METABOLOMICS OF STRESS: ASSESSING BIO-FLUIDS IN RATS AND HUMANS USING PROTON NUCLEAR MAGNETIC RESONANCE (^{1}H NMR) SPECTROSCOPY

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To my Mother and Father for a lifetime
of inspiration and encouragement

ABSTRACT

Stress *in utero* causes epigenetic changes that manifest in offspring physiology and behaviour, becoming risk factors for complex adult diseases. Here we used ^{1}H NMR spectroscopy to analyze two novel cohorts; first, we assessed urine metabolomes of adolescents *in utero* during the largest natural disaster in Canadian history; second, we investigated blood plasma metabolomes of offspring that were transgenerationally or multigenerationally-stressed *in utero*. Metabolomic profiles in offspring of high PNMS mothers were significantly altered. Higher PNMS generated significant alterations in metabolic pathways involving energy metabolism and protein biosynthesis in both rats and humans, particularly in BCAA synthesis, TCA cycle, muscle performance, and immunoregulation. Dysregulation of energy and protein metabolism suggests an increased risk of metabolic diseases, such as insulin resistance, diabetes, and obesity. The present findings provide a novel approach to PNMS outcomes and HPA-axis dysregulation by taking a metabolomics perspective and utilizing novel stressors in animal and human models.

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CHAPTER 1: INTRODUCTION TO METABOLOMICS

"All seats provide equal viewing of the universe"

- Museum Guide, Hayden Planetarium

OVERVIEW

A metabolite is any substance involved in metabolism, whether necessary as an intermediate compound, or formed as a product of metabolism (Dettmer et al., 2011). Metabolomics broadly aims to characterize small-molecule metabolites by measuring the global, dynamic response to biological stimuli, focusing on understanding systemic changes over time (Nicholson & Lindon, 2008). Metabolomics represents a systematic, top-down approach to study unique chemical phenotypes, or "fingerprints", left behind by specific cellular processes that mainly involve enzymatic catalytic activity.

The field of metabolomics reflects the culmination of considerable advances in technology, methodology, and general understanding in physical chemistry, quantum physics, biochemistry, and biological sciences. Metabolomics approaches fit well into systems biology, since interactions between genes, transcripts, proteins, and metabolites in complex organisms can be investigated using a top-down, integrated view (Nicholson & Lindon, 2008). Although genomics, epigenomics, gene and protein expression, and metabolism operate on very different timescales, metabolomics cuts through these layers by investigating and observing interactions and global outcomes of all these systems combined.

Metabolomic responses to environmental stimuli can be measured in almost any biological fluid or tissue, but the most commonly used samples include urine, blood, and cerebrospinal fluid (CSF) (Dumas & Davidovic, 2015; Lindon, Nicholson, Holmes, & Everett, 2000). The sample type chosen for any particular study will depend on accessibility, invasiveness, processing needs, and metabolites or organ systems of interest. For example, while CSF provides the most direct biofluid link to central nervous system functioning, it is cumbersome and invasive to obtain (Wishart et al., 2008). Since metabolites are most often waste-products of metabolic processes occurring in tissues and organ systems, it can be expected that organs with high metabolic activity (or energy consumption) will have a more substantial representation in biological fluids, which function to remove wastes from these systems (Wishart et al., 2013). This

means that the brain, which accounts for almost 25% of the body's energy consumption, will be highly represented metabolically in bio-fluids like blood and urine (Herculano-Houzel, 2011). Thus, metabolomics represents the ideal approach for the discovery of biomarkers related to brain function, development and aging, and stress response.

In addition to a broad selection of biological fluids and tissues to choose from, metabolomics can be performed using multiple independent methods in physical and analytical chemistry. These include techniques such as nuclear magnetic resonance (NMR) spectroscopy, and various modes of mass spectrometry (MS). Metabolomics offers a relatively inexpensive, reasonably simple, high-throughput, and non-invasive method for investigating several biological phenomena, including drug metabolism (Bales, Sadler, Nicholson, & Timbrell, 1984), clinical diagnostics (Bogdanov et al., 2008; Constantinou et al., 2005), cancer research (Chan et al., 2016), plant biology (Kim, Choi, & Verpoorte, 2010; Kim, Saifullah, et al., 2010; Kim et al., 2016), and response to environmental factors (Zucchi, Yao, & Metz, 2012; Zucchi et al., 2013), among many other applications. The following will discuss the impact and role of metabolomics in the biomedical sciences.

A BRIEF HISTORY OF "OMICS" SCIENCES

Following the remarkable discovery of DNA structure in 1953 by Francis Crick, James Watson, and Rosalind Franklin (Watson, 1968; Watson & Crick, 1953), it was understood that humans and other animals contained molecules carrying genetic instructions for growth, development, functioning, and reproduction, otherwise known as "genes". The "genome", a term coined by Hans Winkler in 1920, consists of all genetic material of an organism (Gregory, 2005). The aim of collectively characterizing and quantifying biological molecules that translate into structure and function subsequently came to use the suffix "-ome". As science advanced through the twentieth century, it became clear that genes led to gene transcripts, which were subsequently translated into proteins. The constituents of these proteins, such as amino acids, organic acids,

nucleic acids, amines, sugars, vitamins, enzymes, and co-factors came to be known as metabolites. The "omic" sciences followed, with "proteome" being coined in 1994 by Marc Wilkins (Wilkins, 2009), and "transcriptome"(Kinzler & Vogelstein, 1998) and "metabolome" coming later (Oliver, Winson, Kell, & Baganz, 1998), in 1997 and 1998, respectively.

Individual metabolic profiles were initially conceptualized in the 1940s when Roger Williams (Williams, 1951), using chromatography, noticed characteristic metabolic patterns in urine and saliva of psychiatric patients. In terms of technology, there were two independent starting points that led to the ability of scientists to perform metabolic profiling research. The first was the invention of NMR in 1946 by Felix Bloch and Edward Mills Purcell. Second was the advent of metabolic control analyses in the 1960s, which aimed to model cellular metabolism, requiring the quantification of intracellular metabolite concentration (Nicholson & Lindon, 2008). This led to the development of gas chromatography (GC) and GC-coupled MS.

In 1970, Horning *et al* (1969,1971) coined the term "metabolic profiling" after using GC-MS to describe metabolic profiles of urine. NMR was concurrently being developed rapidly. By the 1980s, NMR was sensitive enough to identify individual metabolites in unmodified biological fluids, allowing David Hoult (1974) to identify metabolites in intact biological tissue using 31-phosphorous (^{31}P) NMR. Technological advances through the 1980s and 1990s continued to revolutionize the field of metabolic research, allowing for more comprehensive profiling techniques (Bjerrum, 2015). More importantly though, applicable bioinformatics software emerged (Gartland, Beddell, Lindon, & Nicholson, 1991; Gartland et al., 1990), allowing for multivariate statistical analyses and identification of multiple analytes in independent sets of biological samples.

In 1998, Stephen Oliver defined the term "metabolomics" for the first time (Bjerrum, 2015). Then in 1999, the field of metabolic profiling was named "metabonomics", defined as the "quantitative measurement of the dynamic, multiparametric, metabolic response of living systems to pathophysiologic stimuli or genetic modification" (Nicholson, Lindon, & Holmes, 1999). The

two terms are often used interchangeably, but there is a defined difference in practice and philosophy that should be pointed out here. Metabonomics aims to measure *global* responses to external stimuli, comparing complete profiles across groups rather than individual compounds (Nicholson & Lindon, 2008). Metabolomics on the other hand, seeks an analytical description of complex bio-molecules, aiming to characterize and quantify all the metabolites in a sample.

THE HUMAN METABOLOME

The Human Genome Project was one of the greatest feats of international collaboration in science ever completed (Kieff, 2003; Palladino, 2006). Beginning in 1990 and finally being declared complete in 2003, the project sought to determine the sequence of nucleotide base pairs that make up human DNA from both a physical and functional perspective, with the goal of identifying common genetic variants responsible for major diseases. This brought about the rapid development of fast, novel, and cost-effective sequencing technologies, enabling an era of personalized human genomics (Gonzaga-Jauregui, Lupski, & Gibbs, 2012; Naidoo, Pawitan, Soong, Cooper, & Ku, 2011).

The genome is defined as an organism's complete set of DNA, including all genes and other information required to build and maintain that organism (Lederberg & McCray, 2001). Similarly, the metabolome refers to the complete set of small-molecule chemicals contained within a biological sample (Oliver et al., 1998), whether that sample is a cellular extract, cell, tissue, bio-fluid, organ, or organism. This metabolome, along with the genome and proteome, fundamentally makes up a human being. Currently, there are approximately 2,651 known, quantifiable metabolites and metabolic species in the human urine metabolome, corresponding to 3,079 distinct structures (Bouatra et al., 2013). When combined, current metabolomic profiling techniques such as NMR, GC-MS, LC-MS, and high-performance liquid chromatography (HPLC) can identify 16%, or 445/2,651 of these urinary metabolites, as well as 3,564/4,229 metabolites in the human serum metabolome (Psychogios et al., 2011).

In contrast to the already mapped and sequenced genome, the human metabolome is only partially defined and identified. This is largely because human metabolism is not only dictated by the genome/transcriptome/proteome, but is also influenced by the environment, including diet, medication, stress, and disease (Feinberg, 2007; King & Laplante, 2005; Zucchi et al., 2012; Zucchi et al., 2013). Both endogenous and exogenous metabolites make up the complete human metabolome, making comprehensive characterization difficult, especially since metabolism is temporally dynamic (Bjerrum, 2015; Pearson, 2007; Suhre et al., 2011). To aid in this endeavor, Wishart *et al* (2013) have created the Human Metabolome Database (HMDB), which is the largest, most comprehensive organism-specific metabolomic database available. It currently contains information on more than 40,000 water- and lipid-soluble metabolites, and is an open-access resource to support the development of metabolomics research worldwide.

Metabolomics has many theoretical advantages over other omics approaches. First, the metabolome is the final downstream product of gene transcription, so metabolomic alterations are amplified compared to those in the transcriptome and proteome (Horgan & Kenny, 2011; Urbanczyk-Wochniak et al., 2003). Second, since the metabolome is the downstream product of these systems, it is closest to the phenotype of the biological system under study. Lastly, despite containing the smallest domain (~5000 endogenous metabolites), the metabolome is more diverse, containing many different biological molecules, making it more physically and chemically complex than other "omes" (Horgan & Kenny, 2011).

ENVIRONMENT – GENE INTERACTIONS AND METABOLISM

Genes contain information beyond their nucleotide sequences (Kaelin & McKnight, 2013). This additional information and its dynamic regulation allows an organism's external environment to interact with gene expression patterns and influence its phenotype, health, and disease (Cao-Lei et al., 2014; Feinberg, 2007; Hullar & Fu, 2014; Jirtle & Tyson, 2013; Kaelin & McKnight, 2013; Pembrey, Saffery, & Bygren, 2014; Scott, Tamashiro, & Sakai, 2012; Zucchi et

al., 2012). Such functionally relevant changes to the genome do not involve a change in the nucleotide sequence and have been coined “epigenetics”. Epigenetic components, which essentially switch genes “on” or “off”, include four main systems that can interact to bring these alterations about: DNA methylation, histone modification, chromatin remodeling, and RNA-associated silencing (Liang, Egger, Jones, & Aparicio, 2004). These changes may become heritable to subsequent generations (Babenko, Kovalchuk, & Metz, 2015; Migicovsky & Kovalchuk, 2011).

DNA methylation involves the addition of a methyl group to a DNA molecule, changing its appearance and structure, and modifying gene interactions with cellular machinery responsible for transcription (Esteller & Herman, 2002). Histones can be modified through either acetylation or methylation, depending on the type of chromatin a histone is associated with, and result in altered chromatin arrangement (Liang et al., 2004). This can determine whether DNA is transcribed or not. Lastly, genes can be switched off through RNA silencing, which affects gene expression. The Common Disease Genetic and Epigenetic (CDGE) hypothesis asserts that epigenetics provides an additional layer of genetic variation, mediating the relationship between genotype and environment (Feinberg, 2007).

Many diseases and negative health outcomes have been linked to epigenetic alterations in plants, animals, and humans following exposure to adverse or harmful environments. In plants for example, Boyko and Kovalchuk (2011) found that stress exposure led to genome destabilization and altered stress tolerance. Ancestral exposure to stress in animal studies has been shown to generate new behavioural traits and lead to changes in the brains of offspring (Ambeskovic et al., 2016), as well as an increased risk of shortened gestational length (Yao et al., 2014), proposing epigenetic mechanisms. It has also been proposed that the disruption of circadian rhythm and breast cancer may have an epigenetic link (Kochan & Kovalchuk, 2015). Michael Skinner’s work on transgenerational epigenetic effects of prenatal exposure to endocrine disruptors, such as pesticides and other environmental toxins, has found negative health effects in both male and

female offspring; namely, reproductive abnormalities, cancer, prostate and kidney disease, and ovarian disease (Anway, Cupp, Uzumcu, & Skinner, 2005; Anway, Leathers, & Skinner, 2006; Anway & Skinner, 2008; Babenko et al., 2015; Nilsson et al., 2012).

In humans, stress during pregnancy has been shown to have significant effects on the physiology and behaviour of offspring (Charil, Laplante, Vaillancourt, & King, 2010; Harris & Seckl, 2011; King & Laplante, 2005; Yong Ping et al., 2015), thought to result from abnormally high levels of maternal glucocorticoids and increased placental corticotropin-releasing hormone. This is thought to lead to dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, as well as to distort neuronal development (Harris & Seckl, 2011; Hayashi et al., 1998). Stress during pregnancy does not lead to direct changes in maternal DNA, yet these physiological and behavioural effects are passed down to offspring, suggesting an epigenetic mechanism.

Under normal conditions, glucocorticoids play an important role in the developing fetus by binding to glucocorticoid and mineralocorticoid receptors, which act as transcription factors to alter gene expression (Harris & Seckl, 2011). Importantly, glucocorticoids play a notable role in promoting maturation of the lungs, and promote correct neurodevelopment by initiating terminal maturation, axon and dendrite remodelling, and cell survival (Meyer, 1983; Yehuda et al., 1989). Despite the clear physiological importance of endogenous glucocorticoids during normal fetal development, there is overwhelming evidence to suggest that excess glucocorticoid exposure *in utero* correlates with negative outcomes in offspring, especially if this exposure occurs during late gestation when growth is accelerating (Harris & Seckl, 2011).

Small changes in the activity of genes or proteins, which may have an unknown impact on the workings of a cell, often create much larger changes in downstream metabolite levels (Pearson, 2007). For this reason, the metabolome arguably represents a reliable investigative measure of environment-gene interactions, and any associated phenotypes (Bouatra et al., 2013).

MAIN GOALS OF METABOLOMICS

The main goal of metabolomics is to provide a top-down, integrated view of biochemical processes in complex organisms, in contrast to traditional bottom-up methods for investigating the network of interactions within and among genes, proteins, and metabolites in individual cell types (Lindon et al., 2000; Nicholson & Lindon, 2008). At its most basic level, metabolomics aims to relate chemical patterns of small-molecule metabolites to the physiology of cells and tissues.

This work is based on the "metabolic theory of disease", which asserts that metabolic phenotypes are altered by intrinsic and environmental factors through changes in signal transduction and catabolic pathways. As a result, experiences and adverse impacts such as stress, brain injury, diet, and lifestyle alter gene methylation patterns, histone modification, or the expression of small non-coding RNA, thus causing a cascade of downstream metabolic changes.

The hypotheses that follow from this theory are: i) that these metabolic changes are measurable in biological fluids or tissue; ii) specific bio-fluid metabolites will be significantly up- or down-regulated compared to control samples; and iii) in clinical settings, a mutually exclusive metabolite profile will serve as a diagnostic marker and aid in prognosticating health outcomes. For clinical applications of metabolomics, such as monitoring disease status, onset, progression, and efficacy of treatment, there are three necessary prerequisites (Beckwith-Hall, Thompson, Nicholson, Lindon, & Holmes, 2003; Liu et al., 2010; Zhang, Sun, & Wang, 2012). First, there should be specific, identifiable biomarkers with a proven link to some diseased state. Second, there should exist a non-invasive approach to detect and monitor these biomarkers. Third, technology should be able to effectively discriminate between biomarkers.

CHAPTER 2: APPROACHES TO PROTON NUCLEAR MAGNETIC RESONANCE (^{1}H NMR) METABOLOMICS

A BRIEF HISTORY OF NMR SPECTROSCOPY

Nuclear magnetic resonance (NMR) spectroscopy is a research technique that exploits the magnetic properties of certain atomic nuclei, determining physical and chemical properties of atoms or molecules containing them. It provides information about the structure, dynamics, reaction state, and chemical environment of these atoms. NMR spectroscopy has evolved as one of the most versatile physical chemistry methods developed in the twentieth century (Cohen, Jaroszewski, Kaplan, Ruiz-Cabello, & Collier, 1995), with a long and dramatic multidisciplinary history.

In 1896, Pieter Zeeman set up an experiment to study the effect of magnetism on light, and found that the magnet caused an apparent line-broadening effect (Zeeman, 1897). This effect was the splitting of emission lines – now referred to as Zeeman splitting. In the mid-1920s, it became apparent that features of atomic spectra could only be accounted for if certain atomic nuclei possessed spin and magnetic moment (Becker, 1993). This concept was soon firmly established by many studies, particularly the Stern-Gerlach experiment (Schmidt-Böcking et al., 2016), in which beams of silver atoms were separated in an inhomogenous magnetic field according to orientation of the electron's magnetic moment (Becker, 1993). The concept of spin was formalized in 1925 when Wolfgang Pauli described a phenomenon that could not be explained by classical physics. His idea of "two-valuedness", now known as the Pauli Exclusion Principle, stated that it is impossible for two electrons of the same atom to occupy the same quantum state simultaneously (Straumann, 2009). This fundamental property of subatomic particles turned out to be a result of what we now know as "spin angular momentum", or spin.

Isador Rabi subsequently had the first observation of magnetic resonance, receiving the 1944 Nobel Prize in physics for his major improvement in beam techniques (Rabi, Millman, Kusch, & Zacharias, 1939). By sending a stream of ^{1}H atoms through both an inhomogenous magnetic field, required for deflection, and a homogenous magnetic field where they were subjected to radio-frequency electromagnetic energy, Rabi observed magnetic resonance. This

effect was applied independently by Felix Bloch at Stanford University and by Edward Mills Purcell at Harvard during the early 1940s. Bloch (1946) and Purcell (1946) experimented with water and paraffin, respectively, both successfully observing magnetic resonance of their respective samples only weeks apart in 1946 (Becker, 1993), and subsequently sharing the Nobel Prize in Physics in 1952. Basic principles and applications of this new method were subsequently explored. The rapid development of NMR owes much to Russel Varian for his early decision to commercialize the manufacture of homogenous electromagnets (Dolcino, Mura, & Paolini, 2009). The dramatic demonstration of 1H chemical shift in ethanol by (Arnold, Dharmatti, & Packard) in 1951 then made it clear to chemists what NMR might do in analytics (Becker, 1993). The first commercial NMR spectrometer (Varian 30MHz) was made available the following year, in 1952.

The decade of discovery from 1946 to 1955 was followed by a decade of chemical application (1956 – 1965) (Becker, 1993). Instruments were primitive by current standards, with observation frequencies fixed by a crystal at a specific frequency (40MHz) – in comparison, the University of Lethbridge's spectrometers range from 300-700MHz (2017). After early NMR experimentation on body bio-fluids and tissues in 1956 (Jardetzky & Wertz), organic chemists soon realized its potential for elucidating the structure of moderate-sized molecules. Around the same time, Lowe and Norberg (1957) made a very important discovery – that a free-induction decay (FID), which is the observable NMR signal generated by non-equilibrium nuclear spin precession, is the Fourier Transform of the corresponding steady-state resonance line shape.

A decade of technology ensued, from 1966 to 1975, which saw data acquisition revolutionized by Richard Ernst, who successfully applied Fourier Transform to NMR and later won the Nobel Prize in Chemistry in 1991 (Cohen et al., 1995; Ernst & Anderson, 1966). This application of Fourier Transform resulted in a much shorter acquisition time and higher sensitivity. The first use of a superconducting magnet was reported by William Phillips in 1967, with Varian rolling out their first commercial superconducting magnet spectrometer (Varian 220MHz) around the same time. A steady stream of improvements in magnet technology

followed, led by Richards (Oxford University) and Bruker Instruments, which expanded NMR operating frequencies to 270MHz, 360MHz, and 400MHz. Software also began to develop as mini-computers were interfaced directly into spectrometers.

The years between 1976 to 1985 finally saw NMR capable of biological applications. Structures of complex organic molecules could now be systematically and efficiently characterized due to advances in Fourier Transform NMR and two-dimensional methods (Becker, 1993). Its first use in medical diagnosis was in 1981, when Ross et al. (1981) successfully diagnosed McArdle's Syndrome – a complication of an inborn error of metabolism caused by a lack of glycogen phosphorylase activity in skeletal muscle. 1986 to 1995 saw applications in medicine, structural biology, and materials science. Determination of the 3-dimensional structure of biopolymers (proteins) was finally possible, and applications in solid-state NMR led to the investigation of polymer ceramics and other composite materials throughout the 1990s (Miller, 1991).

NMR spectroscopy has moved from being a pure research tool to being a major instrument in the development of biotechnology and the advancement of human health. Current spectrometers have operational frequencies at 1GHz or greater, with 950MHz magnets used for NMR in 2006 (Bruker) (Emsley & Feeney, 1995). High-temperature super-conducting magnets will allow for more sensitive observation, and more compact and widely available spectrometers at reduced operating costs over the coming years (Cohen et al., 1995).

NMR THEORY

Properties of Subatomic Particles

In the years since Purcell and Bloch detected weak radio-frequency signals generated by the nuclei of atoms in ordinary bulk materials, NMR has become an incredible physical tool for investigating matter (Levitt, 2008). Matter is composed of atoms, and atoms are made up of electrons and nuclei. The hydrogen atom possesses a single proton in its nucleus, and in 1H NMR,

it is the single proton of interest. Protons and neutrons, collectively known as nucleons, together make up the atomic nucleus and possess fundamental intrinsic properties commonly used to describe matter. Each atomic nucleus has four important physical properties: mass, which is largely due to the mass of atomic nuclei; electric charge, the means by which atoms and molecules are bound together; magnetism; and spin. The last two are much less evident, but these important physical properties mean that nuclei interact with magnetic fields, and that an atomic nucleus behaves somewhat like a tiny, spinning planet.

Mass, Charge, and Spin

Mass is the measure of an object's resistance to acceleration, or any change in state of motion when a net force is applied (Young, Freedman, Ford, & Sears, 2012). It also determines the strength of its mutual gravitational attraction to other bodies. At a rudimentary level, the mass of any substance is the sum of the masses of all nuclei. These nuclei also possess an electric charge, which determines the electromagnetic interaction of subatomic particles. Experimentation in the twentieth century demonstrated that charge is quantized, meaning that it comes in integer multiples of small units called elementary charges (e), which are approximately equal to 1.602×10^{-19} Coulombs. Protons and electrons each have one elementary charge, which is positive and negative, respectively (Mohr & Taylor, 2000). An exception to this is in the case of quarks, which have fractional elementary charges of -e/3 or +2e/3 (Levitt, 2008). Since a neutron is neutral, intuitively it makes sense that it is composed of three quarks; two with charge -e/3 and one with a charge of $+2e/3$, resulting in an electric charge of zero. A proton is also made up of three quarks, but in combination this leads to a net charge of +1. In addition to electric charge, quarks are directly responsible for the subatomic property of spin. In the case of a H atom, the combination of quarks in a single nucleon (proton) leads to a spin of ½.

Most atomic nuclei possess spin, which is a vector since it has both magnitude and direction. Matter existing outside of an external magnetic field will possess completely random

atomic spins, contain a single energy level, and produce a net spin of zero. When an external magnetic field is applied, however, the individual atomic spins will align and experience Zeeman splitting, separating into 2l+1 energy levels where l is the value of an atom's spin. For example, the main nucleus of hydrogen contains a single proton and has spin equal to ½, causing energy states to separate into two distinct energy levels in an external magnetic field. This is precisely what Walter Gerlach and Otto Stern observed using silver atoms in their famous experiment in 1922 (Schmidt-Böcking et al., 2016), as well as the spectral line broadening witnessed by Pieter Zeeman in 1896 (Zeeman, 1897).

Magnetism and Signal Detection

NMR is particularly based on the presence of spin angular momentum and magnetism of atoms (Lipton, 2008). The protons of any atom contain electric charge and spin, therefore generating a magnetic field. Atomic spin determines the gyromagnetic ratio, γ, a constant value unique for each element independent of magnetic field strength, which describes the strength of a nucleus' response to an externally applied magnetic field such as NMR. The spin of a nucleus acts on the mass to produce an angular momentum vector, J, and interacts with electric charge to produce a magnetic moment, μ. An element's gyromagnetic ratio can be characterized by the angular momentum and magnetic moment, shown in Equation 1, where q is electric charge and m is the mass.

$$Equation\ 1.\ \gamma = \frac{\mu}{J} \cong \frac{q}{2m}$$

The magnetic field generated by an NMR spectrometer interacts with atomic gyromagnetic ratios with a force of interaction proportional to the size of the external magnetic field. When the nuclei of atoms experience the force from this magnetic field, nuclear spins move from random to

organized alignment and begin to precess, which is a change in the rotational axis of some rotating body (e.g. when a dreidel or spinning top slows down and begins to tip over). The interaction between the atom's magnetic moment and the applied magnetic field B_o, results in a torque vector, perpendicular to both magnetic vectors (Equation 2).

$$Equation\ 2.\ \ \tau\ =\ \mu\ \text{x}\ B_o$$

The angular momentum vector J precesses about the external field axis with an angular frequency called the Larmor frequency ω, named after its discoverer Joseph Larmor. Crucially, this frequency is independent of the polar angle between the applied magnetic field and the direction of the magnetic moment (Equation 3). Thus, precession rate does not depend on the spatial orientation of the spins prior to applying a magnetic field.

$$Equation\ 3.\ \ \omega\ =\ \gamma B_o$$

The uniform precession of atoms experiencing the external magnetic field causes a net magnetization in the direction of B_o, defined by convention as the positive z-axis in 3-dimensional space (Figure 1). This net magnetization is comprised of two magnetic components; the alignment of nuclear magnetic moments parallel to B_o , designated as α, and those antiparallel to B_o, designated as β.

The parallel orientation is more energetically favourable, and is therefore larger in magnitude, meaning that the net resultant magnetic force is always parallel to the applied field. The difference in energy level between α and β is linearly related to the magnitude of the applied magnetic field, which means that a higher magnetic field will create a greater energy difference between these states. The population difference between the two alignments is given by the Boltzmann distribution. This is a direct manifestation of Zeeman splitting, and explains why higher field spectrometers give greater spectral resolution. The energy of each state is given by the work done to precess, which is the integral of the torque in Equation 2. This gives Equation 4 below, where W represents work.

