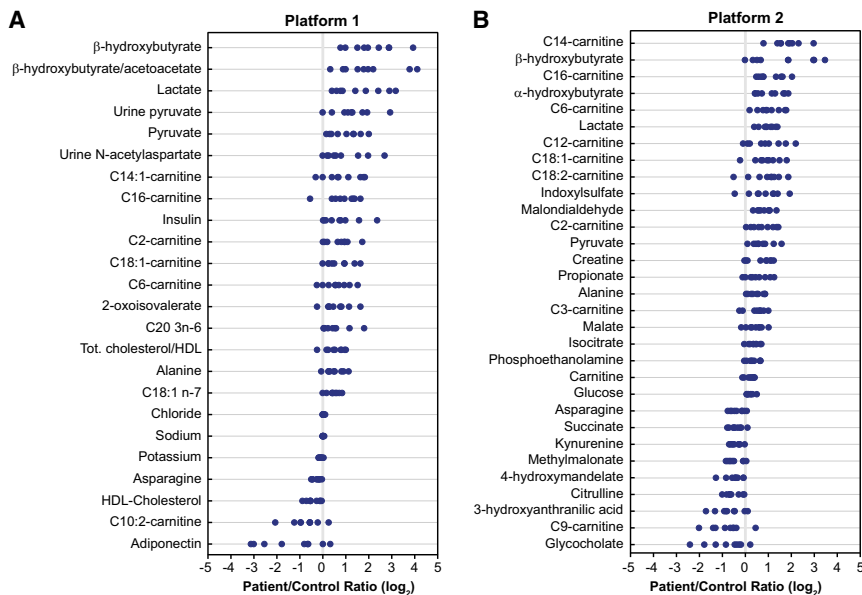


Figure 2. Individual Analytes with Statistical Significance in LSFC Patients versus Controls

(A) Platform 1; (B) Platform 2. Each dot represents a log₂-transformed patient/matched control ratio. Metabolites are ordered by mean log₂-transformed patient/control ratio. See also Figure S2 for data presented as volcano plots, Figure S1 for a summary of measured analytes, and Table S2 for log₂-transformed ratios and p values of all analytes.

metabolites might not be specific to mitochondrial dysfunction (Debray et al., 2008; Haas et al., 2008; Smeitink et al., 2006). Our quantitative analyses further substantiated elevated plasma lactate/pyruvate and β-hydroxybutyrate/acetoacetate ratios, hence reflecting an accumulation of reducing equivalents in the cytosol and mitochondria, respectively. Elevations in blood CAC intermediates and saturated

signature, consisting of 45 compounds spanning indices of cardiometabolic risk, disrupted NADH/NAD⁺, as well as lipid and amino acid metabolism.

Markers of Increased Global Cardiometabolic Risk

Much to our surprise, a disturbed cholesterol profile along with alterations in insulin and adiponectin levels were found, suggesting an increased global cardiometabolic risk in LSFC patients despite their relatively young age (Leiter et al., 2011). Although diabetes has been strongly associated with mutations in some mtDNA-encoded genes (Maassen et al., 2004), dyslipidemia has only been previously reported in few other cohorts of patients with respiratory chain disorders (Clarke et al., 2013; Finsterer et al., 2001; Kaufmann et al., 2009). In LSFC patients, increased cardiometabolic risk is further supported by the higher plasma levels of the fatty acids vaccenic and dihomo-γ-linolenic acids (C18:1n-7 and C20:3n-6, respectively), which have both been recognized as markers of insulin resistance (Lovejoy et al., 2001; Zulyniak et al., 2012). Interestingly, phosphoethanolamine, of which plasma levels were also increased, may also be associated with these alterations. This metabolite is an intermediate in the biosynthesis of phosphatidylethanolamine through the CDP-ethanolamine pathway (“Kennedy pathway”), and transgenic mice heterozygous for *Pcyt2*—a gene involved in this pathway that leads to increased hepatic phosphoethanolamine synthesis—display progressive hypertriglyceridemia, insulin resistance, as well as liver steatosis (Fullerton et al., 2009), which is also a characteristic found in LSFC patients (Morin et al., 1993).

