Metabolic profiling reveals anomalous energy metabolism and oxidative stress pathways in Chronic Fatigue Syndrome patients.

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Data Deposition

The data for this study has been deposited at MetaboLights (http://www.ebi.ac.uk/metabolights) with accession number MTBLS161.

Abstract

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a debilitating long-term multisystem disorder with a central and inexplicably persistent fatigue symptom that is unable to be relieved by rest. Energy metabolism and oxidative stress have been recent focal points of ME/CFS research and in this study we were able to elucidate metabolic pathways that were indicative of their dysfunction. Blood and urine samples were collected from 34 females with ME/CFS (34.9 ± 1.8 SE years old) and 25 non-ME/CFS female participants (33.0 ± 1.6 SE years old). All samples underwent metabolic profiling via 1D ^{1}H Nuclear Magnetic Resonance spectroscopy and quantitated metabolites were assessed for significance. Blood glucose was elevated while blood lactate, urine pyruvate, and urine alanine were reduced indicating an inhibition of glycolysis that may potentially reduce the provision of adequate acetyl-CoA for the citric acid cycle. We propose that amino acids are being increasingly used to provide an adequate carbohydrate source for the citric acid cycle. We suggest that this is via glutamate forming 2-oxoglutarate through an enzyme that deaminates it and subsequently elevates blood aspartate. Dysfunctional energy metabolism appears to have impacted creatinine and its elevation in urine suggests that it may be used as an alternative for anaerobic ATP production within muscle. A decrease in blood hypoxanthine and an increase in urine allantoin further suggest the elevation of ROS in ME/CFS patients. These findings bring new information to the research of energy metabolism, chronic immune activation and oxidative stress issues within ME/CFS.

Keywords

Chronic Fatigue Syndrome; Metabolic; Blood; Urine; Oxidative Stress; Energy metabolism; Amino Acids

Abbreviations

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS)

Principal Components Analysis (PCA)

Branched Chain Amino Acids (BCAAs)

Aspartate Transaminase (AST)

Reactive Oxygen Species (ROS)

1 Introduction

Myalgic Encephalomyelitis/Chronic Fatigue Syndrome (ME/CFS) is a debilitating long-term disorder that is multisystem symptomatic with the primary presentation of fatigue (Bruce M. Carruthers et al. 2003; Christley et al. 2012). The pathological nature of the fatigue experienced by ME/CFS sufferers is its inexplicable persistence, severity and its inability to be sufficiently relieved by rest (Christley et al. 2012). Post-exertional malaise is consistent with the presence of fatigue in all cases of ME/CFS along with a collection of associated symptoms from multiple body systems that vary from patient to patient. These symptoms may be grouped under psychological, cognitive, neurological, motor-sensory, endocrine, cardiovascular, immune, and gastrointestinal issues.

The first case definition of ME/CFS was published in 1988 by the United States Centers for Disease Control and Prevention (Holmes et al. 1988). This was the amalgamation of a plethora of unexplainable long-term fatigue disorders throughout the 20th century (Ax et al. 2001; Johnson et al. 1999). The case definition provides a clinical diagnosis of ME/CFS by essentially forming a threshold marker along a health spectrum that relies upon the patients' personal description of symptoms (B. M. Carruthers 2007) and the absence of any other disease diagnoses (B. M. Carruthers et al. 2011). Its reliance as the only means of diagnosis for the disorder has dictated the priority of research to finding diagnostic biomarkers. In the meantime, the definition has been further refined and improved to the Fukuda definition in 1994 (Fukuda et al. 1994), the 2003 Canadian criteria and the 2011 'international consensus criteria' (B. M. Carruthers et al. 2011; Bruce M. Carruthers et al. 2003). The prevalence of the disorder was found to be 0.42% of the population from a random community-based sample (Jason et al. 1999). The difficulty of accurately determining the prevalence is due to the different definitions used by clinics. This is also reflected in the contradictory research findings in the literature.

The early description of ME/CFS as a flu-like illness has centered a large portion of work on the role of pathogens in the disorder (Buchwald et al. 1996). Many pathogens have been reported to be linked to the onset of the disease but all findings have failed to be reproduced (Johnson et al. 1999; Buchwald et al. 1996) (Ortega-Hernandez and Shoenfeld 2009). The role of pathogens appears to be non-specific in stressing the body before and after the onset of the disorder, which has led to

the idea of an etiological mechanism that entails predisposing, triggering, and maintaining factors (Candy et al. 2004; Candy et al. 2002).

Given the long-term requirements for diagnosis, studies are likely to be most relevant to the maintaining factors of the illness. Fatigability and post-exertional malaise remain the cornerstones of this illness. These symptoms typically involve energy metabolism within the body. Indeed, recent studies have noted abnormalities in energy metabolism, perhaps suggesting a role of the mitochondria in ME/CFS. For example, a 2-day study of oxygen consumption in ME/CFS found normal levels during the first day of exercise that significantly decreased during exercise on the second day (Snell et al. 2013). The reduced use of oxygen inferred a decreased use of aerobic respiration and therefore mitochondrial function. As the prime source of reactive oxygen species (ROS) within the body, there may be a link between mitochondrial dysfunction and ME/CFS through oxidative stress (Morris and Maes 2014a; Meeus et al. 2013).

Oxidative stress has been linked to ME/CFS through the detection of reduced antioxidant levels and increases in signals of oxidative damage including glutathione, peroxidated lipids and damaged tissue (Maes et al. 2011; Kennedy et al. 2005). Typically ROS production may be triggered by cytokines to attack pathogen but can create oxidative stress if production is too high, therefore linking a chronic inflammatory response to oxidative stress as proposed for ME/CFS (Morris and Maes 2014a). In this study we elucidate potential biochemical pathways that may be significant within the ME/CFS cohort by using metabolic profiling to study the blood and urine of ME/CFS and healthy groups. We have performed an untargeted study to broaden our search for a diagnostic by identifying any perturbations in the major biochemical pathways of the body, which include a focus on the energy metabolism and oxidative stress pathways.

2 Materials and Methods

2.1 Study Design and Sample collection

An untargeted metabolomics study was performed on the blood serum and urine of ME/CFS patients as compared to non-ME/CFS subjects. Samples were randomized prior to each analysis step: sample preparation; detection of metabolite concentrations by NMR spectroscopy; and data

analysis. Data analysis focused on finding differences between ME/CFS patients and non-ME/CFS subjects by using Student T-tests, Principal Components Analysis (PCA), and Pearson correlations.

Forty-six patients with ME/CFS and twenty-six non-ME/CFS control subjects were recruited in total. Prior to statistical analysis we removed all males and any females that were above sixty years of age, which reduced the cohort to thirty-four females with ME/CFS (34.9 ± 1.8 SE years old) and twenty-five non-ME/CFS female participants (33.0 ± 1.6 SE years old). This was done to account for age and sex related variables across the cohort.