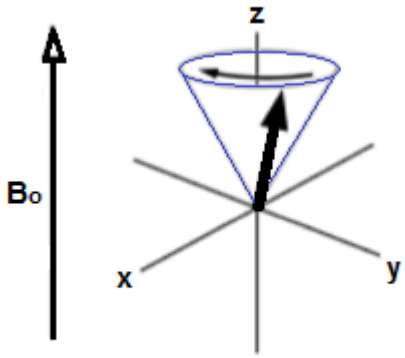

Figure 1. Illustration of precessional motion, in which the vector sweeps out a cone of constant angle to the magnetic field direction. Precession here is shown for a nucleus with positive gyromagnetic ratio, and hence a negative Larmor frequency.

$$Equation\ 4.\ E_{state} = W = \int \tau = \int \mu \ \text{x}\ B_o$$

By rearranging Equation 1 for μ, we can see that $\mu = \gamma J$. If we substitute this into Equation 4, we get a simplified dot product for work in Equation 5.

$$Equation\ 5.\ E_{state} = W = -\gamma(J \circ B_o)$$

Since we are describing angular frequency in terms of radians, we use Planck's constant divided by a factor of 2π. This is represented as $\hbar$, or "h-bar", rather than the commonly known h.

Total angular momentum *J* is equal to the product of $\hbar$ and orbital angular momentum *s* (in this case $\pm$½). With this, our energy equation becomes:

$$Equation\ 6.\ E_{state} = W = -\gamma B_o \hbar s$$

$$based\ on\ Equation\ 3\ above, this\ becomes$$

$$E_{state} = W = -\hbar s \omega$$

Rabi, Purcell, and Bloch had shown that subjecting matter to pulses of radio frequency (RF) electromagnetic radiation could temporarily alter a molecule's bulk magnetization (Bloch et al., 1946; Purcell et al., 1946; Rabi et al., 1939). The energy of this resonance pulse must match the difference in energy between α and β, which depends on the gyromagnetic ratio and magnitude of the external magnetic field and corresponds to the Larmor frequency (Equation 4). This equation describes the energy of transition from one state to the other, rather than the energy of each independent state as described above.

$$Equation\ 7.\ \Delta E = E_{final} - E_{initial} = \hbar\omega$$

$$since\ \omega = \nu 2\pi,\ \ E = \frac{h\omega}{2\pi} = h\nu$$

$$where\ h\ is\ Planck's\ constant\ and\ \nu\ is\ the\ RF\ pulse\ frequency$$

To detect an NMR signal, there must be a change in magnetization over time. This is accomplished by applying an RF pulse perpendicular to the z-axis alignment of precessing atomic nuclei at a frequency that resonates with nuclei of interest (protons in ^{1}H NMR). This results in a temporary change in the magnetization of nuclei in the direction of the RF pulse, called the "transverse plane" (Figure 2).

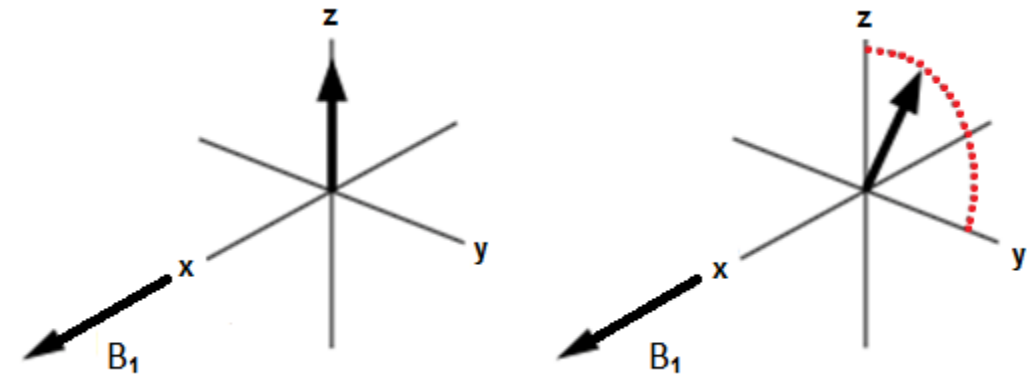

Figure 2. Illustration of a change of magnetization over time. If the magnetic field along the z-axis is quickly replaced by a perpendicular pulse along the x-axis, the resulting magnetization will precess about the x-axis and move towards the transverse plane.

The net nuclear magnetization will subsequently precess back to its original alignment within B_o; this is referred to as "relaxation" and is the detectable component of NMR. Thus, a basic RF pulse-acquire sequence would be a 90-degree RF pulse followed by immediate acquisition. Numerous pulse sequences exist to manipulate magnetic moments and to acquire specific information from various isotopes, including atomic interactions, and intermolecular distances. The RF coil responsible for generating an RF pulse in the NMR spectrometer can also receive an induced electric current from the change in magnetization due to relaxation of the atomic nuclei, which is stored as data on a computer following analog-to-digital conversion (ADC). This is known as the "Principle of Reciprocity", since the coil can both generate an RF field and detect current changes resulting from precession and relaxation in the transverse plane. The raw data acquired by the coil is the free induction decay, or FID, and is the sum of all precessional frequencies of a given sample in the time domain (Figure 3).

As mentioned previously, the FID was shown by Irving Lowe in 1957 to be the Fourier transform of the corresponding steady-state line shape, and vice versa (Lowe & Norberg, 1957). The application of Fourier transform to NMR by Richard Ernst in 1966 effectively revolutionized NMR data acquisition (Ernst & Anderson, 1966). Essentially, an FID acquired and stored in the time domain, when Fourier transformed, becomes a peak in the frequency domain, known as a spectrum. The FID will "decay" as the nuclei precess back to equilibrium following a RF pulse sequence, but the frequency remains constant. Figure 3 illustrates that a rapid decay leads to a broader peak in the corresponding spectrum. By converting a complex FID with numerous overlapping wavelengths to a frequency spectrum, the data become much easier to interpret.

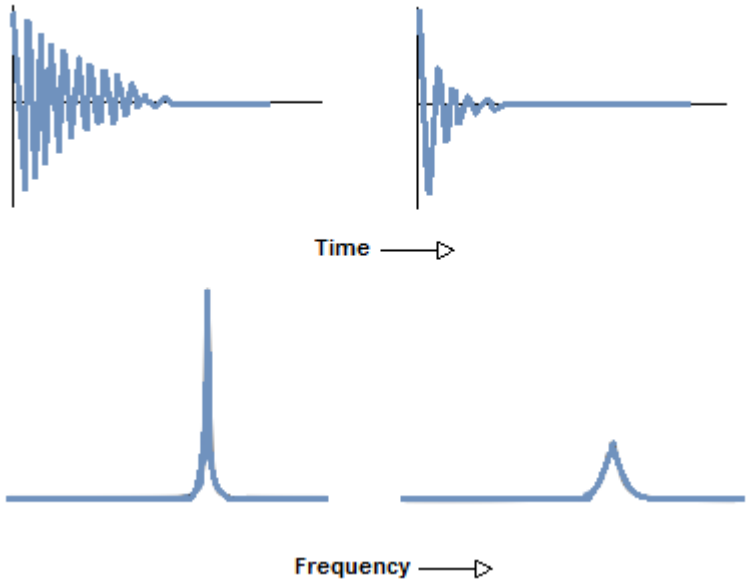

Figure 3.Illustration of free induction decay (FID) of a single frequency in the time domain Fourier transformed to the frequency domain, as well as the phenomenon of rapid FID decay. A rapid FID decay gives a broader line on the corresponding spectrum.

Chemical Shift

Frequencies of NMR spectra are shown in parts per million (ppm or δ), and are relative to an agreed-upon chemical shift reference assigned to 0 ppm (Keeler, 2010). Since NMR resonance frequencies scale linearly with magnetic field strength, we need to specify both to convey the location of a given frequency (e.g. 400.0001255 MHz may correspond with a single peak at 1 ppm). By referencing to a specific compound at 0 PPM, we eliminate this problem.

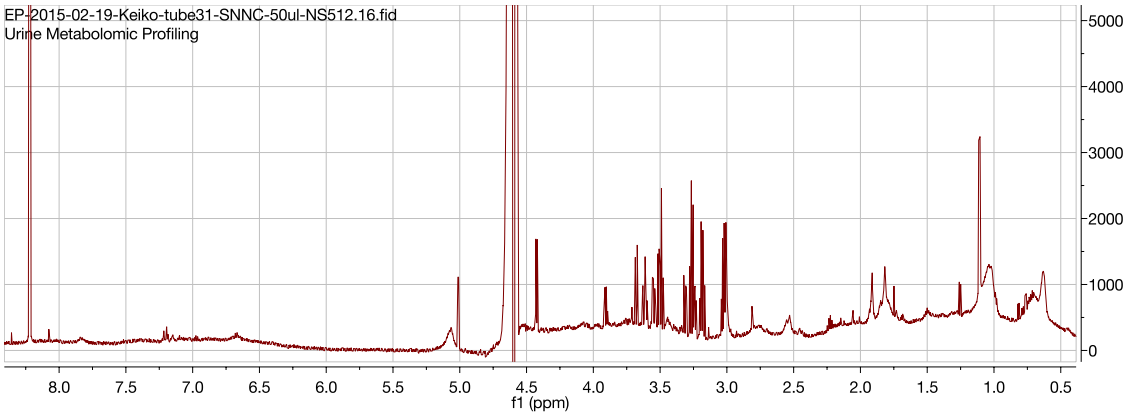

Figure 4. A representative spectrum from transgenerationally stressed rats (see chapter 4). The y-axis represents signal intensity, and the x-axis represents chemical shift (ppm). Formate (δ 8.22) was used as a chemical shift reference.

Since all frequencies scale with the magnetic field, this ratio is independent of magnetic field strength. If the frequency of our peak of interest (ν), is in Hz, and the Larmor frequency of our reference peak (ν_{REF}) is also in Hz, the chemical shift is computed as:

$$Equation\ 8.\ 10^6 \times \delta = \frac{\nu - \nu_{REF}}{\nu_{REF}}$$

The advent of higher-field magnets, especially those containing superconductors, allowed scientists to spread out spectra in frequency units and to distinguish chemical shifts in large molecules that normally coincide at lower magnetic fields (Becker, 1993). However, even spectra from a single molecule such as hydrogen can be very complex and can contain numerous peaks. This is due to "shielding" effects by adjacent electron clouds. Each proton in a molecule will experience both the applied magnetic field B_o and all minute magnetic moments generated by the motion of electrons as they orbit a molecule's nucleus. Magnetic fields generated by moving electrons tend to oppose B_o, resulting in a reduced frequency, or shielding, of the proton in question. Thus, more adjacent electrons will result in a greater shielding effect, and fewer adjacent electrons will result in less shielding. Nuclei tend to be *deshielded* by functional groups

that withdraw electron density, causing them to resonate at a higher ppm. Originally an annoyance to scientists, it was eventually discovered that this could allow for very accurate elucidation of molecular structure due to the consistency in shielding effects of specific functional groups (Balci, 2005).

Signal Intensity

A given NMR spectrum will display multiple spectral peaks in parts per million along the x-axis, and signal intensity along the y-axis. The integrated intensity of an NMR signal is ideally proportional to the ratio of nuclei within a molecule, allowing the user to identify the approximate number of protons in a molecule. Together with chemical shift and coupling constants, integrated intensities allow for structural assignments. It is important to note that NMR signal acquisition inherently produces "noise" in a spectrum, sometimes making it difficult to accurately integrate a set of spectral peaks. The signal-to-noise ratio can be improved, however, by increasing the amount of time spent acquiring the FID through more scans. Since n consecutive scans recorded in digital memory, coherently added, produce a signal n-times as large as one scan, whereas random noise is only $n^{1/2}$ times as large, the signal can be improved through the expenditure of more spectrometer time (Figure 5). Essentially, we need to take four times the number of scans (NS) to get twice the signal-to-noise, 16 times the NS to get four times the signal-to-noise, and so on.

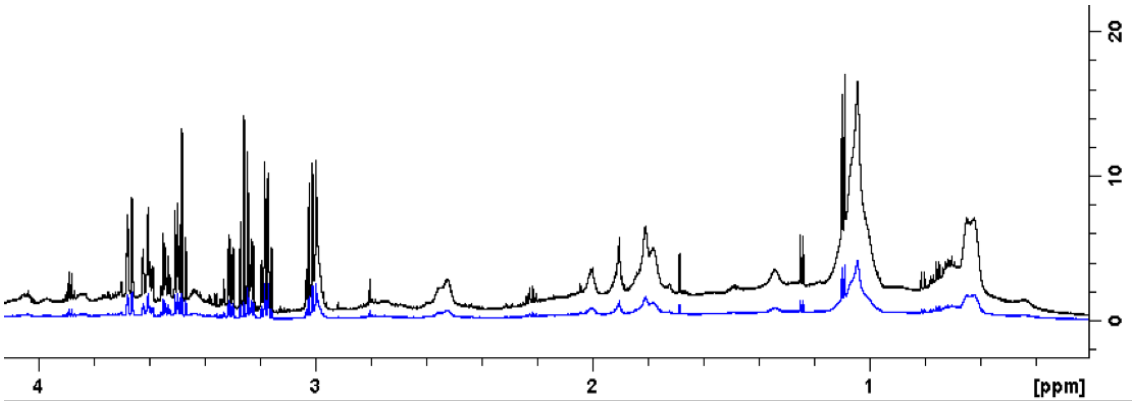

Figure 5. Illustration of the effect of increased number of scans on signal-to-noise in a ^{1}H NMR spectrum, using blood plasma at a concentration of 100uL in 550uL of saline buffer, acquired on a 700MHz Bruker spectrometer (AVANCE III HD). The blue signal was acquired with 128 scans, while the black signal was acquired with 512 scans.

Spin-Spin Coupling

The small magnetic fields of spinning electrons responsible for chemical shift are accountable for another important phenomenon: spin-spin coupling, sometimes referred to as "J" coupling. Essentially, nuclei and local electrons interact, giving an indirect interaction between two nuclear spins. In other words, the alignment of proton H_b in the external magnetic field affects the frequency of neighboring proton H_a. The differing alignment of H_b's magnetic moment causes fine splitting in the signal of H_a (Figure 6).

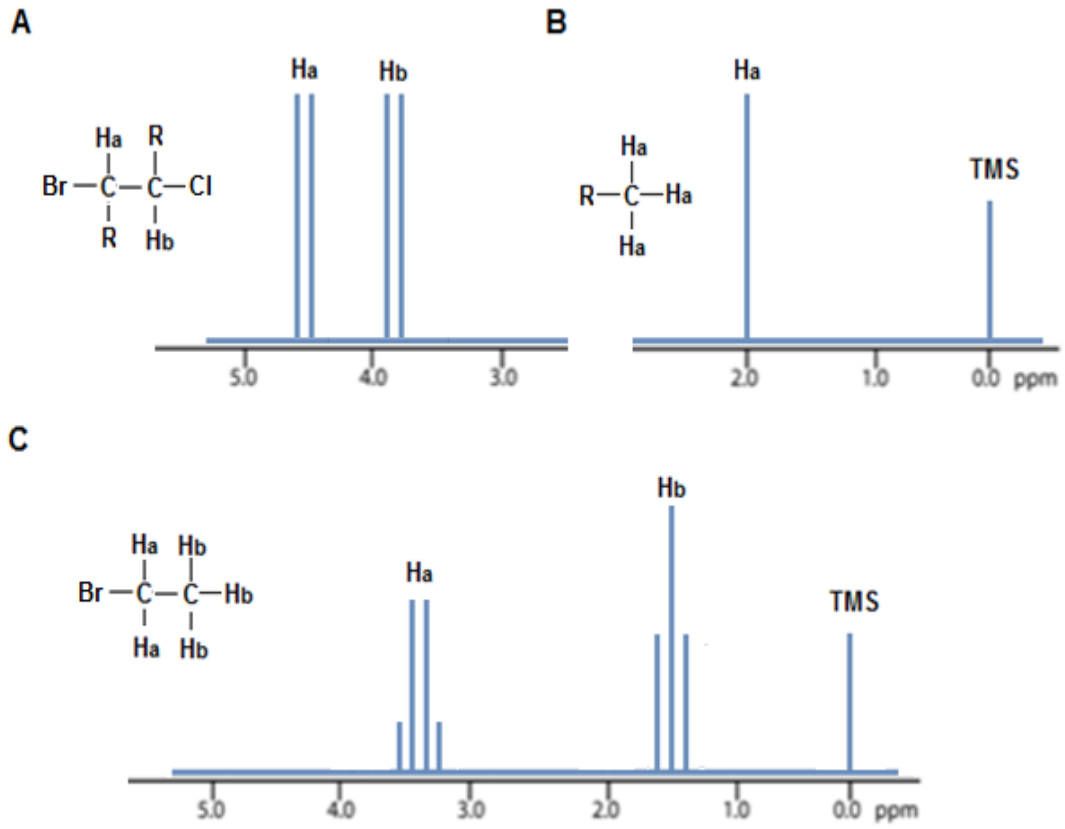

Figure 6. Illustration of spin-spin coupling between identical protons H_a and identical protons H_b in theoretical molecules, where "R" is a non-Hydrogen side group or atom. A: Two doublets result from H_a and H_b existing in slightly different chemical environments due to differences in halide electronegativity. B: One singlet resulting from identical H_a protons. C: The chemical environment created by the bromine atom results in H_a having a deshielded quartet at a higher ppm, and H_b having a shielded triplet at a lower ppm.

Subsequently, the number of signals in a spectrum increases and gains complexity. For example, the effect of scalar coupling, which occurs through chemical bonds between two nuclei, can be understood using a hypothetical proton at 2 ppm. In this hypothetical molecule, another proton exists three bonds away. The adjacent group causes our signal at 2 ppm to split into two, with one peak shifted slightly higher than 2 ppm, and the other shifted slightly lower. These resultant peaks each contain half the area of the former single peak. The magnitude of this splitting is known as a coupling constant and is 7 Hz in this example.

The coupling constant is caused by the magnetic field of an adjacent nucleus, independent of magnetic field strength, and is therefore quoted in frequency (Hz), not chemical shift (ppm).

This means that a greater applied magnetic field will not alter the splitting pattern or distance in Hz of a given molecule. If we add a proton to our hypothetical molecule (e.g. CH_2 from CH), we will get a different splitting pattern: a triplet with an intensity ratio of 1:2:1, with one peak at 7 Hz above 2 ppm, one peak at 2 ppm, and another 7 Hz below 2 ppm. The splitting observed by three identical protons takes the shape of a quartet, with relative intensities of 1:3:3:1. This pattern continues for *n* identical protons whose ratio of peak intensities are given by the *n*th row of Pascal's Triangle. This row contains $n + 1$ components, and splitting is therefore said to follow the "$n + 1$" rule: a proton with *n* adjacent protons gives a peak pattern with $n + 1$ peaks.

If the proton is coupled in two adjacent locations rather than one, the spectrum will contain a doublet of doublets instead of a triplet. These are often collectively labelled as "multiplets" and can become very complex. They do, however, provide important clues about the molecular structure under study. The key point is that J-coupling gives information about bond distance, angles, and connectivity. Signals that have more than one peak (e.g. doublet, triplet, quartet) will have the peak separations determined by J-coupling, which is given by the shape and connectivity of the molecule.

Carbon Satellites

It is not uncommon to see small peaks on the flanks of a main 1H NMR peak. These are not the result of spin-spin proton coupling but result from the coupling of nuclei of hydrogen atoms to nuclei of adjacent carbon-13 (^{13}C) atoms. The natural abundance of 13C is only 1.1% on Earth, which explains why these carbon satellites have such small intensities. As mentioned above, the coupling due to a single spin-1/2 nucleus results in a doublet for the proton attached to a 13C atom, which is why there are two carbon satellites surrounding a proton singlet; one up-field and one down-field. Other NMR-active nuclei, such as ^{19}F and ^{31}P, can cause a similar phenomenon, but they are not nearly as common as ^{13}C in spectra of organic compounds.

METABOLOMICS METHODS

Sample Collection and Storage

Sample collection and storage is one of the most important aspects of experimental design, since it has the potential to throw off an entire study if done improperly. For this reason, in any application of metabolomics, researchers are given a very simple, specific set of instructions to follow when collecting samples from participants. Sample preparation will, however, depend on the specific type of bio-fluid or tissue being collected (Beckonert et al., 2007a). Necessary precautions must be taken regardless of sample type to ensure integrity of the metabolites. One potential problem with improper storage or transport of samples is metabolite production or break-down due to bacterial proliferation. The most common way to prevent this is to store samples at -80°C immediately following sample accession. However, a review by Lauridsen (2007) found that -25°C is sufficient to cease metabolic breakdown by bacterial activity, and that freeze-thaw cycles do not significantly affect sample integrity of samples analyzed by NMR. This is likely because NMR is sensitive to μL quantities of metabolites. Comparatively, GC-MS and LC-MS systems are sensitive to nL quantities, so storage at -25°C may be insufficient to cease metabolic breakdown products in this case.

SAMPLE PREPARATION BY TYPE

A key strength of NMR spectroscopy is its ability to analyze nearly any type of biological fluid or tissue (Suhre et al., 2011). One aspect of the sample preparation common to all sample types is the requirement for a buffer. Without this, cells would lyse and change the levels of extracellular metabolite concentrations, or bacteria would proliferate, also altering extracellular levels of certain compounds. A buffer is also used as a chemical shift reference, usually spiked with some compound with a specific, known chemical shift (not necessarily zero). A common reference compound, and the one used in the following studies, is Trimethylsilylpropanoic acid (TSP) (Pohl & Eckle, 1969). A major reason for buffering a sample, particularly those with high

or low acidity, is that the protonation of a molecule is pH-dependent. It would therefore be impossible to standardize a 1H NMR experiment if the pH were not tightly controlled. Lastly, a metabolomics buffer will contain deuterium oxide (D2O), which is used to monitor the magnetic field while irradiating a sample with an RF pulse sequence.

Urine

Urine is the liquid by-product of metabolism in humans and animals. Cellular metabolism generates numerous by-products, many of which are nitrogenous due to the breakdown of amino acids, requiring clearance from the bloodstream (Rose, Parker, Jefferson, & Cartmell, 2015). Urination is the primary method for excreting water-soluble metabolites from the body. Urine is produced by a process of filtration, reabsorption, and tubular secretion by the kidneys (Strasinger & Di Lorenzo, 2008). Kidneys extract soluble, low molecular weight wastes from the bloodstream, in addition to excess water, sugars, and some toxins. This makes urine ideal for NMR analysis, since it does not contain large macromolecules or lipids that can lead to complex spectra.

Urine can be expected to reflect metabolic activity from most organ systems in the body. Since the brain accounts for almost a quarter of all metabolic activity in humans (Kety, 1957; Rolfe & Brown, 1997), its waste products will be reflected significantly in urinary profiles. Furthermore, urine sample accession is simple and non-invasive, and does not require any specialized training, meaning that study participants can often collect it themselves. Any urine collection is often obtained at first void of the day to attempt to control for diurnal fluctuations in metabolite content (Slupsky et al., 2007). Practical study design should account for other influences that may lead to these fluctuations, such as diet (González-Guardia et al., 2015; Yang et al., 2015), age, sex, and ethnicity (Slupsky et al., 2007), but urine has been shown to maintain a stable phenotype in healthy controls despite these factors.

Glomerular filtrate of blood is usually acidified by the kidneys from physiological pH (7.4) to approximately pH 6, making urine quite acidic. Urine pH can affect the stability of formed metabolic compounds, which means that any obtained sample must be buffered to a consistent physiological pH value. NMR spectroscopy is very sensitive to minor fluctuations in the pH of a sample. A phosphate buffer containing mono- and di-basic potassium salts is an effective way to buffer urine and to prevent these fluctuations in pH. See chapter 3 for a specific urine buffering method.

Blood

Blood is the body fluid in humans and animals responsible for nutrient and oxygen delivery to cells, as well as transport of metabolic waste products. In vertebrates, red blood cells are suspended in blood plasma, which constitutes 55% of blood and is the primary medium for excretory product transportation (Ainslie & Smith, 2011). Blood plasma contains proteins, minerals, glucose, hormones, CO2, electrolytes, and clotting factors (blood serum does not contain clotting factors). Although it is a liquid, blood is considered to be a specialized form of connective tissue, with plasma being akin to an extracellular matrix, representative of the body's metabolic waste content (Alberts et al., 2015).

Preparation of blood for NMR analysis is slightly more involved than that of urine due to the presence of macromolecules, such as protein albumin and lipids. In plasma, intense resonances from lipoproteins can overwhelm signals from small metabolites (Tang, Wang, Nicholson, & Lindon, 2004). An additional step is therefore required to remove these macromolecules, which functions to simplify the resulting spectra. This can be accomplished by removing the signal of macromolecules from the spectrum using an adjusted pulse sequence (Tang et al., 2004), using a macromolecular filter (Ravanbakhsh et al., 2015), or by precipitation using a chemical extraction (Nagana Gowda & Raftery, 2014).

Blood is naturally regulated by the body to remain within a physiological pH of 7.35-7.45 using several mechanisms, including the bicarbonate (HCO3-) system, respiration (via O2 and CO2 partial pressures), and the urinary system (Seldin & Giebisch, 1989). Therefore, to buffer a blood plasma sample for metabolomic analysis, it must only contain NaCl, water, D2O, and sodium azide (NaN3) as an antimicrobial agent (Beckonert et al., 2007a). Blood also contains fewer metabolites identifiable by NMR than urine, making spectra easier to analyze. Despite the ease of buffering and analyzing blood samples, however, it should be noted that it is much more difficult to obtain, requiring at least basic training in phlebotomy, and is much more invasive than alternatives such as urine or saliva (Psychogios et al., 2011).

Saliva

Saliva is 99.5% water, but also contains electrolytes, mucous, white blood cells, epithelial cells, glycoproteins, enzymes, and antimicrobial agents (Hunter, 2013). It is now recognized as an excellent diagnostic medium for detection of disease following major advancements in metabolomic medical diagnostics (Zhang et al., 2012). Silwood et al (2002) report identifying greater than 60 metabolites via NMR in human saliva. As a diagnostic fluid, it is particularly attractive due to its low-invasiveness, minimal cost, and ease of collection and processing, which is rapid with no specialized training necessary. Saliva is functionally similar to blood serum in reflecting the physiological state of the body, since most compounds found in blood are present in saliva; many metabolites, proteins, and other micromolecules enter saliva from the blood by passing through transcellular and paracellular routes (Zhang et al., 2012). Saliva-based diagnostics are still emerging but combined with a NMR-metabolomics approach there is great potential in clinical and research applications.

Cerebrospinal Fluid

Cerebrospinal fluid (CSF) is a clear, colourless body fluid found in the brain and spinal cord that is produced in the choroid plexus of the brain ventricles. It is derived from blood plasma, and is very similar to it, except that CSF is nearly protein-free and contains different electrolyte levels (Felgenhauer, 1974). CSF gives a much more localized profile than blood, urine, or saliva, reflecting nervous system function rather than whole-body metabolism. It allows for removal of waste products from the brain and is critical in the brain's lymphatic system (Iliff et al., 2012). Metabolic waste products diffuse rapidly into the CSF and are removed into the bloodstream as it is absorbed, making cerebrospinal fluid ideal for complimentary metabolomic analyses when compared to metabolite profiles in blood. The downside of using CSF is that it is more invasive and difficult to obtain than any other bio-fluid, making clinical application challenging.

