^{1}H NMR METABOLOMICS OF STRESS

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To Clarissa and Emmitt.

ABSTRACT

Metabolomics provides a method to study the dynamics of cellular function by adopting a systems biology approach to the analysis of small molecule metabolites found in various bio-fluids and tissue locations. Chapters one and two of this thesis explore the theory behind metabolomics and reviews representative studies applying NMR metabolomics to neuroscience. Chapter three presents the results from an experiment investigating the alterations of metabolic by-products found in urine caused by both cumulative and remote ancestral prenatal maternal stress. Chapter four explores the results of a similar experiment where the liver tissue from offspring of either cumulative or remote ancestral prenatal maternal stress were analysed. Chapter five is a general discussion and conclusion of the results from both experiments and explores how both sets of results indicate an alteration in the stress response of the offspring.

ACKNOWLEDGEMENTS

I would first like to thank my wife, whose support and encouragement helped me get to where I am today, and my son who has no idea the extent to which he makes me strive to succeed.

I would also like to thank Anthony Montina for the guidance he provided as we learned together what metabolomics is. Thanks is especially due to Dr. Gerlinde Metz, my supervisor, for taking me on as a student and encouraging me to learn new things.

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CHAPTER 1: INTRODUCTION TO NMR AND METABOLOMICS

1.0 Overview

Metabolomics provides an effective method to study cellular activity [1]. This is accomplished by applying a systems biology approach to analysing the small molecule metabolites found in bio-fluid or tissue samples [2]. Studies can be designed to study specific body functions and organs, or general health. Metabolomics has been proven to be an effective method to use when studying normal and pathological brain function [3]. Multiple bio-fluid types have been involved in metabolomics studies of brain-related disorders and diseases.

The most common bio-fluids used in these studies are blood, urine, and cerebrospinal fluid (CSF) [4,5]. While CSF provides the most direct link to cerebral functioning, it is the least accessible [6]; however, blood and urine are both accessible and valuable samples to use in researching the brain [7-9]. The metabolic profiles from these fluids are global, and as such, all of the various body systems will be represented in these fluids. Metabolic wastes are transported by the blood and filtered by the kidneys into urine. While not all metabolites are waste by-products, wastes do represent a large portion of the metabolome [10]. The degree to which waste by-products originating from a specific organ or body system are present in blood or urine will approximately reflect of energy consumption from the organ of interest. Therefore, as the brain consumes almost a quarter of the body's total energy, the profile of blood and urine will contain a large amount of information filtered directly from the brain [11]. Also, as the brain controls much of the activity of the body, conditions affecting the brain may manifest throughout the body as various other metabolic alterations, which will compound the net metabolic flux measured in bio-fluids. Analysis of these fluids provide an accurate method of

tracking disease progression [12], drug metabolism [13], recovery from injury (traumatic brain injury, TBI) [14], and response to environmental factors [15,16], making metabolomics of bio-fluids a valid experimental technique for studying how an organism responds to a disease or other stimuli.

Tissues may also be used in metabolomics studies [17]. Although they require additional processing steps, they offer a localized perspective on the metabolic response of specific tissues to pathophysiological stimuli [18]. As will be shown, this allows for the possibility to detect and analyze how psychological stressors can impact body systems.

This thesis will introduce the theoretical framework that supports and validates metabolomics research. Introductory theory will also be provided on NMR, an analytical technique frequently utilized to obtain the metabolic profiles in metabolomics. The metabolomics theory will then be applied and illustrated using two case-control rat model studies of ancestral prenatal maternal stress. One involves urine metabolomics, the other uses tissue metabolomics of liver homogenate.

1.1 Systems Biology

Before exploring metabolomics specifically, it is worthwhile to examine systems biology approaches in general, and the primary research modalities that fall under this umbrella. Systems biology refers loosely to any holistic approach of modelling the complexity of biological systems. It differs from traditional reductionist approaches to biology which sought to dissect organisms to the smallest level possible and study the parts of a system in isolation. Systems approaches aim to take all the molecular pieces and reintegrate them to understand how they interact and function within the context of a living system [19].

Living systems are primarily governed by the activity of four classes of biological components: genes, transcripts, proteins, and metabolites. The study of each class of molecule has developed into an independent field of research, and when the study of these components is approached from a systems perspective they are often referred to as the 'omics'. As such, the study of these classes of molecules are referred to as genomics, transcriptomics, proteomics, and metabolomics. The modelling of a system generally involves generating a thorough accounting of all the components, and then attempting to organize them into a network of various biochemical pathways and activities that reflect normal function [20]. The pathways, networks, and profiles that are characteristic of healthy normal function can then be compared to networks and profiles obtained from abnormally functioning systems. This facilitates the targeting of specific molecular processes and networks that go awry or malfunction in response to various stimuli [21].

Genomics, transcriptomics, and proteomics provide information about the structure of system. These methods describe the framework that constrains cellular function. Alternatively, metabolomics provides information on the dynamic functioning of the structures within a cell. Metabolites occupy a unique role as they serve as the building blocks for genes, transcripts, and proteins, as well as the substrates and by-products of metabolic activity. They can also interact with genes, transcripts, and proteins as cofactors and regulators of reactions [22]. Metabolites are the limiting currency within a cell. Whether considering energy needs or the structural component requirements for the macromolecular genes and proteins, the necessary small molecule metabolites must be present in order for functioning to occur. While the traditional reductionist biology tends to describe the information flow within a cell in a linear way, starting with genes and

ending with metabolites, systems biology approaches aim to consolidate the components into a cohesive network of interactive biological machinery to understand the holistic function of a system. The parts of a cell are heavily interconnected, and detailed understanding a system functions requires an appreciation of the relationships between the different biological components.

1.2 Metabolic Phenotype

Of all the 'omics' methods, metabolomics provides the closest measure to phenotype [23,24]. The genome of an organism stays essentially constant throughout a lifespan and a profile of all genes gives an idea of what is possible, not necessarily what is predetermined to happen. The environment interacts with the genome to control gene expression and determine phenotype, which is dynamic throughout a person's life [25]. An illustration of how phenotype changes throughout the lifespan can be given by considering how a person changes as they age. An individual, from infant to elder, possesses the same genome throughout their life. Through varying which genes are expressed, they are able to grow, develop, and change as they age. Therefore, in contrast to the static genome, the expression of the genome, or phenotype, is capable of alterations. This process is mainly controlled by epigenomic regulation [26]. The flexibility of phenotype throughout life results in various proteins being built through different stages of life, as well as in response to environmental cues or disease. When different proteins are expressed, cellular function changes, resulting in a varied utilization of available metabolites and ultimately in distinct metabolic profiles reflective of the current phenotypical state.

The metabolic by-products of an organism reflect the cellular processes that are currently at work [27]. Metabolism is the downstream terminal of the information pipeline that starts with genes and proceeds to protein synthesis and function; therefore, the metabolic profiles will reflect all upstream gene expression and protein function. Beyond this, metabolism also explains many short term effects caused by ingested toxins or drugs that involve a drastic change in cellular function, but no change in gene expression [1]. This is an additional reason why, of all the 'omics', metabolomics is able to most accurately explain and describe phenotype.

Research has been conducted to verify that metabolic phenotypes are consistent in healthy individuals [28], lending validity to the concept of using a metabolic phenotype as a reliable measure to monitor health. Additionally, diseases are accompanied by changes in metabolism [27]. Some studies have even suggested that detectable metabolic changes precede the expression of clinical symptoms of disease [29]. Therefore, there is credible scientific evidence lending support to the efforts pursuing metabolomic screening procedures capable not only of diagnosis, but also early detection of disease. While this has not yet been developed and implemented in a clinical setting, the practicality of it exists and this concept drives a large portion of health related metabolomic research.

1.3 Metabolomics

The goal of metabolomics is to provide a detailed account of the metabolic milieu that accompanies cellular activity [30]. Properly applied, this approach has the ability to determine interactions and activity of up-stream genes and proteins, as well as effects caused by environmental factors. Chemical analysis of samples is combined with multivariate statistical analysis to isolate the metabolites that vary significantly in a

meaningful way with the experimental outcomes. Pathway analysis can then be performed to identify molecular processes that are being affected by stimuli. Metabolomics has successfully been applied to a variety of research areas such as systems biology, drug discovery, disease detection, toxicology, food science, and more [31].

The infancy of the field combined with the broad spectrum of applications translates into there currently being no standardization of the way to conduct these experiments and analyse the data. An initiative has begun that aims to provide standards of experimental practice that will aid in maintaining the integrity of information in metabolomics, which will increase the validity and consistency of inter-laboratory results [32]. However, there are no standardized protocols that are unanimously accepted by researchers. Some suggest that this is ideal, as methods are constantly being improved [33]. Further complicating the lack of precise protocols, there are multiple approaches to take in the research. One example of this is collecting the data. Presently, there are two dominating analytical platforms to use, either mass spectrometry (MS) or nuclear magnetic resonance spectroscopy (NMR). Both techniques are highly informative and offer advantages the other lacks. As the work in this thesis focuses on NMR metabolomics, a brief history of the development of NMR will be explored. A classical description of NMR theory will also be given for the purpose of understanding the theoretical basis of the applicability of NMR to metabolomics. Following the discussion of NMR, an outline of the most common standard practices in metabolomics will be discussed in the following sections.

CHAPTER 2: METHODS OF ^{1}H NMR METABOLOMICS

2.0 Nuclear Magnetic Resonance Spectroscopy

2.0.1 Historical Findings in Magnetic Resonance

The theoretical and experimental groundwork that evolved into modern NMR spectroscopy began in the 1890's. It is worth reviewing some of the milestone discoveries that led up to NMR, as the logical progression of the experiments aid in explaining the theory of magnetic resonance.

Pieter Zeeman, in 1896, wanted to study what effect magnetism could have on visible light. To do this, he placed flaming sodium in an electromagnet and captured two emission spectra from the sodium; one with the magnet turned on and the other with it off. He noticed that with the magnet off the spectrum had more narrow lines than when the magnet was on. Later, using cadmium as light source with higher resolution, he determined that what first appeared as a broadening of the spectral lines was actually the lines splitting. The splitting of the emission lines is now referred to as the Zeeman effect or Zeeman splitting [34]. The explanation of why the splitting occurred would not come for another thirty years when the atomic property of spin was characterized. Zeeman was a co-recipient of the 1902 Nobel Prize in Physics along with Hendrik Lorentz.

In 1922, Otto Stern and Walter Gerlach were experimenting with concepts of quantum mechanics at a time when the quantum theory was new and the idea of mass, energy, and charge existing in discrete quantities was viewed with scepticism. They were attempting to test a hypothesis set forth by Neils Bohr and Arnold Sommerfeld concerning quantization of electron orbitals. Their experiment involved the production of a beam of silver atoms that was passed through slits to collimate the rays (make them parallel so as to have minimal spread during propagation) [35]. The collimated beam of

silver atoms was then passed through a magnetic field. From classical models, the expected result was that the particles passed through the magnetic field would form a continuous random pattern. The quantum model predicted that the particles would cluster in discrete groupings. Unsurprisingly to modern scientists, the result was that expected from the quantum model. This discrete grouping of the silver atoms was due to the spin angular momentum of the unpaired electron in the silver atom interacting with the magnetic field. This property of spin is the same property responsible for the Zeeman splitting. Stern received the Nobel Prize in Physics in 1943 for his continued work in measuring the quantized spin of the proton.

A few years later in 1925 a major breakthrough in quantum theory came with the formalization of the concept of spin. As Wolfgang Pauli was contemplating the splitting of spectral lines observed by Zeeman he proposed that electrons possessed a "classically indescribably two valuedness" [36,37]. This statement means that the electrons seemed to possess the ability to be in either of two states, and that this quality was not accounted for by classical physics. This "two valuedness" is now referred to as spin, or spin angular momentum, and is a fundamental property of matter that is central to the NMR experiment. Though Pauli described the spin of electrons and used it to solidify his theory on the exclusion principle, the same property of spin is possessed by protons and neutrons, and just as the property of atomic mass and charge in atoms are a summation of the masses and charges of the protons and neutrons, so too does atomic spin exist as the net sum of spins of the constituent protons and neutrons.

To this point, it had been discovered that magnetic fields could force atomic particles to assume orientations that were quantized, or limited to discrete values. Isidor

Rabi focused on manipulating the particles to make transitions between the different quantum levels. He accomplished this by applying an oscillating magnetic field to particles being passed through a static magnetic field. He found that when the energy of the oscillating field matched that of the energy difference between the quantized states that he could alter the orientation of the particles and have them transition to the other energy state. This effect is referred to as magnetic resonance and is a core component of the NMR experiment. The Nobel Prize in Physics in 1944 was awarded to Isidor Rabi for his work on magnetic resonance.

The magnetic resonance effect, first observed in beams of particles by Rabi, was put to test in bulk matter by Felix Bloch and Edward Purcell. They worked independently of each other, but both discovered magnetic resonance effects, Bloch in water and Purcell in wax, at almost the same time. The methods employed were similar to those used by Rabi, namely that the sample was placed in a strong external magnetic field and a transverse oscillating magnetic field was applied. However, instead of being able to use a screen to detect deflection of particles, they needed more discrete methods of measurement. Purcell anticipated a change in the conductance of the wax and detected this using an electrical circuit [38]. Bloch arranged an electric coil to detect any electromagnetic radiation that would be emitted from the sample as a result of having the oscillating magnetic field applied [39]. Both detection methods worked and both proved that the magnetic resonance effect could be applied to bulk matter. Bloch and Purcell were joint recipients of the 1952 Nobel Prize in Physics.

2.1 Current NMR Theory

2.1.1 Properties of Subatomic Particles

Following these discoveries, it was understood that the innate magnetization of matter could be manipulated and monitored using strong magnets and precise electronics. These theories matured into the modern fields of NMR and MRI. Here we will provide a basic description of classical NMR theory and the NMR experiment, a more detailed description can be found in a number of NMR textbooks [40-42]. The majority of NMR experiments deal in some way with the hydrogen atom, and it is the hydrogen atoms of a sample that are the focus of most NMR metabonomic experiments; therefore, they will serve as the prototypical nuclei to explain NMR. Hydrogen atoms possess only a single proton in the nucleus, and as such are often referred to simply as protons in NMR jargon. Protons and neutrons, sometimes referred to collectively as nucleons, constitute the nucleus of atoms and possess certain intrinsic properties that are the basic units used to describe matter. The properties under consideration, and which are fundamental to producing the magnetic resonance effect, are mass, charge, and spin.

2.1.2 Mass and Charge

That protons and neutrons have a mass is easily understood, as mass is present in the macroscopic world. The mass of any substance, at an elementary level, is the net sum of the masses of the nucleons. Likewise, nucleons also possess a charge. The charge of a proton is +1 and the charge of a neutron is 0. The net charge of a nucleus is the net sum of the charges of all the constituent protons and neutrons. Therefore, the proton's charge is just +1. At a macroscopic level the positive charge of a nucleus is generally cancelled out by the negative charge of the electrons, making the net charge of the object 0. This is

obviously not always the case, as charge imbalances are the principle behind voltage, the driving force of electric currents. However, in the average desk for example, the charges of the nucleus and electrons will cancel.

2.1.3 Spin

The other intrinsic property of nucleons is spin. Spin is difficult to conceptualize because it does not have a macroscopically observable presence. It is important to remember that spin is a fundamental aspect of the building blocks of matter and cannot be described as a product of mass or charge, it exists independent of the other properties and causes the atom of particles to behave as if they were spinning. Protons, neutrons, electrons, and most of the other subatomic particles have spin. In physics terms, spin is a vector because it has both a magnitude and a direction. In matter outside of a magnetic field the spins align randomly, have a single energy level, and produce a net spin vector of zero. In a magnetic field, the spins will experience Zeeman splitting and separate into 2I+1 different energy levels, where I is the value of the atom's spin. A proton is spin ½, therefore energy states of protons in a magnetic separate into 2 energy levels. These two energy levels are analogous to the splitting of atoms into distinct groups by Stern and Gerlach and the broadening of spectral lines by Zeeman.

2.1.4 Magnetism

From classical mechanics, it is shown that a rotating mass will have an angular momentum vector that points along the axis of rotation. Classical mechanics also provides the information that a moving electric charge will produce a magnetic field. These two concepts are manifested at the atomic level. From the explanation of intrinsic properties, it is known that a nucleus will have a mass, charge, and spin. The spinning

character of the nucleus acts on the mass to produce an angular momentum vector (J) and works on the charge to produce a magnetic moment (μ). The nucleus of an atom can be characterized by the angular momentum and the magnetic moment using a value called the gyromagnetic ration (γ). The relationship between J, μ, and γ is given in equation 1, where q is the charge of the nucleus and m is the mass.