The ME/CFS group comprised patients that are currently symptomatic and diagnosed as having ME/CFS in accordance to the Canadian guidelines (Bruce M. Carruthers et al. 2003) and cared by one clinician. Diagnosis of ME/CFS was made on the nature of the clinical history, in association with the Canadian criteria, having excluded other illnesses. The same clinician also verified that subjects within the non-ME/CFS cohort were not suffering from ME/CFS or any other illness. All subjects were asked to list their current medications and oral supplements. None of the subjects were related to one another nor were they ever living together and none of the subjects were obese or nicotine dependent.

Urine was collected upon rising by each subject and stored at 4 ºC and within 6 hours a blood sample was then taken by venipuncture into BD Vacutainer® blood collection tubes. All blood samples were rested in a refrigerator for 30 minutes to allow blood to coagulate before the serum was separated from the clotted whole blood sample by centrifugation at 4000 rpm for 10 minutes. Five 0.6 mL aliquots of serum from each subject were collected and stored at -80 ºC. All Urine samples were syringe filtered (0.2 µm, 25 mm diameter) and each was dispensed into five 1 mL aliquots and stored at -80 ºC.

2.2 Sample Preparation

All blood and urine samples were prepared for Nuclear Magnetic Resonance (NMR) spectroscopy using a liquid-liquid extraction technique (Sheedy et al. 2010). All 72 blood samples were prepared simultaneously and all 72 urine samples were prepared simultaneously. A 250 µL sample of each serum/urine sample was added to 250 µL ice-cold deuterated chloroform and 250 µL

ice-cold deuterated methanol and mixed by vortexing before being left to sit on ice for 15 minutes. Samples were then centrifuged (16,100 × *g)* at 4 ºC for 10 minutes to produce a biphasic mixture with a hydrophilic phase of water/deuterated methanol and lipophilic phase of deuterated chloroform. A 297 µL sample of the top hydrophilic layer was added to an equal volume of 200 mM sodium phosphate in deuterium oxide (pH 7) and 66 µL of deuterium oxide containing 5 mM DSS and 0.2% (w/v) sodium azide to make a total volume of 660 µL. The sample was then mixed by vortex, centrifuged for 1 minute at 13,000 rpm and 550 µL of supernatant was transferred to a 7-inch 5-mm 507-grade NMR tube for NMR analysis.

2.3 NMR measurements

All NMR spectra of blood sera and urine were collected at 25 ºC on an 800 MHz (18.8 T) Bruker Avance II US ^{2} spectrometer equipped with a 5 mm triple resonance cryoprobe. The 90º pulse width was calibrated and the receiver gain optimized. Standard 1D ^{1}H spectra were acquired using a 1D NOESY pulse sequence (RD-90º-t_1-90º-t_m-90º-acquire) with a Relaxation Delay (RD) of 1.2 seconds and a mixing time (t_m) of 0.1 seconds. Water suppression was achieved by presaturation of the water signal during relaxation delay and mixing time. Each spectrum was collected over 32K of data points and 128 scans with a spectral width of 10.014 ppm. Before Fourier transformation, the free induction decay was zero-filled and multiplied by a 0.3 Hz exponential line broadening. Using the Chenomx 6.0 NMR Suite Professional software package; spectra were manually phased, baseline corrected and referenced to DSS with a chemical shift of 0 ppm.

To confirm metabolite identities in highly overlapped regions of the 1D spectra, 2D TOCSY experiments were collected using WATERGATE solvent suppression and a DIPSI-2 mixing sequence with 60 ms mixing time and 4096 *t*2 and 1024 *t*1 data points. States-TPPI was used for quadrature detection along the indirect dimension and the 2D NMR data was processed using TOPSPIN 1.3 and MestReNova 10.0.1.

The compound libraries in the Chenomx software were used to identify and quantitate metabolites in the NMR 1D ^{1}H spectra based on their characteristic chemical shifts using 0.5 mM DSS as an internal chemical shift reference. Metabolite identities were confirmed using 2D TOCSY

experiments with TOPSPIN 1.3 and MestReNova 10.0.1 software. Twenty-nine metabolites per blood serum sample and thirty metabolites per urine sample were identified.

2.4 Data Analysis

The workflow used for analysis is displayed in Fig. 1A. Concentrations were prepared as absolute concentration data (μM) and as relative abundance data (%) by dividing each metabolite concentration by the total concentration of identified metabolites quantified in each sample. Prior to statistical analysis all data were assessed for normality. Those metabolites not found to be normally distributed were either log converted in the absolute concentration data set or arcsine converted for the relative abundance data set. Bootstrapping was employed to test the stability of the significant results from the t-tests and Pearson correlations (Efron and Tibshirani 1993). Data subsets were created via random sampling, with replacement, from the original dataset, there were 2,000 subsets created that then underwent student t-tests and Pearson correlations, after which a mean, standard error and 95% confidence interval were calculated.

Multivariate and univariate analysis was conducted on all data matrices using an in-house *R* based statistical package. Unsupervised Principal Components Analysis (PCA) was conducted on both the data binned from the NMR spectra and the identified compounds. T-tests and Benjamini-Hochberg adjustments were conducted on the identified compound data to establish significant metabolites. Significant metabolites were then correlated with all metabolites identified in the respective samples using Pearson correlation. Correlation graphs were developed in-house using Microsoft Excel 2010 to overlay the metabolite correlations within both the ME/CFS and non-ME/CFS to highlight discrepancies.

3 Results and Discussion

3.1 Experimental workflow: Sample and Data Collection

We removed age and sex related variables to allow a more cohesive understanding of metabolic anomalies within ME/CFS patients, this required the exclusion of all male subjects and all females over 60 years of age. The resultant cohort was 34 females with ME/CFS (34.9 ± 1.8 SE years

old) and 25 non-ME/CFS female participants (33.0 ± 1.6 SE years old). This study is therefore a representation of ME/CFS within the context of young adult females. The experimental and analytical workflow follows the detection of metabolites within blood and urine, the evaluation of the separation of ME/CFS and non-ME/CFS cohorts, the detection of metabolites that are significantly altered between the cohorts, and the identification of pathways that may be involved with these alterations (Fig. 1A).

The measurement of metabolite concentrations were conducted using 1D ^{1}H NMR experiments in conjunction with Chenomx software (v. 6.1) and an internal standard, DSS, of known concentration (Fig. 1B and 1C). Metabolites were confirmed with 2D TOCSY spectra for blood and urine samples as presented in Fig. 2A and Fig. 2B respectively. Twenty-nine and thirty metabolites were identified and quantitated in blood (Fig. 3A) and urine (Fig. 4A) respectively from NMR experiments. PCA analysis was conducted on the NMR spectra prior to the identification of metabolites by using the 'binned' data (Fig. 1B and 1C). PCA finds an eigenvector that uses metabolites and 'bins' to order the whole dataset and plots each sample accordingly (scores plot) while also plotting the metabolites or 'bins' that were most influential to that order (loadings plot). From unsupervised (without influence) PCA and using the 'binned' data we see a separation of the ME/CFS and non-ME/CFS cohorts (Fig. 1B and 1C). Once we identified the peaks we ensured that no unidentified peaks were found to be important to the separation of the 'binned' data.