Tissue

A biological tissue is made up of similar cells that together carry out a specific function. Tissues can be analyzed via NMR to obtain a very localized and specific metabolite profile. The benefit of using tissue in combination with other bio-fluids is that effects of external environment on particular body systems can be assessed and compared to whole-body responses seen with blood, urine, or saliva. Like blood, however, tissue requires additional steps to separate metabolites from larger macromolecules. The most effective way to isolate these metabolites is to excise and homogenize tissue, subsequently extracting both water- and lipid-soluble metabolites using methanol and chloroform, respectively (Beckonert et al., 2007a). Tissue use is more practical in post-mortem or animal studies, since biopsies are quite invasive, and in some cases, they are not feasible (e.g. brain). Magnetic resonance spectroscopy (MRS) can be used to assess tissues *in vivo* in a non-invasive manner, but this requires the use of magnetic resonance imaging (MRI).

EXPERIMENTAL PLATFORMS

^{1}H NMR Spectroscopy

NMR spectroscopy determines molecular structure of compounds by exploiting electric and magnetic properties of subatomic particles, such as protons. Bouatra et al. (2013) conclude that 1H NMR spectroscopy seems to be the most ideal method for global, untargeted analysis of urinary metabolite profiles. NMR currently permits identification of the largest number of metabolites in urine (209/2651 or 8% of known, quantified metabolites in human urine metabolome), and yields the greatest chemical diversity. 108 of these compounds are unique to NMR. In terms of other fluids, NMR can detect 49 compounds in blood (Psychogios et al., 2011), over 60 in saliva (Silwood et al., 2002) and 53 in CSF (Wishart et al., 2008). One downfall of 1H NMR spectroscopy is its dependence on the presence and location of protons. In other words, a compound that is not at physiological pH may be de-protonated, making it "NMR-invisible". Uric acid, for example, is one of the most highly concentrated waste products found in urine, but it is not detectable by proton NMR.

Mass Spectrometry

Mass spectrometry (MS), rather than exploiting magnetic properties of subatomic particles, is an analytical technique that ionizes chemical species and sorts consequent ions based on their mass-to-charge ratio (Hoffmann & Stroobant, 2002). It essentially measures molecular masses within a given sample and displays them as a spectrum, with ion signal as a function of mass-to-charge ratio. Like NMR, MS can analyze both simple and complex mixtures of solids, liquids, and gases. While MS coupled with gas chromatography (GC) is a close second to NMR in terms of metabolite coverage in urine (179 metabolites), this requires four different analyses on two different GC-MS instruments (Bouatra et al., 2013). Each sample also requires many hours of collection, preparation, and analysis, making this method low-throughput as a metabolomics technique compared to NMR. Direct flow injection (DFI) combined with liquid chromatography

(LC) and MS yields an additional 127 quantified compounds in urine, but lacks chemical diversity (only 6/25 chemical superclasses, compared to 14 in GC-MS and 15 in NMR). However, DFI/LC-MS/MS requires very little sample volume (10μL), is very low-cost, generally automated, and high-throughput. In terms of total urine metabolome coverage, MS techniques combined can identify and quantify 346/2651, or 13% of known metabolites across multiple platforms.

Chromatography

Chromatography is a biochemical technique used to separate proteins, nucleic acids, or small molecules in a mixture based on some chemical property, such as polarity or charge (Vitha & ProQuest, 2017). It is often coupled to mass spectrometry techniques to improve spectral resolution of the sample under study. The two most common types coupled to MS are gas- and liquid-chromatography. Gas chromatography is used for separating and analyzing compounds that can be vaporized without decomposition (Littlewood, 1962). Liquid chromatography (LC), often in the form of high-performance liquid chromatography (HPLC) relies on a pump to pass pressurized liquid solvent containing the sample mixture through a column filled with a solid adsorbent material, allowing for separation of compounds (Katz, 1996). When coupled with MS, chromatographic methods aid in the identification of numerous compounds. However, on their own, HPLC and LC are only able to identify 10/2651, or 0.4% of the human urine metabolome, making it a non-ideal method for analytical metabolomics (Bouatra et al., 2013).

DATA ACQUISITION AND PROCESSING

Data Acquisition and NMR Relaxation

In the NMR experiment, once samples have been prepared and buffered according to a standard protocol, spectra are acquired using a spectrometer of ideal frequency (in this case, Bruker Avance III HD 700MHz). A precisely defined pulse sequence, such as the Nuclear

Overhauser effect spectroscopy (NOESY) sequence irradiates the sample, allowing a signal to be detected while protons precess back to equilibrium along the z-axis. This sequence is typically a set of pulses interspersed with delays. In a simple 90-degree pulse sequence (Figure 7), there is first a recycle delay (D_1), which is an inter-pulse delay that serves to allow the spin system to return to equilibrium along the magnetic field or z-axis (important for achieving maximum signal-to-noise ratio). Following D_1 is the RF pulse, which lasts approximately 5-15μs, with the RF waveform switched on at the start of this pulse and switched off at the end of the pulse. A short delay between the end of the pulse and the start of acquisition then occurs, called the dead time (DE). DE allows the system electronics to stabilize, reducing the likelihood of acquisition artifacts. The spectral window (SW) is a measure of the width of frequency spectrum to be analyzed (in ppm). Signals that possess frequencies within the SW will be detected and all others will be filtered out, thus, the SW must be large enough to contain all observed peaks in a spectrum. One divided by the SW is equivalent to the dwell time (DW), which represents the time between acquisition points; therefore, DW multiplied by the number of data points (TD) provides the total acquisition time (AQ).

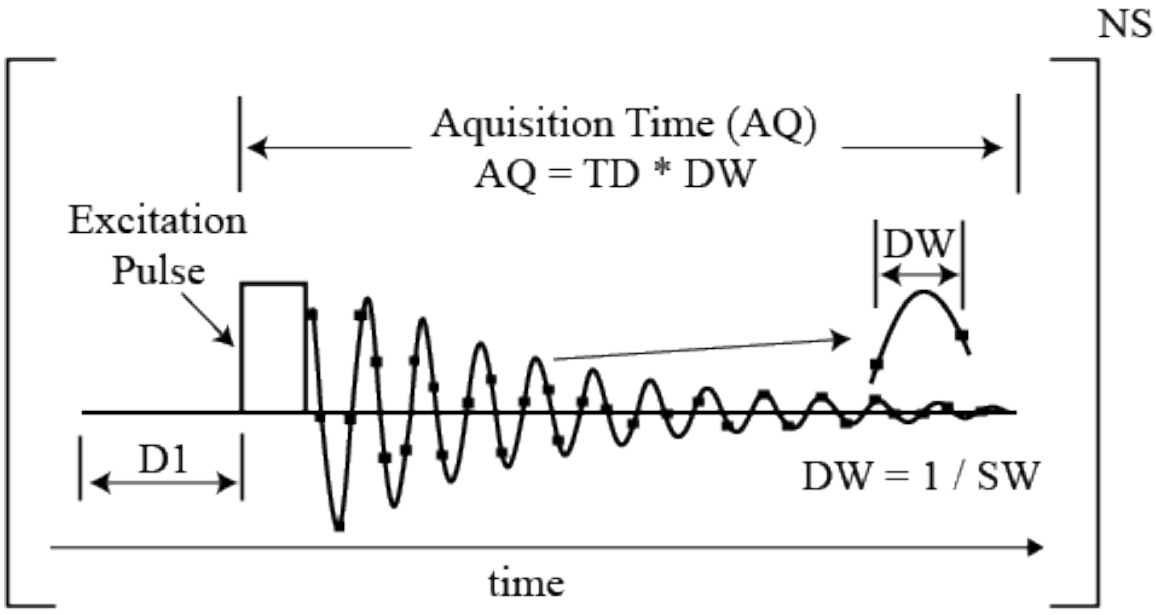

*Figure 7. An illustration of a standard NMR experiment pulse sequence. In this basic pulse sequence there is a recycle delay (D1) followed by the RF pulse, which leads to a signal and its subsequent decay. Total acquisition time (AQ) = total data points (TD) * dwell time (DW). NS is the number of scans; in this case, 1.*

The NOESY is a *through-space correlation method*, which establishes correlations between nuclei that are in close proximity regardless of whether there is a bond between them. The Nuclear Overhauser effect (NOE) is the transfer of nuclear spin polarization from one spin to another through spin-lattice cross relaxation (Friebolin & Kenwright, 1994). It is useful when using NMR to explore atomic structure, since inter-atomic distances derived from the NOE can help to confirm three-dimensional organization of molecules.

Spin-lattice relaxation, also known as the time constant T_1, is the mechanism through which the z-axis component of the magnetization vector reaches thermodynamic equilibrium (Keeler, 2010). Specifically, T_1 characterizes the rate at which the longitudinal magnetization component (z-axis) recovers exponentially back to equilibrium (Equation 9).

$$Equation\ 9.\ \mathrm{M}_z(t) = M_{z,eq} - [M_{z,eq} - M_z(0)]e^{-t/T_1}$$

From Equation 9, T_1 is therefore the time it takes for the M_z longitudinal magnetization to recover approximately 63% of its value following a rotation into the transverse plane by a 90-degree radiofrequency pulse. Thus, the recycle delay, or time between scans of the same experiment, is generally 2-5 times the T_1 value. This contrasts with the *spin-spin relaxation time*, also known as the *transverse relaxation* or T_2, which is the mechanism by which the M_{xy} or transverse component of the magnetization vector exponentially decays back toward equilibrium (Equation 10). This is essentially the time it takes for the FID to decay.

$$Equation\ 10.\ M_{xy} = M_{xy}(0)e^{-t/T_2}$$

T_2 then, is the time it takes for the magnetic resonance signal to decay to 37% of its original value, following an RF pulse. T_2 is generally more rapid than T_1 relaxation, and total acquisition time must be longer than the longest T_2 value. Different sample and tissue types possess different relaxation times, with fluids having the longest (1500-2000ms for T_1 and seconds for T_2), followed by water (400-1200ms for T_1 and 40-100ms for T_2), and fat having the shortest relaxation time (100-150ms for T_1 and 10-100ms for T_2). This phenomenon aids in metabolite identification, since rapid FID decay leads to broad peaks. In other words, large macromolecules like lipids will give broad peaks in NMR spectra, as illustrated in Figure 3.

Dynamic Adaptive Binning

Following data acquisition, spectral datasets must be reduced into a realistic size for statistical analyses. This is done by segmenting or binning the spectrum into a discrete number of bins (Anderson et al., 2011; Ravanbakhsh et al., 2015). NMR raw spectra are collected as a set of many thousands of data points. Binning reduces this dataset to less than 400 points, effectively cutting down time and processing power constraints. While some research groups choose a

predefined number and width of bins (e.g. every 0.04 ppm), it is best practice to adjust individual bins for best fit. This is somewhat subjective and time consuming, but the resulting data integrity is much greater since predefined bins often split peaks or entire compounds into multiple bins. Bins are qualitatively represented as the integral of the spectrum contained within the bounds of one bin, which is approximately equal to the relative concentration of a compound in that bin. This concentration is relative to the known concentration of the reference peak, usually TSP.

Binning, which was originally introduced to allow comparison of NMR data at different magnetic field strengths (Spraul et al., 1994), attempts to minimize effects from peak position variations caused by pH, ionic strength, and compositional differences between samples (Craig, Cloarec, Holmes, Nicholson, & Lindon, 2006). Anderson et al. (2011) have developed an improved spectral binning technique called Dynamic Adaptive Binning (DAB), where bin boundaries are determined by optimizing an objective function using a dynamic programming strategy. This function measures the bin configuration quality based on the number of peaks in each bin. DAB is a significant improvement over other binning techniques and is used here as an initial binning strategy.

Normalization

Normalization is a row operation applied to data from each sample that will apply to an entire spectrum, making these data directly comparable (Craig et al., 2006; Xia & Wishart, 2011). Before statistical analyses can take place, NMR data must be normalized and scaled to account for individual differences in concentration or dilution of metabolites present in a sample (Kohl et al., 2012). This makes any variation in the relative amount of a metabolite more prominent. Normalization is not always needed in blood plasma and tissue samples, however, since metabolite concentrations are highly regulated by homeostatic means. Since biological fluids primarily contain water, which has two protons, any ^{1}H NMR spectra will contain an overbearing water signal even after water suppression. The water region is therefore removed from a spectrum

prior to normalization. Similarly, the highly fluxional urea peak is removed from the normalization when using urine samples.

There are four primary methods used for row-wise normalization in metabolomics analyses: normalization to a constant sum, to a reference sample, to a reference feature, or sample-specific normalization. Constant sum normalization involves setting each spectrum to have a unit total area and expresses each data point (bin) as a fraction of the total spectral integral (Craig et al., 2006). Normalization to a reference sample, also known as *Probabilistic Quotient Normalization (PQN)*, assumes that concentration changes of biologically interesting compounds influence only parts of the NMR spectrum, while changes in dilution will affect all metabolite signals (Kohl et al., 2012). Normalization to a reference feature involves reference to some internal standard, such as creatinine (Prevot, Martini, & Guignard, 2002). A sample-specific normalization compares the dry weight or tissue volume of the original sample to the quantified metabolomic concentration. In the following studies, the constant sum method is used for row-wise normalization.

Scaling

High-concentration metabolites like urinary creatinine tend to have very intense signals in a given spectrum. Due to this phenomenon, bins are scaled to mitigate the statistical tendency to focus on intense signals while ignoring less intense ones (Berg, Hoefsloot, Westerhuis, Smilde, & Werf, 2006; Craig et al., 2006; Kohl et al., 2012). There are many methods for scaling, or column-wise normalization, of multiparametric data. The two most common of these for metabolomics are Auto scaling and Pareto scaling. Auto scaling (Equation 11) compares metabolites based on correlations, thereby making all metabolites equally important. A disadvantage to using Auto-scaling is the potential for inflation of measurement errors.

$$Equation\ 11.\quad x_{ij} = \frac{x_{ij} - \bar{x}_i}{s_i}$$

$$where\ x_{ij}\ is\ the\ spectral\ mean,$$

$$\bar{x}_i\ is\ the\ bin\ mean, and\ s_i\ is\ the\ standard\ deviation.$$

Pareto scaling (Equation 12) aims to reduce the relative importance of large values, while keeping data structure partially intact. This means that the processed data remains closer to the original measurement than Auto scaling, but it also makes data sensitive to large fold changes. Pareto scaling is very comparable to Auto scaling but is instead divided by the square root of the standard deviation as a scaling factor (Berg et al., 2006). Thus, large fold changes are decreased more, and are less dominant than small fold changes. The following studies use Pareto scaling due to its advantages over Auto scaling.

$$Equation\ 12.\quad x_{ij} = \frac{x_{ij} - \bar{x}_i}{\sqrt{s_i}}$$

Multivariate Statistical Analysis

The goal of multivariate statistical tests is to reduce data complexity or dimensionality into a more manageable number of two or three dimensions (Bjerrum, 2015). Following normalization and scaling, data are ready to be analyzed using various multivariate statistical methods, such as principal component analysis (PCA), partial least squares – discriminant analysis (PLS-DA), and/or orthogonal partial least squares – discriminant analysis (OPLS-DA). These tests allow for the visualization and interpretation of datasets containing greater than 100 variables. PCA, which emphasizes variation between individual samples, is an unsupervised technique (Abdi & Williams, 2010; Worley & Powers, 2013). This means that groups and classes are undesignated, eliminating the tendency of statistical methods to over-fit data. Data points in

the PCA that are proximal or clustered share greater similarity than those separated by greater distances, making it ideal for identification of similar biological characteristics within grouped samples. A separation in the diagonal plane represents the greatest variation between variables, and PCA is particularly good at identifying outliers in a set of variables (Bjerrum, 2015).

PLS-DA is probably the most commonly used statistical representation of data in metabolomics studies. In contrast to PCA, PLS-DA and OPLS-DA are supervised techniques, meaning that groups are designated prior to analysis (Szymańska, Saccenti, Smilde, & Westerhuis, 2012). By designating groups, PLS-DA maximizes differences between entire sets, rather than individual samples or organisms (Ren, Hinzman, Kang, Szczesniak, & Lu, 2015). These methods can find the fundamental relations between two matrices; an X matrix containing independent variables, and a Y matrix containing dependent variables. By rotating the PLS-DA scores plot, OPLS-DA places the greatest variance in a single axis, gaining some apparent benefit over standard PLS-DA tests. Thus, these techniques are used to identify biomarkers and variances between different sample groups, such as healthy versus diseased (Bjerrum, 2015). A disadvantage of the supervised approach, however, is that PLS-DA and other similar tests are prone to over-fitting data, which may lead to misleading results (Westerhuis et al., 2008). For this reason, PLS-DA must be coupled with rigorous cross-validation and permutation testing to validate any results that are obtained (Szymańska et al., 2012).

Variable Selection

While it may be possible in ideal situations to base a significant metabolomic alteration on an entire fluidic metabolome, meaning that every detectable metabolite in a given bio-fluid has increased or decreased in relative concentration enough to call it significant, this is highly unlikely. In reality, only a subset of metabolites detectable by some instrument will be up- or down-regulated to any significant degree. This means that these variables must be identified and selected for further analysis. The simplest way to elucidate changes of significance across

variables would be to perform a univariate test, such as a Mann-Whitney U test, or a t-test (Goodpaster, Romick-Rosendale, & Kennedy, 2010). The results of such a test would indicate whether the average of some variable changed significantly between case and control, for example. While the simplest approach is usually the best, especially in cases of biology and statistics, univariate tests are not ideal for metabolomic experiments. This is largely because univariate testing does not account for any possible synergistic and/or compounding effects resulting from the interaction of many metabolites existing in a bio-fluid simultaneously. Another reason that univariate testing is not ideal is the high likelihood of false discoveries (Shaffer, 1995). With multiple, concurrent hypothesis tests occurring in a single experiment, p-values must be Bonferroni-corrected to reduce this false discovery rate.

Given the lack of statistical power in employing univariate methods to select important variables for metabolomics, multivariate tests are generally used. A more common multivariate approach to variable selection is to use the PLS-DA coefficient weightings combined with a Variable Importance in the Projection (VIP) plot (Chong & Jun, 2005). A variable of importance will have a high VIP score (greater than 1) if it significantly contributes to group separation in a PLS-DA analysis. This information is very useful because it directs subsequent functional analyses, along with another recently developed multivariate statistical method known as Variable Importance Analysis based on random Variable Combination (VIAVC).

VIAVC systematically resamples variables to reveal any synergistic effects that may exist between seemingly unimportant variables (Yun et al., 2015), which is unaccounted for in univariate statistical tests. By combining random permutations of variable inclusions with ten-fold cross validation, and by using the Area Under the Curve (AUC) of a Receiver-Operator Characteristic (ROC), VIAVC determines an optimal subset of variables that have led to the most substantial group differences. The combined use of these multivariate tests allows for researchers to dig deeper into the causes of significant metabolome alterations following external or environmental stimuli by providing insight into specific metabolite changes.

Functional Analysis

Following the identification and selection of important variables, functional analysis of these compounds can be performed using Metabolite Set Enrichment Analysis (MSEA) to identify functional pathways of interest that may be affected by alterations in metabolite regulation. In genomic and transcriptomic research, Gene Set Enrichment Analysis (GSEA) has been widely used to successfully analyze databases of predefined gene sets to rank lists of genes identified from microarray studies to identify significant and coordinated changes in gene expression (Xia & Wishart, 2010b). Like GSEA, MSEA helps researchers identify and interpret patterns of metabolite concentration changes in a biologically meaningful context. The key to MSEA is the use of established metabolite libraries, containing thousands of predefined metabolite sets covering various metabolic disease states, pathways, and tissue locations. For example, if a researcher identifies significant regulation disparities of phosphate, succinate, oxaloacetate, and citrate in a disease group compared to controls, MSEA will likely identify the glucose metabolism pathway, otherwise known as the Tricarboxylic Acid Cycle, as possibly involved as a response to the disease or insult. MSEA's main strength is its potential to help researchers identify both obvious and "subtle but coordinated" changes among a group of related metabolic compounds that may remain undetected using conventional approaches (Xia & Wishart, 2010b,2016).

Metabolic pathway analyses were also conducted, which combine pathway enrichment analysis with pathway topology to identify the most relevant pathways involved in study conditions (Xia & Wishart, 2010a). Data were entered as a list of metabolites, with the Human Pathway Library and ORA Algorithm selected, using a hypergeometric test and the Relative-Betweeness Centrality algorithm. These analyses in Metaboanalyst allow for pathway identification to discuss in relation to statistically significant metabolites.

APPLICATIONS OF ^{1}H NMR SPECTROSCOPY

^{1}H NMR has been widely employed in the growing field of metabolomics, and has greatly contributed to agricultural, veterinary, medical, and pharmacological fields in the search for biomarkers of disease (Pontes, Brasil, Cruz, de Souza, & Tasic, 2017). Methods have been developed for use in animal, human, and plant cohorts, which include the acquisition and preparation of many sample types, such as blood plasma, serum, urine, CSF, saliva, feces, and various tissues. The simplicity and versatility of NMR metabolomics makes it very attractive as a method for investigating phenotypical effects of environment-gene interactions and medical treatments or therapies.

Animal Models

Metabolomics studies involving animals are not restricted to only rodents such as rats and mice. There is, in fact, a whole menagerie of animals involved in metabolomic research, including shellfish (Viant, Rosenblum, & Tjeerdema, 2003), piglets (Fanos et al., 2014), and zebrafish (Li et al., 2016) to name a few. For example, Viant *et al.* (2003) assessed the effects of Withering Syndrome in red abalone (Haliotis rufescens), a type of shellfish native to the Pacific Coast, using tissue and hemolymph samples. This study used NMR metabolomics to successfully distinguish between biochemical profiles of controls, food-deprived red abalone, and infected red abalone groups in every type of tissue or bio-fluid studied.

In a second example, newborn piglets were used to investigate whether metabolomic profiles changed significantly according to oxygen concentration administered at resuscitation following perinatal asphyxia (Fanos et al., 2014). The authors found that after separating the piglets into four different oxygen groups, 21% O^2 was the most physiological and effective concentration for resuscitation. Here, urine metabolomes were used to compare and analyze pre- and post-hypoxia samples. In yet another study, metabolomics was used to characterize liver injury following acute arsenic toxicity in zebrafish using GC-MS (Li et al., 2016). Combining

GC-MS metabolomics with histological examination and pathway analyses, a series of significant alterations were seen, including apoptosis, glycogenolysis, accumulation of bile acids, disturbances in glycolysis-related energy metabolism, and changes in amino acid metabolism and fatty acid composition.

Animal studies of particular interest here include those that assess the effects of stress and perinatal environment on the metabolome. One study performed by Shi et al (2013) compared the blood plasma metabolomic profiles of three stress-based models of depression in rats by investigating the effects of acute and chronic stress on the production of endogenous metabolites. The most representative model of human stress was Chronic Unpredictable Mild Stress (CUMS), which was shown to affect glutamate, alanine, aspartate, lactate, valine, lipid metabolism, and inflammatory responses (Shi et al., 2013). These findings are highly translatable to human studies, since they show that animal stress responses are decidedly detectable in metabolomic profiles.

Human Models

Despite being animals, humans tend to fall into their own category in research. Over the past decade, there has been an explosion of literature using metabolomics in various applications involving humans, from drug discovery to diagnostics. On a population level, recent developments in metabolomics are helping to advance drug discovery and development, while on an individual level, these advances are improving the field of precision medicine (Wishart, 2016). For example, Stanley Hazen *et al*. (2011) have established a link between diet, the microbiome, and a plaque-inducing atherotoxin called trimethylamine N-oxide (TMAO) using a plasma metabolomics approach (Wang, Klipfell, et al., 2011). TMAO was determined to be a liver by-product of trimethylamine (TMA), a microbial breakdown product of carnitine, betaine, and choline, by performing targeted metabolomic studies (Koeth et al., 2013; Wang, Klipfell, et al., 2011; Wang et al., 2014). These metabolites are largely derived from meat and phospholipids in the diet. Rats injected with TMAO experienced a rapid increase in arterial plaques, and

subsequent studies in humans showed a strong correlation between high plasma TMAO and adverse myocardial events (Wang et al., 2014).

Probably one of the most widely investigated applications of metabolomics in human disease research has been in the field of oncometabolite discovery. Cancer is widely known as a genetic disease, arising from mutations in fundamental oncogenes or tumour suppressors (Wishart, 2016). A considerable paradigm shift has occurred over the past five years in our understanding of this disease, however, leading it to be viewed increasingly as a metabolic disorder involving disturbances in energy production through respiration and fermentation (Seyfried, 2012; Seyfried, Flores, Poff, & D'Agostino, 2014). The discovery of oncometabolites using metabolomics has substantially affected our understanding of cancer. An oncometabolite is an endogenous metabolite, and any accumulation of these compounds has been shown to initiate and sustain tumour growth and metastases (Wishart, 2016). The first oncometabolite to be discovered was 2-hydroxyglutarate, which is a natural metabolic compound found to be highly concentrated in gliomas (Ward et al., 2010). Metabolomics continues to be used as a method for identifying or reclassifying many other oncometabolites.

As far as metabolic disorders go, Type II diabetes is well-known, although its causes remain poorly understood. A diet rich in simple sugars and an inactive lifestyle can lead to disease development, but individuals who are physically fit can still develop the disease, suggesting that it may have a genetic component (Hu et al., 2001). Several groups over the past 4 years, however, have used both targeted and untargeted metabolomic techniques to identify an unanticipated causal agent – amino acids (Wishart, 2016). High serum levels of branched-chain amino acids (BCAAs; isoleucine, leucine, and valine), as well as the aromatic amino acids phenylalanine and tyrosine, and a little-known compound called 2-aminoadipic acid can be used to identify individuals at risk of developing type II diabetes (Neis, Dejong, & Rensen, 2015; Palmer et al., 2015; Wang, Larson, et al., 2011; Wang et al., 2013; Wurtz et al., 2013). Wang et al (2011) show that these markers are relevant up to fifteen years prior to disease onset and have been found to be

significantly more predictive than genome-wide association studies. BCAAs are insulin analogues, which specifically act on the mammalian target of rapamycin (mTOR) receptor to up-regulate the same pathways and physiological processes as insulin (Li et al., 2013; Wang, Larson, et al., 2011; Wishart, 2016). Chronically high levels of an insulin analogue could potentially lead to insulin resistance and diabetes.

While this is only a sample of the applications of metabolomics in human disease discovery and diagnosis, the approach continues to effectively rewrite our current knowledge of the etiology of many diseases. Some others include Parkinson's disease (Ren, Sun, Zhao, & Pu, 2015), concussion (Daley et al., 2016; Paxman et al., 2017), preeclampsia and gestational hypertension (Austdal et al., 2015), cognitive impairment or Alzheimer's disease (Mapstone et al., 2014), and various forms of cancer (Chan et al., 2016; Seyfried et al., 2014; Wang, Chen, & Jia, 2016). Many of these diseases are now characterized in the Human Metabolome Database (Wishart et al., 2013; Wishart et al., 2009; Wishart et al., 2007). In almost all cases, endogenous metabolites appear to function as direct toxins or signaling molecules that cause a cascade of adverse consequences, leading to some disease phenotype (Wishart, 2016).

Plant Models

With the growing interest of using metabolomic technologies in a wide range of biological focuses, food applications related to nutrition and quality are rapidly emerging (Hall, Brouwer, & Fitzgerald, 2008). The emergent interest in plant metabolomics stems from its potential to broaden our current understanding of plant metabolism and biochemical composition, and to apply this to a variety of fields, particularly plant-based nutrition. The ultimate goal here is to understand the role of exogenous metabolites in human metabolic regulation (Gibney et al., 2005).