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

This equation is a simplified way to view the calculation of γ, and is presented this way to illustrate how γ depends on the magnetic moment and angular momentum of a nucleus, or in more fundamental terms, the mass and charge of a nucleus. Since for any element, the nucleus of different atoms are identical, the γ will be identical for all atoms of the same element. Therefore, all protons will have a γ = 42.5 MHz/T. Gyromagnetic ratios are normally presented in units of rad s^{-1} T^{-1}, but the MHz/T units represent a more user friendly representation of the data. The value of γ determines some of the behaviour of the nucleus when placed in a magnetic field

Magnets interact with each other in a predictable manner through their magnet fields. Applied to NMR, the small magnetic moments of the individual nuclei will interact with any magnetic field they are placed in. The force of interaction will be proportional to the size of the magnetic field vectors being considered. Atomic magnetic moments do interact with the earth's magnetic field, although it is a very weak interaction as the earth's magnetic field is relatively weak. Nuclei cannot have their magnet fields amplified; however, the field they are placed in can be augmented to increase the strength of interaction. This process is enabled by NMR spectrometers. At the heart of every

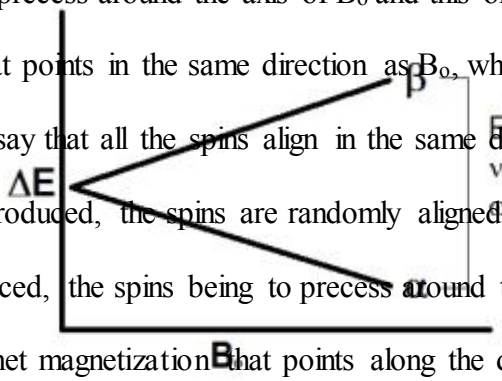
spectrometer is an electromagnet that produces a field which can be up to 100 000 times stronger than earth's field. The magnet needs to be strong enough to interact with the tiny atomic magnetic moments and thereby induce a polarization in the sample. As mentioned earlier, the nuclear spins align randomly in matter. When placed in a magnetic field, the spins begin to precess in an organized manner. The precession is caused by the torque resulting from the interaction of the magnetic moment with the magnetic field of the magnet (B_o). Shown in equation 2, μ and B_o have a vector product (torque, τ) which will be perpendicular to the two vectors. This perpendicular nature of the torque produces a perpetual spinning motion called precession.

Equation 2. $\tau = \mu \times B_o$

The precession can be compared to a spinning top, with the top's angular momentum being analogous to the magnetic moment and the earth's gravitational field being analogous to the static magnetic field of the spectrometer. As the top is spinning in the gravitational field it begins a slow precession as long as the angular momentum is not directly aligned with the gravitational field. If the angular momentum were perfectly aligned with the gravitational field, there would be stable rotation (no perpendicular torque) as the cross product of parallel vectors is zero. This analogy offers a visualization of what precessional motion looks like. The rate at which precession occurs is referred to as the Larmour frequency (ω), and is determined by multiplying the γ for the nuclei by B_o.

Equation 3. $\omega = \gamma B_o$

The spins all precess around the axis of B_o and this oriented motion produces a net magnetic moment that points in the same direction as B_o, which is defined as the positive z-axis. This is not to say that all the spins align in the same direction along the field. Before the field is introduced, the spins are randomly aligned with a no net magnetization. As the field is introduced, the spins being to precess around the field, and this uniform precession creates a net magnetization that points along the direction of B_o (Figure 1). This is a total net magnetization, and is composed of a net magnetization pointing parallel to the field (α) and another net magnetization pointing in opposite direction (β) as shown in Figure 1. The more energetically favourable orientation is that aligned parallel to the field, as such it is larger and the total net magnetization always points parallel to B_o. However, the energy difference between the two states tends to be quite small and the population difference is small as well. As the field strength is increased it becomes more energetically favourable to be in the α state, as such, the population of spins in the α state increases, the difference in energy between α and β states increases, and the net magnetization vector increases. This widening of the energy difference between α and β with an increase in magnetic field strength is a manifestation of the Zeeman Effect (Figure 2).



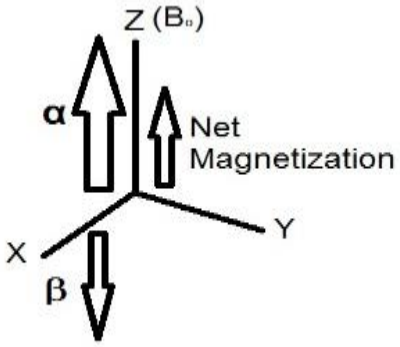


Figure 1. Illustration of the net magnetization being composed of parallel and anti-parallel components, with B_o pointing along the positive z-axis.

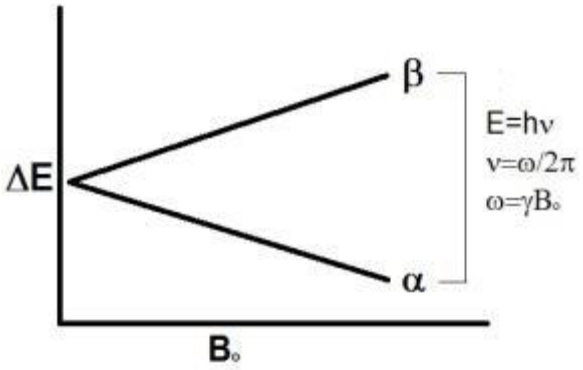


Figure 2. Illustration of Zeeman splitting with formulas correlating the Larmour frequency to the energy difference between the two energy levels.

Population differences between α and β states are also affected by temperature. If the temperature is increased, there is a greater amount of energy available to the spins and the unfavourableness of the anti-parallel state is diminished. Conversely, as the temperature of the sample is lowered, the anti-parallel state becomes even more energetically unfavourable, the parallel state becomes more populated, and the net magnetization of the sample increases. Increases in the net magnetization will in turn increase the strength of the signal obtained during experimentation. The determinants of population difference, and therefore net magnetization of the sample, are summarized in equation 4.

Equation 4. $\frac{P\alpha}{P\beta} = e^{\frac{\gamma B}{kT}}$

Where P_α is the population of spins parallel to the field, P_β is the population of spins anti-parallel to the field, B is the strength of the static magnetic field, T is the temperature in the Kelvin scale, γ is the gyromagnetic ratio, and k is the Boltzmann constant.

The work done by Rabi, Bloch, and Purcell showed that the bulk magnetization of a sample could be manipulated by applying an oscillating electromagnetic pulse. The energy of the pulse must match the energy difference between the α and β spin states, which corresponds to the Larmour frequency. These pulses are referred to as on resonance pulses. Again, this energy difference will depend on the value of γ, or in other words the type of nucleus, and the strength of B_o as in equation 2. This energy difference corresponds to electromagnetic radiation from the radio frequency (RF) range of the spectrum, as such the applied field is often referred to as an RF pulse. Equation 5 shows the relations ship between the frequency of electromagnetic radiation and energy.

Equation 5. $E = h\nu$ (h is Planck's constant and ν is the frequency of the RF pulse)

2.1.5 Detecting the NMR Signal

When simply precessing about the z-axis, there is no way to measure the frequency of the bulk magnetization. This is because NMR measures the changes in magnetization over time. At equilibrium, the vector is precessing symmetrically around the z-axis, leading the transverse component of the magnetization to average to 0 over time. The application of the RF pulse occurs perpendicular to the z-axis in the x, y plane, which is also referred to as the transverse plane. This pulse is generated using an RF coil and applied at a frequency that is on resonance with the nuclear spins of interest; more importantly. It has the effect to force the net magnetization from the z-axis into the transverse plane where it continues to precess around B_o. The physics behind the pulse tipping the magnetization is similar to the torque causing precession. Except instead of a single magnetic moment being caused to precess, the bulk magnetization experiences a torque that forces it into the transverse plane. It is the transverse magnetization that serves

as the source of the NMR signal. Once in the transverse plane, the net magnetization will "relax" back to equilibrium, much as a bobo doll returns upright after being struck. This occurs in an asymmetrical manner, leading to a magnetization vector that does not average to 0 over time, and is therefore detectable. There are two forms of relaxation that govern the return to equilibrium relaxation. Transverse, or T_2 relaxation is a measure of how long it takes for the transverse magnetization signal to diminish. Longitudinal, or T_1 relaxation is a measure of how long it takes for the net magnetization to rebuild along the z-axis. These two processes are both on the order of seconds, with the transverse magnetization typically diminishing faster than the longitudinal magnetization rebuilds.

Pulse sequence is the name given to the structured array of RF pulses applied to a sample before the resulting signal is acquired. The Simplest version of a pulse sequence is the pulse-acquire experiment (described above) in which a 90-degree RF pulse is applied and is immediately followed by acquisition. The longer a pulse is applied for, the farther the magnetization will be tipped, and it is not uncommon for one pulse sequence to incorporate multiple pulses with multiple tip angles in order to manipulate the magnetization to give very specific information. Different pulse sequences are designed to give specific information about the molecules in a sample. Basic experiments are designed to inform on the structural connectivity of functional groups within a molecule. More complex experiments can give information regarding the interaction of two atoms of the same element, interaction of two atoms of different elements, and the distance between atoms in the molecule, to name a few. Other pulse sequences can be designed to remove, or suppress, undesirable signals. The pulse sequences are constrained in duration by the relaxation rates of the nuclei within the sample. As soon as a sample is irradiated

with an RF pulse, it instantly begins to re-establish equilibrium magnetization along the z-axis. Therefore, a pulse sequence needs to be able to apply all the necessary RF pulses fast enough to still have net magnetization present in the transverse plane to detect.

The NMR signal is detected during the relaxation process. The RF pulse does not change the Larmour frequency of the protons, it only forces the magnetization into the transverse plane to be detected. The frequency is detected using the same electric coil that delivered the RF pulse. The principle of reciprocity states that the ability of a substance to deliver an electric current is proportional to its capacity to have an electric current induced in it. The RF pulse is delivered through an electric coil. Since all electric currents emanate a magnetic field, it is the magnetic field produced by sending a current through the coil that generates the RF energy that tips the net magnetization. Once in the transverse plane, the precessing magnetization has the ability to induce a current in the coil. This is exactly what happens. Magnetization in the transverse plane induces an electric current in the coil as it moves to equilibrium. This analogue current is the detected NMR signal and it is digitized using an analogue-to-digital converter (ADC). The resulting digital NMR signal is then sent to a computer for processing.

The raw data from an NMR experiments is called the free induction decay (FID), and represents the sum of all the precessional frequencies of the sample in the time domain. Figure 3 illustrates the FID for a single frequency. In this case, it would be a relatively simple task to measure the period of the time signal and calculate the frequency. However, real data is much more complex and represents hundreds of overlapping frequencies, as shown in Figure 4. In this case, calculating the period of each

time signal is nearly impossible by hand and a computer algorithm that employs a specialized mathematical function is used to determine the resonance frequencies.

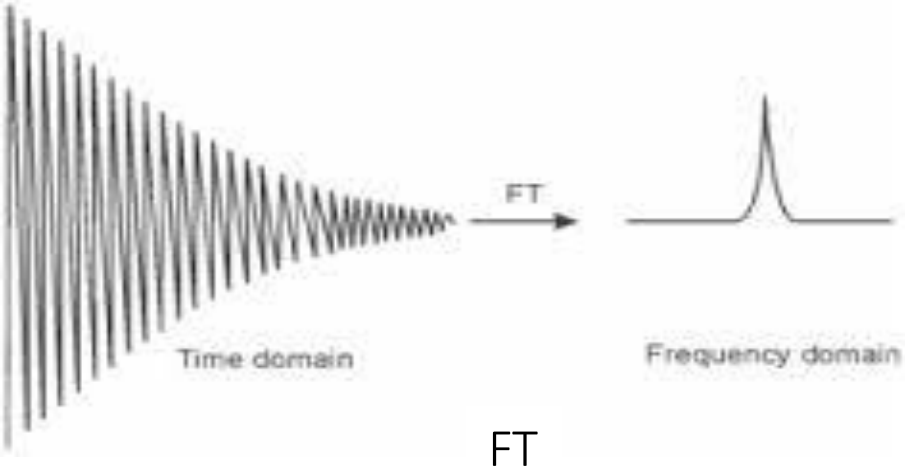


Figure 3. FID of a singfrequency and the resulting FT.

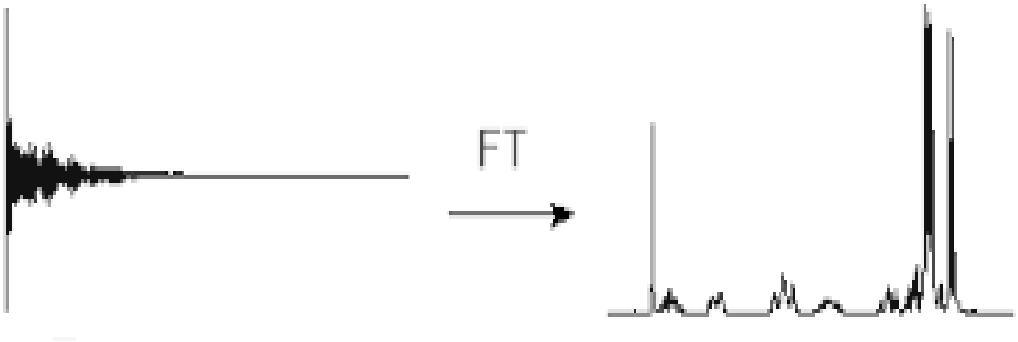


Figure 4. FID of real data and the resulting FT showing a portion of the spectrum.

The mathematical function used by the computer to determine the frequencies in the FID is called a Fourier Transform (FT), named after the French mathematician Jean-Baptiste Joseph Fourier who developed the theory around 1811. The FT is the practical

implementation of the Fourier Relationship, which explains how the time domain is related to frequency domain. Simply put, when a FT is applied to a signal in the time domain, or the FID, the result will be a signal in the frequency domain, known as a spectrum.

When the FID is acquired it is a time domain signal that represents the precession of the bulk magnetization in the transverse plane. As the signal returns to equilibrium, the amplitude of the FID is seen to diminish, but the wavelength, or frequency, remains constant over time. By applying a FT to the FID the signal is converted to a frequency domain. Once represented as frequencies, the information is much simpler to interpret, and this is how NMR data is most often presented.

2.1.6 Chemical Shift

In a basic proton NMR experiment the only type of nucleus being observed is hydrogen. Since all protons have the same γ and during the experiment they are all in the same strength of magnetic field, one would expect from equation 3 that all the protons in the sample would precess at the same frequency and would therefore result in a single resonance peak in the spectrum. This is not the case, and in even a relatively simple spectrum of a single molecule there can be many peaks. This is because the magnetic field that is experienced by each of the protons is not just B_o, but is actually B_o plus the tiny magnetic fields generated by the circulating electrons in the molecule. Electrons tend to have the effect to oppose B_o, effectively lowering the magnitude of the external magnetic field experiences; therefore, the more electrons crowding a proton, the lower its frequency will be (an effect called shielding), and the fewer electrons crowding a proton the higher its frequency will be (an effect called deshielding). This is shown in equation

6. It is because of these minutely different localized magnetic fields that different frequencies are observed for a single type of atom. To give an idea of how small the divergences are, the differences in local magnetic fields of chemically different nuclei are on the order of 1-10 millionths of the magnitude of the static magnetic field strength B_o In NMR, the frequency of each spectral line is known as the chemical shift, and is affected by the local electronic environment of the nuclei. There is a high degree of consistency in what amount of shielding is caused by different functional groups. This knowledge has been applied to help identify structural elements of molecules using NMR.

Equation 6. $\omega = \gamma B_{local}$ where $B_{local} = B_o - B_{eletrons}$

Frequencies of chemical shifts in an NMR spectrum are given in parts per million (ppm) as shown in equation 6. The reference frequency is a frequency that is assigned to be zero ppm. Generally, that frequency is the resonance frequency of protons in trimethylsilane (TMS). In metabonomics, the solvent used is water, TMS is not water soluble; therefore, water soluble trimethylsilylpropionic acid (TSP) or 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) are most often used instead. TSP and DSS have almost identical chemical shifts to TMS and are used as the 0 ppm reference point. A major advantage to using the ppm scale to represent chemical shifts is that as the magnetic field of new spectrometers increases the actual resonant frequency of the nuclei, the relative chemical shift in ppm does not change.

Equation 7. $\delta\ (\text{ppm}) = \frac{\nu ref - \nu}{\nu ref}$

(ν_{ref} is the Larmour frequency of the reference compound (TMS, DSS, or TSP) and will be at 0ppm.