Disturbances in Pathways Related to Energy Metabolism and the NADH/NAD⁺ Ratio

As expected, lactate, pyruvate, and alanine were elevated in LSFC patients as compared with controls, as it has often been reported in patients with mitochondrial diseases, although these

even chain acylcarnitines have previously been reported in patients with mitochondrial disorders (Haas et al., 2008; Smeitink et al., 2006). The elevation in acylcarnitines of all chain lengths in LSFC patients reflects a global disturbance of mitochondrial fatty acid β-oxidation, suggesting an impaired lipid-handling capacity (Adams et al., 2009; Houten and Wanders, 2010). This concurs with our recent finding of an increased susceptibility to palmitate-induced cytotoxicity in LSFC fibroblasts and is consistent with a tissue-specific defect in complex IV alone (for e.g., fibroblasts; Burelle et al., 2015) or combined with complex I (muscle and liver; Sasarman et al., 2010, 2015). The situation is likely to differ for complex I defects alone, which still enable entry of reducing equivalents arising from fatty acid oxidation at complex II and electron flux through complexes II–V and for which a positive effect of high-fat diets has been observed (Schiff et al., 2011).

Our systematic metabolomics approach also revealed an unexpected decrease in decadienoylcarnitine (C10:2) and nonanoylcarnitine (C9). The decrease in C10:2-carnitine, a partial oxidation product of the essential fatty acid linoleate (C18:2n-6), would suggest an increase in 2,4-dienoyl-CoA reductase activity that may be driven by an elevated NADPH/NADP ratio resulting from mitochondrial redox perturbation (Miinalainen et al., 2009). As for C9-carnitine, the metabolic origin of this compound is unclear, although it was suggested to be formed from the combined peroxisomal and mitochondrial catabolism of phytanic and pristanic acids, two branched chain fatty acids (Verhoeven et al., 1998). A decrease in C9-carnitine has also been reported in fibroblasts from patients with mitochondrial trifunctional protein deficiency (Roe et al., 2006), a fatty acid oxidation disorder. The latter findings emphasize the diversity of perturbations in fatty acid oxidation, which can result from mitochondrial dysfunction.

Perturbations in Amino Acid Metabolic Pathways

Beyond markers of mitochondrial energy metabolic pathways, other unexpected changes reflecting perturbations in

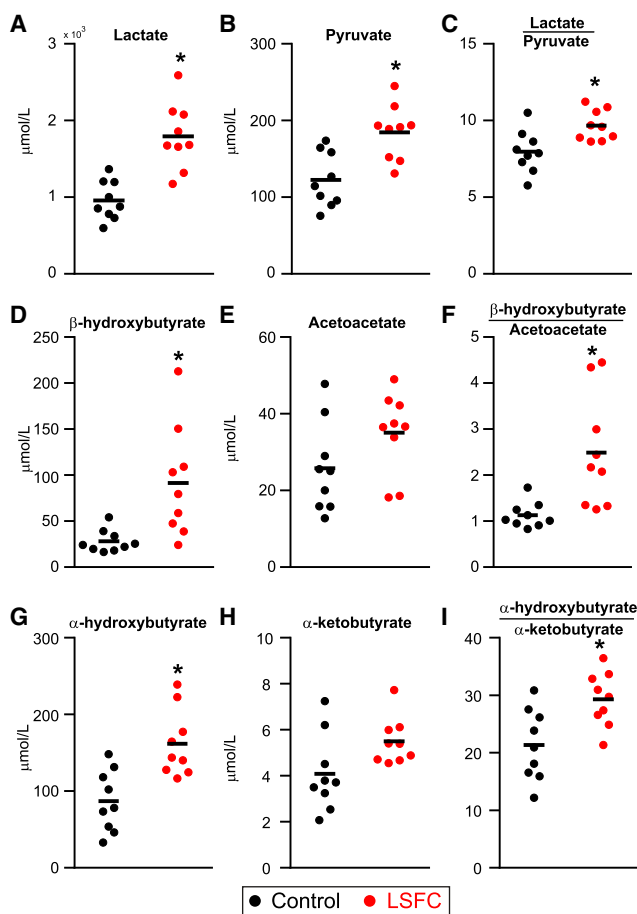


Figure 3. Quantitative Profiling of Metabolites Reflective of NADH/NAD⁺ Redox Status