Metabolomic applications in crop and food analysis are continually growing, using LC-MS and NMR spectroscopy to gain insight into the variation in food composition in terms of

quality and nutrition (Hall et al., 2008). For example, metabolomic profiling of tomato genotypes revealed extensive differences in metabolic composition of sugars, amino acids, and organic acids, despite close genotypic similarities (Carrari et al., 2006). The quality of crop plants, nutritionally or otherwise, is a direct function of metabolite content (Memelink, 2005), making metabolomic investigation imperative for designing new concepts for targeted crop improvement and to guide future development of food products (Rist, Wenzel, & Daniel, 2006).

Other applications in plant metabolomics include the classification and characterization of different species of medicinal plants, such as Cannabis (Choi, Kim, et al., 2004), Ephedra (Kim, Choi, Erkelens, Lefeber, & Verpoorte, 2005), and Ginseng (Yang et al., 2006); monitoring the stress response and infection in plants, such as Arabidopsis (Hendrawati et al., 2006); and discrimination of different plant genotypes in Tobacco (Choi, Choi, et al., 2004), Tomato (Le Gall, Colquhoun, Davis, Collins, & Verhoeyen, 2003), and Maize (Manetti et al., 2004). Apart from animal, human, and plant studies, metabolomics has also found applications in other diverse areas, such as microbiology, food chemistry, and environmental monitoring (Wishart, 2016).

CHAPTER 3: PRENATAL MATERNAL STRESS ALTERS URINARY METABOLOMIC PROFILES IN HUMANS FOLLOWING A NATURAL DISASTER

INTRODUCTION

Stress during pregnancy has been shown to have significant effects on offspring physiology and behaviour (Charil et al., 2010; Harris & Seckl, 2011; King & Laplante, 2005; Yong Ping et al., 2015). Abnormally high levels of circulating maternal glucocorticoids and increased placental corticotropin-releasing hormone may cross the placenta to reach the fetal brain, leading to dysregulation of hypothalamic-pituitary-adrenal (HPA) axis activity and altered neuronal development (Harris & Seckl, 2011; Hayashi et al., 1998). HPA axis programming due to fetal glucocorticoid overexposure has potential lifetime consequences as a risk factor for complex adult diseases, including cardio-metabolic diseases (Khulan & Drake, 2012), altered glucose and insulin metabolism, and adiposity (Dancause et al., 2013; Reynolds, 2010).

The association between HPA axis function and risk of metabolic diseases, such as diabetes and obesity under fetal adversity, may be causally linked to altered genetic and epigenetic regulation. The Common Disease Genetic and Epigenetic hypothesis argues that epigenetics provides an additional layer of variation, mediating the relationship between genotype and internal and external environments (Feinberg, 2007). Accordingly, prenatal maternal stress (PNMS) may alter DNA methylation (Cao-Lei et al., 2014; Meaney & Szyf, 2005) and microRNA signatures in various tissues in exposed offspring (Hollins & Cairns, 2016; Zucchi et al., 2013). Genetic and epigenetic regulation affects cellular functions that ultimately will reflect an altered metabolic output. Hence, the metabolome, i.e., the sum of all metabolites in an organism, arguably represents a direct measure of environment-gene interactions and associated phenotypes (Bouatra et al., 2013).

Aside from primarily metabolic disorders, the majority of other human diseases also involve abnormal metabolic states that reflect altered cellular processes (Feinberg, 2007). For example, it has been proposed that adaptation to chronic stress facilitates visceral fat accumulation (Drapeau, Therrien, Richard, & Tremblay, 2003). Hervey's theory suggests that fat cells take up and catabolize glucocorticoids as a regulatory effect, justifying body fat gain as a

response to stress (Hervey, 1959). In the Project Ice Storm cohort, Dancause et al (2012) (Dancause et al., 2012) found an increased risk of obesity in 5½-year-old children of mothers who had experienced a high level of disaster-related stress during pregnancy, which persisted into adolescence (Liu, Dancause, Elgbeili, Laplante, & King, 2016). Studying natural disasters, or independent, randomly-distributed stressors, such as exposure to the 1998 Quebec Ice Storm, allows for the isolation of the mother's objective degree of exposure from genetic predispositions, socioeconomic biases, and from her own subjective level of distress (Cao-Lei et al., 2014).

The goals of the present study were (a) to detect downstream metabolomic effects in male versus female adolescents resulting from prenatal maternal stress exposure using proton nuclear magnetic resonance (1H NMR) spectroscopy; (b) to differentiate between adolescents exposed *in utero* to either high or low levels of PNMS based on presence and quantity of small-molecule metabolites in urine; and (c) to identify a subset of significantly altered metabolites and metabolic pathways that may be used to predict and determine future health status and disease risk in the study cohort. We hypothesized (i) that normal (low-stress) and pathological (high-stress) phenotypes can be clearly identified by profiling small-molecule metabolites in urine; and (ii) that urinary metabolites will be significantly altered in adolescents of mothers who experienced high subjective and/or objective stress during the natural disaster, compared to those who experienced low subjective and/or objective stress. Such metabolic signatures linked to perinatal programming of disease phenotypes may provide novel prognostic and diagnostic biomarkers and targets for therapeutic strategies.

MATERIALS AND METHODS

Study Design

Sample Development

During the first week of January 1998, the southern region of the Canadian province of Quebec experienced a severe ice storm that decimated the power grid, leaving more than 1.5 million households without electricity for up to 45 days. Damage from the storm made it the costliest disaster in Canadian history and was responsible for 27 deaths.

To identify women who met criteria for inclusion into the study (pregnant on January 9, 1998 or became pregnant during the following 3 months after the storm, at least 18 years of age, and spoke fluent French), physicians who delivered babies from four regional hospitals were asked to mail out an initial recruitment questionnaire to their patients on June 1, 1998. Approximately 15% of women responded (224 of 1,440), of which 178 consented to further contact. These mothers and their children have subsequently been assessed several times: at the ages of 6 months, 2, 4, 5½, 8½, 9½, 11½, 13½, 16½, and 18½ years. Many families have been lost to follow-up over the years. The participants in the present study included 18 males and 14 females at the age of 16-17 years.

Assessment of Objective Hardship and Subjective Distress

Objective hardship was estimated using the mothers' responses to questions about their ice storm experiences from categories of exposure used in other disaster studies: Threat, Loss, Scope, and Change (Bromet & Dew, 1995). Each natural disaster presents unique experiences to the exposed population. Therefore, questions pertaining to each of the four categories were specific to events that occurred during the ice storm (see Laplante (2007) for a detailed description of the items). Each dimension was scored on a scale of 0 – 8, ranging from no exposure to high exposure. A total objective stress score (STORM32) was calculated by summing scores from all four dimensions using McFarlane's approach (McFarlane, 1988). Each dimension

was weighted equally to obtain the total score of our scale because there was no theoretical basis to believe that any one of the four dimensions of exposure has a greater effect than the others.

The mothers' subjective distress related to the ice storm was assessed using a validated French adaptation (Brunet, St-Hilaire, Jehel, & King, 2003) of the widely used Impact of Event Scale – Revised (IES-R) (Weiss & Marmar, 1997). The 22-item instrument provides scores for symptoms in three scales relevant to post-traumatic stress disorder: Intrusive Thoughts, Hyperarousal, and Avoidance (Weiss & Marmar, 1997). Scale items were written to reflect the mothers' symptoms relative to the ice storm crisis, for example: "When I think about the ice storm, my heart beats faster", or "Images of the ice storm suddenly appear in my thoughts." Participants responded on a 5-point (0 – 4) Likert scale, from 'not at all' to 'extremely', the extent to which the behavior describes how they felt over the preceding seven days. We used the total score in all analyses.

Metabolomics Assessment

Sample Collection and Preparation

Thirty-two urine samples were obtained from male (n = 18) and female (n = 14) adolescents (aged 16 to 17 years) from the Project Ice Storm cohort. Urine samples were collected between January 17 and May 9, 2015, by the study participants in their own residences, using an aseptic technique. Study participants were instructed to obtain the sample midstream at their first passage of the day, which were subsequently stored at -80°C until further processing. One female sample was removed after discovering elevated levels of glucose in the urine (see Chemical Examination of Urine below), which indicates that the participant may have diabetes.

To prepare for NMR spectroscopy, samples were thawed at room temperature and 450 µL aliquots were transferred into 1.5 mL centrifuge tubes, with 150 µL of phosphate buffer (see Buffer Preparation below) (Beckonert et al., 2007b). The sample/buffer solution was vortexed and then centrifuged at 12,000 g for 5 minutes at 4°C to precipitate and pellet any particulate matter.

Following centrifugation, 550 µL of supernatant was transferred to a 5-mm high-quality glass NMR tube (Wilmad, SP Industries, PA, USA) to be analyzed immediately.

Chemical Examination of Urine

Commercial reagent strips were used for chemical analysis of urine samples, to screen for bacteria resulting from improper sample storage, as well as confounding diseases in study participants. The Chemstrip 9 (Roche Diagnostics, Indianapolis, IN, USA) was used to detect presence of leukocytes, nitrite, protein, glucose, ketones, urobilinogen, bilirubin, blood, hemoglobin, and to confirm pH value of the pre-buffered sample. Urine was pipetted onto the reagent-impregnated test pads, excess removed, and read within 120 seconds, following the manufacturer's instructions.

Buffer Preparation

Urine pH value can affect the stability of formed elements and metabolites in urine. Any sample obtained, therefore, must be buffered to a consistent pH value. Phosphate urine buffer was prepared as a 4:1 ratio of KH_2PO_4:K_2HPO_4 in a 4:1 H_2O:D_2O solution to obtain a final concentration of 0.5 M. The D2O contained 0.05% v/v trimethylsilyl propanoic acid (TSP) as a chemical shift reference for ^1H NMR spectroscopy. 0.03% w/v of sodium azide (NaN_3) was also added to the buffer as an antimicrobial agent to maintain metabolite integrity. Buffer pH was checked after preparation and titrated to 7.4 using 3M HCl.

NMR Data Acquisition and Processing

Spectra were collected on a 700 MHz Bruker Avance III HD spectrometer (Bruker, ON, Canada). The 1-D NOESY gradient water suppression pulse sequence noesygpr1d was used (Bruker). Each sample was run for 128 scans to a total acquisition size of 128k. The spectra were

zero filled to 256k, automatically phased and baseline corrected, and line-broadened by 0.3 Hz. The processed spectra were then exported to MATLAB (MathWorks, MA, USA) for statistical analysis and binning. Spectra were binned using Dynamic Adaptive Binning, developed by Anderson et al. (2011) to reduce the size of the dataset. The resulting bins were subsequently inspected and modified as required. This method of binning is meant to account for natural drift that occurs in the chemical shift of urine, due to ionic concentration effects.

Each spectrum was normalized to remove effects of imperfect water signal suppression, and spectral regions corresponding to water and urea were removed. The dataset was then Pareto-scaled (mean-centered and divided by the square root of each variable's standard deviation) to reduce the influence of intense peaks, while emphasizing weaker ones. All peaks were referenced to TSP (0.00δ).

Statistical Analysis

Metabolite bins were first analyzed and deemed significant or non-significant using the decision tree algorithm outlined by Goodpaster et al. (2010). In all cases, this algorithm tested for data normality and concluded by running a Mann-Whitney U test. Data visualization to determine sample structure and presence of distinct groups within the dataset was conducted using Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA). These tests were performed using the online chemometrics software, Metaboanalyst (Xia, Broadhurst, Wilson, & Wishart, 2013; Xia, Mandal, Sinelnikov, Broadhurst, & Wishart, 2012; Xia, Psychogios, Young, & Wishart, 2009; Xia, Sinelnikov, Han, & Wishart, 2015; Xia, Sinelnikov, & Wishart, 2011; Xia & Wishart, 2002,2010a,2010b,2011,2016). Double cross-validation and permutation testing (minimum 1,000 iterations) were performed to verify and support all statistically significant PLS-DA results. Variable Importance in the Projection (VIP) plots were subsequently made using the weighted sum of squares of the PLS loadings, based on explained Y-variance in each dimension.

Variable Importance Analysis based on random Variable Combination (VIAVC) was used as a feature selection method to aid in this analysis (Yun et al., 2015). VIAVC provides a method for systematically resampling variables to determine whether synergistic effects exist between seemingly unimportant variables. The algorithm combines random permutations of variable inclusion with a ten-fold cross validation of models to determine the optimal subset of variables providing the most information about group differences. It should be mentioned that, although the VIAVC script uses cross validation, the structure of the algorithm produces results that are no different from the double cross-validation (DCV) method suggested for validation of metabolomics datasets (Szymańska et al., 2012; Westerhuis et al., 2008). Reported p-values were calculated from a t-test of distribution scores based on whether a metabolite was included in the model. All VIAVC tests were carried out using MATLAB. A more detailed description of this method is available by Yun et al. (2015).

Biological significance of important metabolites was investigated using the Metabolite Set Enrichment Analysis (MSEA) and the Over-Representation Analysis (ORA) available through Metaboanalyst. Sets of predefined metabolic pathways were used by MSEA to recognize significant and meaningful changes in functionally related metabolites within a biologically pertinent context (Xia & Wishart, 2010b). Data were entered into MSEA as a list of metabolic compounds by name, and the ORA algorithm was used. Metabolic pathway analyses were also conducted, which combine pathway enrichment analysis with pathway topology to identify the most relevant pathways involved in study conditions (Xia & Wishart, 2010a). Data were entered as a list of metabolites, with the Human Pathway Library and ORA Algorithm selected, using a hypergeometric test and the Relative-Betweeness Centrality algorithm. These analyses in Metaboanalyst allow for pathway identification to discuss in relation to statistically significant metabolites.

Metabolite Identification

A spectral database of pure metabolite substances was generated and used to identify most metabolites found in NMR spectra. The Human Metabolome Database (Wishart et al., 2013; Wishart et al., 2009; Wishart et al., 2007) was used to supplement and aid the creation of our spectral library, and to identify some substances that were not obtained for the creation of this database. Furthermore, tools for identifying and quantifying NMR metabolites were used in the Chenomx 8.2 NMR Suite (Chenomx Inc., Edmonton, Alberta, Canada) which allowed for spectral deconvolution of biofluid samples into individual components.

RESULTS

For this project, 32 subjects were separated into High and Low stress groups using a median split for both objective hardship (STORM32) and subjective distress (IES-R); for objective hardship, there were 17 high stress (9 males, 8 females) and 15 low stress (9 males, 6 females), and for subjective distress there were 16 high stress (9 males, 7 females) and 16 low stress (9 males, 7 females).

Exploratory Statistical Analysis

Both unsupervised (PCA) and supervised (PLS-DA) chemometric tests were used to identify adolescents exposed *in utero* to high or low levels of PNMS based on significantly altered urinary metabolites. In males, the unsupervised separation was less apparent (Figure 7A and 7B), particularly when they were split by their level of objective hardship exposure (Figure 7B).

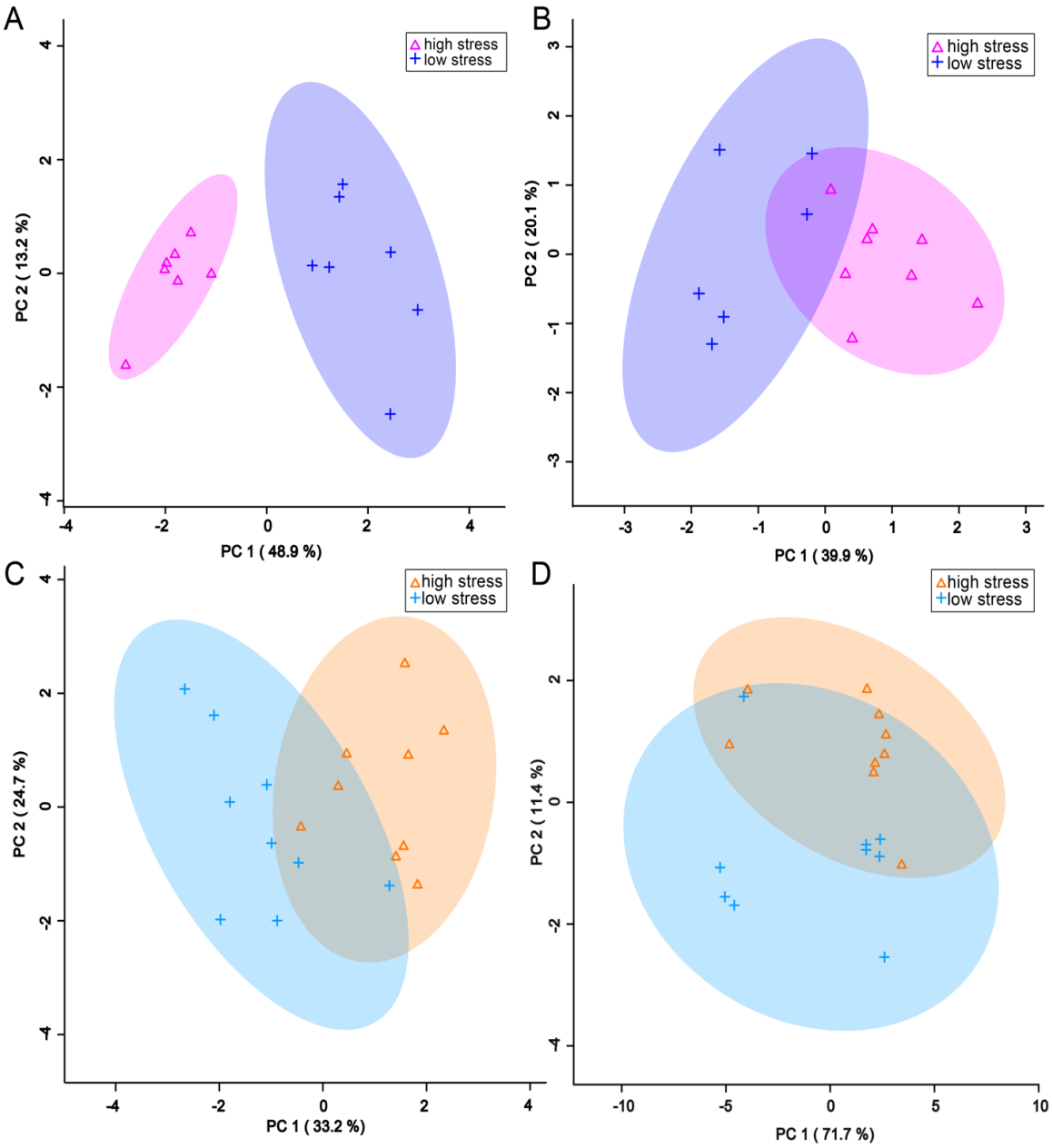

Figure 8. Principle Component Analysis (PCA) plots showing unsupervised separation for male (A and B) and female (C and D) adolescents exposed in utero to high or low levels of PNMS. A/C: Subjective distress measured by the Impact of Events Scale – Revised (IES-R) in mothers exposed to stress during pregnancy. B/D: Objective hardship measured by the Storm 32 survey (ST32) in mothers exposed to stress during pregnancy. Each triangle or cross represents one individual under study, plotted using a list of urinary metabolites found to be statistically significant via a Mann-Whitney U test. Coloured ellipses represent 95% confidence intervals. X and Y axes show principal components 1 and 2, respectively, with brackets indicating percent variance between groups.

However, the supervised groups separated very well (Figure 9A and 9B). For females, clear unsupervised separation was observed when the sample was split by *in utero* exposure to levels of subjective distress and objective hardship (Figure 8C and 8D). Well-defined supervised separation for females was also observed (Figure 9C and 9D). Double cross-validation and

permutation tests validated the observed supervised separation results as a function of subjective distress (male p=0.034, female p<0.001) and objective hardship exposure levels (male p=0.01, female p=0.011).

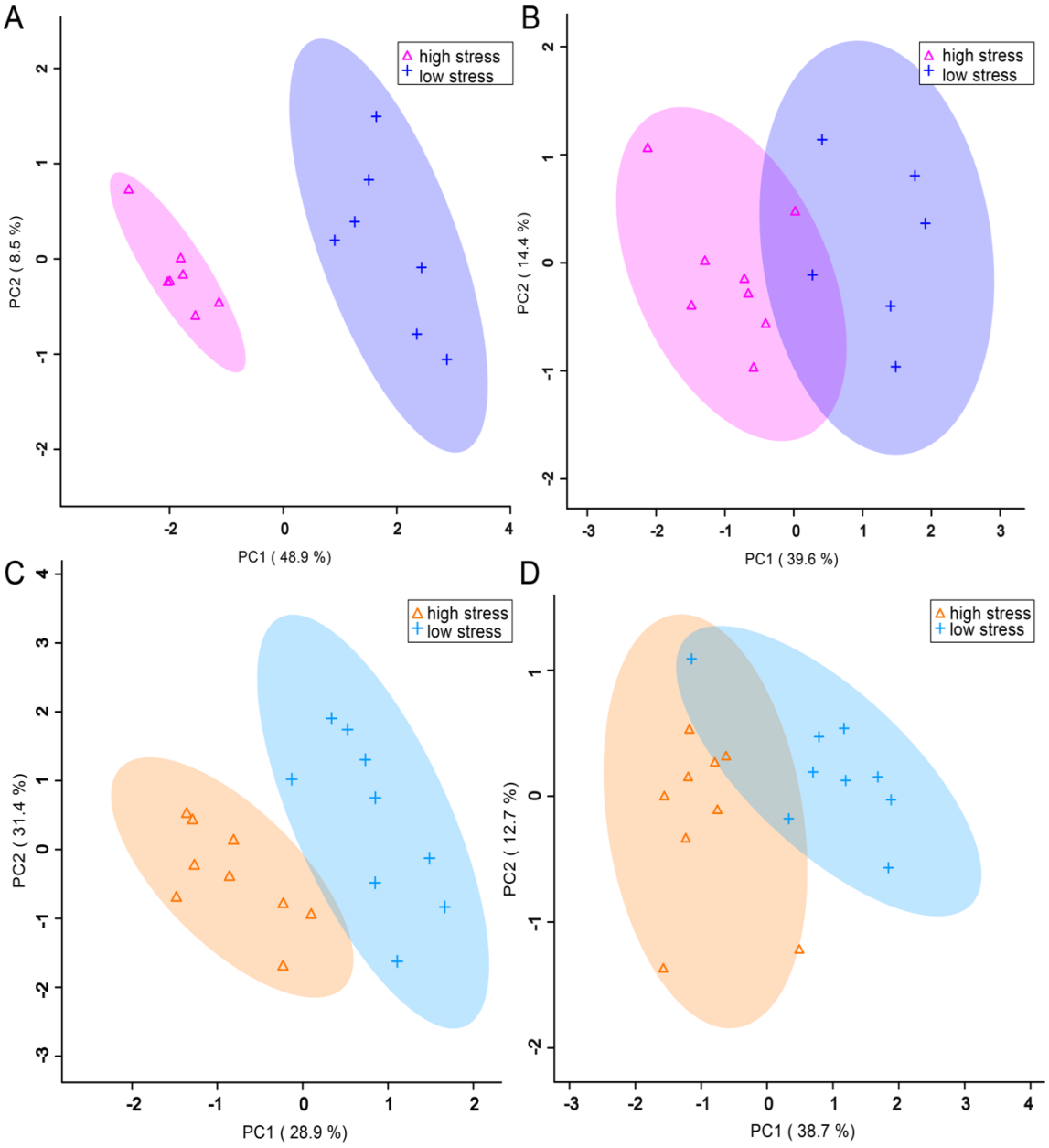

Figure 9. Partial Least Squares – Discriminant Analysis (PLS-DA) plots showing supervised separation for male (A and B) and female (C and D) adolescents exposed in utero to high or low levels of PNMS. A/C: Subjective distress measured by the Impact of Events Scale – Revised (IES-R) in mothers exposed to stress during pregnancy. B/D: Objective hardship measured by the Storm 32 survey (ST32) in mothers exposed to stress during pregnancy. Each triangle or cross represents one individual under study, plotted using a list of urinary metabolites found to be statistically significant via a Mann-Whitney U test. Coloured ellipses represent 95% confidence intervals. X and Y axes show principal components 1 and 2, respectively, with brackets indicating percent variance between groups.

Variable Importance in Projection (VIP) plots (Figure 10) show which variables contributed most to significant supervised separation results, while the accompanying heat maps indicate whether separation resulted from up- or down-regulation of that metabolite. It should be noted that some compounds on the y-axis contain multiple metabolites separated by commas – this indicates that the ^{1}H NMR spectrum showed overlap of those metabolites at the same chemical shift. In males, the metabolites contributing most to the separation when subjective distress was included in the analysis were 1,7-dimethylxanthine, malonate, and 2-hydroxyisobutyrate (Figure 10A), with VIP scores of 1.47, 1.35, and 1.15, respectively (Table 1). Metabolites that contributed most to the separation when objective hardship was included in the analysis were the 3-hydroxymandelate/3-chlorotyrosine cluster and the 2-aminobutyrate/2-ethylacrylate/leucine cluster (Figure 10B), with VIP scores of 1.39 and 1.31, respectively (Table 1). In females, the top three compounds that contributed most to separation when subjective distress was included in the analysis were adenine, hypoxanthine, and 3-methyladipate/isocaproate (Figure 10C), with VIP scores of 1.65, 1.65, and 1.50, respectively, as shown in Table 2. The top three compounds contributing to the separation when objective hardship was included in the analysis (Figure 10D) were malonate, 2-hydroxy-3-methylvalerate, and adenine with VIP scores of 1.71, 1.18, and 1.04, respectively (Table 2).

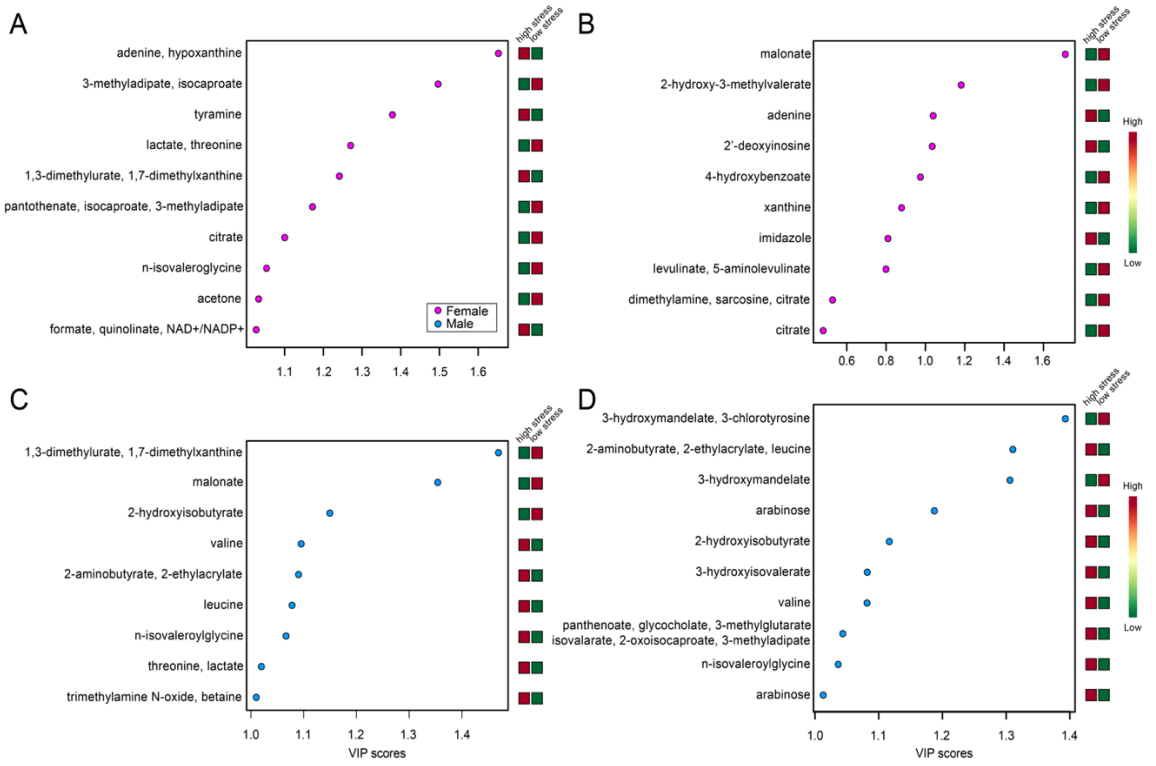

Figure 10. Variable Importance in Projection (VIP) plots of subjective distress and objective hardship comparison groups, showing the relative contribution of metabolites to the variance between high- and low-stress groups in the Project Ice Storm human stress cohort. A: Female subjective distress. B: Female objective hardship. C: Male subjective distress. D: Male objective hardship. High VIP values indicate greater contribution of these metabolites to group separation, shown in PLS-DA plots. Green and red boxes to the right indicate whether the relative metabolite concentration decreased or increased, respectively, in the human urine metabolome of offspring of high-stressed versus low-stressed mothers. A VIP score of 1.0 is considered able to discriminate between two phenotypes.