Direct quantitation of metabolites from the NMR data is presented here as absolute concentrations. Alterations in the absolute concentration of a metabolite in the blood suggests that the disruption of the homeostasis of biological processes has occurred, whereas, alterations in the absolute concentration of urine metabolites reflect a deficiency or an excess in the blood that has been excreted in order to maintain homeostasis, but this may vary with the total amount of metabolite excreted. In this regard blood metabolites will indicate the extent of an acute perturbation at the time of collection and urine will indicate the chronic nature of a metabolite or alteration to a pathway over a period of time (from last urination). There is a shared relationship between metabolites within the blood and urine, such that if they are disconnected then this may suggest a disruption at the point of excretion or a rapidly changing blood homeostasis. Alterations in

the absolute concentration therefore reveal potential strain on biochemical systems in the blood and the chronic extent of that strain through metabolite excretion or end-point production or potentially alterations in metabolite concentration associated with altered renal function.

Creatinine has been used to standardize urinary metabolite concentrations to account for variations in urinary accumulation and dilution factors. In this instance we deemed it an inappropriate method as creatinine was trending towards a significant decrease within the absolute concentration blood data of ME/CFS patients (Fig. 3A). In this study when the urinary data was normalized to urinary creatinine all metabolites were significantly altered, which we concluded was an artificial result (Fig. 4A). To remove the influence of dilution factor on both the urine and the blood we normalized each sample to the total metabolite concentration, therefore producing relative abundance data. This method of normalization focuses analysis on the ratio of metabolites within either the blood or urine.

Pearson correlational analysis is used to find the correlation coefficients (r) that quantitate how metabolites fluctuate with one another in the ME/CFS cohort compared to the non-ME/CFS cohort (Fig. 5). This allows us to compare the relationship of the two groups in terms of positive ($r > 0.4$), neutral ($-0.4 < r < 0.4$) and negative ($r < -0.4$) relationships.

3.2 Altered blood metabolites in patients with ME/CFS

The absolute concentrations of six blood metabolites from the ^{1}H NMR analysis were significantly altered. Glucose levels were increased whereas acetate, glutamate, hypoxanthine, lactate, and phenylalanine were decreased in ME/CFS patients (Fig. 3A). When blood metabolites were analyzed as a function of total metabolite concentrations (relative abundance data) there were again, six metabolites that were significantly different (Fig. 3A). For this analysis aspartate and glucose were increased whereas acetate, glutamate, hypoxanthine, lactate, and phenylalanine decreased in ME/CFS patients.

These distinct metabolic anomalies in ME/CFS patients could be separated clearly from the control non-ME/CFS subjects by the unsupervised PCA analysis on the relative abundance distribution for blood (Fig. 3B). The scores plot displays a significant separation of the cohorts

described by two principal components defining 30.0% and 15.0% of the dataset. The loadings plot revealed that the significant metabolites involved in the separation are indicative of those that were found by student T-tests to be altered (Fig. 3A). Formate and acetate were indicated by the PCA analysis as being involved in the separation of the cohorts despite not being seen to be significant in the relative abundance blood dataset.

3.3 Altered urinary metabolites in patients with ME/CFS

The absolute concentrations of five urinary metabolites from the ^{1}H NMR analysis significantly differed between the ME/CFS and non-ME/CFS cohorts (Fig. 4A). All urinary absolute concentration metabolites were decreased in ME/CFS patients: acetate; alanine; formate; pyruvate; and serine. Eight metabolites were significantly altered within the relative abundance dataset for urine. These include the five that were decreased in ME/CFS in the absolute concentration data, along with decreased valine and increased allantoin and creatinine.

The unsupervised PCA analysis of relative abundance data for urine demonstrated a moderate separation of the two cohorts as shown by the scores plot described by two principal components defining 25.0% and 10.8% of the dataset (Fig. 4B). The PCA loadings plot revealed that only five of the significant metabolites involved in the separation of the cohorts were found to be altered by student T-tests. These metabolites are creatinine, allantoin, pyruvate, serine, and acetate.

3.4 Correlations of blood metabolites: Differences in the energy metabolism pathways in ME/CFS

The absolute concentration of four blood metabolites, glutamate, hypoxanthine, lactate and phenylalanine were decreased in ME/CFS patients (Fig. 3) and were found to be positively correlated with one another in both the ME/CFS and non-ME/CFS cohorts (Fig. 5). In addition, they also all positively correlated with alanine, glycine, ornithine, serine and total metabolites in both ME/CFS and non-ME/CFS cohorts. Similarly, absolute concentration blood acetate and glucose, which respectively decreased and increased in ME/CFS patients (Fig. 3), were also found to correlate with these metabolites (Fig. 5). Glucose showed negative correlations in the non-ME/CFS for hypoxanthine, lactate and phenylalanine; whereas, acetate showed positive correlations with

glutamate, hypoxanthine, lactate and phenylalanine in the ME/CFS. The amino acids and components interacting with the citric acid cycle appear to be maintained but the input of carbohydrates from glucose via glycolysis into the cycle appears to have markedly decreased. These correlations, paired with a decrease of amino acid concentrations, implicate an increasing utilization of amino acids as a source of energy production through the citric acid cycle, largely via glutamate (Fig. 6B).

The absolute concentrations of blood acetate, creatinine and formate were observed to positively correlate with several significantly decreased blood metabolites in ME/CFS patients only. Acetate and formate are typically considered intestinal microbial metabolites but are also products of lipid oxidation of the host. The absolute concentration of creatinine in blood was trending towards a significant decrease in ME/CFS patients; however, it has significantly increased in the relative abundance data in urine of ME/CFS. Creatine is used to carry phosphate from the liver to muscles for the rapid synthesis of ATP from ADP, which may be a compensatory mechanism for tissues requiring anaerobic respiration given the proposed dysregulation of glycolysis.

While glycolysis relationships remain maintained in both cohorts, only in the ME/CFS cohort a negative correlation between glucose and non-essential amino acids, alanine, glutamate and proline, was observed. In non-ME/CFS, and not the ME/CFS cohort, the key product of glycolysis, lactate, and the ATP breakdown molecule, hypoxanthine, are both negatively correlated with the essential amino acids including the branched chain amino acids (BCAAs) and threonine. The lack of relationship with ME/CFS indicates that perhaps essential amino acids are maintained while lactate and hypoxanthine are depleted. The disparity between essential and non-essential amino acids and their relationship with glycolytic metabolites suggests in ME/CFS a depletion of nonessential amino acids in the blood is being used to fuel the citric acid cycle and produce ATP in the absence of sufficient glucose usage via glycolysis (Fig. 6).

In non-ME/CFS subjects, relative abundance blood aspartate positively correlated with the blood BCAAs and tyrosine. In the ME/CFS cohort aspartate positively correlated with glutamine, threonine, tryptophan and total amino acids. Aspartate Transaminase (AST) produces aspartate from oxaloacetate when glutamate is deaminated to 2-oxoglutarate. In non-ME/CFS only, relative

abundance blood glutamate was positively correlated with alanine and aspartate while in ME/CFS glutamate was positively correlated with arginine and negatively correlated with glucose. This highlights the compensatory relationship of glutamate for glucose while also verifying the altered relationship of key urea cycle metabolites within the ME/CFS cohort (Fig. 6).