Metabolites are shown in scatter dot plots with line indicating the mean. **p* < 0.05 patients versus controls. See also [Table S2](#) for raw data.

the metabolism of several amino acids were observed in LSFC patients. These include higher plasma levels of α -hydroxybutyrate. However, its oxidized metabolite counterpart, namely α -ketobutyrate, which arises from the catabolism of the essential amino acids threonine and/or methionine in the cytosol (Landaas, 1975), did not differ; consequently, the α -hydroxybutyrate/ α -ketobutyrate ratio was also significantly elevated. Whereas these findings likely represent another consequence of an elevated NADH/NAD⁺ ratio, the higher level of α -hydroxybutyrate also highlights major critical perturbations in its metabolism. The latter is depicted in [Figure 4](#) along with changes in closely related metabolites. Similarly to pyruvate, α -ketobutyrate is at a metabolic crossroad between the cytosol and the mitochondria. It may be transported to the mitochondria and subjected to oxidative decarboxylation to form propionyl-CoA; these steps are likely catalyzed by the pyruvate transporter and the branched-chain α -keto acid dehydrogenase complex (Jakobs et al., 1977; Paxton et al., 1986; Smith and Strang, 1958; Steele et al., 1984). Alternatively, α -ketobutyrate may be reduced to α -hydroxybutyrate by lactate dehydroge-

nase or β -hydroxybutyrate dehydrogenase (Rosalki and Wilkinson, 1960).

A few studies have reported increased α -hydroxybutyrate in patients with inherited metabolic diseases, such as lactic acidosis (Pettersen et al., 1973) or maple syrup urine disease (Jakobs et al., 1977; Smith and Strang, 1958). More recently, this metabolite has been reported to represent a strong and early marker of insulin resistance and glucose intolerance (Adams, 2011; Ferrannini et al., 2013; Fiehn et al., 2010; Gall et al., 2010; Xu et al., 2013) and was suggested as a potential biomarker for the diagnosis of metabolic syndrome (Demine et al., 2014; Lin et al., 2014). Factors proposed to play a role in α -hydroxybutyrate accumulation in plasma include perturbations in branched-chain amino acid catabolism, hepatic glutathione synthesis, or redox status (Gall et al., 2010; Landaas, 1975). Whereas the exact mechanisms underlying its elevation in LSFC patients remain to be elucidated, the present findings do not support a central role for branched-chain amino acids as proposed for insulin-resistant subjects (Adams, 2011; Newgard, 2012), because their levels were similar between LSFC patients and controls. Furthermore, whereas impaired glutathione metabolism has been reported in children with classical Leigh syndrome (Pastore et al., 2013), levels of reduced and oxidized glutathione in our LSFC cohort were similar to controls, suggesting that oxidative stress was not increased. This observation is consistent with our recent finding in LSFC fibroblasts (Burelle et al., 2015) but contrasts with those reported for other OXPHOS defects, namely complex I and III (Diaz et al., 2012; Distelmaier et al., 2009; Koene et al., 2011). It is noteworthy that the plasma level of malondialdehyde was significantly increased in LSFC patients, albeit this is not a specific oxidative stress marker, because it is also generated by cyclo-oxygenases in thromboxane metabolism (Kadiiska et al., 2005). An accelerated methionine metabolism through the S-adenosylmethionine cycle in LSFC patients is, however, suggested by the increased plasma levels of creatine, which may reflect a low intracellular energy status (Shaham et al., 2010). This may also suggest a perturbation in methylation reactions, which could impact on signaling pathways regulating cellular survival or mitochondrial biogenesis such as Sirt1 and/or PGC-1 α (Guéant Rodriguez et al., 2013).