The strength of an NMR magnetic field is often quoted as the Larmour frequency of a proton in that magnetic field. Therefore, a magnet with a strength of 7.04 Tesla would be referred to as a 300 MHz magnet as the protons would rotate 300 million times each second in that magnetic field. This value of 300 MHz is obtained by multiplying the magnetic field strength by the γ for proton as in equation 3. Similarly, a magnet with a field strength of 16.4 Tesla would be referred to as a 700 MHz magnet because the protons would be rotating 700 million times each second.

2.1.7 J-Coupling

In the NMR world, magnet manufacturers have constantly been pushing to develop more powerful magnets. This may seem an unimportant advancement as the chemical shift of the signals will not change. What does change, and why increased magnet strength is important, is that the overall obtained signal intensity is greater (equation 4) and the number of Hz within each part per million is increased. This has the effect of spreading out the chemical shift of each signal. Inspection of an NMR spectrum will show that there is often some sort of multiplet structure. These multiplets retain the splitting pattern and splitting distance in Hz with an increasing B_o field strength. This means that as the chemical shifts separate further with increasing B_o, the splitting of each signal will remain constant, leading to a more easily interpreted spectrum. This is well illustrated in the proton spectrum of arginine acquired at 300 and 700 MHz (Figure 5 and 6, respectively). At 300 MHz there appears to be a single multiplet structure at the far right of the spectrum and this is shown to be two clearly separate multiplet signals at 700 MHz. This is because the two multiplet signals are overlapping at 300 MHz and the increased magnetic field at 700 MHz has allowed for the separation of these two signals.

Interestingly, the two triplets at the left of the spectra do not appear to change between the two different magnetic field strengths; furthermore, if the splitting of these triplets were measured, they would be the same in both spectra. This is just one illustration of many demonstrating the increased ability to analyze a spectrum that comes with an increase in magnet strength.

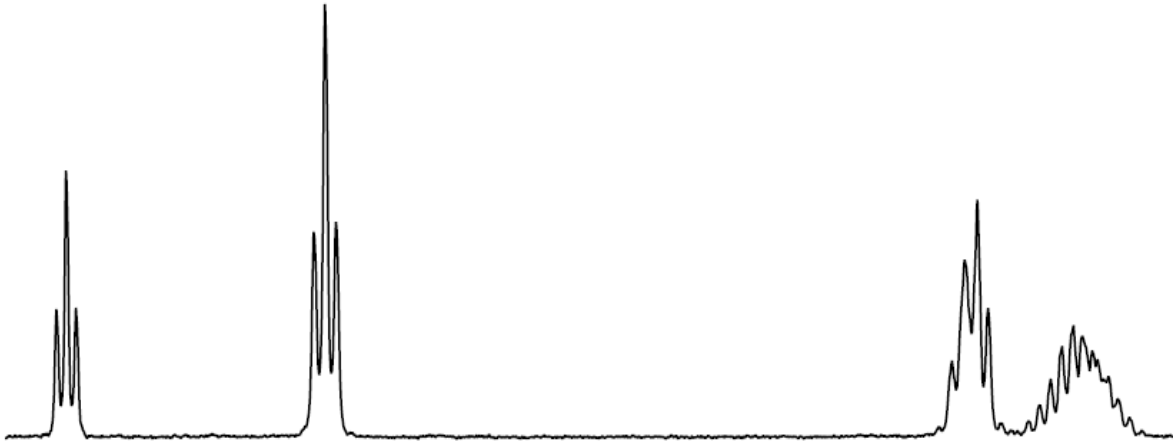


Figure 5. Arginine proton spectrum at 300MHz.

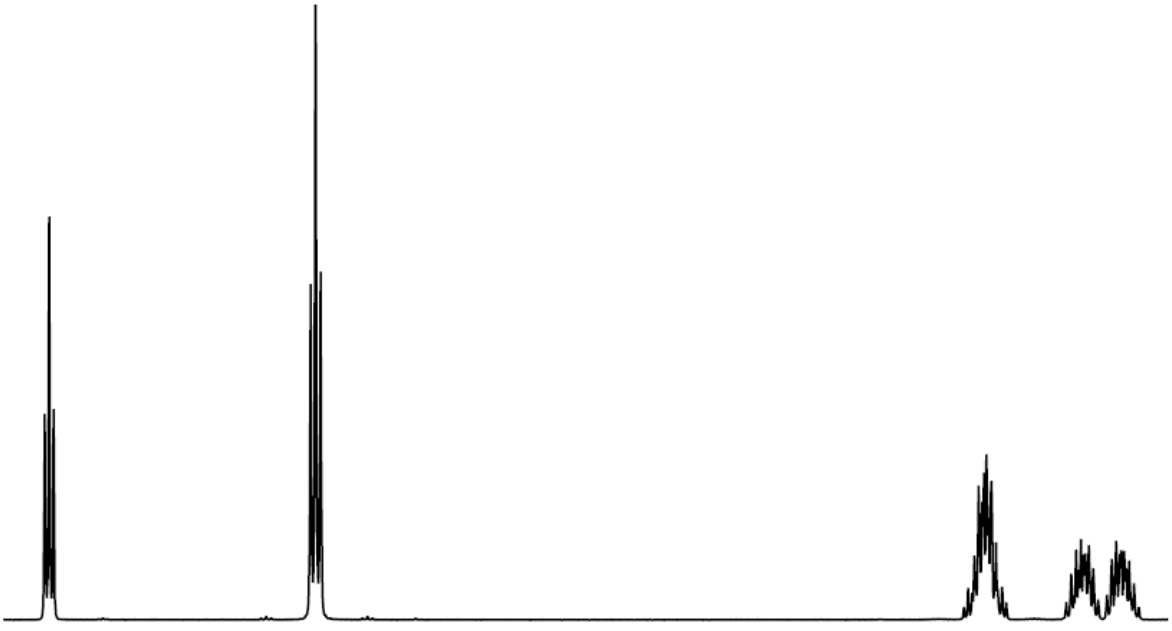


Figure 6. Arginine proton spectrum at 700MHz.

The multiplet structure gives information regarding the connectivity of the molecule. This is because each proton, or unique peak in the spectrum experiences a small magnetic field from adjacent non-equivalent proton spins, as each spin is essentially a little bar magnet. These small magnetic fields cause the single peak to be split into 2*N*I +1 lines, where N is the number of non-equivalent proton nuclei adjacent to nuclei and I is the spin of the nuclei. For Protons where I=1/2 this equation simply becomes N+1. Thus, if you have no proton neighbors (N=0) you will get a single peak (singlet), if you have 1 non-equivalent proton neighbor (N=1) you will get 2 peaks (doublet), and so on and so forth. The intensity of each peak in the multiplet pattern follows the rules of Pascals' Triangle. This coupling to neighboring nonequivalent protons is known as J-coupling and is a much smaller effect than the shielding or deshielding, as it is given in Hz; furthermore, as mentioned above, the splitting observed due to J-coupling is independent of the magnetic field strength.

When the signal is received as an FID, all of the information about chemical shift and coupling is in the FID. Upon FT of the data, the frequency information is easily interpreted and the structure and connectivity of the molecule being studied can be determined. The magnitude of signal coming from each peak in the spectrum is proportional to the number of spins contributing to that signal in the sample. Integration of a peak in the spectrum is a way of measuring this magnitude and, in a sample of a single compound, will correspond to the number of protons making that signal. From this, peaks in an NMR spectrum from methyl protons are easily distinguished from peaks arising from methylene or methine protons as the integration values will scale as 3, 2, and 1 respectively. In metabolomic samples containing multiple molecules, the integration

values of the different peaks can be adapted to be used as a measure of relative concentration, as a more concentrated molecule will produce more signal and therefore a larger integral than a less concentrated molecule. If a peak is split into a multiplet structure, the entire multiplet must be integrated to obtain an accurate value.

2.1.8 Receiver Gain

In order to acquire all the information from the FID the spectrometer's electronics must be calibrated properly. One value that changes frequently is the receiver gain of the analogue-to-digital converter (ADC). The receiver gain is adjusted to be able to detect the entire strongest signal in the spectrum. In metabolomics this is problematic as the sample is almost entirely water at about 55M. The dissolved metabolites will constitute only a small portion of the NMR signal, with concentrations in the mili- to micromolar range. The problem arises because when the signal is acquired it must be digitized. An effect of the digitization can be visualized as follows. Consider the NMR peak to be a certain height, say two feet. Upon digitization, these two feet are segmented into a defined number of equally spaced pieces, like ticks on a ruler or measuring tape. If the two feet were to be segmented into only eight pieces, the rest of the signals would be defined as being values corresponding to one of those eight segments. If a signal were to be too small to be taller than the first segment (less than 3 inches in this example) then the signal would not be visible in the spectrum. The reason the water signal is problematic in metabolomics is that it is literally thousands of times stronger than the metabolite signals. This means that once the receiver gain is set to accommodate the massive water signal, there is a loss of sensitivity to the lower concentration signals. The spectrometers digitize

into much more than eight pieces, but if nothing is done to the water signal there is a large portion of information lost due to the decreased sensitivity.

Modern pulse sequences are able to manipulate the net magnetization in such a way that the signal from the water is almost completely removed. The benefit in this is that the sensitivity of the digitization to the low level metabolites is increased. Back to the two-foot signal example, each segment of digitization with the two-foot signal is three inches. If that signal is removed and the next largest signal is only one inch, then segmentation into eight pieces leaves a digitizer that is sensitive to within an eighth of an inch. Again, the actual method of how this happens is more sophisticated and involves more knowledge of electronics, but the analogy of how removal of the water signal improves sensitivity to low concentration metabolites is valid.

2.2 Metabolomics Methods

This section will discuss metabolomics methods and procedures in the order that an experiment is conducted. A normal experiment would first have the sample collected and stored, the sample would then be prepared for experimentation and the data collected. Following this is statistical analysis and interpretation of the results. With this workflow in mind, sample collection recommendations will first be discussed. Following this, the strengths, weaknesses, and preparation methods of the most common sample types will be discussed. This will be followed by a comparison of NMR and MS for data collection. The last section will be on the various statistical approaches that are often adopted in metabolomics. As the focus of this thesis is NMR metabolomics, the discussion will focus on NMR, with exception to the section comparing NMR to MS.

2.2.1 Sample Collection and Storage

Sample collection protocols will vary depending on the type of sample being analysed and the procedures followed by a particular lab [43]. Regardless of the sample type, there are necessary precautions that must be taken to ensure that once the sample has left the organism the integrity of the metabolic profile is maintained [44]. The profile is most often affected by bacterial digestion of the metabolites. A common way to protect the metabolites from this is to immediately freeze the sample once it is collected. Normally stored at -80°C, a review of storage procedures indicated that -25°C is sufficient to halt the breakdown of metabolites due to bacterial activity [44]. Others recommend adding an antimicrobial agent to the sample prior to storage. Once stored properly, the samples may be good indefinitely, but at least for a six months as indicated by a study on the topic. On occasion, samples will end up being thawed and refrozen prior to metabolomics analysis. Although not encouraged, in the event this does happen, the freeze thaw cycle will not significantly impact the results [44]. When the time does come to prepare the sample, most sample types have similar preparation procedures.

2.3 Sample Types and Preparation

A major strength of NMR is that almost any bio-fluid can be used and sample preparation for analysis is simple. When preparing the samples, one thing that remains consistent is the need to combine the bio-fluid with a buffer solution, this also applies to tissue samples [43,45]. The buffer generally contains D_2O, sodium azide, a chemical shift reference, and buffer salts. D_2O, sometimes called heavy water, is structurally similar to normal water, but instead of two hydrogen atoms, there are two deuterium atoms in the molecule. Deuterium, like hydrogen, has a single proton in the nucleus. Unlike hydrogen,

deuterium also has a neutron. The presence of this neutron changes the γ of the nuclei resulting in D_2O having a different precessional frequency than water and not appearing in a proton spectrum. There is still an NMR signal from D_2O, and it is used to monitor the magnetic field of the spectrometer. All electromagnets experience minor fluctuations, or drift, that minutely alter the strength of the magnetic field over time. The spectrometer continuously analyzes the signal from D_2O and uses additional magnet coils to make adjustments to the spectrometer's magnetic field and keep D_2O at the same frequency. This allows the spectrometer to maintain the same net field strength and correct for the drift in the magnetic field during an experiment. Sodium azide is added as an antimicrobial agent in order to prevent any bacteria that may be present from digesting the metabolites. The chemical shift reference is necessary to properly assign the chemical shifts in the spectrum and was discussed in section 2.1.6. While blood and CSF are physiologically constrained to a narrow pH range, urine has a broad range of normal possibilities. This can be problematic as many metabolites have chemical shifts that are pH dependent, making it is necessary to adjust all the samples to have a uniform pH by using the buffer salts.

Common bio-fluids that get studied are urine [23], blood [46], CSF [6], saliva [47], breath condensate [48], amniotic fluid [49], and breast milk [50]. When trying to determine which fluid is best suited to a particular study, it should be remembered that bio-fluids offer complimentary information. For example, urine will contain metabolites that are filtered out of the blood, while the blood will contain the unfiltered metabolites. For this reason, it is worthwhile to combine information from multiple sample types to increase the power of a study. The fluids that have received the most attention and

therefore are the most characterized are urine, blood, and CSF. The use of these three fluid types, as well as the use of tissues, will be explored.

2.3.1 Urine

Urine is the produced as the kidneys filter wastes and excess materials from the blood and concentrate them in the bladder. This collection and concentration translates into multiple benefits to analysing urine. One is that the sample is concentrated enough to give excellent signal in a small amount of experiment time. After filtration through the kidneys, samples are also relatively free of large macromolecules that tend to obstruct the spectra, and any cellular debris that may be present is easily removed following centrifugation during sample preparation. Another benefit is that the urinary metabolic profile gives a global perspective of the waste products from the entire body. Considering that almost a quarter of human energy consumption is from the brain the waste products from the brain will be highly represented in the urinary profile. Urine is also one of the least invasive samples to obtain from a subject, making the implementation of this type of research in a clinical setting realistic for researchers and stress free for patients.

Importantly, Profiles from urine do exhibit diurnal fluctuations [51]. Most studies collect samples either as the first pass of the day after a night of fasting, or as a one-day average of multiple samples from a single person to avoid this innate variation in urinary profiles.

Diet also impacts the urinary metabolic profile [52,53]. Some researchers implement a diet normalization in their subjects for one day prior to sample collection in order to minimize dietary fluctuations all study participants are fed the exact same diet for that day [54]. Results indicate that this indeed helps to mitigate some of the individual

variation normally seen in human studies. Other factors that have been shown to impact the urinary metabolic profile are age, sex, and ethnicity [51]. Even with these sources of variation, urine has also been shown to maintain a stable representative phenotype in healthy controls [55,56], making it a valid medium for tracking disease development and progression.

Preparation of urine for an NMR experiment is incredibly simple. The sample needs only be combined with the proper buffer, centrifuged, and transferred to an NMR tube for experimentation.

2.3.2 Blood

Blood is essentially the highway down which many metabolites, wastes, and other materials travel from one place in the body to another. Using similar logic as with urine, since the brain is such a large consumer of resources in the body, a large portion of the blood is always in the brain and large portion of by-products in the blood will be from the brain. Both blood plasma and serum are regularly used for metabolomics analysis. Temporally, it exhibits less diurnal variation than urine, leading some to prefer it over the use of urine [57]. Blood also contains fewer detectable metabolites and consequently is more simple to analyze than urine [46].

One complicating factor that does exist with blood is that it has a high protein and fat content [58]. This adds unwanted signals to the spectrum and obstructs low concentration metabolites. The macromolecules can be left in the sample, but they are generally considered to detract from the information present. The signals from these molecules tend to be very broad and are reminiscent of a large hill blocking the view of a skyline. The chemical shift reference TSP also binds to the macromolecules [43]. This

will result in erroneous quantification if the TSP is to be used for concentration calculations during analysis. For this reason, many blood metabolomics studies use an alternate reference material such as formate. If the macromolecules are removed, TSP again becomes a suitable reference molecule. There are different ways of removing the signal from the proteins and fats, which will essentially create an unobstructed skyline view of the spectrum.