Functional Analysis

Figures 11-12 show metabolite sets that are most significantly implicated by the identified compounds, with significant variables agglomerated to compare total effects of high stress on both males and females. These metabolic sets are predicted to be changed in the case of dysfunctional enzymes, using a genome-scale network model of human metabolism. High PNMS exposure in male adolescents affected leucine, isoleucine, and valine biosynthesis significantly ($p < 0.01$) (Figure 11A), which are the branched chain amino acids (BCAA). Additionally, numerous energy metabolism systems were affected, including pathways in the Citric Acid cycle ($p = 0.0014$) and gluconeogenesis ($p = 0.005$) (Figure 11B).

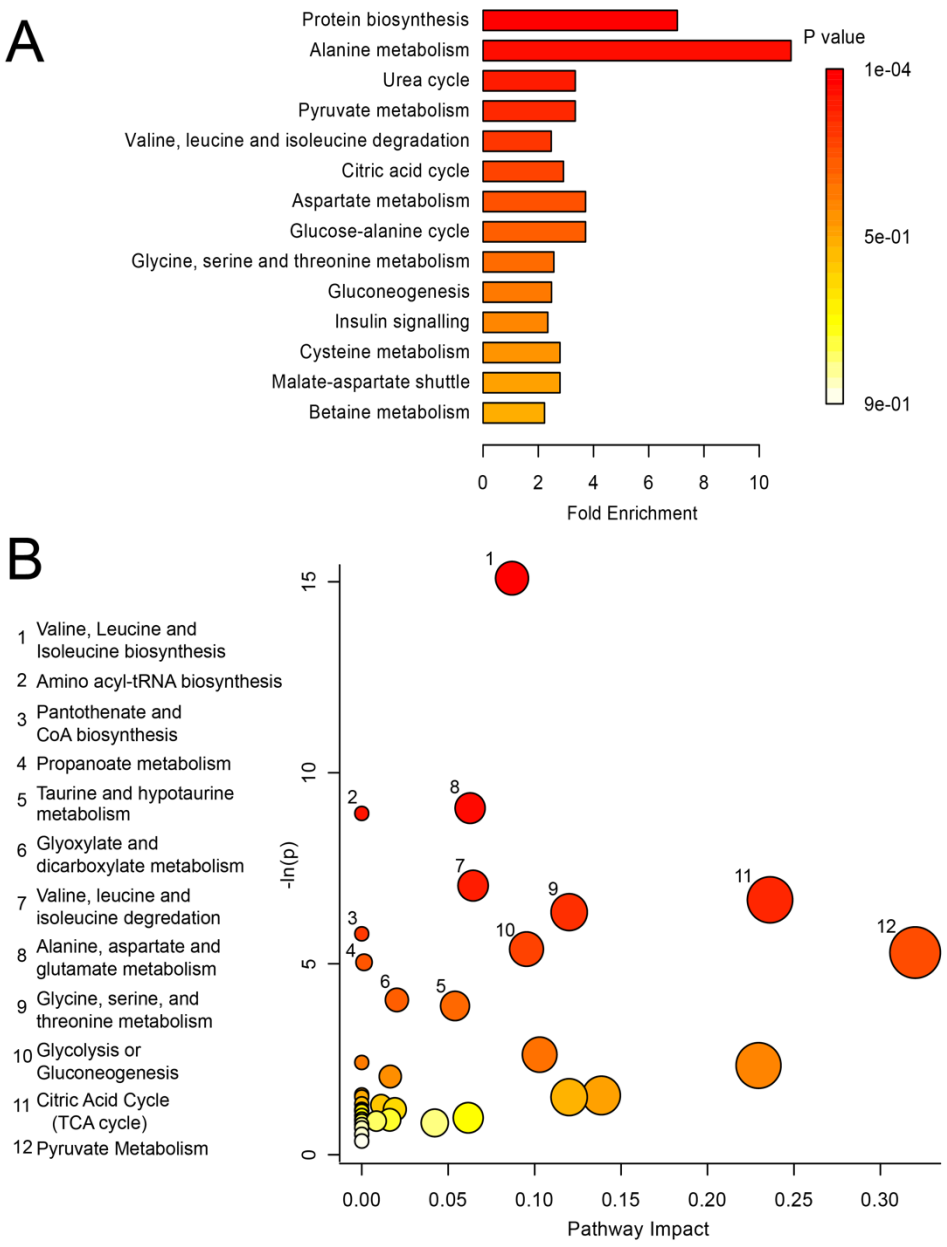

Figure 11. A: Pathway-Associated Metabolite Set Enrichment Analysis (MSEA) plot showing affected biological processes in male offspring of stressed mothers, based on metabolites identified as significantly altered between high- and low-stress groups. A library containing 912 metabolic sets, predicted to be changed in the case of dysfunctional enzymes using a genome-scale network model of human metabolism was used to acquire these data. Fold enrichment shows how many times greater than chance the process is involved. B: Metabolomic Pathway Analysis showing all matched pathways according to p-values from pathway enrichment analysis and pathway impact values from pathway topology analysis in males. Y-axis shows the negative natural log of p, such that a higher value on the y-axis gives a lower p-value. The x-axis gives the Pathway Impact, which correlates to the number of metabolite hits in a particular pathway. Only metabolic pathways with p<0.05 are labeled.

High PNMS exposure in females also had the greatest impact on protein biosynthesis ($p < 0.01$), as well as nucleotide sugar metabolism ($p < 0.01$) and ketone body metabolism ($p = 0.016$)

(Figure 5A). This is supported by the pathway topology analysis (Figure 12B), showing significant effects on valine, leucine, and isoleucine/BCAA biosynthesis/degradation ($p < 0.01$), propanoate metabolism ($p = 0.0016$), and ketone body biosynthesis/degradation ($p = 0.0031$).

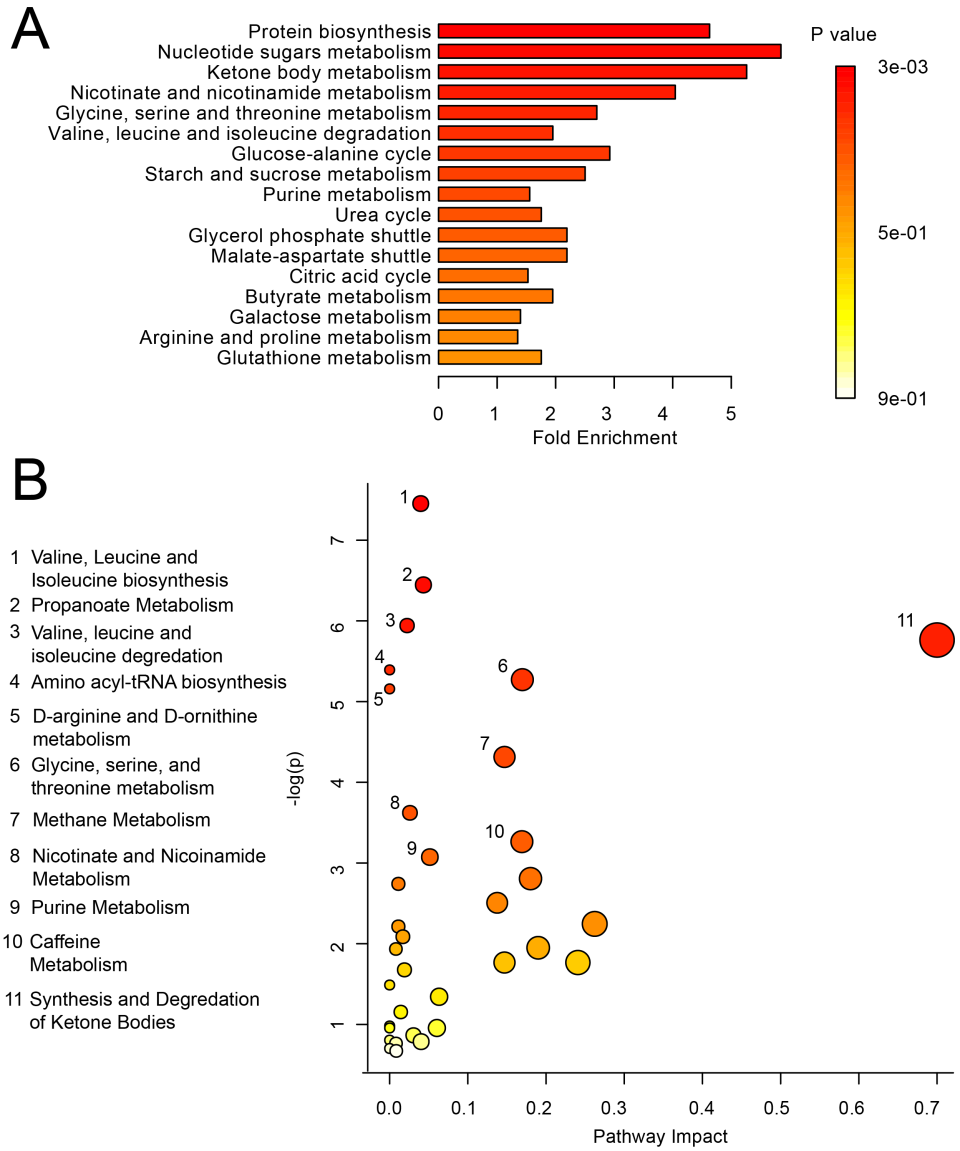

Figure 12. A: Pathway-Associated Metabolite Set Enrichment Analysis (MSEA) plot showing affected biological processes in female offspring of stressed mothers, based on metabolites identified as significantly altered between high- and low-stress groups. A library containing 912 metabolic sets, predicted to be changed in the case of dysfunctional enzymes using a genome-scale network model of human metabolism was used to acquire these data. Fold enrichment shows how many times greater than chance the process is involved. B: Metabolomic Pathway Analysis showing all matched pathways according to p-values from pathway enrichment analysis and pathway impact values from pathway topology analysis in females. Y-axis shows the negative natural log of p, such that a higher value on the y-axis gives a lower p-value. The x-axis gives the Pathway Impact, which correlates to the number of metabolite hits in a particular pathway. Only metabolic pathways with $p<0.05$ are labeled.

Table 1. P-values of urinary metabolites found to be significant in male subjective distress and objective hardship groups (IESR and ST32, respectively) in either a Mann-Whitney U test, the Variable Importance Analysis based on random Variable Combination (VIAVC), or both. VIP scores, shown in descending order, correspond to figures 9A and 9B. Metabolites with multiple peaks are represented as metabolite.1, metabolite.2… metabolite.n.

Group	Metabolite	Mann-Whitney U Test	VIAVC	VIP Score	Regulation
Male IESR	1,3-Dimethylurate	-	2.94E-88	1.47	Up
	Malonate	-	2.17E-53	1.35	Up
	2-Hydroxybutyrate.1	3.99E-03	1.68E-09	1.15	Down
	Valine.1	1.85E-02	5.19E-01	1.1	Down
	Isoleucine.1	1.88E-02	2.37E-04	1.09	Down
	Leucine.1	2.76E-03	2.27E-07	1.08	Down
	N-Isovaleroylglycine	3.99E-03	5.23E-06	1.07	Down
	Threonine, Lactate	3.99E-03	1.59E-10	1.02	Down
	Trimethylamine N-Oxide, Betaine	4.00E-02	4.99E-17	1.01	Down
	2,3,4-Trihydroxybenzoate, 2,6-Dihydroxybenzoate	1.88E-02	1.78E-03	0.96	Down
	Leucine.2	1.42E-02	2.96E-03	0.96	Down
	5-Hydroxyindole-3-Acetate	-	3.54E-01	0.94	Down
	Leucine.3	2.43E-02	1.93E-04	0.94	Down
	Valine.2	3.15E-02	2.01E-02	0.92	Down
	1,3-Dimethylurate	4.00E-02	4.24E-13	0.89	Up
	Alanine	-	1.35E-01	0.88	Down
	3-Hydroxy-3-Methylglutarate	3.15E-02	2.53E-12	0.87	Down
	Panthenoate, Isovalerate.1	1.06E-02	1.26E-03	0.84	Down
	Isoleucine.2	1.42E-02	1.04E-03	0.8	Down
	Isovalerate.2, 3-Methyladipate	2.44E-02	1.35E-02	0.76	Down
	2-Hydroxybutyrate.2	4.00E-02	1.89E-02	0.75	Down
	3-Hydroxyisovalerate	-	1.49E-03	0.66	Down
	Betaine	-	8.35E-15	0.64	Down
Male ST32	3-Hydroxymandelate.1, 3-Chlorotyrosine	3.99E-03	1.40E-05	1.39	Up
	3-Hydroxymandelate.2	3.99E-03	1.27E-04	1.31	Up
	Isoleucine.1	5.64E-03	2.04E-03	1.31	Down
	Arabinose.1	1.42E-02	1.52E-03	1.19	Down
	2-Hydroxybutyrate	1.42E-02	7.71E-06	1.12	Down
	3-Hydroxyisovalerate	3.15E-02	3.63E-08	1.08	Down
	Valine	3.15E-02	1.50E-06	1.08	Down
	N-Isovalerylglycine	1.88E-02	1.50E-07	1.04	Down
	Panthenoate, Isovalerate, 3-Methyladipate	5.64E-03	6.54E-04	1.04	Down
	Arabinose.2	1.42E-02	4.31E-05	1.01	Down
	Leucine.1	3.15E-02	3.01E-04	0.96	Down
	Isoleucine.2	2.44E-02	4.87E-07	0.9	Down
	Leucine.2	3.99E-03	7.65E-02	0.88	Down
	Pyruvate, Succinate, Oxalacetic Acid	3.15E-02	3.30E-07	0.84	Down
	Isoleucine.3, Glycocolate.2	2.44E-02	3.27E-02	0.79	Down
	N6-Acetyllysine	3.99E-03	1.29E-03	0.65	Down
	3-Indoxylsulfate, Tryptophan	-	3.10E-03	0.36	Down
	Methylsuccinate	3.99E-03	1.00E+00	0.25	Up

Table 2. P-values of urinary metabolites found to be significant in female subjective distress and objective hardship groups (IESR and ST32, respectively) in either a Mann-Whitney U test, the Variable Importance Analysis based on random Variable Combination (VIAVC), or both. VIP scores, shown in descending order, correspond to figures 10C and 10D. Metabolites with multiple peaks are represented as metabolite.1, metabolite.2… metabolite.n.

Group	Metabolite	Mann-Whitney U Test	VIAVC	VIP Score	Regulation
Female IESR	Hypoxanthine, Adenine	2.62E-02	3.73E-83	1.65	Down
	3-Methyladipate.1	5.83E-04	1.65E-80	1.5	Up
	Tyramine	1.11E-02	1.44E-17	1.38	Down
	Lactate, Threonine	4.08E-03	5.10E-59	1.27	Up
	1,3-Dimethylurate	2.62E-02	1.87E-42	1.24	Down
	Pantothenate.1, 3-Methyladipate.2	2.33E-03	3.30E-62	1.17	Up
	Citrate	3.79E-02	5.04E-02	1.1	Up
	N-Isovalerylglycine	1.11E-02	1.98E-13	1.05	Up
	Formate, Quinolinate, NAD+/NADP+	1.75E-02	7.69E-05	1.03	Down
	Acetone	1.11E-02	2.50E-14	1.03	Up
	Leucine	2.62E-02	5.45E-08	0.99	Up
	Isoleucine.1, Valine	6.99E-03	1.80E-07	0.95	Up
	2-Hydroxybutyrate.2	6.99E-03	4.04E-12	0.91	Up
	Pantothenate.2	6.99E-03	1.20E-02	0.9	Up
	Acetoacetate	1.11E-02	1.66E-18	0.89	Up
	UDP-Galactose, UDP-Glucose, UDP-Glucuronate	4.08E-03	2.26E-18	0.85	Up
	3-Hydroxyisovalerate	2.62E-02	1.40E-06	0.82	Up
	3-Methyladipate.3, 4-Pyridoxic Acid	1.75E-02	1.50E-01	0.79	Up
	3-Methyladipate.4	6.99E-03	2.35E-03	0.79	Up
	2-Hydroxybutyrate.2	1.11E-02	1.26E-05	0.77	Up
	Isoleucine.2	1.75E-02	1.19E-01	0.76	Up
	Trimethylamine N-Oxide, Betaine	3.79E-02	2.17E-05	0.74	Down
	Succinylacetone, 5-Aminolevulinate, Levulinate	2.62E-02	1.78E+00	0.74	Up
	Singlet At 1.67ppm	1.11E-02	2.91E-02	0.74	Up
	3-Methyladipate.5	3.79E-02	9.09E-08	0.71	Up
	Arginine	6.99E-03	5.80E-29	0.69	Up
	3-Methyladipate.6	2.62E-02	3.57E-03	0.62	Up
Female ST32	Malonate	1.27E-02	5.05E-138	1.71	Up
	2-Hydroxy-3-Methylvalerate	2.93E-02	1.31E-07	1.18	Up
	Adenine	2.94E-02	3.66E-04	1.04	Down
	2'-Deoxyinosine	-	1.07E-05	1.03	Down
	4-Hydroxybenzoate	2.00E-02	1.51E-02	0.97	Up
	Xanthine	4.26E-02	1.29E+00	0.88	Up
	Imidazole	2.93E-02	1.99E-02	0.81	Down
	Succinylacetone, 5-Aminolevulinate, Levulinate	4.26E-02	1.52E+00	0.8	Up
	Dimethylamine, Sarcosine, Citrate.1	-	7.86E-08	0.53	Up
	Citrate.2	-	1.73E-02	0.48	Up

DISCUSSION

Many previous metabolomic analyses have been used to successfully investigate systemic metabolic responses to both internal and external factors, such as stress, disease, medical treatment, and depression (DeBerardinis & Thompson, 2012; Feigin et al., 2007; Laborde et al., 2012; Wang et al., 2009; Zheng et al., 2010). It is now known that these events alter cell signaling and other important functions related to metabolism, causing a cascade of metabolic changes. In this study, we set out to assess whether normal (low-stress) and pathological (high-stress) phenotypes of PNMS can be measured using ^{1}H NMR spectroscopy to profile small-molecule metabolites in human urine of adolescents who were *in utero* during a sudden-onset severe weather event – the 1998 Quebec Ice Storm.

Both normal and pathological metabolomic phenotypes were successfully profiled and measured using urinary metabolites, supporting the study's primary hypothesis. A select set of these metabolites was significantly altered in the male and female adolescents of mothers who experienced high subjective distress and/or objective hardship during the ice storm, compared to adolescents whose mothers experienced lower PNMS levels, thus supporting our secondary hypothesis. Many of the metabolites found to be significantly altered belong to metabolic pathways involved in energy metabolism and protein biosynthesis, supporting a link between early adverse life events and risk of metabolic illness later in life (Nicholson et al., 2011).

Sixty-eight metabolites known to be present in urine (Bouatra et al., 2013) were identified as significant, related to the level of PNMS experienced *in utero* (Tables 1 and 2). When only these metabolites were selected for chemometric analysis, both unsupervised and supervised separation was observed as a function of PNMS exposure (subjective distress or objective hardship) and sex (male or female), which were validated by cross validation and permutation tests. These significant class separations demonstrate that *in utero* exposure to PNMS may lead to genetic or epigenetic changes in offspring that manifest as downstream alterations to the metabolome.

While only the mothers experienced the stressful event directly, there may be gender-related differences in genetics, physiology or metabolism of the fetus that lead to differential downstream effects manifesting in the metabolome. For example, physiologically, women tend to have higher rates of chronic pain, depression, and anxiety disorders compared to men. These differences span from puberty to menopause, indicating that sex hormones are likely involved, and thus, probably metabolism (Wang et al., 2007). Furthermore, in a prenatal flood stress study, Yong Ping et al (2015) have shown that 2½ year-old females involved in a maternal separation protocol exhibited significantly higher cortisol levels following a brief separation period, whereas males did not, suggesting a gender difference in stress hormone secretion.

Sex differences in the brain uniquely affect biochemical processes, disease susceptibility, pharmacokinetics, and specific behaviours (Gandhi, Aweeka, Greenblatt, & Blaschke, 2004; Ngun, Ghahramani, Sánchez, Bocklandt, & Vilain, 2011). For example, Krumsiek et al (2015) show blood serum pathway analyses revealing strong gender differences in steroid and lipid metabolism, oxidative phosphorylation, and purine metabolism. While our results show similarities in effects on protein metabolism in males and females, particularly with branched-chain amino acid (BCAA) biosynthesis (Figures 11 and 12), these data support gender differences in lipid and ketone body metabolism, nucleotide sugar metabolism, and energy metabolism. These results are further supported by exercise physiology studies showing that females oxidize proportionately more fat and less carbohydrates than males, during exercise (Knechtle et al., 2004; Riddell et al., 2003). Kochhar et al (2006) suggest that females may have a metabolic advantage in using fatty acids as an energy source, likely due to circulating levels of estrogen and progesterone, or due to lower muscle mass and a reduced ability to store glycogen.

Almost all metabolites in the present study with a significant VIP score are involved in energy metabolism. Abnormal levels of metabolites involved in the Krebs cycle, such as citrate and malonate, can lead to energy deficiency, which is an important factor in fatigue – one of the most frequently represented symptoms in major depressive disorder (Zheng et al., 2010).

Interestingly, branched chain amino acids (leucine, isoleucine, and valine), which are critical to human life and are particularly involved in stress, energy, immunity, and muscle metabolism (Monirujjaman & Ferdouse, 2014), are highly represented in the VIP plots for males, and in pathway analyses for both males and females.

A more in-depth analysis of variables identified as significant in the VIP plots further elucidated the extent of metabolomic alterations in males and females resulting from the Ice Storm. In both genders, malonate, which is a competitive inhibitor of succinate dehydrogenase in the electron transport chain (ETC) (Oyedotun & Lemire, 2004), was highly significant in contributing to the observed unsupervised and supervised separations (Figure 10). In females, the purine derivative and adenosine reaction intermediate hypoxanthine, as well as adenine, a purine base, were found to significantly contribute to group separation. Both metabolites are involved in purine and energy metabolism, supporting gender differences in these systems (Kaya et al., 2006). Hypoxanthine has also been found to increase significantly in blood serum of obese individuals during exercise (Saiki, Sato, Kohzuki, Kamimoto, & Yosida, 2001).

In males, 3-hydroxymandelate, 3-chlorotyrosine, 2-aminobutyrate, and 2-ethylacrylate were identified as highly important metabolites in the unsupervised and supervised separations (Figures 10C and 10D). These appear to be breakdown products or intermediates in many oxidation pathways (Mamer Oa Fau - Tjoa, Tjoa Ss Fau - Scriver, Scriver Cr Fau - Klassen, & Klassen, 1976; Soga et al., 2006). Interestingly, 3-chlorotyrosine has been shown to be markedly elevated in low-density lipoprotein isolated from atherosclerotic intima (Hazen & Heinecke, 1997), suggesting a link to atherogenesis and prenatal stress in male offspring.

The metabolites identified for chemometric analyses, and those that are significantly altered following *in utero* PNMS exposure, are known to be involved in numerous metabolic pathways throughout the human body. MSEA was used to illuminate these pathways. Similar to Gene Set Enrichment Analysis used in transcriptomic research, MSEA helps to identify and interpret patterns of human metabolite concentration changes in a biologically meaningful

framework (Xia & Wishart, 2010b). A group of metabolites are considered to constitute a set if they meet the following criteria: they are involved in the same bio-processes; they change significantly under the same pathological or physiological conditions, and; they are present in the same locations (organs, tissues, cells) (Xia & Wishart, 2010a). The most significant and consistent pathway involved with the current stress cohort is protein biosynthesis (Figures 11 and 12), with 6 metabolite hits in males ($p = 0.000096$) and 5 metabolite hits in females ($p = 0.0031$). This means that the formation and breakdown of amino acids is somehow involved in response to high PNMS exposure.

Significant pathways in the female MSEA (Figure 12A), apart from protein biosynthesis, include ketone body metabolism, nucleotide sugar metabolism, and nicotinate and nicotinamide metabolism. Nicotinate and nicotinamide play several essential roles in respiration, glycolysis, and fatty acid synthesis, acting as coenzymes and ADP-ribose donors (Billington et al., 2006; Kobayashi & Shimizu, 1999). Disruption in protein biosynthesis and energy metabolism supports the finding that adolescents who were *in utero* during the 1998 Quebec Ice Storm are at an increased risk for developing obesity, independent of size at birth and several maternal characteristics, such as height or body mass index (Dancause et al., 2012; Liu et al., 2016). Furthermore, the most common pathological process known to cause ketone body dysregulation in the blood is diabetes (Laffel, 1999).

Consistent with MSEA results and VIP scores, both the male and female pathway topology analyses show valine, leucine, and isoleucine synthesis and degradation as significantly affected (Figures 11B and 12B). In combination with the up-regulation of branched chain amino acids (BCAAs) in the male VIP heat maps (Figure 10), this draws attention to a possible metabolic dysregulation of BCAAs in prenatally stressed male adolescents. Circulating levels of BCAAs have been shown to be increased in obese individuals and are associated with poor metabolic health and future insulin resistance, suggesting the possibility that these amino acids contribute to pathogenesis of obesity and diabetes (Lynch & Adams, 2014; Newgard et al., 2009).

Additionally, the alanine pathway, which involves the breakdown of pyruvate and some dipeptides, was significantly altered in male adolescents (Figure 11). Alanine is one of the most important amino acids released by muscle, acting as a major energy source and an important regulator in glucose metabolism, lymphocyte production, and immunity (Nicholson et al., 1984; Wishart et al., 2013). Alterations in the alanine cycle that increase levels of serum alanine aminotransferase (ALT) have been linked to the development of type II diabetes (Sattar et al., 2004). Altogether, the results of this study suggest that adolescents whose mothers experienced high stress during pregnancy, particularly males due to BCAA and alanine pathway changes, may be at an increased risk for developing obesity and diabetes later in life.