Our results also demonstrate alterations in the metabolism of tryptophan (reduced kynurenine and 3-hydroxyanthranilic acid) and aspartate (increased N-acetylaspartate), which have been linked to neurodegeneration (Sas et al., 2007; Tan et al., 2012), an important clinical feature of LSFC. Among intermediates of the tryptophan catabolic pathways that lead to NAD⁺ synthesis, kynurenine and 3-hydroxyanthranilic acid were significantly modified ([Figure 4](#)), although tryptophan itself or other metabolites of this pathway were unchanged. As for N-acetylaspartate, it is found exclusively in neurons and arises from the condensation of aspartate and acetyl-CoA in the mitochondria. Its increased level in urine has been reported in patients with aspartoacylase deficiency (Canavan disease), a leukodystrophy characterized by neurodegeneration and early death (Al-Dirbashi et al., 2007). In the brain, its elevation has been reported as a marker of compromised neuronal integrity, but reduction suggests neurodegenerative disorders (Knapman et al., 2012; Moreno et al., 2001).

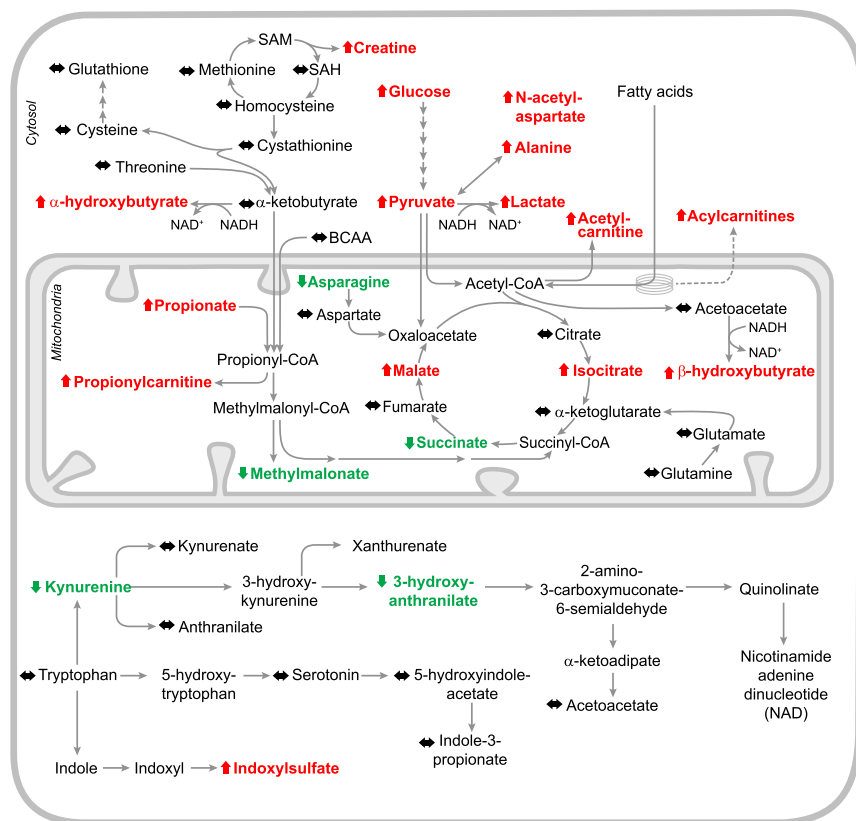


Figure 4. Pathway Relationship of Major Metabolic Alterations in LSFC Patients

This scheme depicts pathways related to reported perturbations in cytosolic and mitochondrial NADH accumulation, disrupted citric acid cycle (CAC), fatty acid β -oxidation, and amino acid metabolism, with a specific emphasis on those linked to α -hydroxybutyrate formation. Metabolites whose levels increased are indicated in red and with an upward arrow; those whose levels decreased are indicated in green and with a downward arrow. Metabolites whose levels did not change significantly are indicated in black with a sideways arrow. Those metabolites that were not measured are indicated in black (with no arrow). See also [Figure S1](#) for an overview of analytes measured in the study and [Table S2](#) for raw data and p values.

controls included gender, age (± 2 years for children < 18 years; ± 5 years for adults), BMI (± 10 percentiles of BMI for age for children; ± 3 kg/m² for adults), and physical activity level (see the [Supplemental Experimental Procedures](#) for details). Carrying A354V or C1277STOP mutation was an exclusion criterion for controls. The protocol was approved by the Human Ethics and Research Committee of the Centre de Santé et de Services Sociaux de Chicoutimi. Written informed consent was obtained for all study participants or their legal guardians, and assent was obtained when applicable. Metabolic profiling was performed on venous blood and urine samples collected after

an overnight fast of minimum 12 hr, during which water was allowed. Genotyping was performed on saliva samples. Samples were processed as described in the [Supplemental Experimental Procedures](#).