The three most common approaches to removing the macromolecular signals are to adjust the NMR pulse sequence to remove the signal from these molecules [59], physically remove the molecules using a filter [60], or to precipitate the proteins and fats using a chemical extraction [61]. The most common pulse sequence used to remove these broad signals is a Car-Purcell-Meiboom-Gill (CPMG). It works well to make the spectrum appear less obstructed. However, a side effect of this experiment is signals from the metabolites are also attenuated. This happens because the CPMG experiment exploits the different relaxation rates of small and large molecules. As mentioned previously, the NMR signal is detected in the time period between application of the RF pulse and the net magnetization returning to equilibrium. Not all nuclei relax at the same rate, and in general, signals from large molecules will relax faster than signals from small molecules. The CPMG pule sequence is relatively long, and through a series of pulse manipulations, the signals from large molecules are removed. During this time though, signals from smaller molecules have time to diminish as well. The appeal to this method is there are no additional preprocessing steps or alterations made to the sample. However, the signals from fast relaxing metabolites will be less, or even gone if this pulse sequence is used.

Filtering works well to physically remove the macromolecules from each sample. This approach has been shown to be highly reproducible, but it is a costly in both time and money. Alternatively, a variety of different precipitation techniques, all using common chemicals, can be performed for little money. To do this, a blood sample is typically combined with a chemical such as methanol, perchloric acid, or acetonitrile. After a short incubation period, the mixture is centrifuged and the pellet removed, leaving behind a sample free of macromolecules. A recent review recommended using methanol to perform the extraction [61]; however, each method of removing these molecules has strengths and weaknesses that vary greatly. Also, each method does not detect signals from all metabolites to the same degree. This makes it important to study the effects of the different sample pre-treatment protocols to pick the method best suited to recovering the information of most worth to the experiment.

Once the decision is made on whether or not the macromolecules will be removed, the blood is mixed with a buffer. If the fats and proteins are removed, the same buffer as for urine can be used. If the macromolecules are left behind, a modified buffer must be used which incorporates an alternate chemical shift reference. Some groups simply mix blood with a saline solution, instead of a buffered solution, as blood is self buffering in the body to approximately pH 7.35-7.4. Again, similar to urine, the use of blood is translatable to a clinical setting as the collection of blood samples for a number of assays are already common.

2.3.3 Cerebrospinal Fluid

CSF provides a more localized metabolic profile. Contained within the central nervous system, it reflects brain functioning more closely than blood or urine. CSF

contains fewer metabolites than urine, making it more simple to analyze and attempts are being made at automating its analysis [60]. CSF can be collected at will in animal models; however, in a clinical setting it is much more challenging to obtain a sample without great need for it. This makes CSF a more challenging sample type to translate to clinical work. Just like blood and urine, NMR analysis of CSF requires only that it be mixed with the buffer and centrifuged prior to experimentation.

2.3.4 Solid Tissue

Tissues provide the most localized and specific metabolic profile. There are three primary ways of analyzing tissue for metabolomics. One is to excise and homogenize the tissue, then extract the metabolites [18]. This typically gives the best resolution of metabolites in the spectrum from NMR of tissue samples. Another method is called high resolution magic angle spinning (HR MAS), which uses intact tissue in a solid state spectrometer [62]. Solid state spectroscopy is arguably more complicated than solution work and the spectral resolution tends to be lower than with homogenized tissues. However, by using intact tissues, it reduces the variation of the data caused by the lab procedures required to homogenize tissue. Lastly, an MRI can be used to perform a procedure known as magnetic resonance spectroscopy (MRS). This process involves collecting a standard one dimensional proton spectrum from a single voxel in the MRI data [63]. An obvious strength of this technique is that it is non-invasive and leaves the tissue intact in a living organism. It does suffer from poor spectral resolution, and requires an MRI, which are less accessible than NMR spectrometers.

2.4 Experimental Platforms

The primary analytical techniques used in metabolomics are NMR and MS. There will be a brief summary of the two techniques, followed by a comparison of the relative strengths and weaknesses of the techniques.

2.4.1 Nuclear Magnetic Resonance Protocols

As discussed in the beginning of this chapter, NMR spectroscopy works by manipulating the minute magnetic fields that exist at the nuclei of atoms. This is accomplished by first aligning all the nuclei using a strong magnetic field. Within this field, the minute magnetic fields of the nuclei precess, or 'spin'. The rate of precession is product of the strength of the spectrometers magnetic field and the nuclei's charge and mass; therefore, atoms of different elements will have different precessional frequencies. Although NMR experiments can be conducted on many different types of nuclei, the most common in metabolomics is hydrogen (^{1}H) because it is so abundant in biological molecules. The NMR experiments require that a transverse magnetic field is applied to the sample to disrupt the nuclei from their equilibrium state along the magnetic field lines. This transverse magnetization is applied at the same frequency as the nuclei's precession. Because the pulse to disrupt the nuclei needs to be on resonance with the nuclei of interest, the spectrum is usually free from interference from nuclei of other elements in the sample. As the nuclei return to equilibrium, their magnetic field induces an electric current in the circuitry of the spectrometer. This is the origin of the NMR signal. The signal is composed of the net current produced by the nuclei and is proportional to the total number of nuclei in the sample. Each metabolite in a sample will

contribute a unique signal to the spectrum. In metabolomics, these signals are compared to a library of pure substances to determine what compounds are present in the sample.

2.4.2 Mass Spectrometry Approaches

Mass spectrometry works by breaking a molecule into ionized fragments and measuring the mass to charge ratio of those fragments. On a basic level, every mass spectrometer has three main parts: a source where ions are formed, an analyzer that separates the ions based on the mass to charge ratio, and a detector that produces a signal each time an ion contacts it. The first step is to ionize the sample, which can be done in a number of different ways [64]. Ions are then accelerated through a vacuum while various magnetic and electric fields are applied in order to separate the ions by their mass to charge ration. The kinetic energy of the particles determines how they are deflected in the analyzer. The separated ions are electrostatically propelled into the detector and can now be detected as discrete mass fragments [65]. MS is routinely used to determine the exact mass of a molecule and can help to determine the structure of molecules. Similar to NMR, when used in metabolomics, MS spectra are compared to spectra of pure compounds to determine what metabolites are present. It is standard practice to separate the sample based on some chemical property to improve the resolution of the results. The most common separation techniques are liquid chromatography and gas chromatography. By introducing the separation, it is typically necessary to run multiple experiments for a single sample in order to analyze all the different fractions from the separation [31].

2.4.3 Technological Comparisons

Considering the brief overview of how the primary analytical techniques of metabolomics function, their respective strengths will now be highlighted. The most

notable differences between the techniques are how the samples are prepared, the reproducibility of the experiments, how quantitative the results are, recovery of the sample after experimentation, the sensitivity or minimum concentration for detection, number of experiments required, and the time and money to perform the experiments. A more thorough exploration of the comparisons between techniques is available from a number of reviews [22,30,31]

2.4.4 Sample Preparation

With regard to NMR, minimal treatment of samples is necessary before analysis. For fluid samples, the preparation is as simple as combining the bio-fluid with a buffer and transferring the mixture to an NMR tube, ready for the spectrometer. Additional to the buffer salts, the solution generally has D_2O to provide a locking frequency signal for the spectrometer, sodium azide or some other antimicrobial agent to prevent the metabolites in the sample from being metabolized by bacteria, and a chemical shift reference indicator such as DSS or TSP. The chemical reference compound can also double as reference for concentration measurements of all other metabolites in the sample. Contrastingly, MS typically requires chemical extractions or derivatization to separate each sample into multiple fractions based on pKa, hydrophobicity, or other chemical properties. This need to separate the sample leads less reliable experiment reproducibility.

2.4.5 Reproducibility of Results

The simple preparation protocol of samples for the NMR experiment aides in the results being exceptionally reproducible. As mentioned, in typical biofluid NMR metabolomics there are no chemical reactions prior to analysis, reducing the possibility of

experimenter error affecting the results. As more steps are added to any experiment the opportunity for experimenter error and differential results is introduced. Since MS can be a multi-step process, involving extraction and ionization of metabolites, it is inherently less reproducible than NMR analysis and results are less consistent.

2.4.6 Quantitative Analysis

The nature of the NMR signal makes quantification very simple. The signal derives from protons in the sample that interact with the magnetic fields of the spectrometer. The signal obtained is proportional to the number of protons producing that signal. The signals in the spectrum can then be measured and compared to either a known reference compound that is added to the sample or to an electronic reference spectrum to calculate the concentration of known compounds in the spectrum [66]. Determination of the correct concentration requires that the identity of the signal is known. Theoretically simple, it is quite difficult and labour intensive to implement quantitation. This is evidenced by the large number of qualitative NMR metabolomics studies compared to quantitative studies [54]. Quantitation of mass spectra is more complicated and requires an internal standard in the sample for each metabolite to be quantified [22]. The addition of these specific internal standards becomes quite expensive and labour intensive.

2.4.7 Non-Destructive Procedures

The MS experiment requires that the sample be ionized (fragmented into pieces) in order to analyzed, which eliminates the chance of reusing the sample for future uses. Conversely, NMR requires no such destruction of the sample and the sample integrity is maintained throughout the experiment. However, MS experiments require micromolar volumes, whereas NMR experiments typically use about half a millilitre of sample. The

need for large volumes in NMR makes some research, such as rodent model urine or CSF obtained via microdialysis, difficult to perform.

2.4.8 Sensitivity

The largest strength that MS has over NMR is that it is a much more sensitive technique. With a room temperature probe, NMR can detect metabolite concentrations in the low millimolar range, while MS is orders of magnitude more sensitive with a lower detection limit in the nanomolar range. NMR sensitivity can be increased in a number of ways. A cryo-probe can be used to reduce thermal noise in the NMR signal, this decreases the noise, thereby increasing the signal. Microcoil probes can also be used, which are probes designed to use a smaller amount of sample as well as to have a more sensitive detection ability. The NMR experiment can be also run for a longer period of time. A typical NMR experiment takes only a few seconds to perform a single scan, but to get informative results the experiment is repeated multiple times and the results are combined into a single spectrum. The relationship between signal and noise when summating multiple repeats of the same experiment is that the signal increases as a function of root N, where N is equal to the number of scans. Therefore, to get twice the signal to noise, the experiment time must be quadrupled. There are obvious diminishing returns with trying to increase sensitivity this way and it is impractical to expect to obtain the same sensitivity in NMR as in MS just by extending the experiment. Another way to increase the signal to noise and sensitivity is to increase the strength of the NMR magnet. This has the effect to increase the number of nuclei in the α state, thereby increasing the net magnetization vector of the sample. The different techniques mentioned can also be combined to increase the NMR signal even more.

Related to the sensitivity, MS can also detect many more metabolites in a sample, primarily because it detects metabolites that are below the detection limit of NMR. The typical NMR metabolomic sample can anticipate to identify between 40-100 metabolites in a sample, whereas MS experiments detect over 500. Incidentally, an MS spectrum has far more unknown compounds in them than an NMR spectrum.

2.4.9 Single-Step Analysis Procedures

NMR experiments are able to collect information of all metabolites at once. In urine metabolomics, almost all metabolites are water soluble and present together in the sample; therefore, only a single experiment is necessary to obtain all the metabolite information. With tissues and blood analysis, the sample can be separated into water soluble and fat soluble fractions, which would then require two experiments to obtain all the information. Mass spectrometry samples that undergo a separation will typically require multiple different fractions to be analysed separately. The fractions are generally separated on the basis of various chemical properties of the metabolites, and the property determining the separation will change with the separation technique used. There are multiple chromatography techniques that can be used to separate the different classes of metabolites [64]. Acquisition of a comprehensive metabolite profile will require that more than one method be used [20,65]. This is because the chemical properties, such as pKa or polarity, of metabolites cover a wide range, and no single separation method (liquid chromatography, gas chromotography) will separate all metabolites. Additionally, no one detector can measure all metabolites because the dynamic range of the detector is less than the concentration ranges of metabolites in biological samples. These factors lead to

MS needing many more separate experiments to be run in order to obtain all the information from a single sample.

2.4.10 Time Considerations

Preparation of a sample for NMR takes less time than MS. Once in the machine, NMR experiments can take as little as 5 to ten minutes to acquire a spectrum. MS can take longer, and with the need to run multiple experiments on a single sample to get the full complement of metabolites present, MS takes much longer than NMR when coupled with popular LC/GC-MS techniques.

2.4.11 Cost Effectiveness

Given that analysis of a sample by MS requires separation prior to analysis and multiple standards for quantification, it is not surprising that MS costs more money per sample than NMR. Although the cost of the experiments is not often seen in reviews, it is worthwhile to note that labs offering to perform metabolomics for a price will perform analysis by NMR for a fraction of the price of MS. When evaluating the quality of information versus the cost of the experiment, NMR is a more valuable per dollar technique.

2.5 Data Analysis

2.5.1 Profiling Approach

Analysis of metabolomics data can be divided into two main streams, profiling and targeted analysis [22]. Although many of the steps involved with these two approaches are extremely similar, they offer fundamentally different ways of looking at the data, and answer different types of research questions. Profiling, sometimes called untargeted or chemometric studies, is done when there is limited information available

about a topic and proposing an explicit scientific hypothesis is not feasible [67]. There is the general hypothesis that condition A and B will exhibit distinct metabolic profiles, but no specifics are given as to what metabolites are expected to change with the experimental conditions. The goal of these studies are to acquire valid quantitative or qualitative measures of as wide a range of metabolites as possible with the intent of being able to distill the results into something meaningful that can then be re-tested and verified in the future. Qualitative or chemometric approaches are often considered to be hypothesis generating for this reason. During analysis, the whole profile can be used as a classification tool to separate cases from controls, or significant features can be identified and proposed as potential bio-markers for the condition under study. Since the entire profile is being used, these studies regularly return significant features that are not currently identifiable.

2.5.2 Targeted Analysis

Alternatively, targeted approaches require that all metabolites involved in analysis are accurately quantified [54,68,69]. This method selects a subset of metabolites that are anticipated to have been effected by the experimental conditions and focuses the analysis on them. Usually, only the subset will be quantified and statistically analyzed.

2.5.3 Binning

Whether a targeted or profiling approach is adopted, the sample preparation and data acquisition for NMR is the same. Data processing and statistical analysis also remains somewhat similar. If the metabolites are to be quantified, there are various software packages available that are capable of this, with the final output being a matrix of the concentrations of metabolites found in each sample [60,70]. If quantification is

unavailable, then qualitative methods are used. This begins with segmenting the spectra into a discrete number of bins [71]. This serves to reduce the size of the data set. NMR spectra are collected as a set of hundreds of thousands of data points. Working with data this large would be unnecessarily time consuming and require large amounts of computer processing power. Binning reduces these hundreds of thousands of points down to 100-400 discrete spectral segments referred to as buckets or bins. The bins can be all of the same width across the entire spectrum or adapted to precisely include metabolite signals (Figure 7). Adapting the bins is time consuming, but usually provides a more accurate representation of the data. Bins of equal width are prone to splitting peaks in half or putting the same metabolite from different spectra in different bins if the data sets are not aligned perfectly. Each bin is qualitatively represented as the integral of the spectrum within the bin boundary. This translates approximately into the relative concentration of the metabolite within the bin, but not the actual concentration value.

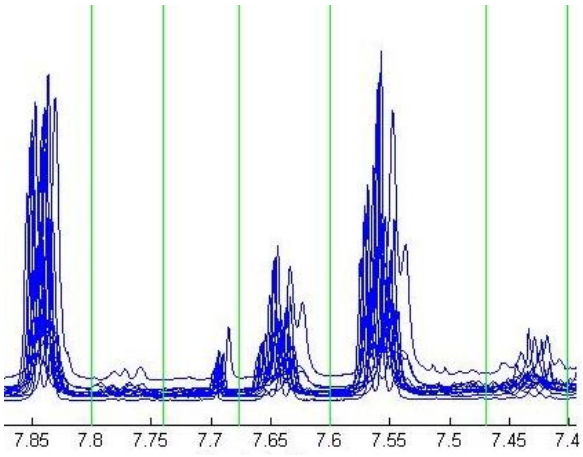


Figure 7. A region of multiple spectra stacked on top of each other and binned to include entire signals in a single bin.

2.5.4 Normalization

Once the spectra are binned, there are a number of data transformations that can applied. If it is a targeted study, these transformations can be applied to the concentration values. It is standard to apply a normalization method to each spectrum and a scaling function to each bin. Normalization can be to a reference spectrum, total spectrum area, or to a reference feature [72]. The importance of normalization is best expressed by giving an example. In urine metabolomics, samples from many different subjects are compared. If one person normally passes more water in their urine than another, their sample will appear to be much more dilute, even if they both subjects have the same total metabolite content present in their sample. To generalize this idea, by normalizing the data, individual differences in the dilution of metabolites are adjusted for. Hence, the variation in the relative amounts of metabolite present is more prominent [73]. Generally, the region of the spectrum corresponding to water is removed prior to normalization to remove effects of imperfect removal of the water signal.