3.5 Correlations of urine metabolites: Altered glycolysis, lipid oxidation, creatinine production and the potential role of gut microbiota

Excluding acetate and formate, the remaining significant urine metabolites in the absolute concentration data are generally positively correlated with the majority of the dataset of absolute concentration metabolites, excepting 1-methylhistidine, acetate, creatine, formate, maltose, mannitol, trigonelline and tyrosine (Fig. 5). The absolute concentrations of acetate and formate were significantly decreased in the urine of ME/CFS patients (Fig. 4) and in the blood of ME/CFS patients they were found to be positively correlated with blood metabolites that were significantly decreased, including glutamate, hypoxanthine, lactate, and phenylalanine. This reflects a biological pathway reduction of these two products of lipid oxidation. In the relative abundance data, urine acetate positively correlated with other decreased glycolysis metabolites (alanine, lactate and pyruvate) in non-ME/CFS subjects (Fig. 5). However, formate had no relationship with any other urine metabolites. Both acetate and formate were found to be positively correlated with the microbial metabolite mannitol in ME/CFS patients, which suggests that the formate and acetate in urine may be largely related to metabolism of the microbiota in the gut.

Normalization of the absolute concentration dataset from urine by total metabolite concentration can account for possible dilution effects within urine without using creatinine, which was recognized as potentially being altered within this disorder (Fig. 4). Creatinine, which is increased in ME/CFS patients, is negatively correlated with the key amino acid component of its synthesis in the relative abundance data – glycine. In the non-ME/CFS cohort there is a negative correlation of relative abundance creatinine with glucose, lactate, pyruvate, serine, citrate and succinate. This correlation indicates that the production of ATP for muscles that produces creatinine for excretion occurs when the level of glucose is limited in the non-ME/CFS cohort, while in the

ME/CFS patients the increase in creatinine may occur because of a dysfunctional use of glucose. End-point metabolites of glycolysis, such as alanine, pyruvate and serine are decreased within the urine of ME/CFS patients and they all correlate with one another when observed as relative abundance data. This suggests that their alterations are likely related to the same cause - impaired glycolysis (Fig. 6A).

3.6 Correlations of blood and urine metabolites: dysregulated allantoin production and the use of amino acids via glutamate and creatine for energy production

Correlations of blood metabolites with urine metabolites from individuals allow us to understand the relationships that exist between metabolites maintained in the blood and excreted in the urine. Distinct differences are observed between the ME/CFS and non-ME/CFS cohorts (Fig. 5). Relative abundance blood glutamate was observed to be negatively correlated with relative abundance urine total amino acids in non-ME/CFS but positively correlated in ME/CFS. This difference is perhaps due to blood glutamate in ME/CFS being converted to glutamine to deaminate amino acids for energy metabolism and therefore reducing urine amino acid excretion. Relative abundance blood acetate and relative abundance urine creatinine are positively correlated in non-ME/CFS but are negatively correlated in ME/CFS which may be attributed to the dysregulation of the metabolism of acetate and creatinine within the ME/CFS cohort. Relative abundance blood lactate and relative abundance urine glycine are negatively correlated in non-ME/CFS but are positively correlated in ME/CFS. The decrease of lactate in the blood may be a result of reduced glycolysis (Fig. 6A) that increases creatinine production therefore decreasing glycine in the production of more creatine (Fig. 6C).

In the absolute concentration blood data of non-ME/CFS subjects, acetate, hypoxanthine and lactate are largely positively correlated with most urine metabolites, signifying their importance as indicators for excretion. In ME/CFS subjects blood phenylalanine was negatively correlated with urinary metabolites including urine phenylalanine suggesting that the level of phenylalanine is related to the rate of excretion. Importantly, in ME/CFS patients, blood glutamate is negatively

correlated with urine creatinine and blood glucose with urine creatine, which highlights the use of creatine to form creatinine and ATP in these patients.

Relative abundance blood and urine correlations for ME/CFS show a positive correlation of urine creatinine with blood glucose and a negative correlation of urine creatinine with blood formate, glycine, hypoxanthine, and lactate. This data supports a reliance of rapid ATP production through the conversion of creatine phosphate to creatinine in ME/CFS. Relative abundance allantoin was positively correlated with blood alanine, aspartate, glutamate, histidine and proline in non-ME/CFS only. Allantoin was not related to any other metabolite in ME/CFS patients. Allantoin is produced from hypoxanthine via a reaction that also produces ROS species that may contribute to oxidative stress in ME/CFS patients (Fig. 6C). The absence of correlation indicates that its production may be sporadic and not reflected in the blood data.

3.7 Reduced use of glycolysis to fuel aerobic respiration in ME/CFS patients

The increase of glucose and decrease of lactate within the blood of ME/CFS patients highlighted the potential significance of glycolysis in this cohort (Fig. 3). The decrease of alanine, pyruvate and serine in the urine of ME/CFS patients (Fig. 4) supports a reduced level of glycolysis (Fig. 6A). The blood was taken mid-morning while the urine was taken upon waking. Therefore, the urine is an indication of metabolism at rest over time while the blood is a snapshot of metabolism during the day. The urine indicates a general reduction of glycolysis metabolites, but with the maintenance of normal glucose reabsorption by the kidneys. Studies of ME/CFS have reported a decrease of alanine and serine in the urine (Niblett et al. 2007; McGregor et al. 1996) and a decrease of lactate in the blood during and following exercise (Georgiades et al. 2003). Increased levels of glucose have been found in blood and urine, however, they have not been reported as significant (Jones et al. 2005; Niblett et al. 2007; Armstrong et al. 2012; Georgiades et al. 2003). No previous study has measured pyruvate.

The increase of glucose in the blood of ME/CFS is trending towards hyperglycemia, which is linked to critical illnesses as a response to stress (Brealey and Singer 2009) and the development of insulin resistance (Robinson and van Soeren 2004). The elevation of glucose in the blood may be

resultant of either the upregulation of glucose production or the down-regulation of glucose uptake. The latter may involve a reduction in insulin levels or its function, while the former may involve increased glycogenolysis and gluconeogenesis as a stress response via glucagon, catecholamines, growth hormone, and cortisol. Alternatively, an inhibited glycolysis pathway may have slowed down the degradation of glucose resulting in its accumulation.

The absolute concentrations of metabolites in the blood showed a correlation between glucose and the end-products of glycolysis in non-ME/CFS but not in ME/CFS. When the proportions of the metabolites (relative abundance data) were considered, it was observed that these relationships were maintained in both ME/CFS and non-ME/CFS subjects (Fig. 5). This suggests that fluctuations in blood glucose are independent of the end-products in ME/CFS patients, potentially implicating an insulin issue, such as insulin resistance. Glutamate potentiates the sustained release of insulin and glucagon (Fahien and Macdonald 2011; Cabrera et al. 2008), its decrease may be indicative of a poorer level of glucose homeostasis indicated by the negative correlation of glutamate and glucose in the relative abundance blood data (Fig. 5).