Study Limitations

In the growing field of metabolomics, there are numerous methods by which assessments of metabolomic alterations following environmental events, such as natural disasters, can be carried out. Alternative techniques including Mass Spectrometry (MS), Gas Chromatography – Mass Spectrometry (GC-MS), and Liquid Chromatography – Mass Spectrometry (LC-MS), as well as alternative bio-fluids such as blood plasma, serum, cerebrospinal fluid, and even tissues may be used to assess the metabolome from different perspectives. It may be a limitation of this study then, that only Nuclear Magnetic Resonance (NMR) spectroscopy and urine were used. However, NMR spectroscopy appears to be the method of choice for global, untargeted metabolomic analysis of urine (Bouatra et al., 2013), as it permits measurement of the largest number of metabolites (209, compared to 179 in GC-MS and 127 in LC-MS methods), and yields the greatest chemical diversity. Furthermore, NMR is non-destructive, so samples can be saved and re-used for further analysis. Lastly, urine is naturally an ideal bio-fluid for stress metabolomic studies since it is very easy to obtain, non-invasive, does not easily transmit infectious diseases, and it contains many identifiable metabolites (209 compared to 53 in CSF and 49 in blood).

Obtaining blood or tissue for analysis may cause agitation in study participants, resulting in an acute stress reaction that could lead to confounding results.

A final comment on drawing conclusions about potential disease biomarkers using urinary metabolomics analyses is the fact that previous research has shown metabolite concentration (scaled to creatinine) of the average compound in normal human urine can vary by ±50%, with some varying by as much as 350% (Bouatra et al., 2013). However, these large ranges in metabolite concentration are due to several factors, including age, gender, genetic background, diet, and activity level (Psihogios, Gazi, Elisaf, Seferiadis, & Bairaktari, 2008; Saude, Slupsky, & Sykes, 2006; Slupsky et al., 2007). In this study, adolescents were separated into sex classifications (male and female), were approximately the same age, and were from the same geographical region, mitigating much of the normal variance in metabolite quantities seen across individuals.

CONCLUSION

Using an independent random stressor, the 1998 Quebec Ice Storm, epigenetic alterations resulting from PNMS were detected as downstream metabolomic changes using ^{1}H NMR spectroscopy. Here, we differentiate and discriminate between pathologically high or low prenatal stress groups, based on a subset of significantly altered metabolites and/or metabolic pathways. There are strong links to a risk of metabolic illness, such as insulin resistance, diabetes, and obesity. Known metabolomic differences in males and females, such as ketone body production and energy metabolism, are supported by these results. Some similarities between high-stress male and female adolescents were also identified, which include alterations in branched-chain amino acid biosynthetic pathways. Possibilities exist to correlate future work, such as body composition analyses and other health outcomes, with the findings of this study. Ultimately, we have shown here that stress *in utero* has long-lasting effects on the metabolome, showing

significant alterations to amino acid synthesis and energy metabolism in children up to 17 years after prenatal exposure to a severe weather event.

CHAPTER 4: TRANS- AND MULTI-GENERATIONAL STRESS ALTERS BLOOD PLASMA METABOLOME IN RATS ASSESSED USING ^{1}H NMR SPECTROSCOPY

INTRODUCTION

It is currently estimated that one in five Canadians will personally experience a mental illness or disorder, while all Canadians will be directly or indirectly affected by mental illness in their lifetime. According to the Canadian Mental Health Association (Canadian Electronic & Canada, 2013), it is anticipated that 10-20% of Canadian youth are affected by a mental illness or psychiatric disorder, making them the single most disabling group of disorders worldwide. The economic cost of mental illness in Canada was estimated to be $7.9 billion in 1998. Very little is known about specific causes of these illnesses, but links have been established between abnormal cognitive, behavioural, and psychosocial outcomes, and stress *in utero* (Charil et al., 2010).

Metabolic disorders such as obesity, insulin resistance, and diabetes have also been linked to prenatal maternal stress (PNMS) (Bose, Oliván, & Laferrère, 2009). PNMS has a significant effect on offspring physiology and behaviour (Charil et al., 2010; Harris & Seckl, 2011; King & Laplante, 2005; Yong Ping et al., 2015). Abnormally high levels of circulating maternal glucocorticoids and increased placental corticotropin-releasing hormone may cross the placenta to reach the fetal brain, leading to dysregulated hypothalamic-pituitary-adrenal (HPA) axis activity and altered neuronal development (Harris & Seckl, 2011; Hayashi et al., 1998). HPA axis programming due to fetal glucocorticoid overexposure has potential lifetime consequences as a risk factor for complex adult diseases, including cardio-metabolic diseases (Khulan & Drake, 2012), altered glucose and insulin metabolism, and adiposity (Dancause et al., 2013; Reynolds, 2010).

The association between HPA axis function and risk of metabolic diseases may be causally linked to altered genetic and epigenetic regulation. The Common Disease Genetic and Epigenetic hypothesis argues that epigenetics provides an additional layer of variation, mediating the relationship between genotype and internal and external environments (Feinberg, 2007). Accordingly, PNMS may alter DNA methylation (Cao-Lei et al., 2014; Meaney & Szyf, 2005) and microRNA signatures in various tissues in exposed offspring (Hollins & Cairns, 2016; Zucchi

et al., 2013). Genetic and epigenetic regulation affects cellular functions that ultimately will reflect an altered metabolic output. Hence, the metabolome, i.e., the sum of all metabolites in an organism, arguably represents a direct measure of environment-gene interactions and associated phenotypes (Bouatra et al., 2013).

Aside from primarily metabolic disorders, the majority of other human diseases also involve abnormal metabolic states that reflect altered cellular processes, particularly neuropsychiatric illnesses (Feinberg, 2007; Harris & Seckl, 2011). Maternal stress has been shown to reduce offspring neurogenesis and lead to faster aging through a decline in cell proliferation associated with learning, which correlates to cognitive deficits (Lemaire, Koehl, Moal, & Abrous, 2000). Animal studies using the same cohort of trans- and multi-generationally stressed rats utilized here found that ancestral stress led to higher stress sensitivity and precocious onset of motor hyperactivity and risk assessment behaviours (McCreary et al., 2016). In addition to poor cognitive and behavioural outcomes, maternal anxiety and prenatal stress have been linked to low birth weight in both humans and animals (Lederman et al., 2004; Maric, Dunjic, Stojiljkovic, Britvic, & Jasovic-Gasic, 2010), which results in a high risk for postnatal growth failure, and the development of metabolic syndromes like type 2 diabetes, cardiovascular disease, and obesity later in life (Li, Wang, Wu, & Wang, 2017).

The goals of this study were (a) to identify metabolic signatures generated by cumulative multi-generational prenatal stress (MPS) and acute trans-generational prenatal stress (TPS) that may predict offspring health outcomes; (b) to differentiate between offspring of rats exposed to MPS, TPS, or an enriched environment (EE), based on presence and quantity of small-molecule metabolites in blood plasma using ^{1}H NMR spectroscopy; and (c) to identify a subset of significantly altered metabolites and metabolic pathways that may be used to predict and determine future health status and disease risk in translational human studies. By using a metabolomics approach to the study of PNMS in an animal model, we hope to provide insight into potential metabolic mechanisms underlying well-documented health outcomes of offspring.

MATERIALS AND METHODS

Experimental Design

Forty-eight male Long Evans rats were obtained from one of three stress treatment groups: controls (n = 16), transgenerational prenatal stress (TPS; n = 16), and multigenerational prenatal stress (MPS; n = 16). TPS rats were from the third filial (F3) generation of a line in which only the first filial (F1) generation was stressed prenatally (i.e. a SNN filial line, Figure 13). MPS rats were the F3 generation of a filial line in which each consecutive generation was prenatally stressed (i.e. a SSS filial line). Pregnant dams were stressed using a social isolation stressor, which has been shown to result in mild psychosocial stress in rats (Hawkley, Gu, Luo, & Cacioppo, 2012). Each dam was housed alone and did not experience any direct contact with any other rats from postnatal (P) day 90 until offspring were weaned. Control rats were not stressed and were housed in pairs until gestational day 21.

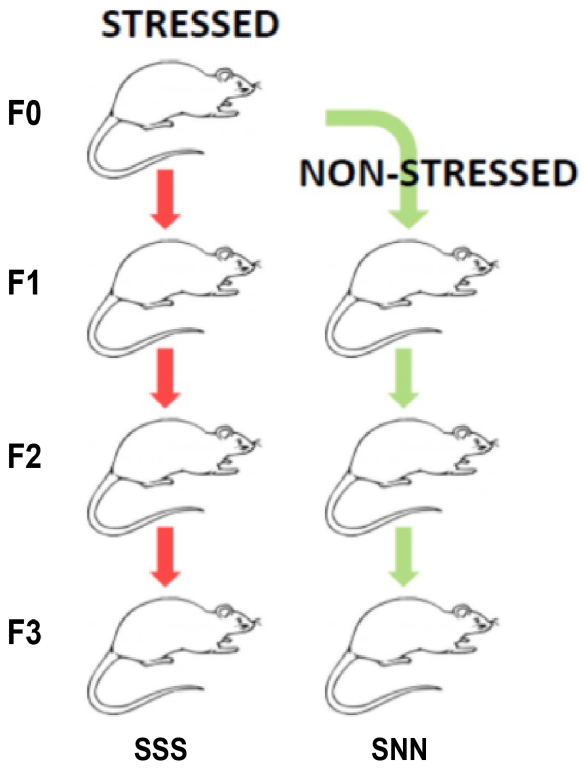

Figure 13. Illustration of transgenerational prenatal stress (TPS) and multigenerational prenatal stress (MPS) groups through filial generation zero (F0) to F3.

In addition to prenatal stress, half of each stress group was randomly assigned to one of two environmental conditions: standard housing or enriched environment (EE). In the standard housing condition, rats were housed in non-sibling pairs in a standard shoebox housing unit. In total, animals were gathered from 12 litters (4 control, 4 TPS, 4 MPS), and animals in each litter were then split into standard or enriched housing. From P 21 to P 35, the EE rats were housed in groups of four, living in a standard housing unit. At P 35, each group of four EE rats was moved to large circular

condominiums (Figure 14).

Figure 14. A: A standard housing shoebox unit, showing a non-sibling pair. B: An enriched environment condominium containing multiple shelters, toys, and novel foods. C: A group of rats housed together in an enriched environment condominium. Recreated with permission from J. McCreary.

In addition to the increased social interactions and living space, the EE was filled with multiple shelters and enrichment toys. EE rats were provided with novel foods in addition to standard rat chow, such as raw pasta, non-sweetened breakfast cereal, and seeds. In total, three identically arranged environments were used in the study, each housing all rats from a single stress treatment group. The animals were housed under a 12 h light/dark cycle with lights on at 7:30 AM. The room temperature was maintained at 20℃ with relative humidity at 30%. Corticosterone levels were assessed through blood collections obtained at three separate time points. The pre-stress time point was assessed one day prior to beginning social isolation stress; the acute stress time point was assessed on the first day of stress; and the chronic stress time point was assessed on day fourteen of stress. All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by the University of Lethbridge Animal Welfare Committee.

Buffer Preparation

Blood is regulated by mammalian bodies to remain within a physiological pH of 7.35-7.45 using several mechanisms, including the bicarbonate system, respiration, and the urinary system (Seldin & Giebisch, 1989). Thus, to buffer a blood plasma sample for metabolomic

analysis, it must only contain NaCl 0.9%, water, D_2O 10%, and sodium azide (NaN_3, 0.05% w/v) as an antimicrobial agent (Beckonert et al., 2007a).

pH value can affect the stability of formed elements and metabolites in fluids (Strasinger & Di Lorenzo, 2008). Samples must therefore be buffered to a consistent pH value. Phosphate urine buffer was prepared as a 4:1 ratio of KH2PO4:K2HPO4 in a 4:1 H2O:D2O solution to obtain a final concentration of 0.5 M. The D2O contained 0.05% v/v trimethylsilyl propanoic acid (TSP) as a chemical shift reference for 1H NMR spectroscopy. 0.03% w/v of sodium azide (NaN3) was also added to the buffer as an antimicrobial agent to maintain metabolite integrity. Buffer pH was checked after preparation and titrated to 7.4 using 3M HCl. Blood plasma samples that were protein precipitated using the methanol method and saline buffer were subsequently resuspended in this phosphate buffer for final data acquisition.

Sample Collection and Preparation

At 140 days old, 600µl blood samples were collected from the lateral tail vein using a 23-gauge butterfly needle coated with lithium heparin, while rats were anaesthetized using 4% isoflurane. EDTA and citrate collection tubes were avoided, as they give additional spectral signals in ^1H NMR spectroscopy (Barton et al., 2010). Blood was transferred to centrifuge tubes and plasma was obtained by centrifugation at 1600g for 15 minutes at 4°C. Samples were then stored at –80°C until further processing.

50µL aliquots of blood plasma were added to 550µL of Blood Saline Buffer solution in 1.5mL Eppendorf microfuge tubes. Samples were then vortexed for 5 seconds each, and centrifuged at 12,000g for 5 minutes at 4°C. Following centrifugation, 550µL of supernatant from each sample was added to 7-inch, high quality glass NMR tubes (Wilmad). These were either analyzed immediately or stored at -20°C until ready for analysis. Samples were kept at -80°C for long-term storage greater than 24 hours.

Following data acquisition of whole plasma/buffer mixtures, water soluble metabolites were extracted using a methanol precipitation protocol adapted from (Nagana Gowda & Raftery, 2014). Methanol was added to buffered plasma samples in a 2:1 ratio (550µL buffered plasma + 1.10mL methanol) in 2.0mL Eppendorf tubes. These were then vortexed for 5 seconds and incubated at -20°C for 20 minutes. Samples were subsequently centrifuged at 12,000g for 30 minutes at 4°C. Supernatant was decanted to fresh tubes and allowed to dry in a fume hood until all liquid had evaporated. Dried pellets were then re-suspended in 600µL of phosphate buffer calibrated to pH 7.4 and vortexed for 10 seconds each, or until pellet was completely dissolved. Finally, these were centrifuged again at 12,000g for 5 minutes at 4°C and 550µL of re-suspended supernatant was added to 7-inch high quality glass NMR tubes for data acquisition, or stored at -20°C.

NMR Data Acquisition and Processing

NMR spectra were collected on a 700 MHz Bruker Avance III HD spectrometer. The 1-D NOESY gradient water suppression pulse sequence noesygpr1d was used (Bruker). Each sample was run for 512 scans to a total acquisition size of 256k. The spectra were zero filled to 512k, automatically phased and baseline corrected, and line-broadened by 0.3Hz. The processed spectra were then exported to MATLAB for statistical analysis. Spectra were binned using Dynamic Adaptive Binning to reduce the size of the dataset. Each spectrum was normalized to remove effects of imperfect water signal suppression, and the spectral region corresponding to water was removed from data normalization. The data set was then Pareto scaled to reduce the influence of intense peaks, while emphasizing weaker ones. All peaks were referenced to formate (8.22δ) in whole plasma samples and to TSP (0.00δ) in methanol-precipitated samples.

Statistical Analysis

The overall structure of scaled spectra was visualized and compared across control and experimental groups using Principal Component Analysis (PCA). A *supervised* statistical method known as Partial Least Squares Discriminant Analysis (PLS-DA) was then employed to maximize the observed differences between groups. A Mann-Whitney U test was subsequently used to determine which spectral bins were significantly altered in comparison groups. Bins with p-values less than or equal to 0.05 were deemed significant and considered as potential markers defining substantial alterations in metabolite concentration between control and experimental groups. According to the Goodpaster *et al.* decision tree algorithm, a Shapiro-Wilk test for normality was used. Non-normal data with a p-value <0.05 was deemed "possibly significant". Data analyses, including dynamic adaptive binning, PCA, PLS-DA, and Mann-Whitney U tests were carried out using MATLAB®.

Metabolite Identification

A spectral database of pure metabolites was generated and used to identify most metabolites found in NMR spectra. The Human Metabolome Database (Wishart et al., 2013; Wishart et al., 2009; Wishart et al., 2007) was used to supplement and aid the creation of our spectral library, and to identify some substances that were not obtained for the creation of this database. Furthermore, tools for identifying and quantifying NMR metabolites were used in the Chenomx 8.2 NMR Suite (Chenomx Inc., Edmonton, Alberta, Canada) which allowed for spectral deconvolution of biofluid samples into individual components.

RESULTS

Exploratory Statistical Analysis

Principle Component Analysis (PCA) and Partial Least Squares – Discriminant Analysis (PLS-DA) were both utilized to identify patterns in group separation of male rats that were offspring of either non-stressed controls (CC), non-stressed controls housed in an enriched environment (CEE), transgenerationally-stressed (SNNC), or multigenerationally-stressed (SSSC). These tests were performed on a subset of metabolites that were deemed significant in both a Mann-Whitney U test ($p<0.05$) and a VIAVC analysis. Figure 15A shows poor separation between CC and CEE groups, with a large confidence interval overlap. Both trans- and multi-generational stress groups, on the other hand, showed excellent separation when compared to non-stressed controls (Figure 15B and 15C). While there appears to be similarities in the separation of SNNC and SSSC groups compared to controls, Figure 15D shows that enough variance remained to result in appreciable separation between SNNC and SSSC groups, with virtually no confidence interval overlap.

Double-cross validation and permutation tests were used to validate supervised PLS-DA separation results (Figure 16). While all cross-validation tests passed, three of the four permutation tests did not (CC vs CEE $p = 0.46$; CC vs SSSC $p = 0.25$; CC vs SNNC $p = 0.032$; SNNC vs SSSC $p = 0.19$). Thus, only the CC vs SNNC group is shown in Figure 16 below.

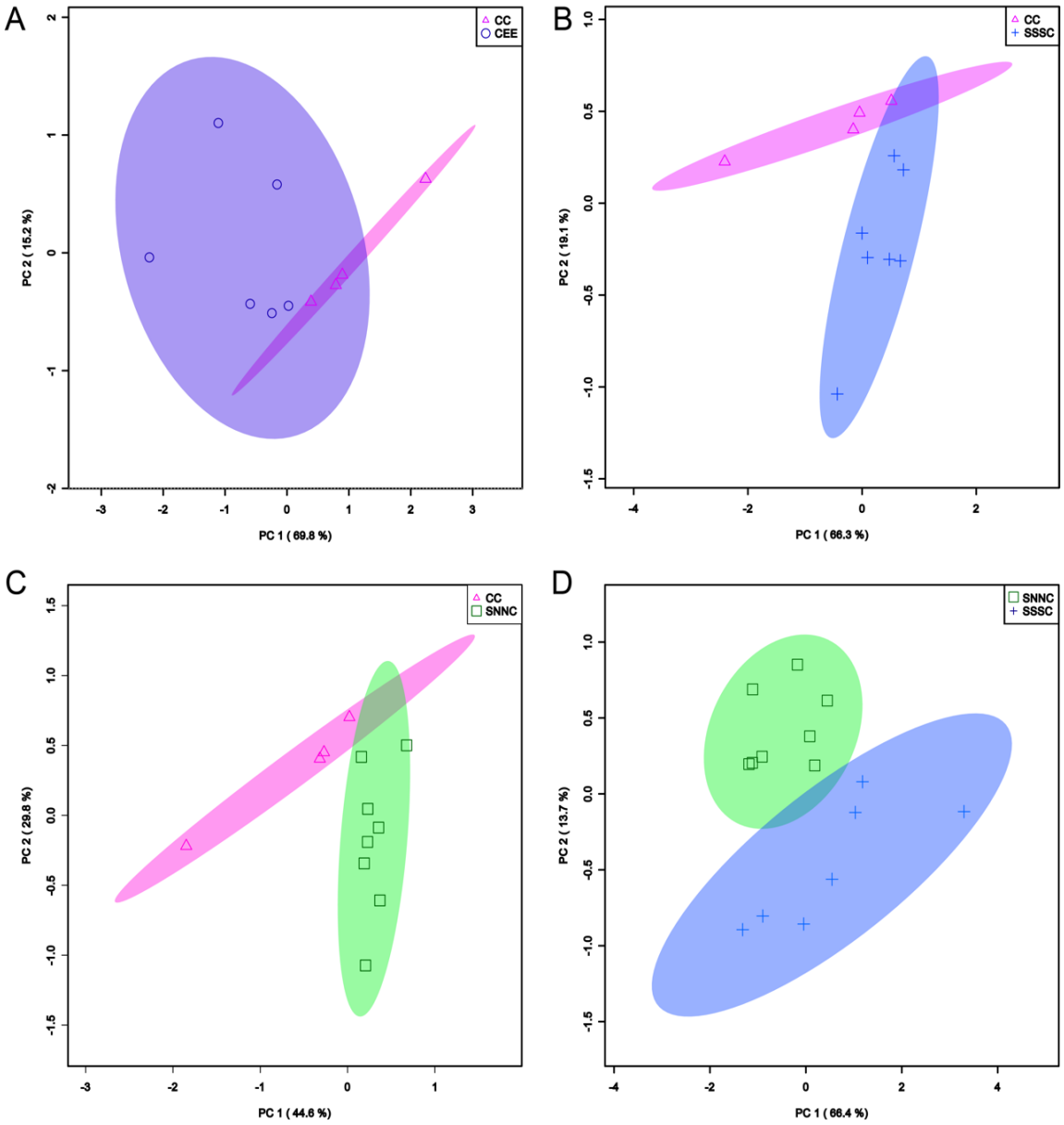

Figure 15. Principle Component Analysis (PCA) plots showing unsupervised separation in comparison groups. A: Non-stressed controls (CC) vs. Non-stressed enriched environment (CEE); B: Non-stressed controls (CC) vs. Multigenerationally stressed rats (SSSC); C: Non-stressed controls (CC) vs. Transgenerationally stressed rats (SNNC); and D: Transgenerationally stressed rats (SNNC) vs. Multigenerationally stressed rats (SSSC). All rats in the SNNC and SSSC groups were stressed using a social isolation stress paradigm. Each point (triangle, cross, circle, or square) represents one individual under study, plotted using a list of blood plasma metabolites found to be statistically significant via a Mann-Whitney U test and a VIAVC analysis. Coloured ellipses represent 95% confidence intervals. X and Y axes show principal components 1 and 2, respectively, with brackets indicating percent variance between groups.

Since PLS-DA analyses in most groups did not pass permutation testing, only a Variable Importance in the Projection (VIP) plot for the CC vs SNNC group is shown in Figure 17, which indicates which variables contributed most to separation. The heat map to the right of the VIP plot conveys whether separation resulted from up- or down-regulation of the metabolite, based on relative concentration changes found in the PCA-matrix. Some compounds along the y-axis are separated by commas, indicating an overlap of multiple compounds at the same chemical shift region in the NMR spectrum.

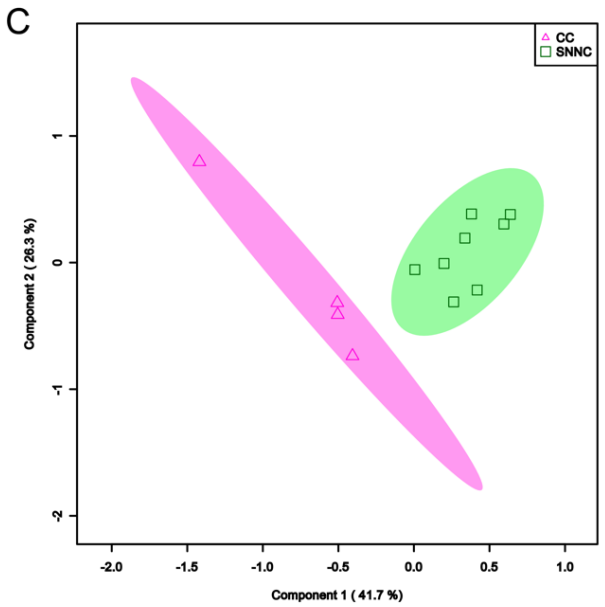

Figure 16. Partial Least Squares – Discriminant Analysis (PLS-DA) plots showing supervised separation in CC vs SNNC comparison group. All rats in the SNNC group were stressed using a social isolation stress paradigm. Each point (triangle or cross) represents one individual under study, plotted using a list of blood plasma metabolites found to be statistically significant via a Mann-Whitney U test and a VIAVC analysis. Coloured ellipses represent 95% confidence intervals. X and Y axes show principal components 1 and 2, respectively, with brackets indicating percent variance between groups.

In the CC vs. CEE comparison group, metabolites showing the largest percent difference were citrate, 3-methylxanthine, theophylline, and formate, with -26.30%, 24.06%, 19.19%, and 18.94%, respectively (Table 3). An unidentifiable singlet at chemical shift 8.38ppm, as well as creatine phosphate and formate, had the greatest percent difference in the multigenerational stress cohort (CC vs SSSC, Table 3), with changes of 23.32%, 13.91%, and 13.25%, respectively. In the transgenerationally-stressed group (CC vs SNNC, Figure 17 and Table 3), formate, creatine phosphate, and glucose were the top three metabolites showing highest percent differences, with respective percentages of 16.39%, 15.17%, and -6.31. Lastly, the transgenerational vs. multigenerational stress group had a single metabolite, citrate, contribute most to the group separation. Since citrate has four peaks, each with its own bin in the PCA matrix, these peaks

claimed the top four spots for percent difference, with relative concentration changes ranging from -16.82% to -34.68% in the TPS (SNNC) group compared to MPS (SSSC).

There were similarities in the trans- and multi-generational stress groups regarding significant changes in metabolites. This can be seen with both formate and creatine phosphate having among the largest percent differences in the CC vs. SNNC and SNNC vs. SSSC groups. The heat map in these figures also indicates that in both groups this is due to a relative up-regulation in formate and creatine phosphate concentration in the stressed group (either SNNC or SSSC). Formate also appeared to increase in relative concentration in the enriched environment group but was not a significant variable in the SNNC vs. SSSC comparison group.

In the SNNC vs. SSSC group, citrate contributed most to separation and was due to a relative down-regulation in transgenerationally-stressed rats and a relative up-regulation in multigenerationally-stressed rats (Table 3). While citrate was also the top metabolite in the CC vs. CEE comparison, the enriched environment group had a decrease in the relative concentration of citrate (Table 3).

Hierarchical clustering analysis (HCA) was applied to reveal differences between classes without supervision. Two of the four comparison groups were successfully separated into their respective groups using HCA (Figures 18 and 19). Rats housed in an enriched environment (CEE) separated from non-stressed controls (CC), showing a pattern of decreased relative concentration of citrate, lactate, threonine, and the branched-chain amino acids (BCAAs)

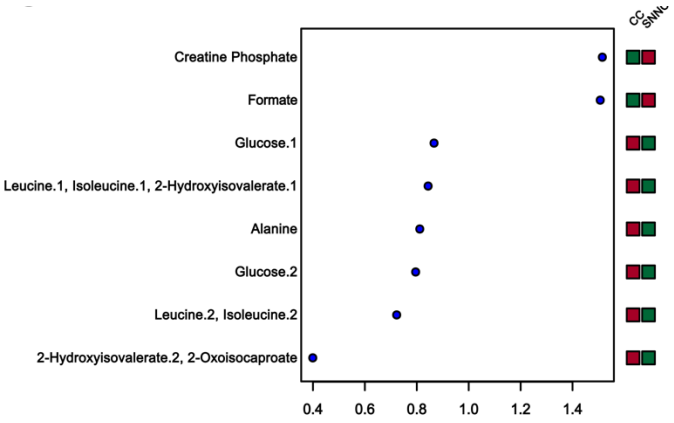

Figure 17. Variable Importance in Projection (VIP) plot of Non-stressed controls (CC) vs. Transgenerationally stressed rats (SNNC), showing the relative contribution of metabolites to the variance between these groups. High VIP values indicate greater contribution of these metabolites to group separation, shown in PLS-DA plots. Green and red boxes to the right indicate whether the relative metabolite concentration decreased or increased, respectively, in the blood plasma metabolome of rat offspring. A VIP score of 1.0 is considered able to discriminate between two phenotypes.

compared to the control group (Figure 18). 3-Hyroxybutyrate, formate, methylxanthine, and theophylline on the other hand, had a relative concentration increase in the CEE group compared to CC.