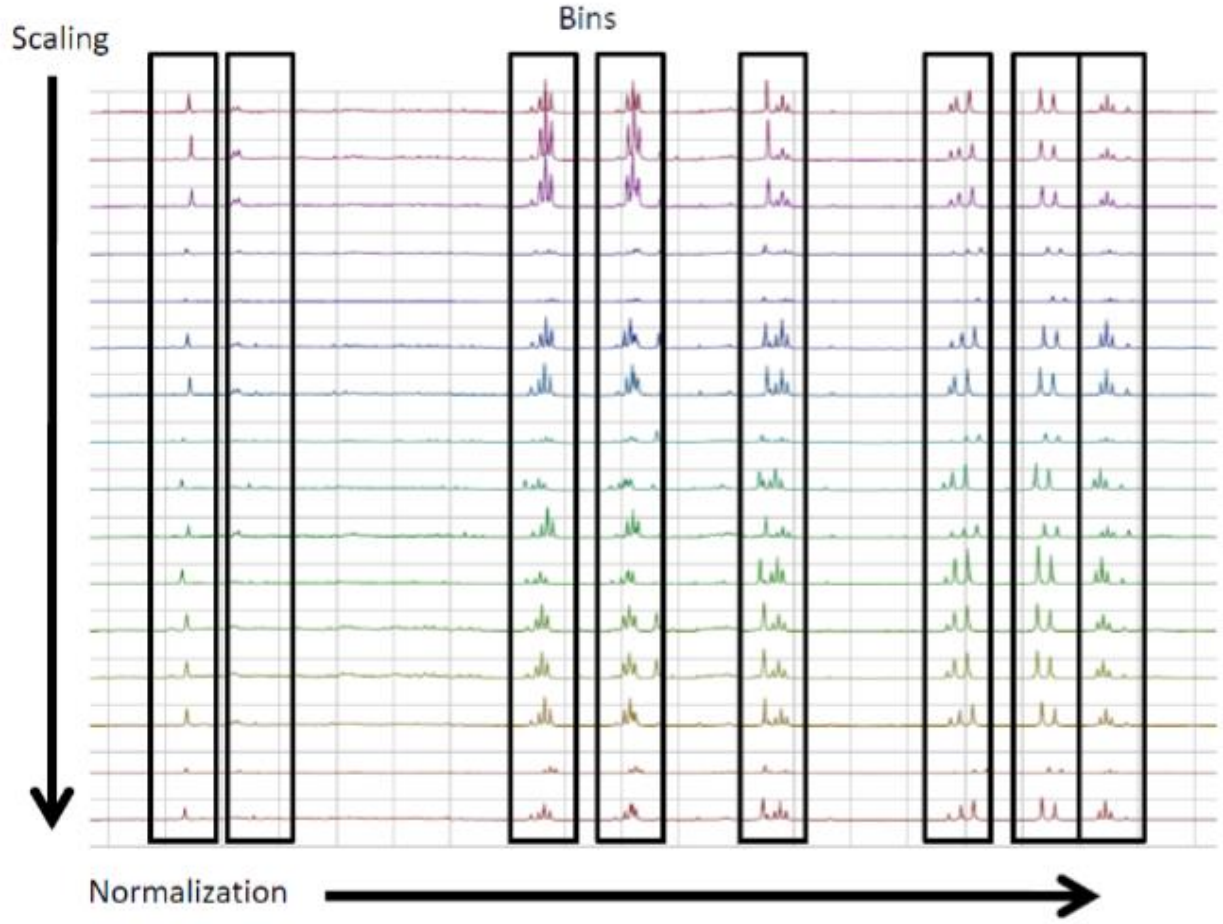


Figure 8. Illustration of spectra being binned and the dimensions that normalization and scaling work it. Normalization functions on each individual spectrum, while scaling functions on a bin single bin across all the spectra.

2.5.5 Scaling

Scaling helps to offset the tendency for metabolites of higher concentration to dominate the results [74]. Major differences between concentrations and variances of different metabolites tend to force most multivariate statistical techniques to focus on the most intense signals, ignoring valuable information that is potentially held in the smaller signals. By scaling the data, this effect can be mitigated or removed, depending on the choice of scaling function that is applied. One common method of scaling is called unit variance, or auto scaling, which sets the mean of each metabolite to zero and the standard deviation to 1. The formula for unit variance is given in equation 7 and its effect is to

weight all metabolites the same, completely removing issues related to disparate concentrations. Another method often employed is Pareto scaling. In Pareto scaling the mean is also adjusted to be zero, and the data is then divided by the square root of the standard deviation as shown in equation 8. Pareto scaling retains a greater similarity to the original data structure than with auto scaling. Both methods help to increase the visibility of variation in low concentration metabolites. This is important because the concentrations of metabolites cover a range of values that vary by several orders of magnitude. By scaling, low concentration data is not lost [75].

Equation 8. Auto Scaling $= \frac{\text{x average} - \text{x bin}}{\text{SD}}$

Equation 9. Pareto Scaling $= \frac{\text{x average} - \text{x bin}}{\sqrt{\text{SD}}}$

2.5.6 Multivariate Statistics

After the data is normalized and scaled, it is analysed using multivariate statistical tests. The large number of variables, or bins, in metabolomic data sets leads to difficulty in identifying patterns because graphical representation of samples composed 100+ variables is not possible. Luckily, multiple tests exist that offer a way to visualize and interpret the data. Principal component analysis (PCA) is most often the first test used because it expresses the data in a way that highlights differences and similarities between the samples and aids in pattern identification [76,77]. Importantly, it is an unsupervised technique, meaning that the statistical analysis does not involve designating which group a sample belongs to and there is no possibility to over-fit the data or conclude that non-existent patterns are real. PCA takes all the variables and calculates new principal components, which mathematically are the product of the original dataset and the

eigenvectors of the covariance matrix of the data [78]. Graphing of the components provides the scores plot, in which each data point represents a single sample, and points that group together have more similar characteristics than points that are distant. As the data size is reduced, some information is lost; however, the percent of the total variance explained by a principal component can be calculated, and when two or three components are used to make a graph, the total variance explained by the graph is the sum of the variance explained by each component. It is not uncommon to have a data set with hundreds of variables have more than 60% of the total information of the data explained by two principal components. Studies that want to determine if metabolic profiles can be used to discriminate between two groups perform PCA in order to visualize if their experimental groups cluster separately in the scores plot.

Generally, if PCA shows separation, partial least squares discriminant analysis (PLS-DA) is then used. PLS-DA is similar to PCA in that it is a clustering algorithm that reduces the dimensional space of a data set [79]. However, it is a supervised technique, meaning that the group identity of the samples is given [80]. As an aside, if in the formula each sample is defined as an independent group, the PLS-DA scores will be identical to PCA scores. The value of PLS-DA is that it specifically maximizes the differences between groups, as opposed to exposing differences between all the samples. Due to the supervision of the grouping the results are potentially more informative for answering the research question; however, it is prone to over-fit the data and produce grouping that is not real [81]. This is why PCA is run first to see if the groups cluster independently without supervision. Even with prior PCA, PLS-DA is routinely couple with rigorous cross validation and permutation testing in order to validate the results [81,80]. A similar

approach to PLS-DA is orthogonal partial least squares discriminant analysis (OPLS-DA). The predictive value of OPLS-DA has been shown to be the same as that for PLS-DA [82]. The benefit of using this technique is that it places greatest variance in a single axis, which is accomplished simply by rotating the PLS-DA scores plot [83].

2.5.7 Variable Selection

The multi-variate techniques mentioned above are used in both profiling and targeted studies to determine class membership and see if there is an overall difference between the groups. Some studies end with this type of analysis and conclude that the whole profile can be used to classify samples into groups and no attempt is made to find a subset of metabolites that could function as biomarkers [84]. Most metabolomics studies go beyond saying simply that the samples cluster correctly between the groups, and identify metabolites that are predictive of group membership. There are a number of ways to determine which metabolites are important. The simplest method is to perform univariate hypothesis testing, such as a t-test of Mann-Whitney U test [85]. These will indicate which metabolites have an average that varies significantly between the cases and controls. A weakness of univariate statistics is that it has no way of accounting for synergetic and compounding effects caused by multiple metabolites interacting with each other. Also, since multiple hypothesis tests are being conducted, p-values need to be corrected for multiple comparisons using methods such as Bonferoni correction in order to reduce the number of false discoveries [86]. Most experiments either stay away from univariate testing due to its lack of power, or combine it with some form of multivariate metabolite identification.

There are many multivariate methods that exist for determining the most important variables. Most commonly, PLS-DA coefficient weights or variable importance in the projection (VIP) values can be used to determine whether or not a metabolite is important [72]. With these methods, each variable has an associated value calculated for it and threshold value is decided as to what will be considered important or not. For VIP, it has been suggested that any value greater than 1 is enough to classify a variable as important, but this is not a rule [87]. In general, the coefficients and VIP will identify the same metabolites as being important.

Receiver operator characteristic (ROC) curves can be used as a test for evaluating the predictive power of a small set of metabolites [88]. The worth of most current biomarkers is determined by the area under the curve (AUC) that they obtain. ROC curves plot the number of true positives against the number of false positives. The AUC is a measure of how accurate the model is at classifying cases and controls, with an AUC of 1 being perfect classification and 0.5 being random chance. Applied to metabolomics, this test can be applied to demonstrate the strength of a proposed bio-marker [68].

A new method has recently been developed called variable importance analysis based on random variable combination (VIAVC), which systematically resamples variables to determine if potentially synergetic effects exist between seemingly unimportant variables [89]. The algorithm combines random permutation of variables with a 10-fold cross validation (CV) of models to determine the optimal subset of variables that provide the most information about the differences between groups. With each random permutation of variables, an AUC for the ROC model is calculated and used as the measure of how good the metabolites are at predicting the class of the samples.

2.5.8 Pathway Analysis

There is currently no accepted standard for the optimal method for analysing data. Even though this is the case, a recent study that used the same data in multiple labs found that consistent results for a metabolomic experiment were obtained in different labs using different processing techniques and different statistical analysis [90]. This finding is supported by the work done on this thesis. Several different metabolite selection techniques were used when analyzing the data, and it was found that in general, each method provided very similar results. So even though the methods of experimentation are not standardized, the results tend to be reliable and similar regardless off the differences in workflow.

Once important metabolites are identified it is then possible to link them to functional pathways in an organism. Web based platforms exist that are capable of searching online databases of biochemical pathways and disease profiles in order to link the metabolites to possible functional origins [21,91]. This is valuable in adding context and meaning to results beyond a list of differential metabolites.

2.6 Applications of NMR Metabolomics to Neuroscience

NMR Metabolomics has experienced widespread applications in drug discovery, systems biology, pharmaceuticals, disease detection, and toxicology [31]. Part of what makes metabolomics so versatile in studying biological systems is the simplicity of the experimental preparation and the ability to analyse all components in a solution at once. With most living organisms being a salt bag, it is easy to take almost any biological sample and find a way to obtain a profile of all the small molecule metabolites. In studies of the brain, NMR metabolomics has been applied in a number of ways using the

different sample types discussed. An overview of some representative studies will be given that illustrate the power and applicability of NMR metabolomics to research of brain related diseases and disorders. The studies cover both rodent and human models that used blood, urine, CSF, or tissue for analysis.

2.6.1 Animal Models using NMR Metabolomics

Blood plasma from a rat model was used to evaluate the different stress models commonly used to induce depression like symptoms in rats. Three different stress paradigms were compared and all three types of stress had distinguishably different metabolic responses. Chronic unpredictable mild stress (CUMS) affected glutamate metabolism, energy metabolism, and inflammatory responses. The one day forced swim test group showed changes in metabolites affected by intestinal micro-organisms, and the fourteen day forced swim test group showed impacts to lipid metabolism. From this study the group concluded that metabolomics and clinical data supports CUMS as the most representative model of human depression that was studied [92]. This shows that metabolism is highly linked to the stress response of the animals, to the extent that different forms of stress were detectable in the metabolic profiles. This is important to understand as stress related disorders affect a large portion of the world's population.

NMR metabolomics of urine from a mouse model of Alzheimer's disease (AD) found markers of oxidative stress in urinary profiles prior to becoming symptomatic. The markers discovered were 3-hydroxykynurenine and homogentisate, which are both precursor molecules of reactive oxidizing species (ROS) [93]. ROS are involved in the pathogenesis of AD and this finding helps point to a potential biomarker that could be used to help early diagnosis of the disease. This would greatly improve the quality of life

of many people and families as early detection and therapies have been shown to slow the progression of the disease.

Recently, a mouse model of closed head injury was used to study traumatic brain injury (TBI). Whole brains were homogenized and analyzed using quantitative metabolomics analysis. A ROC model was constructed using the concentrations of ADP, AMP, NAD+ and IMP which had perfect class discrimination. This model was validated using further statistical testing and proven to be significant. The analysis also revealed that TBI leads to disturbances in mitochondrial function, energy metabolism, and neurotransmitter function [94]. These are timely results as there is a growing concern over the side effects of mild TBI experienced by so many people, and further knowledge on this topic is being sought after.

Using a mouse model of Batten's disease, brain tissue from multiple brain regions was analyzed. Batten's disease is a fatal autosomal recessive neurodegenerative disorder that typically begins in childhood. All regions studied showed a deficit in cycling of neurotransmitters indicated by imbalances of glutamate, glutamine, and GABA. These differences were detected at one month old, which is significant because neuronal loss characteristic of this disease does not typically begin for another six months in the mouse model of this disease [95].

How drugs affect brain metabolism has recently been explored in the brain homogenates of mice. Specifically, the metabolic response to diazepam in a anisatin-induced convulsive seizure model in homogenized brain samples from specific brain regions. Diazepam is a benzodiazepine frequently prescribed as an anxiolytic and also as to treat seizures. Previous studies on diazepam have focussed on how it effects the amino

butyrate system. This study was able to show that diazepam helps to recover damaged neurotransmitter metabolism as well as lessening oxidative stress, minimizing energy metabolism disturbances, and rebalancing amino acid metabolism. The results from the metabolomic experiment were integrated with histopathological and behavioural data to provide a more holistic understanding of the treatment effects of diazepam [96].

2.6.2 Human Studies of NMR Metabolomics

As informative as animal models are, human studies provide information that is so much more intimately connected to the concerns of society. The following studies all used human samples and again illustrate the diversity of applications of metabolomics and the wealth of information recovered from these studies.

Major depressive disorder (MDD) is a pervasive mental health disorder with no empirical lab test to support its diagnosis. A study involving NMR metabolomics of blood plasma from drug naïve MDD patients was able to create an OPLS-DA model that successfully distinguished between MDD patients and controls, as well as accurately diagnosing blinded samples that were added to the model. The metabolites integral to the classification accuracy were involved in lipid and energy metabolism [97]. These results suggest the possibility of generating an empirical test to aid in the diagnoses MDD and potentially even develop and screening test for people at risk of developing this disease.

Multiple sclerosis (MS) was investigated using plasma from human patients suffering from the disease. The study used PCA and OPLS-DA to create a model of the data and select metabolites important to discriminating between the MS and control samples. The metabolites that were selected are involved in tryptophan metabolism and energy metabolism. The ROC model created from the identified metabolites had almost

perfect class discrimination with an AUC of 0.98 when applied to a set of samples not included in building the model. The study pointed out that while the disease tends to have a heterogenous expression in the population, sometimes making clinical diagnosis quite difficult, the results from this study point to a very consistent alteration in energy metabolism, which could be utilized to increase the accuracy with which people are diagnosed with this disease [98].

A recent review of urinary markers of brain disease illustrated that human urine has been largely ignored compared to other sample types when searching for markers of brain disease [7] Even with this, the limited studies on urinary based bio-marker of brain diseases that do exist indicate that urine has significant value when searching biomarkers of some brain diseases. The studies reviewed in this article provided some promising evidences, indicating that clinically applicable urine biomarkers of brain diseases may largely exist and should be useful in future diagnoses. If proven reliable, urinary biomarkers of brain diseases would be an informative and convenient tool with clinical applications.

In another study of MDD, similar to the one using blood plasma, urine was analysed to determine its diagnostic power when separating classes from controls [99]. Multivariate analysis of the data yielded a panel of six candidate bio-markers of MDD. These six markers were included in an ROC model and yielded an AUC of more than 0.917 when tested against samples not included in the model. This is another study showing the plausibility of developing a lab test for depression.

An investigation into the differences of urinary metabolic profiles associated with neuropathic and nociceptive pain was able to classify between the types of pain with high

specificity [100]. The classification required use of the entire metabolic fingerprint; however, specific metabolites were identified that suggested that neuropathic pain was accompanied by neuronal damage. This was evident by the metabolites that were key to the discrimination between groups. Information such as this is crucial when trying to determine the best course of action to take when treating different types of pain.