Mitochondrial dysfunction has been suggested as a potential cause of depleted ATP and fatigue-like symptoms (Filler et al. 2014). However, in our study the quantitation of blood glycolytic metabolites revealed that glycolysis inhibition is occurring implying a lower level of acetyl-CoA production. This may indicate a reduced use of mitochondria in ME/CFS patients which is consistent with a recent study that found lower levels of citrate synthase in the muscle cells of ME/CFS patients, possibly implying lower numbers of mitochondria (Smits et al. 2011).

3.8 Increased use of amino acids to fuel aerobic respiration in ME/CFS patients

The decrease of glutamate and increase of aspartate within the blood of ME/CFS indicates an altered role of amino acid metabolism (Fig. 3). Glutamate was observed to be positively correlated with total amino acids in both cohorts. Glutamate may accept or donate an amino group to deaminate another amino acid as fuel for the citric acid cycle. It is also a key neurotransmitter of the body and has been proposed to have a potentiating role in the release of insulin and glucagon (Meldrum 2000; Cabrera et al. 2008; Fahien and Macdonald 2011). Glutamate has been reported to

be lowered in the urine of ME/CFS patients (McGregor et al. 1996) but no change has been previously observed in the blood (Armstrong et al. 2012; Jones et al. 2005).

The increase in aspartate may be linked to the decrease of glutamate via AST, which converts aspartate and 2-oxoglutarate to glutamate and oxaloacetate (Fig. 6B). This allows glutamate to enter the citric acid cycle and due to the proposed lack of acetyl CoA contribution from glycolysis inhibition we may be observing a forward reaction that results in the accumulation of aspartate. Both aspartate and glutamate are involved in the donation of amino groups that supply the urea cycle - responsible for the creation of creatine, creatinine, urea, and nitric oxide (Fig. 6C). Dysregulation of the urea cycle in ME/CFS patients was previously suggested by a smaller study (Armstrong et al. 2012). Although there are discrepancies in the significant metabolite changes, this study highlights the depletion of glucogenic amino acids linked through the metabolism of glutamine. The discrepancies in metabolites may be attributed to an older cohort and a smaller sample size (Armstrong et al. 2012). Nitric oxide has been observed to be heightened in ME/CFS patients (Suarez et al. 2010), and the elevated levels of aspartate that we have observed may increase the level of nitric oxide produced through creation of arginine from citrulline via argininosuccinate synthase, arginine is then converted back to citrulline with the release of nitric oxide. Elevated argininosuccinate synthase is considered a marker for inflammatory events often mediated by cytokines which have been reported in ME/CFS patients (Husson et al. 2003; Cao et al. 2013; White et al. 2010).

The relationships of glycolysis with essential amino acids and non-essential amino acids trend towards the depletion of the latter and maintenance of the former. Similar results have been observed previously in the urine (Niblett et al. 2007) and perhaps suggest a prioritised depletion of non-essential amino acids to synthesise more glutamate in ME/CFS.

3.9 Increased synthesis of creatine to carry phosphate to muscles for quick ATP production and subsequent creatinine creation in ME/CFS patients

The proportion of creatinine in the urine was found to be increased in ME/CFS patients and evidence from correlations suggests that the apparent reduction in glycolysis was met with an

increased production and excretion of creatinine. Creatinine is produced as a breakdown product of creatine phosphate as it converts ADP to ATP – an efficient mechanism for the rapid production of anaerobic energy (Fig. 6C). When we used urinary creatinine to standardize our urine metabolites we found that all were considered significant suggesting that the production of creatinine itself may be dysregulated in ME/CFS patients as it trended towards a significant decrease in the blood. The increased use of creatine phosphate to form ATP may be due to the inefficient anaerobic glycolysis observed in ME/CFS.

3.10 Increased level of oxidative stress in ME/CFS patients

Mounting evidence in the literature suggests that ME/CFS is marked by both oxidative stress and a chronically activated innate immune system (Morris and Maes 2014c). Several studies have elucidated elements of oxidative stress including a recent study that found a significant increase of lactate in cerebrospinal fluid that was seen to be linked to oxidative stress with a lower levels of glutathione (Shungu et al. 2012). Another report links ME/CFS and oxidative stress through dysfunctional energy metabolism (Booth et al. 2012) while others suggest a role of gene polymorphisms that have altered mitochondrial aspects of energy metabolism (White et al. 2012). In our study we observed a consistent positive correlation between lactate and hypoxanthine. Hypoxanthine is the breakdown product of purine metabolism that may either be resalvaged or excreted as allantoin (Fig. 6C), a conversion that produces superoxide in the presence of Xanthine Oxidase (XO). The proportion of allantoin was shown to increase in the urine of ME/CFS patients indicating increased oxidative stress. Studies on allantoin have suggested it to be a reliable indicator of *in vivo* oxidative stress levels tested during exercise and reperfusion injury, both citing the importance of an increased oxidative environment for the activation of XO (Mikami et al. 2000; Serkova et al. 2005). Although blood hypoxanthine and urine allantoin were not shown to correlate with one another, the discrepancy between overnight accumulation of allantoin in the urine and the snapshot of hypoxanthine in the blood may be too disconnected in terms of fluctuations in hypoxanthine synthesis and degradation. This therefore requires further studies to elucidate the mechanism of this pathway in ME/CFS patients. There have been many indications of oxidative

stress in ME/CFS but this is the first to report of allantoin as a marker and the first to suggest a pathway linking ATP degradation and oxidative stress.

ME/CFS has been described to be the product of a chronically activated immune response linked with oxidative stress (Maes 2009). Stressed-induced hyperglycemia has been correlated with a pro-inflammatory immune response (Xiu et al. 2014) and the production of ROS (Yu et al. 2006). Our observations of a mild hyperglycemic state and glycolysis inhibition are consistent with the hypothesis that chronic innate immune system activation and oxidative stress may underpin ME/CFS. More research into the involvement of mitochondrial dysfunction is required given these results show an inhibition of glycolysis that may precede mitochondrial function.

4 Conclusion

Here we have presented a metabolomics analysis of the blood and urine of ME/CFS patients compared to non-ME/CFS subjects. The analysis reveals an inhibited glycolysis pathway exists in ME/CFS patients along with an oxidative stress pathway and a reduced level of amino acids. We have highlighted the possible role this plays in ME/CFS patients and it supports the current literature that proposes a chronic immune activation and oxidative stress phenotype exists in ME/CFS patients. The cause of this dysregulation remains unknown but the reduced glycolysis infers a reduced use of pyruvate and acetyl CoA is entering the citric acid cycle. The use of amino acids may be through glutamate forming 2-oxoglutarate via AST, which transfers the amino group to oxaloacetate to form and accumulate aspartate. This inefficient energy production and lowering of amino acid levels may both be important in producing a fatigue phenotype in patients with ME/CFS. The observed increased creatinine production may provide a means of anaerobic ATP energy for muscles as well as nitrogen removal from deaminated amino acids. This study has also identified allantoin as an ROS marker in ME/CFS linking the degradation of ATP and other purines to the oxidative stress phenotype that has been linked to the disorder.

Conflict of Interest Statement

There were no conflicts of interest.