While the trans- and multi-generational stress groups clustered successfully, the pattern is less clear (Figure 19). Citrate, lactate, and alanine appear to have generally increased in relative concentration in the SSSC group compared to a relative decrease in most SNNC rats.

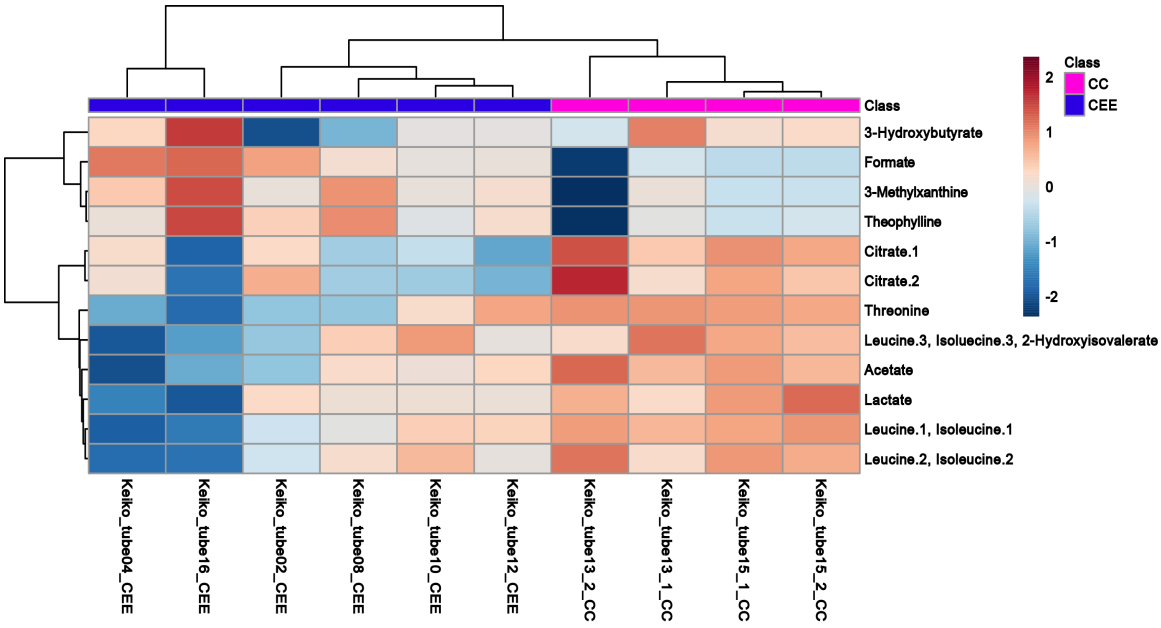

Figure 18. Heat map showing hierarchical clustering of 11 blood plasma metabolites found to be significant in male Long Evans rats reared in an enriched environment (CEE), compared to non-stressed controls (CC). Controls are shown as pink and EE rats as purple in the dendrogram (top). Each coloured cell corresponds to an up- (red) or down-regulation (blue) in relative concentration, with samples on the x-axis and metabolites on the y-axis.

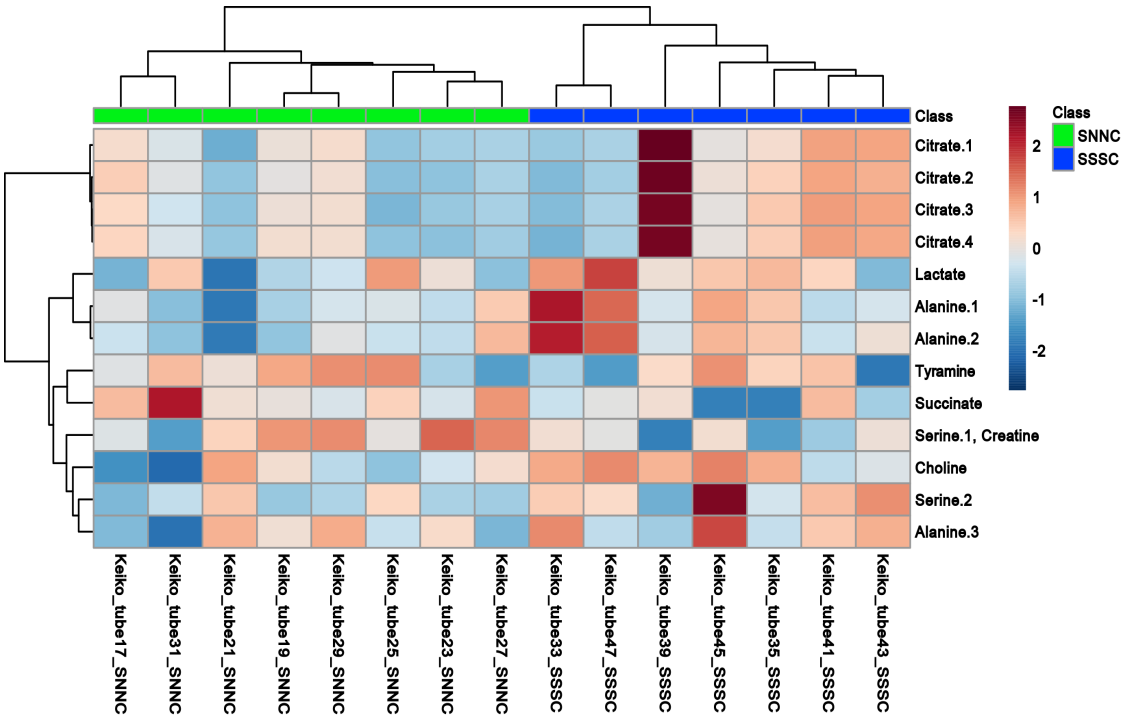

Figure 19. Heat map showing hierarchical clustering of 8 blood plasma metabolites found to be significant in male Long Evans rats that were transgenerationally-stressed (SNNC), compared to rats that were multigenerationally-stressed (SSSC). SNNC rats are shown as green and SSSC rats as blue in the dendrogram (top). Each coloured cell corresponds to an up- (red) or down-regulation (blue) in relative concentration, with samples on the x-axis and metabolites on the y-axis.

Functional Analysis

Figures 20A to 23A show metabolite set enrichment analyses, with metabolite sets that are most implicated by the compounds identified as significant by Mann-Whitney U tests and VIAVC. These metabolic sets are predicted to be changed in the case of dysfunctional enzymes, using a genome-scale network model of rodent metabolism. Figures 20B to 23B show metabolomic pathway analyses displaying all matched pathways according to p-values from pathway enrichment analysis and pathway impact values from pathway topology analysis.

Rats that were housed in an enriched environment, compared to controls, showed significantly affected protein biosynthesis ($p < 0.01$), particularly with valine, leucine, and isoleucine (or branched-chain amino acid) synthesis (Figure 20A). Other energy metabolism systems were significantly affected, including the glucose-alanine cycle ($p = 0.016$), and the citric

acid cycle ($p = 0.05$) (Figure 20A). This is supported by the pathway topology analysis (Figure 20B), showing significant alterations in the aminoacyl tRNA synthesis pathway ($p < 0.01$), branched-chain amino acid synthesis pathway ($p < 0.01$), and the tricarboxylic acid (TCA) pathway ($p = 0.014$). The butanoate metabolic pathway was also found to be significant ($p = 0.014$) in the CC versus CEE comparison group topology analysis.

Multigenerational stress had a significant impact on glycine, serine, and threonine metabolism ($p = 0.013$), compared to non-stressed controls (Figure 21A). The pathway topology analysis reflected this significance (Figure 21B), showing changes to the glycine, serine, and threonine metabolic pathway ($p < 0.01$). No other pathways were found to be significant in this group, although betaine metabolism was somewhat significant in the metabolite set enrichment ($p = 0.07$).

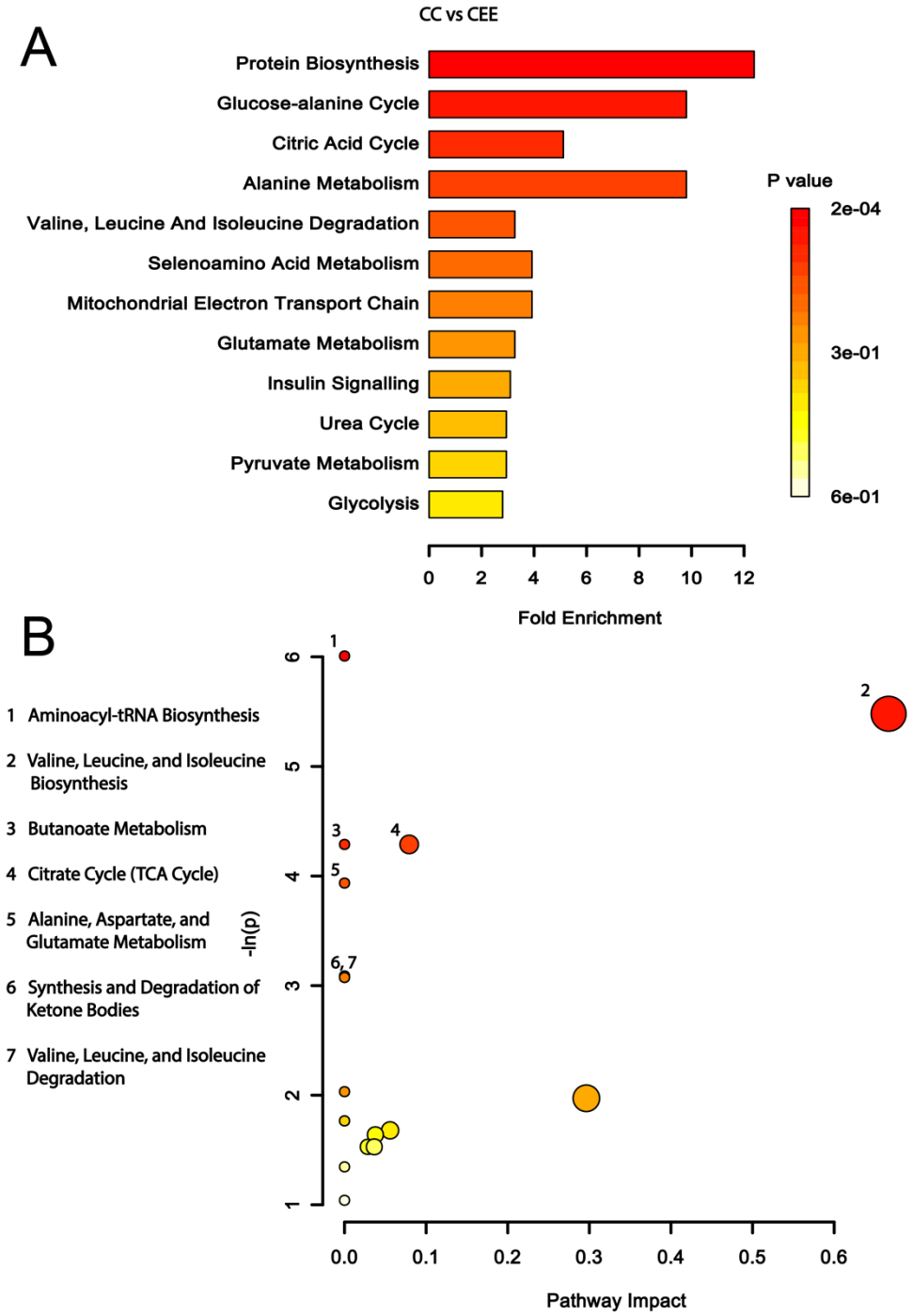

Figure 20. A: Pathway-Associated Metabolite Set Enrichment Analysis (MSEA) plot showing affected biological processes in non-stressed rats (CC) compared to non-stressed rats housed in an enriched environment (CEE), based on metabolites identified as significantly altered between these groups. A library containing 88 metabolite sets based on normal metabolic pathways was used to acquire these data. Fold enrichment shows how many times greater than chance the process is involved. B: Metabolomic Pathway Analysis showing all matched pathways according to p-values from pathway enrichment analysis and pathway impact values from pathway topology analysis in non-stressed controls and rats housed in an enriched environment. Y-axis shows the negative natural log of p, such that a higher value on the y-axis gives a lower p-value. The x-axis gives the Pathway Impact, which correlates to the number of metabolite hits in a particular pathway. Only metabolic pathways with $p<0.05$ are labeled.

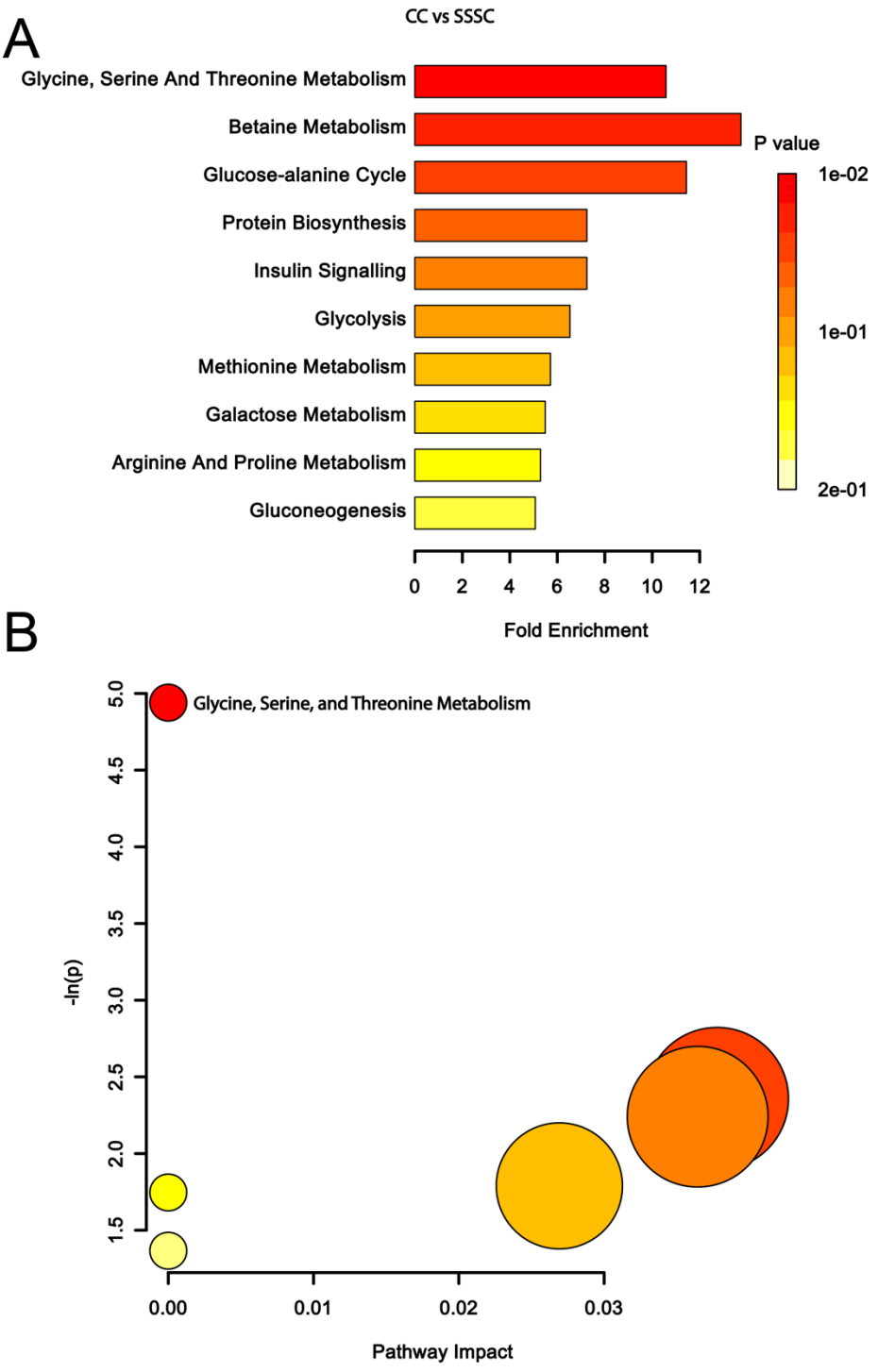

Figure 21. A: Pathway-Associated Metabolite Set Enrichment Analysis (MSEA) plot showing affected biological processes in non-stressed rats (CC) compared to multigenerationally-stressed rats (SSSC), based on metabolites identified as significantly altered between these groups. A library containing 88 metabolite sets based on normal metabolic pathways was used to acquire these data. Fold enrichment shows how many times greater than chance the process is involved. B: Metabolomic Pathway Analysis showing all matched pathways according to p-values from pathway enrichment analysis and pathway impact values from pathway topology analysis in non-stressed controls and rats housed in an enriched environment. Y-axis shows the negative natural log of p, such that a higher value on the y-axis gives a lower p-value. The x-axis gives the Pathway Impact, which correlates to the number of metabolite hits in a particular pathway. Only metabolic pathways with $p<0.05$ are labeled.

In the transgenerationally-stressed group, protein biosynthesis was significantly altered in the metabolite set enrichment (Figure 22A). Specifically, valine, leucine, and isoleucine biosynthesis ($p < 0.01$) and degradation ($p < 0.01$) were found to have significant changes in transgenerationally-stressed rats, compared to non-stressed controls. The glucose-alanine cycle ($p < 0.01$), and alanine metabolism were also found to be altered in the MSEA. Pathway topology supported the effects on branched-chain amino acid synthesis ($p < 0.01$) and degradation ($p < 0.01$) pathways but did not reveal significant pathway hits in the glucose-alanine cycle or alanine metabolic pathways (Figure 22B). The aminoacyl tRNA pathway ($p < 0.01$), however, was also shown to be affected by transgenerational stress.

In the trans- versus multi-generational stress comparison group (SNNC vs. SSSC, Figure 23), the metabolite set enrichment analysis found the TCA cycle ($p < 0.01$), methionine metabolism ($p < 0.01$), glycine, serine, and threonine metabolism ($p < 0.01$), and alanine metabolism ($p = 0.05$) to be significantly affected. The SNNC vs. SSSC topology analysis (Figure 23B) shows the glycine, serine, and threonine metabolic pathway ($p <0.01$) to be most significantly affected, followed by the TCA cycle ($p < 0.01$), the alanine, aspartate, and glutamate metabolic pathway ($p < 0.01$), cyanoamino acid pathway ($p = 0.03$), the methane metabolic pathway ($p = 0.05$), and the aminoacyl tRNA pathway ($p = 0.05$).

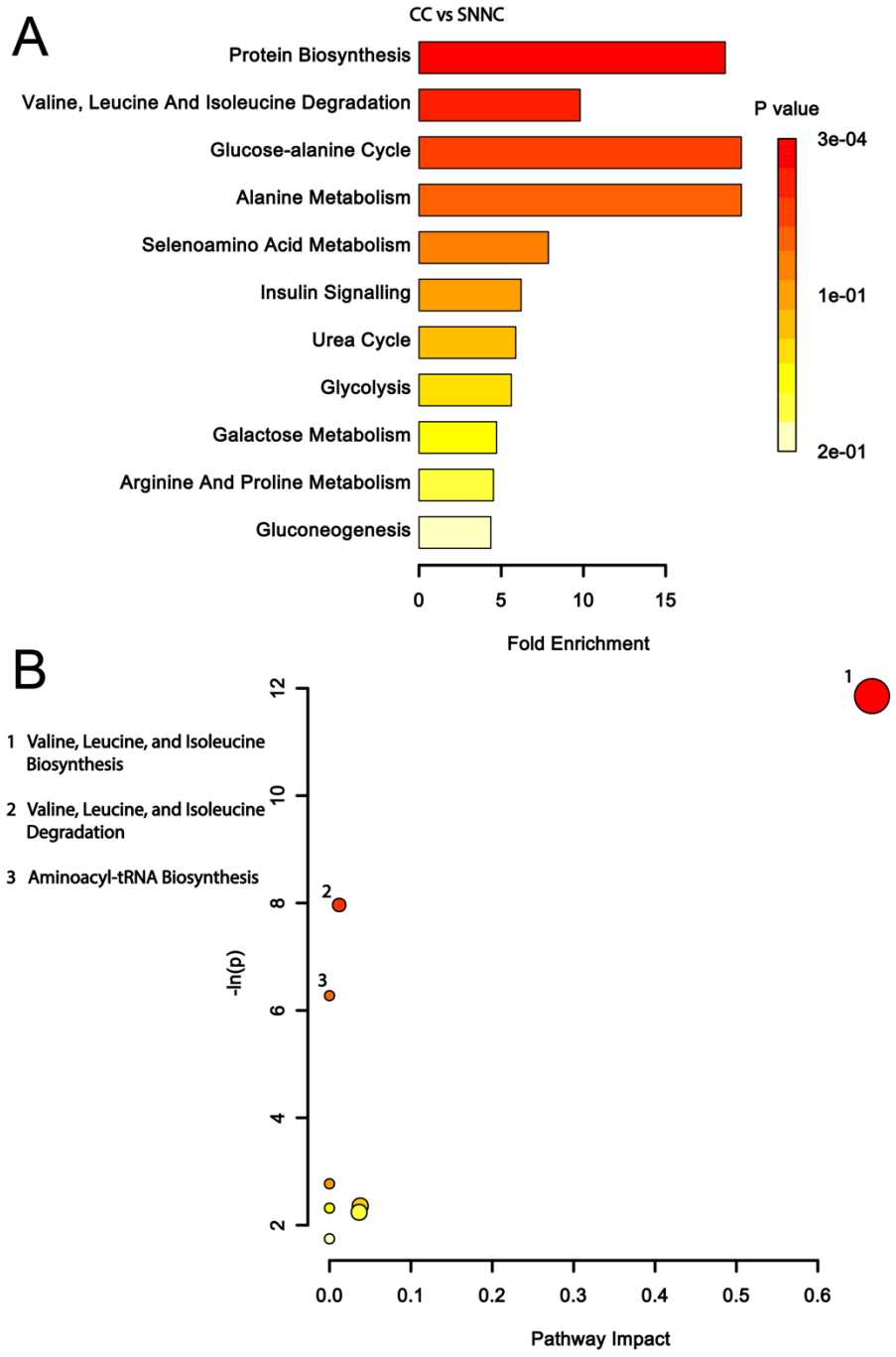

Figure 22. A: Pathway-Associated Metabolite Set Enrichment Analysis (MSEA) plot showing affected biological processes in non-stressed rats (CC) compared to transgenerationally-stressed rats (SNNC), based on metabolites identified as significantly altered between these groups. A library containing 88 metabolite sets based on normal metabolic pathways was used to acquire these data. Fold enrichment shows how many times greater than chance the process is involved. B: Metabolomic Pathway Analysis showing all matched pathways according to p-values from pathway enrichment analysis and pathway impact values from pathway topology analysis in non-stressed controls and rats housed in an enriched environment. Y-axis shows the negative natural log of p, such that a higher value on the y-axis gives a lower p-value. The x-axis gives the Pathway Impact, which correlates to the number of metabolite hits in a particular pathway. Only metabolic pathways with $p<0.05$ are labeled.

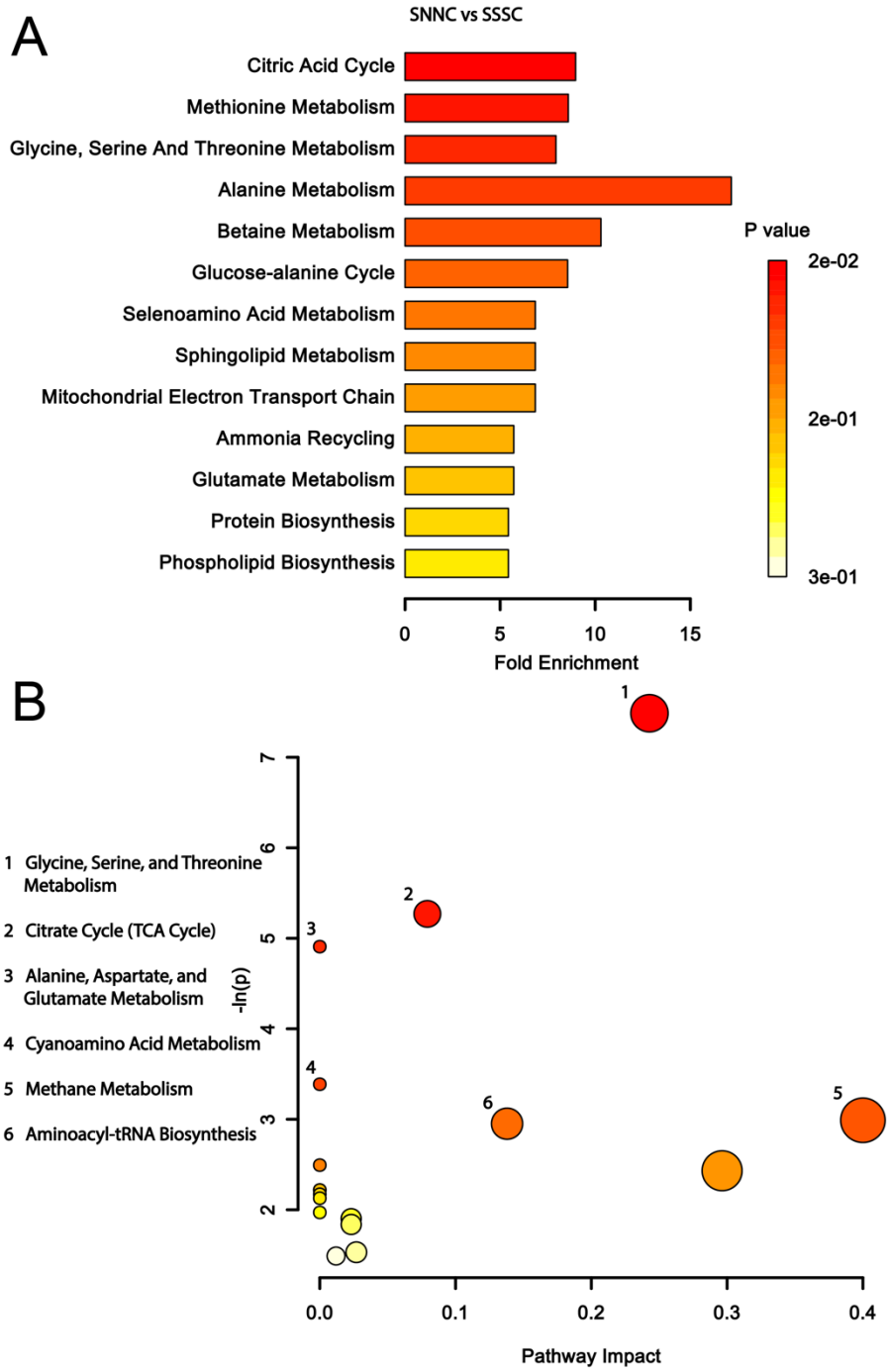

Figure 23. A: Pathway-Associated Metabolite Set Enrichment Analysis (MSEA) plot showing affected biological processes in transgenerationally-stressed rats (SNNC) compared to multigenerationally-stressed rats (SSSC), based on metabolites identified as significantly altered between these groups. A library containing 88 metabolite sets based on normal metabolic pathways was used to acquire these data. Fold enrichment shows how many times greater than chance the process is involved. B: Metabolomic Pathway Analysis showing all matched pathways according to p-values from pathway enrichment analysis and pathway impact values from pathway topology analysis in non-stressed controls and rats housed in an enriched environment. Y-axis shows the negative natural log of p, such that a higher value on the y-axis gives a lower p-value. The x-axis gives the Pathway Impact, which correlates to the number of metabolite hits in a particular pathway. Only metabolic pathways with $p<0.05$ are labeled.