Most people don't hesitate too much to give a blood sample, and even fewer think twice about giving a urine sample. However, CSF sample collection is a painful and unpleasant experience, making it harder to get permission to obtain and consequently less accessible for experimental purposes. Even with these hindrances, there have been a number of studies analyzing human CSF.

A study using CSF from over 152 participants was able to distinguish between drug naïve individuals with a recent diagnosis of schizophrenia and controls [101]. Interestingly, the study also showed that medication was able to return the metabolic profile to normal, but only if the disease had been caught and medicated early. This emphasizes the need for an early detection and treatment of the disease. Beyond being able to classify the different groups in the experiment, the results also showed that brain specific alterations to glucoregulatory activity in the brain are part of the pathogenesis of schizophrenia.

Using human CSF samples again, PD patients and healthy controls were able classified separately with high fidelity [102]. The primary metabolites that were altered between groups were involved in glucose metabolism. It is encouraging that the results of this study correspond with other research on PD.

As mentioned earlier, there are a number of elements that can be analysed using NMR. This study used carbon instead of hydrogen. The researchers used labelled carbon and were able to determine which metabolic pathway is used for energy production in the brain following TBI [103]. CSF was collected from the brains of participants using a microdialysis pump. These samples indicated that following injury the amount of anaerobic glucose metabolism in the brain increases and that this increase is achieved through activation of the pentose phosphate metabolic pathway of energy metabolism.

Using CSF 1H-NMR metabolomics and unsupervised statistical methods, distinct differences in the metabolic profiles of MS and non-MS control patients were observed [104]. The metabolites that varied significantly between the groups were involved in energy metabolism and phospholipid metabolism. The study did have a small sample size, but provides reasonable grounds to continue research targeted at developing a metabolic biomarker of MS.

In a 2015 study, ALS patients had their CSF compared to that of healthy controls using NMR metabolomics. The results pointed towards ALS being partially characterized by a hyper-catabolic state with evidence that this state could possibly involve mitochondrial function [12]. The team proposed ethanol as a putative biomarker for neuronal tissue damage in ALS.

Brain tissue samples in rodent models have provided incredible information regarding the pathogenesis of various condition, and it would be nice to have similar studies in humans, but there are obvious barriers to excising brain tissue from people just for experiments. Much more common is to utilize MRS, which allows in-vivo analysis of

metabolic profiles by using an MRI. There is at least one study; however, that was able to obtain human brain samples for analysis.

In 2014, the metabolome of post mortem brain tissue from AD patients was analyzed. The results showed that AD brain tissue revealed dysregulation of taurine and hypotaurine metabolism, as well as alanine, aspartate, and glutamate metabolism [105]. The metabolite concentrations were fair (ROC AUC = 0.67) at discriminating cases from controls; however, by comparing pairs of metabolites ratios, opposed to absolute concentrations, a more powerful discrimination was achieved.

Entirely non-invasive, MRS uses an MRI instead of a NMR spectrometer. It should be noted that the use of MRS instead of NMR does come with a cost, as the resolution of metabolites is greatly reduced when compared to NMR spectroscopy. An MRS study of concussion tracked the progression and recovery of metabolic fluctuations following injury [106]. The soonest analysis after a concussion occurred 3 days post injury for all subjects. This time frame exhibited the largest deviation from baseline of the metabolic profile. The profiles were seen to normalize of the course of a 30 days, with a normal profile being obtained on day 30. This study provides information that could help guide sports medicine doctors on when an appropriate time to return to sport would be following an injury. Another MRS study measured the concentration of NAA in relation to cerebral vasculopathy [107]. The results indicated that NAA was reduced in this condition.

2.6.3 Discussion

NMR metabolomics is an adaptable technique that can be applied to a wide range of experimental designs. It provides significant and often novel insights into metabolic

responses to various stimuli. The results are often meaningful on their own, leading to the increasing popularity of this branch of systems biology [24,108]. Metabolism is also considered to provide the closest measure to phenotype, giving the study of metabolic profiles great value for use monitoring and measuring the health of an organism [109,110]. The ability to analyze all metabolites present in a single experiment offers the possibility of developing an extensive screening test that could replace a battery of assays and tests with a single experiment. This is a reasonable goal to work towards as diseases are often accompanied by altered metabolism [27] and metabolic changes precede disease symptoms in some cases [29] leading to the possibility of screening for early detection and treatment of disease.

Beyond simply using one sample type in a study, results from multiple bio-fluids or sample locations can provide a wider perspective on the dynamic response to diseases [31,111-115]. NMR metabolomics can also be combined with MS metabolomics to harness the strengths of both techniques and identify previously unknown metabolites [116]. Other systems biology approaches have been combined with metabolomics so that information on both the structure and dynamic functioning of the system is obtained in a single study [117]. Metabolomics testing has also been applied alongside existing clinical methods [118] to increase the sensitivity of current diagnostic tests.

The synergetic combination of omics data has potential to vastly improve personalize medicine. By combining omics platforms, it opens the possibility to consider complex interactions within the individual. Metabolomics potentially offers a key in determining why drugs affect people differently, which is a major hurdle in modern

medicine. Indeed, there are some toxicological effects, such as drunkenness, that can only be explained by metabolomics [1].

The potential for metabolomics to help answer questions in neurological [119], and psychiatric [120] disorders has been explored in recent reviews. These reviews, and others, illustrate the fundamental link between brain diseases and metabolism [121], and suggest the possibility of exploiting this connection to develop therapies [122].

An instigator of many mental health disorders is stress [123,124]. Chronic stress has been shown to precipitate the development of several diseases and negative symptoms such as depression, anxiety, decreased cognitive abilities, and erratic behaviours [125]. Additionally, stress experienced by pregnant mothers has been shown to cause a ripple of effects through multiple subsequent generations [126]. The metabolic effects of ancestral prenatal maternal stress have not received much focus. Chapter two of this thesis will analyze how prenatal maternal stress effects the urinary metabolic profile of F4 male offspring, and chapter three will also analyze the effects of prenatal maternal stress, this time in the liver metabolic profile of F4 male and female offspring.

CHAPTER 3: STRESS TRANSGENERATIONALLY PROGRAMS METABOLIC PATHWAYS LINKED TO ALTERED MENTAL HEALTH

3.0 Introduction

Mental health disorders are the leading cause of disability worldwide. A recent survey of representative communities from 28 countries conducted by the World Health Organization (WHO) World Mental Health initiative found the prevalence of mental health disorders to be up to 36% in the population [127]. Among these, anxiety was the most common condition, followed by mood disorders, externalizing disorders, such as attention deficit hyperactive disorder and oppositional defiant disorder, and substance abuse disorders [127]. Although these disorders differ in their symptoms, all of them are commonly influenced by the experience of stress or exposure to an adverse environment [123,128]. Treatments for these disorders are often ineffective and the lack of understanding concerning their etiology is hindering the ability to develop more effective cures.

Lifetime stress experienced by the individual has long been considered as a possible cause for mental health disorders. Additionally, recent discoveries have emphasized that stress experienced by the mother during pregnancy can impact the developing fetal brain and increase the probability of the offspring developing mental illness [129-131]. Support for this argument comes from human cohort studies of individuals whose mothers were pregnant during a traumatic event or natural disaster [132,133]. Results from these studies showed that maternal exposure to adversity during pregnancy was associated with accelerated cognitive decline in later life [134], higher lifetime incidences of schizophrenia [135], poor cognitive health [136,137], altered immune function [138], and unique DNA-methylation signatures [139]. Other studies on prenatal stress during human development support a link between the mother's mood

during pregnancy and increased risk of attention deficit hyperactive disorder, anxiety, and cognitive impairment [140]. Experimental studies have isolated the influence of prenatal stress (PS) on higher lifetime incidence of altered stress response, anxiety, reduced attention, learning deficits [140] and that these changes may propagate to subsequent generations [141-143].

Recent studies have documented that an early adverse environment affects more than one generation of offspring [142-144]. For example, human studies involving the Dutch Famine Birth Cohort have suggested that prenatal undernutrition can significantly affect the health of children (F1) and grand-children (F2) [145]. Experimental data have shown that prenatal exposure to toxins [144,146,147] and stress [142] alter behaviour and stress response across several generations (F1-F3). Long-term outcomes include altered affective state and physiology [131,141-143,148] and the development of new behavioural traits based on multigenerational stress programming in the F4 generation [149-151]. Potential mechanisms of transgenerational programming involve variations in maternal care [152,153], epigenetic regulation by DNA methylation [130,139] and microRNAs [142,143].

Metabolic profiles reflect cellular functioning; therefore, up-stream epigenetically regulated gene and protein expression will be detectable in metabolic profiles [1,4,30,109,110]. Clearly identifiable metabolic signatures linked to mental health disorders have been identified using ^{1}H nuclear magnetic resonance (NMR) spectroscopy [99,101]. Connecting altered epigenetic states, due to PS, to functional pathways reveals that its neurodevelopmental consequences are linked to altered brain [5,154,155] and amniotic fluid [156] metabolic signatures. Here we used ^{1}H NMR spectroscopy to determine if epigenetic programming caused by a single exposure to experimental PS four

generations removed permanently alters metabolic activity. In addition, we identified clearly distinguishable metabolic fingerprints in urine to discriminate the consequences of multigenerational versus transgenerational prenatal stress in the filial F4 generation (Figure 1). These metabolic profiles were used to determine metabolic pathways that are implicated in mental health disorders. These findings are an important demonstration that ancestral origins of altered mental health can be indicated by metabolic signatures that are of clinical predictive and diagnostic value.

3.1 Materials and Methods

3.1.1 Animals

This study involved Long-Evans rats raised at the Canadian Centre for Behavioural Neuroscience, University of Lethbridge vivarium. The animals were housed in pairs under a 12:12 h light/dark cycle with light starting at 07:30 h and the room temperature set at 22 °C. Rat chow food and water were available at libitum. All procedures were approved by the University of Lethbridge Animal Care Committee in compliance with the guidelines of the Canadian Council on Animal Care.

3.1.2 Experimental Design

To investigate unambiguous transgenerational programming of an adult metabolic phenotype [40], this study focuses on six-month old males from the F4 generation. The use of males minimized the potential impact of female hormonal fluctuations [157]. Four successive generations of timed-pregnant female rats were bred under consistent laboratory conditions. Parental female rats (F0) were exposed to stress during pregnancy. For the multigenerational stress lineage (F4-SSSS; n=6) the pregnant daughters (F1), granddaughters (F2), and great granddaughters (F3) were also stressed during pregnancy.

The transgenerational stress lineage (F4-SNNN; n=6) was bred by stressing only the F0 mothers and not the F1 – F3 mothers (see Figure 1; S = stress, N = non-stress condition). Yolked controls were bred in parallel for each generation (Control, n=7).

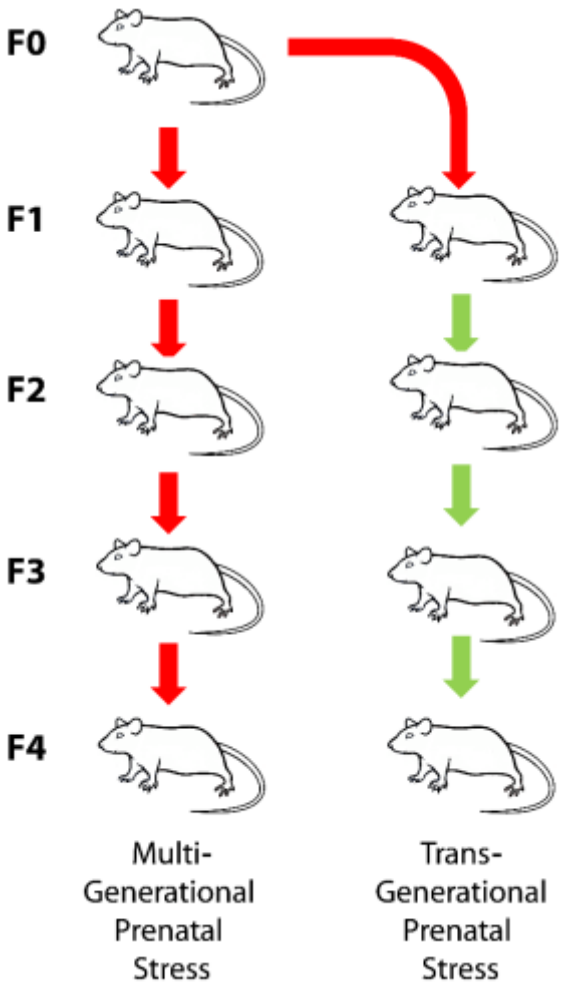


Figure 9. Illustration of the experimental design that tested the F4 offspring of a lineage in which stress occurred in each generation (multigenerational stress, SSSS) or in which stress was limited to the first parental generation (transgenerational stress, SNNN.

3.1.3 Stress Procedure

Pregnant dams were subjected to stress daily from gestational day (GD) 12 to GD 18. This timeframe was used as it corresponds to the third trimester in human pregnancy when substantial neural development occurs [158]. Stressors included restraint in a

Plexiglas cylinder for 20 min and forced swimming in warm water at 21°C for 5 min. Stressors were administered each day in a semi-random alternating order; in the morning between 8:00-9:00 hours or in the afternoon between 16:00-17:00 hours.

3.1.4 Behavioural Testing

Open field locomotor activity was used to measure the exploratory behaviour, which serves as a standard parameter in the assessment of stress-induced emotional state [150,159]. Animals were placed individually into Accuscan activity monitoring Plexiglas boxes (length 42 cm, width 42 cm, height 30cm) and recorded for 10 min. The boxes attached to the computer recorded the activity based on sensor beam breaks. The horizontal beam breaks were recorded on the computer with the VersaMax™ program and converted to spread sheets using VersaDat™ software (AccuScan Instruments Inc., OH, USA). Distance traveled in the center of the open field arena was averaged to obtain the total distance traveled (in cm) per minute.

3.1.5 Sample Collection and Preparation

Subjects for analysis were euthanized with an injection of Euthanosol (Merck, QC, Canada) and perfused transcardially with phosphate-buffered saline (approximately 200 ml) followed by a transcardial injection of approximately 200 ml of 4% paraformaldahyde (Sigma-Aldrich, MO, USA). Urine samples were obtained via bladder puncture with a 1.5 ml sterile syringe at time of euthanization and stored at -80°C. To prepare the samples for NMR spectroscopy, urine samples were thawed at room temperature and 450 µl aliquots were transferred into 1.5 ml centrifuge tubes with 250 µl of phosphate buffer. The phosphate 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 a final concentration of 0.5 M. The D_2O

came with 0.05 % by weight trimethylsilyl propanoic acid (TSP) as a chemical shift reference. To protect the metabolite profile integrity, 0.02 % w/v of sodium azide was added to the buffer solution as an antimicrobial agent. Once the sample was mixed with the buffer, it was centrifuged at 12,000 rpm for 10 minutes to precipitate any particulate matter. After centrifugation, 550 µl of the supernatant was transferred to a 5 mm NMR tube for NMR analysis.

3.1.6 NMR Data Acquisition and Processing

NMR spectra were collected on a 700 MHz Bruker Avance III HD spectrometer (Bruker, ON, Canada). The Bruker 1-D NOESY gradient water suppression pulse sequence 'noesygppr1d' was used. Each sample was run for 128 scans to a total acquisition size of 128 k. The spectra were zero filled to 256 k, automatically phased, baseline corrected, and line-broadened by 0.3 Hz. The processed spectra were then exported to MATLAB (The MathWorks, MA, USA) for statistical analysis. Spectra were manually binned to reduce the size of the dataset. Each spectrum had the areas corresponding to water and urea removed before being normalized to remove effects of imperfect water signal suppression. The data set was then auto-scaled.

3.1.7 Statistical Analysis

Data visualization to determine sample structure and the presence of distinct groups within the dataset was conducted using principal component analysis (PCA). Hierarchical clustering analysis was also performed and is presented as a dendrogram coupled to a heatmap. Both PCA and the clustering analysis were performed using the online chemometrics software Metaboanalyst [160]. Percent differences for each

metabolite in each comparison group were also calculated using MATLAB (The MathWorks, MA, USA).

Variable importance analysis based on random variable combination (VIAVC) [89] is a new feature selection method and was used for this analysis. Briefly, VIAVC provides a method for systematically resampling variables to determine if potentially synergetic effects exist between seemingly unimportant variables. The algorithm combines random permutation of variable inclusion or exclusion with a 10-fold cross validation (CV) of models to determine the optimal subset of variables that provide the most information about the differences between groups. It should be noted that even though the VIAVC script only employs CV, the way the algorithm is structured produces results that are no different from the method of double cross validation (DCV) that has been recommended elsewhere for the validation of metabolomics data sets [81]. The P-values reported are calculated from a t-test of the distribution of scores based on whether the particular metabolite was included or excluded from the model. All of the VIAVC tests were carried out using MATLAB. A more detailed description of this complex method is available by Yun et al. [89]. Furthermore, for the behavioural analysis the average centre distance traveled in an open field was analyzed using a t-test and its relationship to metabolic outcomes was determined using Pearson R correlations in MATLAB.