Compliance with Ethical Requirements

This study was approved by the University of Melbourne human research ethics committee (HREC# 0723086).

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Figure Legends

Fig. 1. Metabolic workflow and the raw spectral NMR data obtained from blood and urine. A) The basic workflow of analysis of the NMR-detected metabolites within the urine and blood of ME/CFS patients. B) A spectrum of the blood and its metabolites using 1D ^{1}H NMR experiments, identifications (matched to Fig. 3A) were verified using 2D ^{1}H NMR experiments and quantitated using Chenomx NMR suite 6.1. The PCA plot displays the separation of the ME/CFS and control cohorts using the area under the curve from 250 'bins' (0.04 ppm wide) of the blood NMR spectra. C) A spectrum of the urine and its metabolites using 1D ^{1}H NMR experiments, identifications (matched to Fig. 4A) were verified using 2D ^{1}H NMR experiments and quantitated using Chenomx NMR suite 6.1. The PCA plot displays the separation of the ME/CFS and control cohorts using the area under the curve from 250 'bins' (0.04 ppm wide) of the urine NMR spectra.

Fig. 2 2D TOCSY spectra of (A) blood serum and (B) urine. Crosspeaks used to identify metabolites are labelled. Twenty-nine metabolites per blood serum sample and thirty metabolites per urine sample were confirmed.

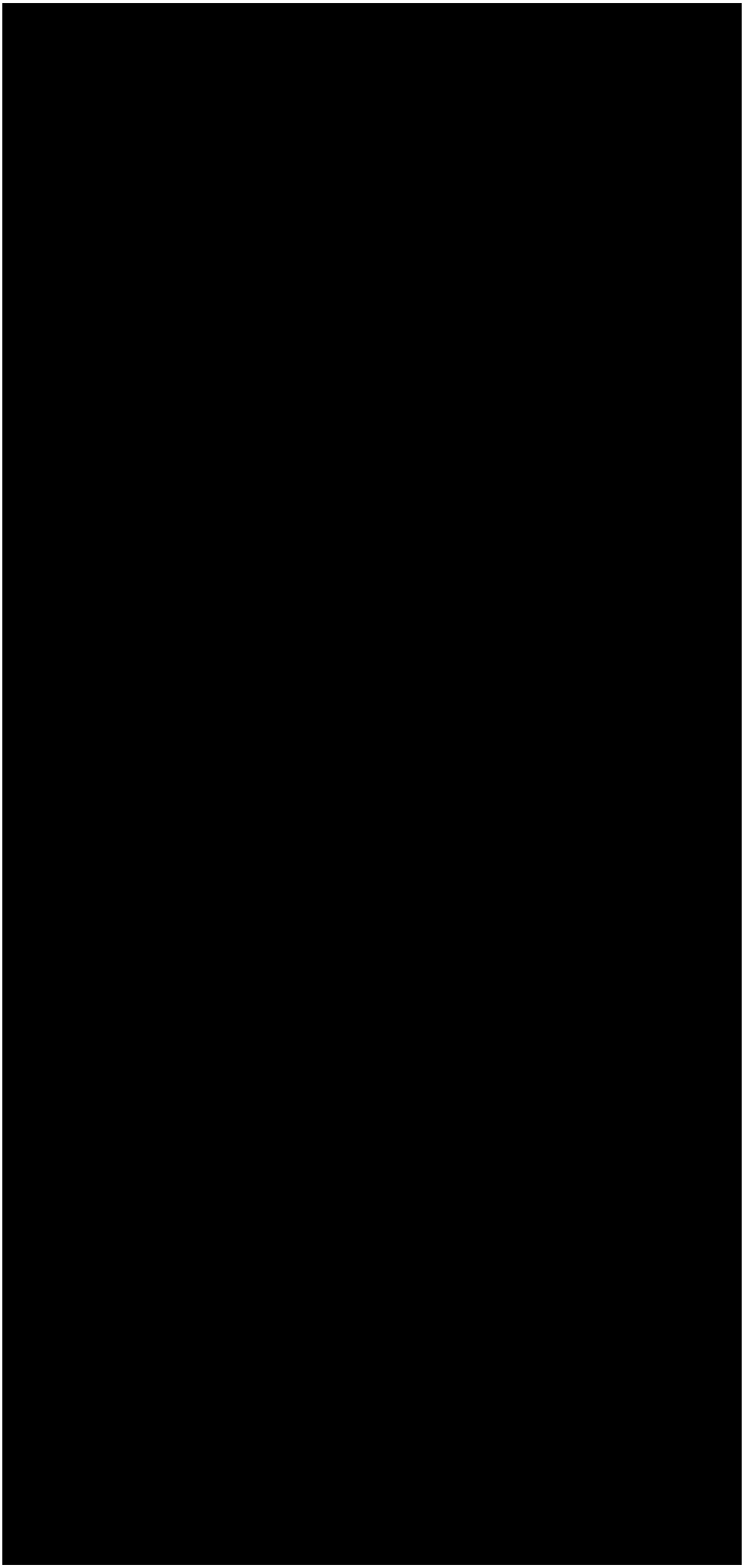
Fig. 3. Blood metabolite anomalies in ME/CFS patients. A) The fold change (FoldC) and *P*-value (*P*) of metabolites identified within the blood of ME/CFS patients compared to non-ME/CFS subjects. Metabolites were detected by 1D ^{1}H NMR experiments and quantitated using Chenomx NMR suite 6.1. Data is presented in both absolute and relative distribution forms. *P*-values have been adjusted using the Benjamini-Hochberg equation and are denoted significance by *. B) PCA plots using the relatively distributed blood metabolite data present a scores plot with separation of the ME/CFS and control cohorts and a loadings plot with the metabolites distribution that contributed to the scores plot separation.

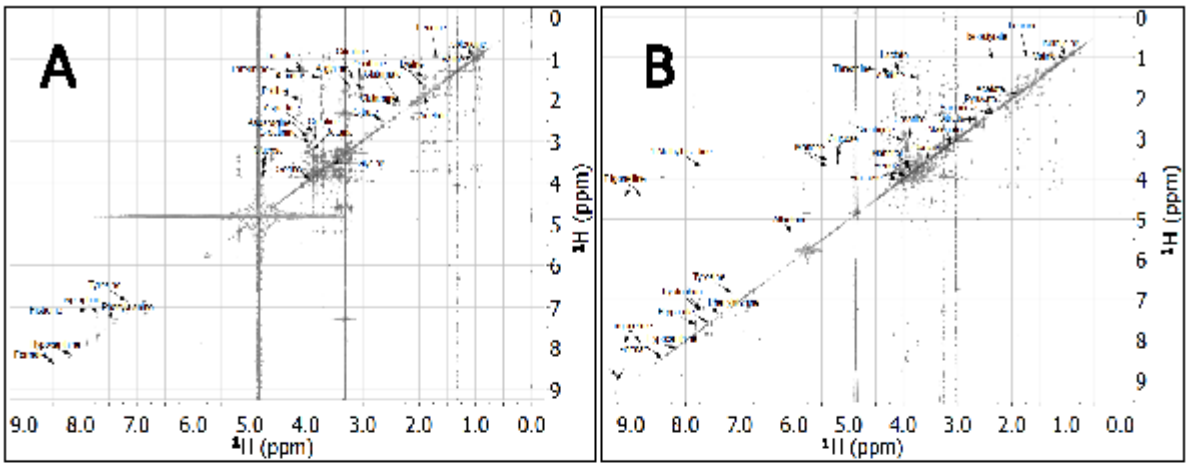
Fig. 4. Urine metabolite anomalies in ME/CFS patients. A) The fold change (FoldC) and *P*-value (*P*) of metabolites identified within the urine of ME/CFS patients compared to non-ME/CFS subjects. Metabolites were detected by 1D ^{1}H NMR experiments and quantitated using Chenomx NMR suite