Table 3. P-values of blood plasma metabolites found to be significant in male Long Evans rats in either a Mann-Whitney U test, the Variable Importance Analysis based on random Variable Combination (VIAVC), or both. Rats were either non-stressed (CC), non-stressed and housed in an enriched environment (CEE), transgenerationally-stressed (SNNC), or multigenerationally-stressed (SSSC). VIP scores, shown in descending order, correspond to Figure 17. Metabolites with multiple peaks are represented as metabolite.1, metabolite.2… metabolite.n.

Group	Metabolite	Mann-Whitney U Test (p-value)	VIAVC (p-value)	VIP Score	Percent Difference (%)
CC vs CEE	Citrate.1	3.81E-02	2.69E-15		-26.30
	3-Methylxanthine	9.52E-03	1.58E-11		24.06
	Formate	9.52E-03	3.52E-18		18.94
	Theophylline	1.90E-02	8.96E-32		19.19
	Threonine	1.90E-02	3.32E-17		-15.15
	Citrate.2	9.52E-03	9.72E-12		-14.37
	Lactate	1.90E-02	1.17E-41		-14.96
	Leucine.1, Isoleucine.1	1.90E-02	1.30E-03		-11.34
	Leucine.2, Isoleucine.2	9.52E-03	2.05E-06		-10.03
	Acetate	9.52E-03	2.18E-32		-8.07
	Leucine.3, Isoleucine.3, 2-Hydroxyisovalerate	1.14E-01	2.27E-05		-8.89
	3-Hydroxybutyrate	6.10E-01	1.54E-07		-4.70
CC vs SNNC	Creatine Phosphate	1.62E-02	4.40E-09	1.51	15.17
	Formate	2.83E-02	2.09E-06	1.51	16.39
	Glucose.1	1.09E-01	7.02E-06	0.87	-6.31
	Leucine.1, Isoleucine.1, 2-Hydroxyisovalerate.1	7.27E-02	1.12E-47	0.84	-5.62
	Alanine	4.85E-02	2.35E-20	0.81	-5.45
	Glucose.2	3.68E-01	1.20E-07	0.80	-8.84
	Leucine.2, Isoleucine.2	4.85E-02	1.85E-30	0.72	-5.14
	2-Hydroxyisovalerate.2, 2-Oxoisocaproate	5.70E-01	6.83E-22	0.40	-4.63
CC vs SSSC	Singlet at 8.38ppm	1.09E-01	3.46E-37		23.31
	Formate	7.27E-02	3.03E-54		13.25
	Creatine Phosphate	2.83E-02	2.39E-02		13.91
	3-Methylxanthine	3.68E-01	4.02E-31		13.49
	Threonine	4.85E-02	1.66E-20		10.79

	Tyramine.1	6.83E-01	1.25E-20	11.82
	Glucose, Betaine	7.27E-02	2.81E-31	-5.69
	Tyramine.2	9.33E-01	3.90E-27	3.54
SNNC vs SSSC	Citrate.1	6.50E-02	5.94E-11	-34.68
	Citrate.2	8.30E-02	3.34E-12	-27.34
	Citrate.3	1.05E-01	7.66E-11	-23.31
	Citrate.4	1.05E-01	2.25E-08	-16.82
	Choline	2.07E-02	1.75E-13	-7.24
	Lactate	3.79E-02	-	-8.70
	Succinate	1.95E-01	1.01E-10	7.24
	Alanine.1	8.30E-02	9.29E-16	-6.66
	Alanine.2	2.34E-01	3.88E-10	-6.17
	Serine.1, Creatine	2.34E-01	4.95E-08	5.83
	Serine.2	4.99E-02	0.0053	-4.33
	Alanine.3	1.61E-01	1.27E-08	-2.86
	Tyramine	2.34E-01	2.99E-08	2.85

DISCUSSION

Early life events such as PNMS have long-lasting impacts on tissue structure and function, cognition, and behaviour in offspring (Charil et al., 2010; McCreary et al., 2016). It is well-established that, apart from the effects of genes and environment, adverse conditions such as malnutrition, stress, and excess glucocorticoid exposure during fetal development may cause permanent cardiometabolic, neuroendocrine, and cognitive effects (Harris & Seckl, 2011; Meaney & Szyf, 2005; Meaney, Szyf, & Seckl, 2007; Seckl & Meaney, 2006). Previous metabolomic analyses have successfully investigated systemic metabolic responses to these types of stressors (DeBerardinis & Thompson, 2012; Wang et al., 2009; Zheng et al., 2010), and it is now known that these events alter cell signaling and other important metabolic functions, leading to a cascade of downstream changes in metabolism.

The purpose of the present study was to identify metabolic signatures generated by cumulative (MPS) or acute (TPS) ancestral PNMS that may predict offspring health outcomes. Our results show that it is possible to differentiate between offspring of rats exposed to social isolation MPS, TPS, or no stress, based on the presence and quantity of small-molecule metabolites found in blood plasma, using ^{1}H NMR spectroscopy. Compared to non-stressed controls, rats that were housed in an EE were also found to have an altered blood plasma metabolome. The results provide evidence that transgenerational inheritance of the altered stress response is linked to altered metabolic activity, particularly those involved in energy metabolism, protein biosynthesis, and immunity.

Twenty-one independent metabolites known to be present in blood serum/plasma (Psychogios et al., 2011) were identified as significantly altered across all comparison groups. When interfering and non-significant metabolites were excluded from analyses, both unsupervised PCA and hierarchical clustering analyses showed successful separation as a function of TPS, MPS, or EE. This class separation may reflect underlying epigenetic changes and related cellular functions that become evident as downstream alterations in the metabolome. While previous studies have shown EE to promote positive behaviours and outcomes such as skilled movement (Jadavji, Kolb, & Metz, 2006; Jadavji & Metz, 2009; Knieling, Metz, Antonow-Schlorke, & Witte, 2009), learning and memory (Falkenberg et al., 1992; Gibb, Gonzalez, & Kolb, 2014), and affective behaviour (Galani et al., 2007), the present study was unable to find any clear mechanism for these outcomes. For example, metabolites found to be significantly altered by EE include down-regulated citrate and lactate levels, and up-regulated formate levels. Both a decrease in citrate, which is involved in the TCA cycle, and an increase in formate, which can disrupt mitochondrial electron transport (Wishart et al., 2013), can lead to energy deficiency (Zheng et al., 2010). Additionally, evidence suggests that lactate is an important metabolite at early stages of brain development in prenatal and early postnatal subjects, and it has been shown to be preferentially utilized over glucose in the brain (Zilberter, Zilberter, & Bregestovski, 2010).

Although these findings appear to defy well-established positive after-effects of environmental enrichment, it may be that these metabolic pathways are involved in an energy repossessing process. Of the major metabolic pathways affected by EE were BCAA biosynthesis and aminoacyl tRNA synthesis, suggesting some alteration in amino acid and/or protein metabolism (Monirujjaman & Ferdouse, 2014). Furthermore, alterations in the glucose-alanine cycle, which is utilized when the body is in a state of muscle catabolism (Fromm & Hargrove, 2012), support the notion that there is some high demand for energy. Since EE has been shown to restore and promote dendritic and synaptic morphology (Pascual, Valencia, & Bustamante, 2015; Yaojin Peng Xiaohong Jian Lihua Liu Jianbin Tong Deliang, 2011), and to increase hippocampal neurogenesis (Bhagya, Srikumar, Veena, & Shankaranarayana Rao, 2017), these animals may be utilizing energy from other systems to account for the high energy demands of these functions. Although this view is speculative, there appears to be one system that is clearly supported by the metabolomic analysis of EE rats, specifically, improved immune function. EE rats showed a statistically significant up-regulation of multiple xanthines, which lower inflammation and mediate the innate immune response (Peters-Golden, Canetti, Mancuso, & Coffey, 2005).

While the effects of environmental enrichment on the metabolic relationship with health outcomes remains vague, mechanisms of PNMS appear more evident. In both the MPS and TPS groups, creatine phosphate and formate were significantly up-regulated compared to non-stressed controls. As mentioned previously, formate is responsible for disrupting the electron transport chain, and therefore energy production, by inhibiting the terminal electron acceptor cytochrome oxidase (Nicholls, 1975). Similarly, creatine phosphate plays a vital role in the regeneration of ATP in skeletal muscle, and its availability likely limits muscle performance during brief, high-power activity (Baker, McCormick, & Robergs, 2010; Wishart et al., 2013). The involvement of these metabolites in both TPS and MPS rats indicates the probable involvement of energy metabolism systems in response to PNMS, supporting a link between early adverse events and documented low birth weights in offspring exposed to stress *in utero*.

In humans, low birth weight has been associated with increased maternal anxiety and prenatal stress resulting from both man-made and natural disasters (Lederman et al., 2004; Maric et al., 2010; Xiong et al., 2008). There are two proposed mechanisms for this phenomenon. The first is that an increased fetal exposure to maternal glucocorticoids, facilitated by alterations in placental permeability due to the reduction of 11ß-hydroxysteroid dehydrogenase type 2 (HSD2; the placental barrier to glucocorticoids) reduces birth weight through catabolic action of stress steroids (Harris & Seckl, 2011; Maric et al., 2010; Van den Bergh, Mulder, Mennes, & Glover, 2005). This mechanism is supported by animal studies demonstrating reduced brain weight at birth (Huang et al., 1999), altered synaptogenesis (Antonow-Schlorke, Schwab, Li, & Nathanielsz, 2003), and permanently altered brain structure (Matthews, 2000). The second mechanism proposes stress-related changes to blood vessels resulting from an adverse prenatal environment, leading to decreased fetal blood supply (Maric et al., 2010). This vasoconstriction of placental vessels is thought to result from a stress-induced increase in norepinephrine levels (Teixeira, Fisk, & Glover, 1999), which may be an adaptive mechanism to protect the mother during times of stress at the expense of her fetus.

Low birth weight is considered a risk factor for various metabolic diseases, such as diabetes mellitus (Harris & Seckl, 2011; Raaijmakers et al., 2017), and mental disorders such as schizophrenia (Gunther, Drukker, Feron, & Van Os, 2005), depression (Gale & Martyn, 2004), and attention deficit hyperactivity disorder (Breslau, 1995; Maric et al., 2010). Since reduced birth weight likely has a metabolic component, and we have shown here that PNMS alters metabolic pathways important in energy metabolism, such as BCAA biosynthesis and the TCA cycle, it appears neurological and behavioural outcomes are probably mediated by metabolic mechanisms. This is further supported by the finding that maternal glucocorticoids mediate effects of diet on fetal biology. For example, feeding rats a low-protein diet during pregnancy leads to higher blood pressure in offspring from the time of weaning. However, blocking maternal glucocorticoid synthesis removes this protein restriction effect (Gardner, Jackson, & Langley-

Evans, 1997; Langley & Jackson, 1994; Langley-Evans, 1997; Langley-Evans, Gardner, & Jackson, 1996; Langley-Evans, Phillips, et al., 1996).

In addition to the effects of MPS and TPS compared to non-stressed controls, the differential effects of PNMS were assessed by comparing these two groups. Interestingly, citrate was found to be significantly down-regulated in the TPS group compared to MPS, but neither had a significantly altered citrate level compared to controls. Likewise, lactate levels in the TPS group were down-regulated compared to MPS rats, but showed no significant change compared to non-stressed controls. Both citrate and lactate are involved in energy generation, particularly in the production of ATP in the TCA cycle, and in muscular ATP regeneration, respectively. If lactate levels are low, and production did not occur, then metabolic acidosis and muscular fatigue will occur more quickly (Robergs, Ghiasvand, & Parker, 2004). Similarly, if citrate levels are low, an energy deficiency may exist (Zheng et al., 2010).

TPS rats also had significantly down-regulated choline and serine levels, compared to the MPS group. Choline and its metabolites are required for several physiological functions, such as cell membrane signaling and cholinergic transmission (Fischer, da Costa, Kwock, Galanko, & Zeisel, 2010), and may be involved in the development of cardiovascular disease, cognitive decline in aging, and blood lipid regulation (Leermakers et al., 2015). Serine plays a central role in cellular proliferation and in the development and functioning of the central nervous system (Tabatabaie, Klomp, Berger, & de Koning, 2010). Altered levels of serine in patients with psychiatric disorders and severe neurological abnormalities underscore its importance in brain development and function (de Koning et al., 2003). Abnormal levels of these metabolites may help to explain cognitive deficits in PNMS offspring, but it remains unclear why the TPS group has significantly lower levels than the MPS group. It has been hypothesized that cumulative stress across generations is mediated by an adaptive response to the constant presence of a stressor (Nederhof, Ormel, & Oldehinkel, 2014), which may explain this effect.

The most significant and consistently affected metabolic pathways in the current stress cohort are involved in protein biosynthesis, energy generation, and immunity. This means that the formation and breakdown of amino acids, as well as energy metabolism, are likely involved in the response to high PNMS. Altogether, the results of this study shed light on some of the potential metabolic mechanisms underlying neuroendocrine, cognitive, and behavioural outcomes in offspring borne of HPA-axis dysregulation resulting from prenatal maternal stress.

CONCLUSION

This study provides a novel approach to outcomes of PNMS HPA-axis dysregulation by taking a metabolomic perspective and providing possible mechanisms for PNMS outcomes in an animal model utilizing a social isolation stressor. More research is needed to elucidate the role of an enriched environment in metabolic and neurologic outcomes. However, the present findings suggest a link between cognitive and behavioural outcomes of PNMS and metabolism.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS

SUMMARY OF FINDINGS

The present findings indicate that metabolomic phenotypes assessed by ^{1}H NMR spectroscopy serve as clinically accessible predictive biomarkers of disease. Compared to other methods, NMR spectroscopy appears to be the method of choice for global, untargeted metabolomic analysis of urine as it permits measurement of the largest number of metabolites (209, compared to 179 in gas chromatography - mass spectrometry and 127 in liquid chromatography - mass spectrometry), and yields the largest chemical diversity. This is not the case for blood, unfortunately, where NMR is only able to detect 49 compounds out of more than 3500 known metabolites that are detectable using platforms such as thin-layer chromatography/gas chromatography – flame ionization detection (TLC/GC-FID) (3381 compounds) and GC-MS (99 compounds) (Psychogios et al., 2011). However, NMR is non-destructive, so samples can be saved and re-used for further analysis. As body fluids go, urine is an ideal biofluid for metabolomic studies since it is very easy to obtain, non-invasive, does not easily transmit infectious diseases, and it contains many clearly identifiable metabolites (209 compared to 53 in cerebrospinal fluid and 49 in blood). As the primary carrier of small molecules in the body, and a critical medium for transporting dissolved gases, nutrients, hormones, and metabolic wastes, blood is a close second in terms of ideal biofluids for metabolomic analyses (Martini, Nath, & Bartholomew, 2015).

In the first study presented (chapter 3), we successfully used an isolated traumatic stressor, the 1998 Quebec Ice Storm, to detect experience-dependent biomarker signatures resulting from PNMS as downstream metabolomic changes using ^{1}H NMR spectroscopy in a human cohort. We accurately differentiated pathologically high and low prenatal stress groups based on a subset of significantly altered metabolites and/or metabolic pathways, which are potentially linked to mental health risks and metabolic illnesses, such as insulin resistance, diabetes, and obesity. A subset of significantly altered metabolites and pathways were also identified, which may help to predict the likelihood of these health risks. The results support long-

lasting metabolomic differences in males and females, such as ketone body production and energy metabolism. Some similarities between high-stress male and female adolescents were also identified, which include alterations in BCAA biosynthetic pathways.

In the second study presented here (chapter 4), we successfully used a social isolation stress paradigm to show that ancestral prenatal maternal stress can lead to metabolic alterations in the offspring of future generations in a rodent model. We identified metabolic signatures generated by cumulative (MPS) and acute (TPS) ancestral PNMS that may predict offspring health outcomes and were able to differentiate between offspring of rats exposed to MPS, TPS, or EE, based on presence and quantity of small-molecule metabolites in blood plasma. ^{1}H NMR spectroscopy was used to identify a subset of significantly altered metabolites and metabolic pathways that may be used to predict and determine future health status and disease risk in translational human studies. By using a metabolomics approach to the study of PNMS in an animal model, our goal was to provide insight into potential metabolic mechanisms underlying well-documented health outcomes of offspring.

EVOLUTION OF METHODS

Since the University of Lethbridge metabolomics program began in 2013-14, started by Tony Montina and Douglas Kiss, our methods have evolved considerably. These changes have mostly occurred in the metabolite identification and statistical analysis stages. For the purpose of documenting these changes through the duration of my master's thesis, I will briefly outline these former methods.

At the metabolite identification stage, to predict where each metabolite will appear in the NMR spectra of bio-fluids such as blood plasma and urine, a reference metabolite library was used. These libraries contained representative spectra of small molecule metabolites expected to be found in blood, urine, and soft tissues. Experimental spectra were compared to these libraries once constructed. Approximately 3.000µg of each metabolite (obtained in pure form through

Sigma Aldrich) were added to 550μL of blood saline buffer, or urine phosphate buffer, in 8-inch high-quality glass NMR tubes (Wilmad) and were analyzed using the 1-D NOESY gradient water suppression pulse sequence noesygpr1d (Bruker, ON, Canada). After assessing a trial of the chemometric mixture analysis software Chenomx NMR Suite (Edmonton, AB), our meagre metabolite library was abandoned for this much larger and more consistent metabolite database, which includes any metabolite currently characterized by the Human Metabolome Database (HMDB). By using this software, we can quickly and consistently identify multiple metabolites at once, across various buffer systems and biological fluids. This is very important in metabolomics, since many spectral signals overlap, making it virtually impossible to correctly classify metabolites one-by-one.

In the statistical analysis stage, the overall structure has remained quite consistent. Scaled and normalized spectra were visualized and compared across experimental groups using PCA and PLS-DA, followed by cross validation and permutation testing. A Mann-Whitney U test was employed to determine which metabolites were significantly altered across these groups. However, there were two key advances in our data analyses that significantly contributed to a more reliable, high-throughput method to performing our statistics. The first was the advent of Yun et al's Variable Importance Analysis based on random Variable Combination, or VIAVC, which systematically resamples variables to reveal synergistic effects that may exist between seemingly unimportant variables (Yun et al., 2015), unaccounted for in univariate statistical tests. By combining random permutations of variable inclusions with ten-fold cross validation, it determines an optimal subset of variables that have led to the most substantial group differences, while removing interfering and non-significant variables.

The second was the introduction of Jianguo Xia's online metabolomics software, Metaboanalyst (Xia et al., 2012; Xia et al., 2009; Xia et al., 2015; Xia & Wishart, 2002,2011,2016). Prior to our use of this software, all statistics were coded and run using MATLAB. Metaboanalyst allowed us to quickly and efficiently visualize data, producing PCA

plots, heat maps, and VIP scores easily. It also allowed us to expand our analyses by incorporating metabolite set enrichment analysis (MSEA), pathway topology analysis (MetPA), and receiver/operator (ROC) analyses. With the help of Michael Opyr, who wrote code that allowed for the direct uploading of NMR data into MATLAB, we eliminated our use of another free software program, called matNMR. With these changes to our statistical methods, we greatly improved throughput and consistency in this stage of our metabolomics analysis.

PRENATAL MATERNAL STRESS AND METABOLISM

Stress is one of the most critical determinants of lifetime health. Here we have shown that (i) offspring whose mothers experienced stress during pregnancy can be clearly identified by profiling small-molecule metabolites in urine and blood plasma in both an animal and human study; (ii) that urinary metabolites are significantly altered in adolescents of mothers who experienced high objective and/or subjective stress during a natural disaster, compared to those who experienced low objective and/or subjective stress using an independent, randomly stressed human cohort; and (iii) that plasma metabolites are significantly altered in MPS, TPS, and EE offspring compared to non-stressed controls, using a rodent model. The majority of the metabolites found to be significantly altered belong to metabolic pathways involved in energy metabolism and protein biosynthesis in both human and rat cohorts, supporting a link between early adverse life events and risk of metabolic illness later in life (Nicholson et al., 2011).

Forty-nine metabolites known to be present in human urine (Bouatra et al., 2013) were identified as significantly related to the level and type of PNMS experienced *in utero* for the Project Ice Storm study. While the results here showed opposite effects in protein metabolism among males and females, particularly in regulation of branched-chain amino acids (BCAAs) and protein biosynthesis, the results also suggest gender differences in lipid and ketone body metabolism, nucleotide sugar metabolism, and energy metabolism. This finding supports known

gender-related fetal differences in genetics, physiology or metabolism that may lead to differential downstream effects manifesting in the metabolome.

Twenty-one metabolites known to be present in blood serum/plasma (Psychogios et al., 2011) were identified as significantly altered across all comparison groups in our rodent model of prenatal maternal stress. Interestingly, while previous studies have shown environmental enrichment to promote positive behaviours and outcomes such as skilled movement (Jadavji et al., 2006; Jadavji & Metz, 2009; Knieling et al., 2009), learning and memory (Falkenberg et al., 1992; Gibb et al., 2014), and affective behaviour (Galani et al., 2007), this study was unable to find any clear mechanism for these outcomes. Metabolites found to be significantly altered by EE were similar to those found in comparing MPS to TPS rats, and included down-regulated citrate and lactate levels, and up-regulated formate levels. These metabolic systems are involved in energy generation and early stages of brain development, respectively.

While the effects of environmental enrichment on the metabolic relationship with health outcomes remained vague in the rat cohort, mechanisms of PNMS appeared to be much more evident. In both the MPS and TPS groups, numerous systems suggesting links to metabolic and neurologic illnesses were found. These include metabolites involved in electron transport chain disruption (formate), muscle performance (creatine phosphate, alanine), protein biosynthesis (branched-chain amino acids), and the TCA cycle (lactate). Furthermore, serine, threonine and choline fluctuations suggest a connection to neurological disorders and disturbed immune system function, since these metabolites play central roles in cellular proliferation, CNS development, and immuno-stimulation.

Using the 1998 Quebec Ice Storm as an independent random stressor, and a social isolation rodent cohort, epigenetic alterations resulting from PNMS were detected as downstream metabolomic changes using ^{1}H NMR spectroscopy. Here, we differentiated between pathologically high or low prenatal stress groups, based on a subset of significantly altered metabolites and/or metabolic pathways. Strong links to a risk of metabolic illnesses, such as

insulin resistance, diabetes, and obesity, were found in both human and rat studies. Additionally, known metabolomic differences in males and females, such as ketone body production and energy metabolism, were supported by Project Ice Storm results. Some similarities between high-stress male and female adolescents were also identified, which included alterations in branched-chain amino acid biosynthetic pathways. Ultimately, we have shown here that stress *in utero* has long-lasting effects on the metabolome, showing significant alterations to amino acid synthesis and energy metabolism in children up to 17 years after prenatal exposure to a severe weather event. Lastly, our trans- and multi-generational stress study provides a novel approach to outcomes of PNMS HPA-axis dysregulation by taking a metabolomic perspective and providing possible mechanisms for PNMS outcomes in an animal model utilizing a social isolation stressor. More research is needed to elucidate the role of an enriched environment in metabolic and neurologic outcomes. However, the present findings suggest a link between cognitive and behavioural outcomes of PNMS and metabolism.

STUDY LIMITATIONS

Alternative techniques including Mass Spectrometry (MS), Gas Chromatography – Mass Spectrometry (GC-MS), and Liquid Chromatography – Mass Spectrometry (LC-MS), as well as alternative bio-fluids such as blood plasma, serum, cerebrospinal fluid, and even tissues may be used to assess the metabolome from different perspectives. A combination of these biofluid types would form a more complete picture of the total metabolomic response. A 'biomarker set', including metabolites from multiple biofluids and platforms is more likely to result in an effective and reliable determinant of future health outcomes than any single biomarker could. It may be a limitation of this study then, that only Nuclear Magnetic Resonance (NMR) spectroscopy was used. However, NMR spectroscopy appears to be ideal for global, untargeted metabolomic analysis of urine (Bouatra et al., 2013), as it permits measurement of the largest number of

metabolites, and yields the greatest chemical diversity. Furthermore, NMR is non-destructive, so samples can be saved and re-used for further analysis.

Urine is naturally an ideal bio-fluid for stress metabolomic studies since it is very easy to obtain, non-invasive, does not easily transmit infectious diseases, and it contains many identifiable metabolites (209 compared to 53 in CSF and 49 in blood). Obtaining blood or tissue for analysis may cause agitation in study participants, resulting in an acute stress reaction that could lead to confounding results. While taking blood for plasma analyses in animal models may be more acceptable, as it can be taken when the animal is sacrificed, avoiding any acute alterations in stress hormones, it is very difficult to attain sufficient amounts for metabolomics purposes. This may result in an inability to recover lost samples and takes up an excessive amount of spectrometer time to make up for low volumes in the signal-to-noise ratio. Finally, there are probably slight deviations in translational findings using blood plasma from rats, since Psychogios *et al.* (2011) have published the Human Serum metabolome, which is dissimilar in two ways; first, humans utilize slightly different metabolic systems than rats, and second, the plasma metabolome may differ slightly from that of serum due to the presence of clotting factors and other metabolites involved in the clotting process.

A final comment on drawing conclusions about potential disease biomarkers using urinary metabolomics analyses is the fact that previous research has shown metabolite concentration (scaled to creatinine) of the average compound in normal human urine can vary by ±50%, with some varying by as much as 350% (Bouatra et al., 2013). This variation in human urine metabolite content most likely extends to blood plasma as well, although plasma may be less acutely affected by dietary changes and activity level. These large ranges in metabolite concentration in urine are due to several factors, including age, gender, genetic background, diet, and activity level (Psihogios et al., 2008; Saude et al., 2006; Slupsky et al., 2007; Stella et al., 2006). In the Ice Storm study, adolescents were separated into gender classifications (male and female), were approximately the same age, and were from the same geographical region,

mitigating much of the normal variance in metabolite quantities seen across individuals. As for the rat cohort, these factors are well-controlled for, with the exception of novel food items in the EE groups. Lastly, the findings of our PNMS rat study support most major findings in our human study, suggesting that these metabolomic alterations are quite robust since our rodent model is much less susceptible to changing environmental conditions (i.e. rat dietary changes, activity levels, age, and geographical origins are very consistent and well-controlled).

CONSIDERATIONS FOR FUTURE WORK

In the Project Ice Storm cohort, possibilities exist to correlate future work, such as body composition analyses and other health outcomes, with the metabolomic findings of the study. A more in-depth gender comparison of this study would also be very interesting, since the results presented here only offer minimal correlative findings of differential PNMS effects in males and females.

An analysis of chronic and acute stress in the rat cohort would be very supportive of the findings presented here and is already underway. Also, since the effects of environmental enrichment were only compared to controls here, further analyses are needed to compare stress and EE groups to parse out additional details regarding these effects.

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