The biological significance of the important metabolites was investigated using two tools offered by Metaboanalyst. Metabolite set enrichment analysis (MSEA) uses a set of predefined metabolic pathways to identify significant and meaningful changes in functionally related metabolites within a biologically relevant context [91]. Data were entered into the MSEA as a list of metabolites and the Over-Representation Analysis

(ORA) algorithm was used. Pathway analysis was also conducted, which combines the results of pathway enrichment analysis with pathway topology to aid in the identification of the most relevant pathways involved in the conditions of the study [21]. The data were input as a list of metabolites, the Rat pathway library was chosen, and the ORA algorithm was selected using hypergeometric test. These two analysis tools were used in the identification of pathways to discuss in connection with significant metabolites.

3.1.8 Metabolite Identification

An in-house spectral database of pure metabolite substances was used to identify the majority of the metabolites in the spectra. Any metabolites not in our database were referenced using the online Human Metabolome Database [10].

3.2 Results and Discussion

3.2.1 Metabolite Identification and Selection

The purpose of this research was to identify urine metabolic signatures generated by cumulative or transgenerational ancestral stress that associate with or predict mental health outcomes. Urine represents the combined collection and concentration of metabolic wastes from an organism and thus it reflects individual phenotype, which is frequently affected by adverse experiences such as stress [23]. The initial results of PCA, when all metabolites were included, are shown in Figures 2A and 2B and revealed no separation of groups. The present approach used variable importance analysis based on random variable combination (VIAVC) as the feature selection method because it incorporates 10-fold cross-validation (CV) and random permutation methods in order to address the tendency of partial least squares discriminant analysis (PLS-DA) to over-fit data [80,81]. Furthermore, VIAVC method also allows extraction of informative

metabolites, exploits synergetic effects between metabolites, and excludes metabolites that vary randomly between samples. The P-value of each metabolite identified as significant by VIAVC, along with the percent differences for each, are displayed in Table 1. Once the important metabolites were identified, they were used to re-run PCA (Figure 10C and 10D) and hierarchical clustering (Figure 11A and 11B) analysis. Both of these unsupervised grouping methods correctly separated the experimental groups from the controls when considering only the variables identified by VIAVC. Not all of the metabolites identified by the VIAVC method for the two experimental groups were identical; however, there were a number of metabolites that were similar and these will be discussed later.

The present results demonstrate that repeated prenatal stress in each of four generations (multigenerational stress, F4-SSSS group) and a single exposure to prenatal stress four generations removed (transgenerational stress, F4-SNNN group) produce urinary metabolic profiles that are unique when compared to a non-stress control lineage. This is evident by the clear separation of each of the stress lineages from the control group in the PCA scores plots that include only the metabolites identified by the VIAVC method (Figure 10C and 10D). Thus, the impact of stress in each stress lineage has caused a distinct alteration in metabolic activity. The multigenerational lineage demonstrates an impact of both direct and indirect stress exposure [148,161] while the transgenerational stress lineage unambiguously isolates the impact of inherited transgenerational programming, which transmits to the F4 generation [131,161]. The unique metabolic footprint of ancestral stress included 21 metabolites that were

significantly altered (Table 1). The heat map for each of the altered metabolites among the two stress lineages (Figure 11A and 11B) provides a graphical indication of whether

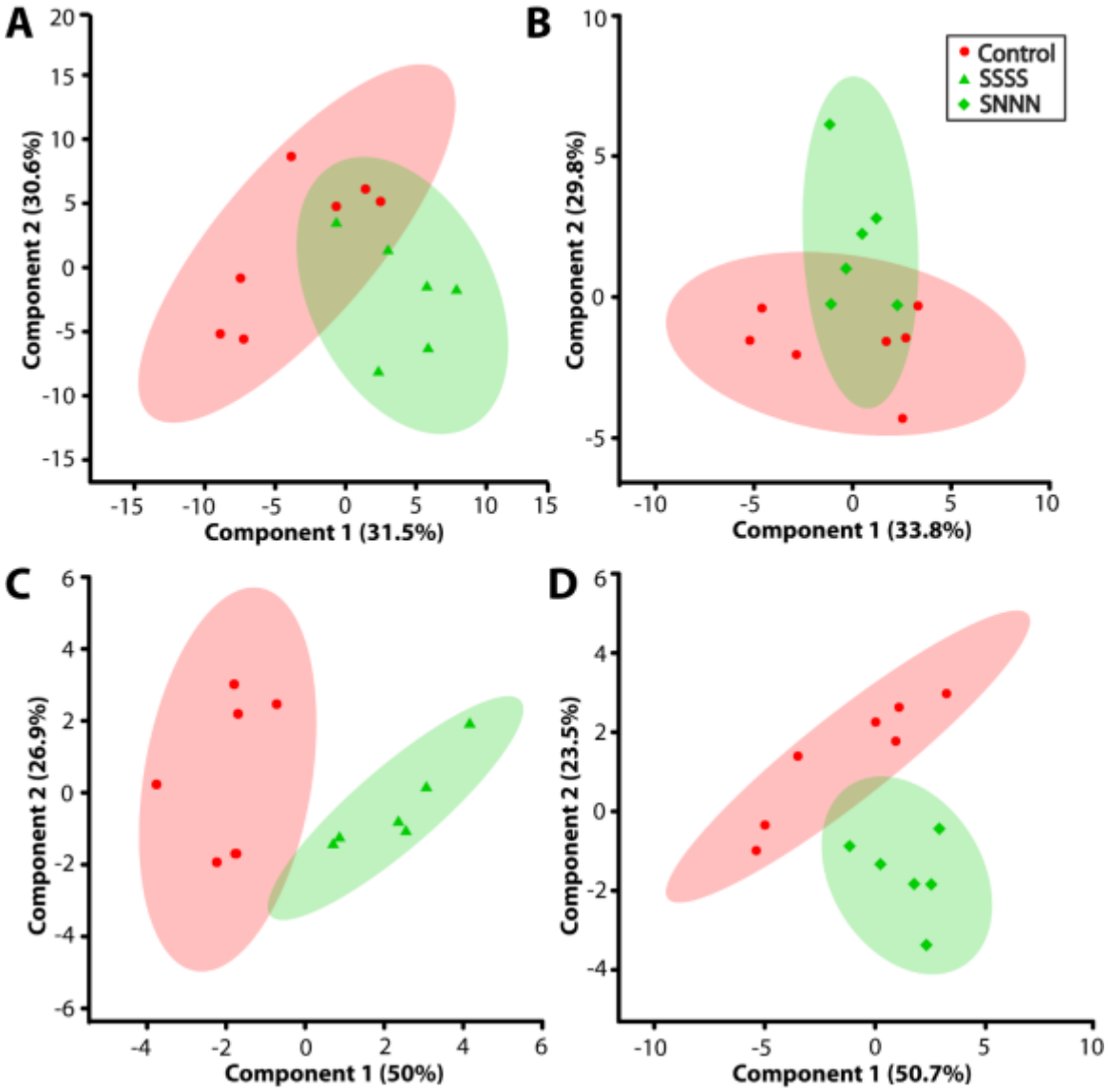


Figure 10. Scores plots showing components 1 (X-axis) and 2 (Y-axis) of the PCA analysis for (A and C) F4-SSSS multigenerationally stressed lineage versus controls and (B and D) F4-SNNN transgenerationally stressed lineage versus controls. A and B provide the result of the analysis when on the metabolites identified by VIAVC were considered. The percentages shown along each axis indicate the amount of variance in the data set given by each componenet and the shaded ellipses designate the 95% confidence interval for each group. The controls are indicated by the red circles, the F4-SSSS by green triangles, and the F4-SNNN by green diamonds.

the metabolites were up- or down-regulated with respect to the control group. Furthermore, the percent differences given in Table 1 show that, when considering all metabolites identified from both stress lineages, a history of ancestral stress consistently reduces nine metabolites and elevates nine metabolites (Table 1). The dendrograms shown above the heat maps (Figure 11A and 11B) illustrate the results of hierarchical clustering analysis; this unsupervised method was able to correctly separate each stress lineage from the controls.

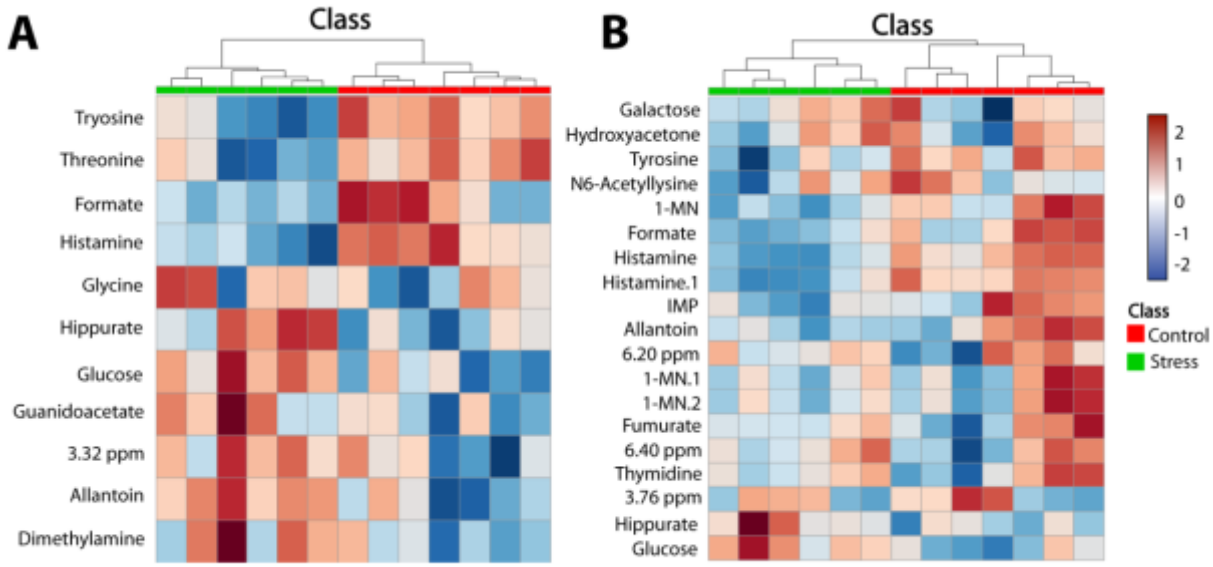


Figure 11. Heat maps for (A) F4-SSSS multigenerationally stressed lineage versus controls and (B) F4-SNNN transgenerationally stressed lineage versus controls. The X- and Y-axis show the class and the metabolite identity, respectively. These heat maps visually indicate either up- or down-regulation of the metabolites presented in Table 1. The legend corresponding to the class label and the heat map for both figures is shown to the right of figure B. Abbreviations: 1-MN, 1-methylnicotinamide, IMP, inosine monophospate. The labels 1-MN, 1-MN.1 and 1-MN.2, as well as Histamine and Histamine.1, correspond to different resonance peaks of the same metabolite. The dendrogram at the top of each heatmap illustrates the results of the unsupervised hierarchical clustering analysis.

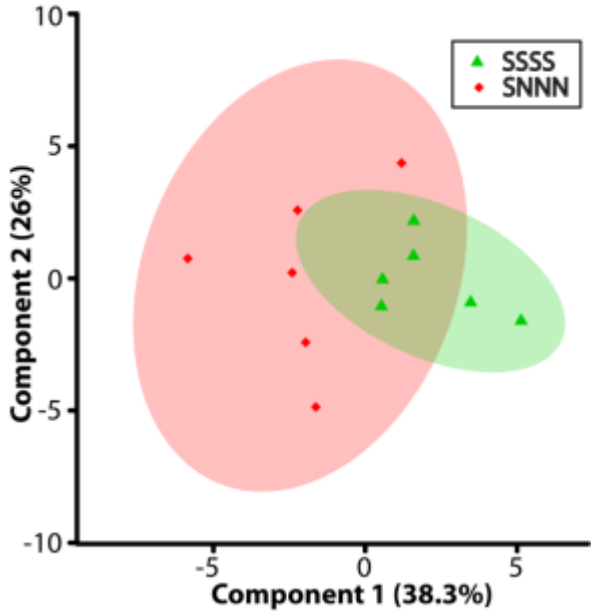


Figure 12. Summary plot for the over representation analysis of metabolic pathways associated with the complete list of metabolites shown in table 1. The p-values for the metabolic pathways are color coded with dark red being highly significant and white being least significant. The category "Intracellular Signalling" stands for intracellular signalling through the H2 receptor and histamine.

The metabolites identified as varying significantly in the SSSS and SNNN lineages (Table 1) concur with our previous findings that variations induced by multi- vs. transgenerational stress [142,162] tend to be reflected in the intensity of the phenotype expression, and not the directionality. Accordingly, the directionality of the percent differences in 18 of 21 identified metabolites, either up- or down-regulation, was consistent between groups. Furthermore, when the two experimental groups (multi- and transgenerational) were plotted using a PCA scores plot (Figure 4) they highly overlap, suggesting similarity of metabolic profiles induced by multigenerational and transgenerational stress programming. Thus, irrespective of the frequency of prenatal stress exposure among the ancestral lineage, common metabolic pathways were reprogrammed that reflect the altered phenotype [142,162]. This observation aligns with

the finding of a coordinated pattern of epigenetic changes, including microRNAs that propagate from one generation to the next [142,143]. Accordingly, metabolic activity is dictated by genetic expression and epigenetic regulation of gene expression will be reflected by distinct levels of metabolic end products.

Table 1. Display of the percent differences and P-values of metabolites that were determined to be significant for at least one of the stress lineages based on the VIAVC analysis (Control, n=7; F4-SSSS, n=6; F4-SNNN, n=6).

	SSSS vs Control		SNNN vs Control	
	P-value	**Percent Difference**	**P-value**	**Percent Difference**
Formate	3.22×10^{-18}	-25.06	3.08×10^{-12}	-31.19
IMP		-6.98	1.61×10^{-9}	-41.78
1-MN	6.64×10^{-13}	-11.66	6.64×10^{-13}	-29.02
Histamine	3.30×10^{-24}	-16.82	5.03×10^{-16}	-39.14
Hippurate	1.84×10^{-11}	26.9	3.56×10^{-6}	27.64
Tyrosine	8.37×10^{-17}	-27.89	6.35×10^{-7}	-20.43
Fumarate		-8.48	0.36	-3.01
Singlet 6.40 ppm		-13.96	2.84×10^{-6}	4.75
Thymidine		-3.69	5.07×10^{-3}	-1.0
Singlet 6.20 ppm		9.78	0.03	2.95
Allantoin	4.94×10^{-8}	32.58	1.54×10^{-4}	17.2
Galactose		-11.12	0.24	3.95
Glucose	5.33×10^{-7}	15.31	2.69×10^{-5}	15.91
Hydroxyacetone		-18.14	1.16×10^{-3}	1.4
Guanidoacetate	2.56×10^{-9}	8.94		12.99
Singlet 3.76 ppm	2.51×10^{-10}	2.23	0.24	2.84
Glycine	2.24×10^{-22}	8.83		17.58
Singlet 3.32ppm	2.5×10^{-4}	19.64		45.87

Dimethylamine	$1.75x10^{-10}$	62.07		15.17
N6-Acetylysine		-11.01	0.017	-3.30
Threonine	$4.31x10^{-10}$	-24.75		-10.4

3.2.2 Metabolic Pathway Analysis

The metabolites that were significantly altered by ancestral stress (Table 1) are diverse and involved in various pathways. Although one cannot conclusively rule out which of the potential metabolic pathways are relevant to phenotype, Pathway Analysis (PA) and Metabolite Set Enrichment Analysis (MSEA) identified a number of key metabolic pathways that these metabolites are involved in (Table 2 and Figure 5, respectively). The following pathways were selected from the outputs of PA and MSEA based on their biological relevance to mental health: catecholamine biosynthesis and tyrosine metabolism; intracellular signalling through the H2 receptor and histamine; histidine metabolism; and glycine, serine and threonine metabolism. The following list of metabolites from Table 1 are associated with the above pathways and will now be discussed individually: tyrosine, threonine, and histamine.