6.1. Data is presented in both absolute and relative distribution forms. *P*-values have been adjusted using the Benjamini-Hochberg equation and are denoted significance by *. B) PCA plots using the relatively distributed urine metabolite data present a scores plot with separation of the ME/CFS and control cohorts and a loadings plot with the metabolites distribution that contributed to the scores plot separation.

Fig. 5. Anomalous metabolites correlated with all metabolites in ME/CFS and non-ME/CFS cohorts. Pearson correlations were conducted on the blood and urine data in both absolute and relative forms. Correlations were calculated within the ME/CFS cohort and then in the control cohort. The correlations are displayed as colours in terms of positive ($r > 0.4$), no change ($-0.4 < r < 0.4$), and negative ($-0.4 > r$) correlations in both ME/CFS and non-ME/CFS groups, these include: dark blue – ME/CFS positive/non-ME/CFS positive; fluorescent blue – ME/CFS no change/non-ME/CFS positive; light blue – ME/CFS negative/non-ME/CFS positive; dark green – ME/CFS positive/non-ME/CFS no change; light green – ME/CFS negative/non-ME/CFS no change; dark red – ME/CFS positive/non-ME/CFS negative; fluorescent pink – ME/CFS no change/non-ME/CFS negative; light red – ME/CFS negative/non-ME/CFS negative; white – ME/CFS no change e/non-ME/CFS no change.

Fig. 6. Key metabolic pathways including (A) glycolysis and pentose phosphate pathway, (B) citrate cycle and amino acid catabolism, (C) urea cycle, creatinine production and ROS production pathways. Significant metabolites are highlighted if increased (red) or decreased (blue) in ME/CFS patients. Non-significant or non-detected metabolites that link a pathway are also highlighted (green).

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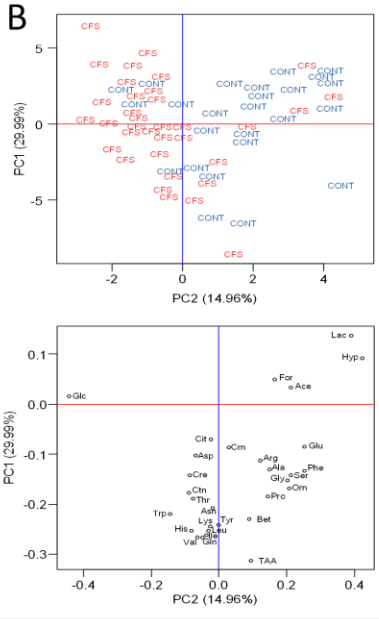
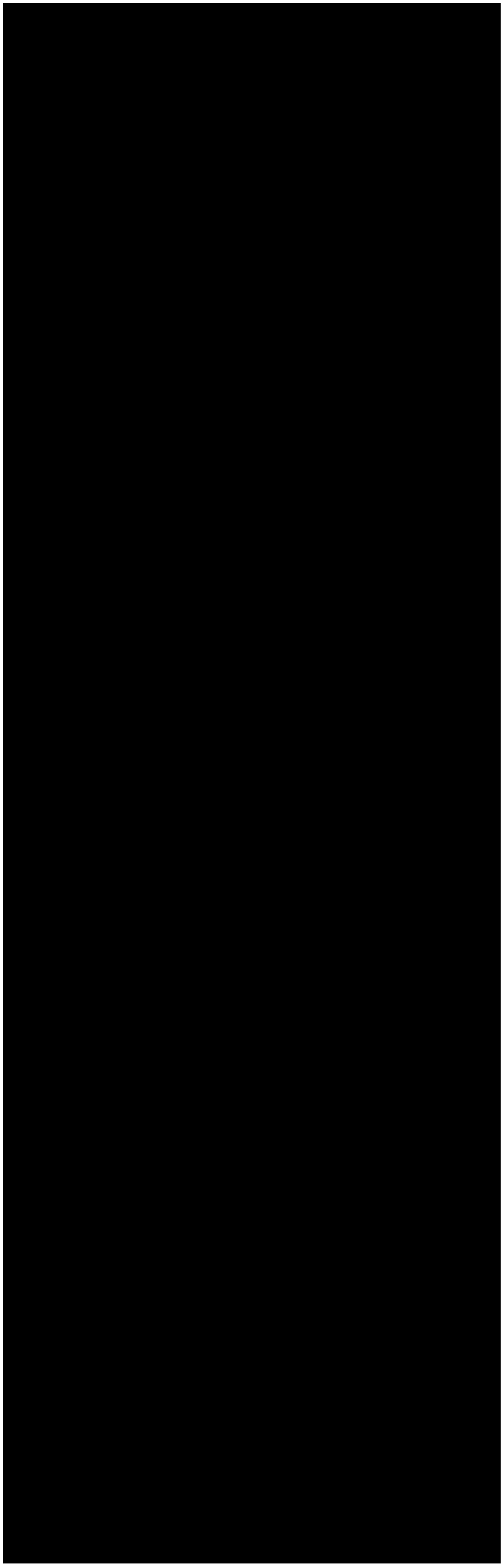
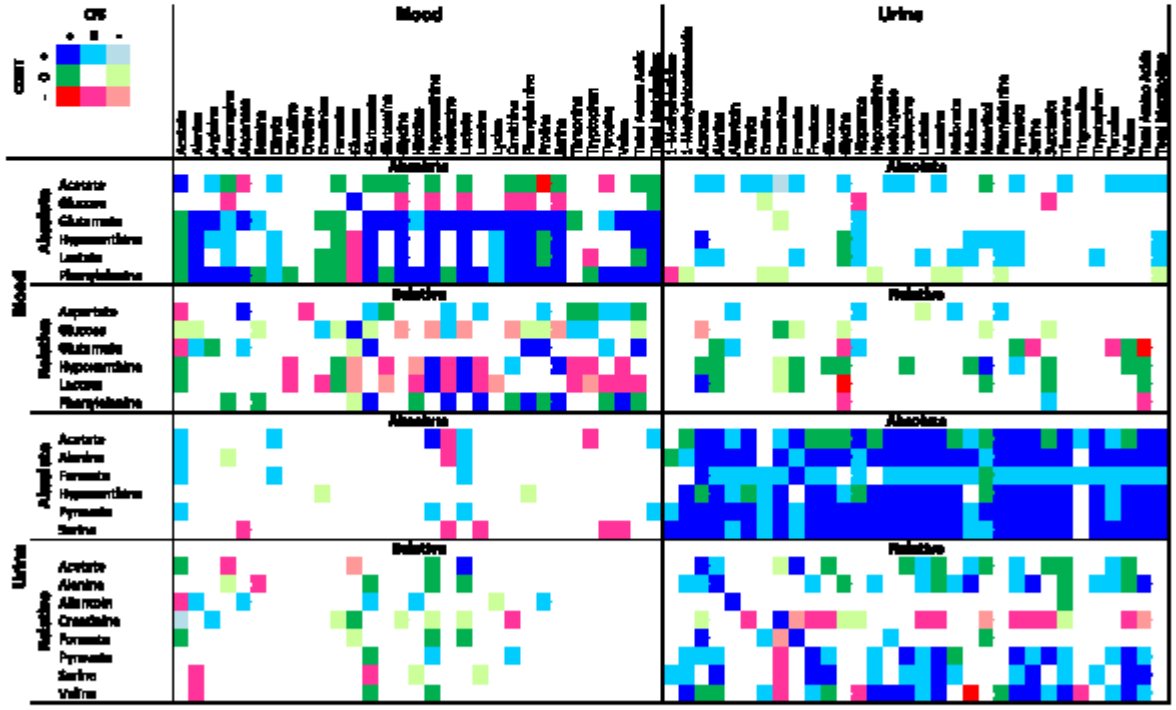
METABOLITE		ABSOLUTE		RELATIVE	
		FoldC	*P*	FoldC	*P*
Acetate	Ace	0.75	0.040*	0.78	0.11
Alanine	Ala	0.87	0.14	0.94	0.61
Arginine	Arg	0.88	0.18	0.93	0.69
Asparagine	Asn	0.95	0.36	1.02	0.81
Aspartate	Asp	1.08	0.27	1.17	0.049*
Betaine	Bet	1.04	0.84	1.14	0.66
Citrate	Cit	1.01	0.71	1.08	0.63
Citrulline	Ctn	1.03	0.90	1.10	0.61
Creatine	Cre	1.16	0.95	1.24	0.61
Creatinine	Crn	0.87	0.08	0.91	0.61
Formate	For	0.71	0.14	0.78	0.61
Glucose	Glc	1.24	0.011*	1.29	0.002*
Glutamate	Glu	0.80	0.029*	0.85	0.036*
Glutamine	Gln	0.95	0.37	1.01	0.89
Glycine	Gly	0.96	0.68	1.03	0.81
Histidine	His	0.96	0.36	1.03	0.76
Hypoxanthine	Hyp	0.38	0.001*	0.39	0.003*
Isoleucine	Ile	0.97	0.37	1.02	0.89
Lactate	Lac	0.57	0.006*	0.61	0.004*
Leucine	Leu	0.95	0.25	1.00	0.99
Lysine	Lys	0.94	0.18	1.00	0.95
Ornithine	Orn	0.90	0.18	0.97	0.75
Phenylalanine	Phe	0.81	0.001*	0.87	0.003*
Proline	Pro	0.85	0.14	0.90	0.61
Serine	Ser	0.98	0.71	1.05	0.68
Threonine	Thr	0.90	0.08	0.96	0.74
Tryptophan	Trp	0.95	0.25	1.01	0.95
Tyrosine	Tyr	0.88	0.14	0.94	0.67
Valine	Val	0.91	0.34	0.96	0.89
Total Amino Acids	TAA	0.96	0.14	1.02	0.81
Total Metabolites	Tme	0.92	0.18		