Conclusions

This study highlights the power of metabolomics when applied in a prospective case-control design to a genetically homogenous form of Leigh syndrome presenting with many of the hallmark features of mitochondrial respiratory chain disease. Results uncovered a LSFC metabolic signature including both classic and unexpected metabolites, which collectively highlight the complexity of perturbations that may result from mitochondrial dysfunction but also have implications for understanding potential contributions of mitochondrial dysfunction to common disease. Altogether, this signature concurs with the reported tissue-specific impact of mutated *LRPPRC* on mitochondrial OXPHOS complexes ([Mourier et al., 2014](#); [Sasarman et al., 2010, 2015](#)) and function in LSFC fibroblasts ([Burelle et al., 2015](#)), while representing also possible targets for intervention. Future studies will be needed in order to further delineate the underlying metabolic mechanisms and further characterize whether the identified metabolites may be relevant as biological markers for the assessment of severity, prediction of lactic acidosis crises, monitoring of disease progression, or response to interventions in patients with LSFC and, possibly, other inherited mitochondrial disorders.

EXPERIMENTAL PROCEDURES

Subjects, Sample Collection, and Genotyping

LSFC patients were included based on medical history and genetic testing for the A354V and C1277STOP mutations in *LRPPRC*. Matching criteria for

Metabolite Profiling and Quantitative Analysis

The overall workflow for metabolic profiling is depicted in [Figure S1](#) and described in detail in the [Supplemental Experimental Procedures](#). This was performed using a combination of standard biochemical and hormonal assays as well as established targeted GC-MS and LC-MS methods, altogether encompassing 407 analytes, which were treated as two distinct platforms. Platform 1 included clinical laboratory assays, hormonal assays, and MS-based profiling of amino acids, fatty acids, organic acids, and acylcarnitines. For Platform 2, two LC-MS methods were used to profile polar metabolites. After obtaining data from Platforms 1 and 2, a quantitative isotope dilution GC-MS method was developed to quantify plasma metabolites specifically reflecting cellular redox state, based on a previously published method ([Lauzier et al., 2013](#)).

Data Mining and Statistical Analysis

Results from Platform 1 and Platform 2 were treated as two distinct data sets to which the statistical workflow was applied using R 3.0.2 (R Foundation for Statistical Computing) for quality control filtering, log₂-transformation, and missing data imputation (see the [Supplemental Experimental Procedures](#)). Raw data for all measured metabolites are provided in [Table S2](#). The post-quality control, imputed data sets were submitted to PCA (SIMCA-P+ 13.0; Umetrics) and permutation test between the two groups. All possible distinct permutations were conducted within patients/control pairs, for a total of 256 (2⁸). The significance threshold was determined according to the estimation of true and false positives and was established in order to correspond to an estimated false discovery rate of 10%. This led to a p threshold of < 0.03 for Platform 1 and 0.023 for Platform 2. For the quantitative profiling of selected metabolites, p values were generated by a permutation test, and a threshold

of 0.05, corresponding to a false discovery rate of 5.7%, was used to control for type 1 error.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and four tables and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.09.054>.

CONSORTIA

At the time of recruitment, the members of the LSFC Consortium were, in alphabetical order, Azadeh Aliskashani, Bruce G. Allen, Chantale Aubut, Claudine Beauchamp, Chantal Bemeur, Yan Burelle, Guy Charron, Lise Coderre, Christine Des Rosiers, Sonia Deschênes, François Labarthe, Jeannine Landry, Catherine Laprise, Geneviève Lavallée, Pierre Lavoie, Bruno Maranda, Charles Morin, Yvette Mukaneza, Tamiko Nishimura, John D. Rioux, Marie-Ève Rivard, Florin Sasarman, Eric A. Shoubridge, Jessica Tardif, Julie Thompson Legault, Nancy Tremblay, Vanessa Tremblay-Vaillancourt, Luc Vachon, and Josée Villeneuve. Affiliations are listed in the Supplemental Information along with individual author contributions.

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