B



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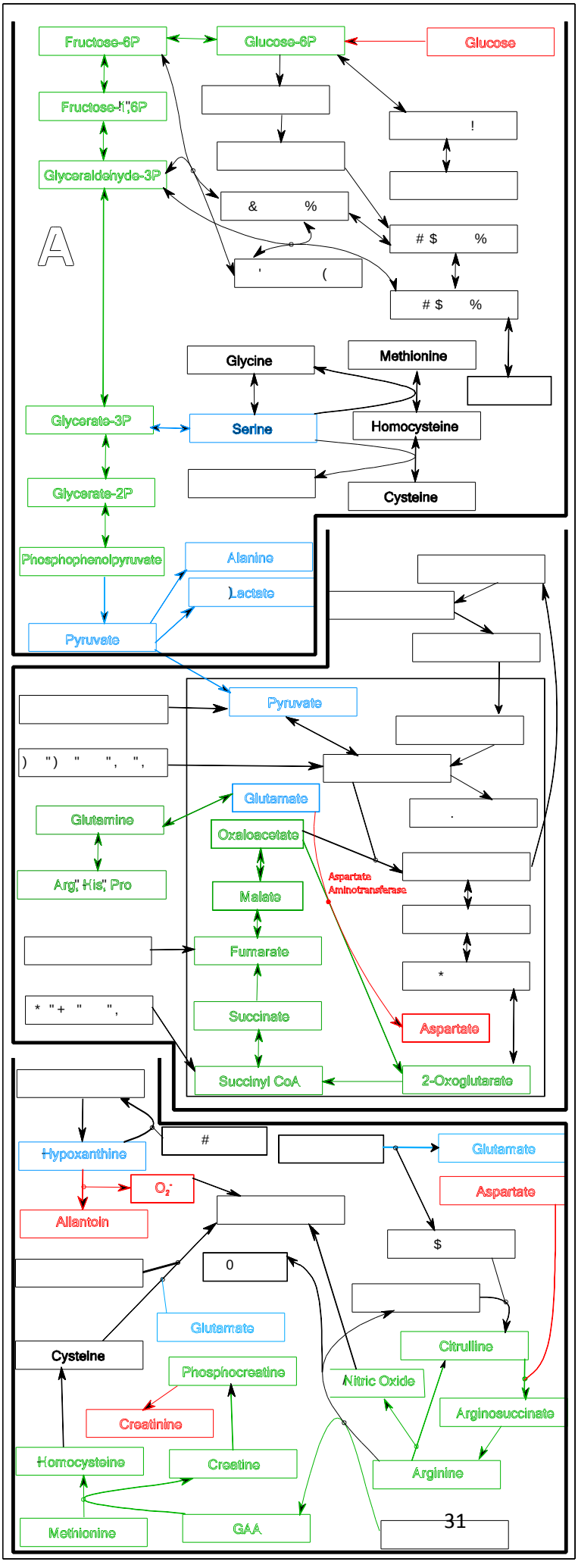
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Glucose-6P
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Glycine
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Glycerate-2P
Cysteine
Phosphophenolpyruvate
Alanine
Lactate
Pyruvate
Pyruvate
Glutamate
Glutamine
Oxaloacetate
Arg", His", Pro
Aspartate
Aminotransferase
Malate
Fumarate
Succinate
Aspartate
Succinyl CoA
2-Oxoglutarate
Hypoxanthine
Glutamate
Aspartate
Allantoin
Glutamate
Cysteine
Citrulline
Phosphocreatine
Nitric Oxide
Creatinine
Arginosuccinate
Homocysteine
Creatine
Arginine
Methionine
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