Ancestral stress-induced up-regulation of tyrosine excretion supports the link to mental health and stress programming. Amino acid L-tyrosine is the precursor of multiple monoamine neurotransmitters including norepinephrine (NE), which is a central molecule directing the immediate physiological response to stress [125]. Obtained from the diet, animals in our study should had relatively identical levels of L-tyrosine [163]. However, both SSSS and SNNN groups had a significantly lower level of tyrosine being excreted, alluding to an elevated stress response [130,143] which consumes larger amounts of tyrosine to maintain elevated NE production in the stressed lineages.

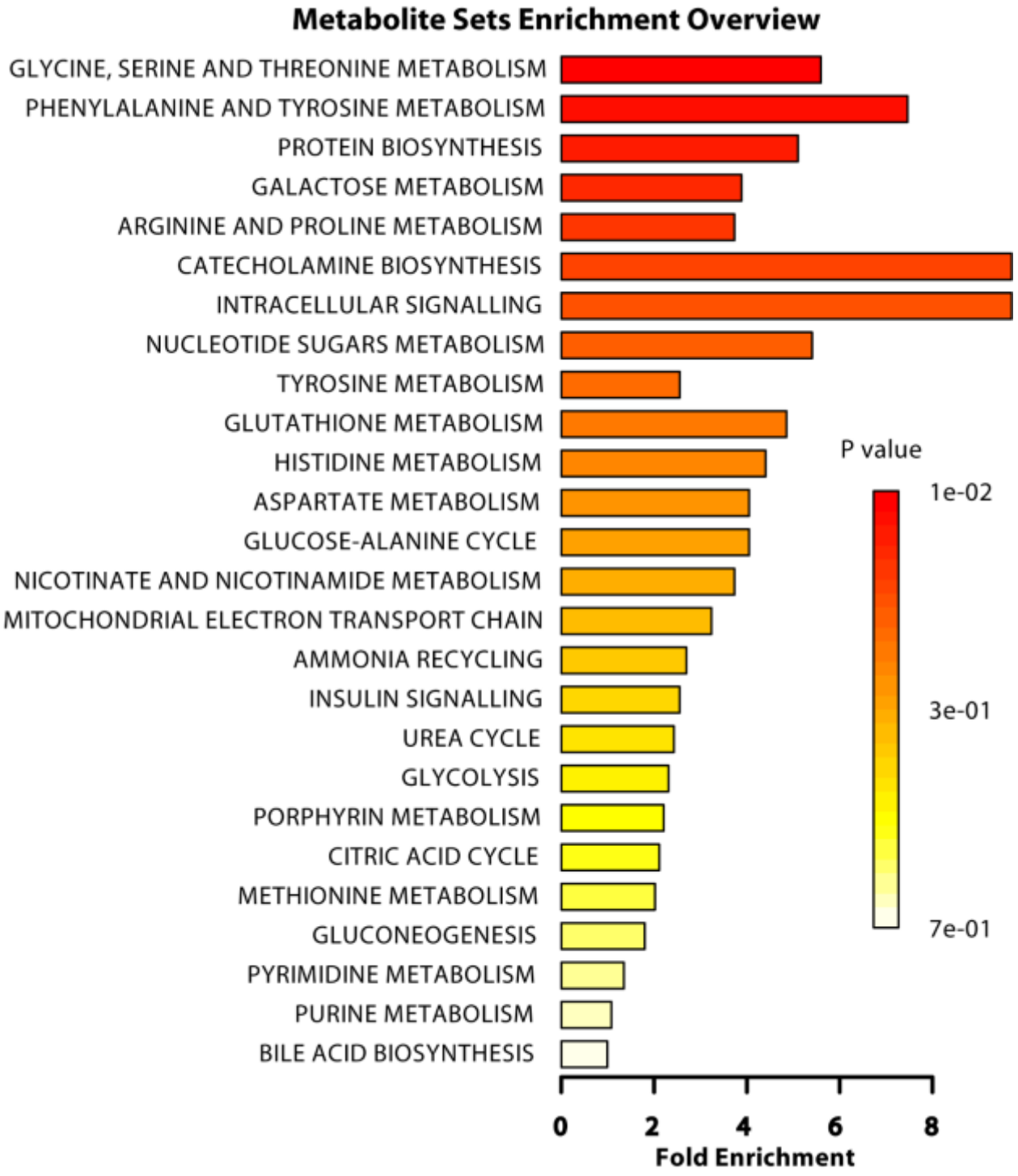


Figure 13. Summary plot for the over representation analysis of metabolic pathways associated with the complete list of metabolites shown in table 1. The p-values for the metabolic pathways are color coded with dark red being highly significant and white being least significant. The category "Intracellular Signalling" stands for intracellular signalling through the H2 receptor and histamine.

Table 2. Overview of the metabolic pathways that were altered by ancestral stress compared to non-stress controls. All indicates the total number of metabolites listed in the pathways; hits indicate the number of significant metabolites identified in the pathways; the P-value is based on the enrichment analysis; FDR indicates false discovery rate;

Pathway Name	All	Hits	P-value	FDR	Impact
Aminoacyl-tRNA biosynthesis	67	3	0.043	0.77	0.10
Methane metabolism	9	2	0.0047	0.23	0.17
Glycine, serine and threonine metabolism	32	3	0.0059	0.23	0.32
Histidine metabolism	15	1	0.168	0.85	0.15
Phenylalanine, tyrosine and tryptophan biosynthesis	4	1	0.047	0.77	1.0
Tyrosine Metabolism	42	2	0.089	0.84	0.14

impact designates the pathway impact as determined by pathway topology analysis.

Stress may increase susceptibility to disease and allergies, autoimmune disease, and inflammation [164]. Threonine, an essential amino acid, stimulates thymus development and is a major building block of immunoglobulins [165]. In line with the notion of elevated stress responses induced by ancestral stress, threonine levels were reduced in both groups. As an important immuno-stimulant, low threonine excretion may be linked to higher demand by the immune system.

Another immunomodulator that was reduced by a history of stress is histamine. Histamine is produced by decarboxylation of the essential amino acid L-histidine. It is active at sites of inflammation, stimulates gastric secretion, and also regulates immune function. Even low concentrations of histamine can be highly active in biological systems and therefore its synthesis, transport, storage, and release are carefully regulated [166].

The stress-induced reduction in histamine in both the SSSS and SNNN groups may reflect either a lower conversion of L-histidine into histamine or a higher usage of histamine by the organism. Given that stress is often accompanied by exacerbated immune function [164], it is likely that histamine usage has been up-regulated, reflected by decreased excretion.

The VIAVC method and the percent differences both identified hippurate as a significant metabolite; however, hippurate, which is a common urinary metabolite in mammals, is not related to metabolic pathways identified by MSEA and PA. There are a number of possible initial substrates for the production of hippurate, all of which are large polyphenols that enter the organism via the diet and undergo a series of reactions catalyzed by various enzymes and endogenous microbiota. The final step in the production of hippurate occurs in the mitochondrial matrix where benzoic acid, a remnant of the polyphenols, is conjugated with glycine [167]. Hippurate then enters the blood stream and is filtered out of vascular circulation by the kidneys. Its excretion is upregulated in human subjects with diabetes [167] and high levels of anxiety [168], both of which are common symptoms associated with prenatal stress [140,169,170]. Both experimental groups in this study displayed up-regulated hippurate excretion, which suggests discrete metabolic alteration linked to metabolic disorders and/or mental health.

3.2.3 Behavioural Phenotype

The distinct metabolic profiles of the SSSS and SNNN groups were associated with mental health outcomes. Open field locomotor profiles indicate a phenotype of higher emotional state and increased anxiety-like behaviour in the stressed lineages, as reflected by distance travelled in the centre of the open field arena. Rats that are less anxious will generally spend more time in the illuminated centre of the open field arena

whereas rats that are more anxious will avoid this open, lit space [171]. In the present study, an independent samples t-test compared average centre distance scores for male offspring from stressed and control lineages. There was a significant difference in the scores for control ($M = 159.4$, $SD = 33.8$) and stressed ($M = 119.7$, $SD = 39.5$) lineages ($t(29) = -2.41$, $p < 0.05$). The relationship between average distance scores and the relative concentrations of histamine and tyrosine indicated positive correlations for histamine [$r = 0.55$, $p < 0.01$ (one tailed)] and tyrosine [$r = 0.21$, $p = 0.177$ (one-tailed); Figure 6]. Thus, higher anxiety-like states were associated with lower histamine and lower tyrosine concentrations.

Figure 14. Pearson correlations to assess the relationship between anxiety-like behaviour (i.e., average centre distance traveled) and the relative concentrations of histamine (A) and tyrosine (B). There were positive correlations between the distance score and histamine ($r = 0.55$, $p = 0.0045$), and tyrosine ($r = 0.21$, $p = 0.177$), indicating that a higher anxiety-like state was linked to lower histamine and tyrosine concentrations.

3.3 Conclusions

Using a ^{1}H NMR-based metabonomics approach, perinatal programming caused by both remote and cumulative ancestral stress was manifested in altered metabolic profiles and anxious behaviour in the F4 generation. The programming was evident by changes in relative concentrations of a number of key metabolites, particularly hippurate, tyrosine, threonine and histamine. These metabolites are implicated in catecholamine biosynthesis, immune responses, and microbial host interactions in mammalian systems. The similarities between the metabolic profiles of multi- and transgenerational stress indicate that both cases share common metabolic pathways. When altered, these common metabolic pathways may either lead to or be reflective of pathophysiological processes

resulting in impaired health. Identification of metabolic pathways affected by ancestral stress will help to determine epigenetically regulated gene targets leading to stress-associated diseases, such as mental health disorders. Ultimately, this will lead to a better understanding of the etiology of these diseases and potential biomarker discovery.

CHAPTER 4: TRANSGENERATIONAL STRESS PROGRAMMING OF LIVER METABOLOME

4.0 Introduction

Social isolation acts as a mild to moderate stressor, activating the stress response, and leading to negative outcomes in humans [172,173] and animals [174]. The effects associated with stress are capable of impacting more than just the individual to whom the stress is applied. As discussed in the introduction to Chapter 3, stressors experienced during pregnancy have the ability to cause altered developmental outcomes in subsequent generations of offspring. Additional to the already discussed aspects of prenatal stress on offspring, there have also been specific studies analyzing how social isolation in pregnant animals impacts the lifespan of the offspring [175-177].

Activation of the stress response results in the release of corticosteroids (cortisol in humans, corticosterone in rats) from the adrenal glands [125]. The stress response of offspring who experienced pre-natal maternal stress (PNMS) in utero is shown to be altered [129,140]. This alteration in the stress response will result in an altered pattern of corticosteroid release. Corticosteroids act on cells to convert energy stored in the cell into glucose through a process called gluconeogenesis [178]. As the liver is the primary site of gluconeogenesis, it is a logical tissue to analyze the metabolic effects of stress [179].

We performed ^{1}H NMR metabolomics on liver homogenate samples from rats bred in both a trans- and multigeneration stress model (same experimental model as described in Chapter 3) using social isolation during pregnancy as the stressor. Our aim was to identify distinct profiles that could be used to discriminate between healthy controls and subjects whose ancestors were stressed.

4.1 Methods

4.1.1 Animals

This study involved Long-Evans rats raised at the Canadian Centre for Behavioural Neuroscience, University of Lethbridge vivarium. The animals were housed in pairs under a 12:12 h light/dark cycle with light starting at 07:30 h and the room temperature set at 22 °C. Rat chow food and water were available at libitum. All procedures were approved by the University of Lethbridge Animal Care Committee in compliance with the guidelines of the Canadian Council on Animal Care.

4.1.2 Experimental Design

To investigate the truly heritable metabolic phenotype, this study focuses on six-month old male and female offspring from the F4 generation (reference for importance of F4). Four successive generations of timed-pregnant female rats were bred under consistent laboratory conditions. Parental female rats (F0) were socially isolated before and during pregnancy. For the multigenerational stress lineage (F4-SSSS; n=6) the pregnant daughters (F1), granddaughters (F2), and great granddaughters (F3) were also stressed before and during pregnancy. The transgenerational stress lineage (F4-SNNN; n=6) was bred by stressing only the F0 mothers and not the F1 – F3 mothers (see Figure 1; S = stress, N = non-stress condition). Yolked controls were bred in parallel for each generation (Control, n=7), the mothers were housed in pairs until gestational day 21.

4.1.3 Stress Procedure

Pregnant dams were stressed using a social isolation stressor, which has been shown to result in mild psychosocial stress in rats [175]. Each dam was housed alone and

did not experience direct contact with any other rats from P 90 until her offspring were weaned. Control rats were housed in pairs until gestational day 21.

4.1.4 Sample Collection and Preparation

For fresh tissue collection, animals were placed into an induction chamber filled with 5% isoflurane (oxygen at 1.5 l/min). Upon successful deep anesthesia, the animals were administered 0.2 ml of sodium pentobarbital (Euthansol, Merck QC, Canada) by intracardiac infusion. After cardiac arrest, the liver (along with additional organ samples) were collected and immediately placed on dry ice at -80^{o}C.

4.1.5 Liver Homogenization

Homogenization of the liver followed the protocols recommended by Beckonert et al. for extraction of polar and non-polar metabolites [43]. Briefly, frozen samples were separated into 150mg pieces and combined with 4ml/g methanol and 0.85ml/g of water. These were homogenized until a uniform mixture was obtained. An additional 4ml/g of water and 2ml/g of chloroform were added to the sample tube and vortexed for 30 seconds. The sample was then centrifuged at 4°C for 15 minutes at 1500 rpm. One milliliter of supernatant was then removed and lyophilised overnight. The metabolites were reconstituted in 1ml of D_2O. To prepare the samples for NMR spectroscopy, 450 µl aliquots of homogenate were transferred into 1.5 ml centrifuge tubes with 250 µl of phosphate buffer. The phosphate 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 a final concentration of 0.5 M. The D_2O came with 0.05 % by weight trimethylsilyl propanoic acid (TSP) as a chemical shift reference. To protect the metabolite profile integrity, 3mM of sodium azide was added to the buffer solution as an antimicrobial agent. Once the sample was mixed with the buffer, it was centrifuged at

12,000 rpm for 10 minutes to precipitate any particulate matter. After centrifugation, 550 µl of the supernatant was transferred to a 5 mm NMR tube for NMR analysis. The remaining homogenate was stored at -80°C for future use. The lipid layer was not separated or collected, inclusion of the lipid solvent chloroform in the homogenization was used as it produced a cleaner spectrum.

4.1.6 NMR Data Acquisition and Processing

NMR spectra were collected on a 700 MHz Bruker Avance III HD spectrometer (Bruker, ON, Canada). The Bruker 1-D NOESY gradient water suppression pulse sequence 'noesygppr1d' was used. Each sample was run for 512 scans to a total acquisition size of 128 k. The spectra were zero filled to 256 k, automatically phased, baseline corrected, and line-broadened by 0.3 Hz. The processed spectra were then exported to MATLAB (The MathWorks, MA, USA) for statistical analysis. Spectra were manually binned to reduce the size of the dataset. Each spectrum had the areas corresponding to water and acetone removed before being normalized to remove effects of imperfect water signal suppression. The data set was then auto-scaled.

4.1.7 Statistical Analysis

Data visualization to determine sample structure and the presence of distinct groups within the dataset was conducted using PCA. Hierarchical clustering analysis was also performed, and is presented as a dendrogram coupled to a heatmap. Both PCA and the clustering analysis were performed using the online chemometrics software Metaboanalyst. Percent differences for each metabolite in each comparison group were also calculated using MATLAB (The MathWorks, MA, USA).

Variable importance analysis based on random variable combination (VIAVC) [89] is a new feature selection method and was used for this analysis. Briefly, VIAVC provides a method for systematically resampling variables to determine if potentially synergetic effects exist between seemingly unimportant variables. The algorithm combines random permutation of variable inclusion or exclusion with a 10-fold cross validation (CV) of models to determine the optimal subset of variables that provide the most information about the differences between groups. It should be noted that even though the VIAVC script only employs CV, the way the algorithm is structured produces results that are no different from the method of double cross validation (DCV) that has been recommended elsewhere for the validation of metabolomics data sets [81]. The P-values reported are calculated from a t-test of the distribution of scores based on whether the particular metabolite was included or excluded from the model. All of the VIAVC tests were carried out using MATLAB.

Similar to the previous study analyzing the urine metabolome, the biological significance of the important metabolites was investigated using MSEA and pathway analysis.

4.1.8 Metabolite Identification

An in-house spectral database of pure metabolite substances was used to identify the majority of the metabolites in the spectra. Any metabolites not in our database were referenced using the online Human Metabolome Database [10].

4.2 Results and Discussion

Initial multivariate analysis of the data using PCA did not produce separation between the control and experimental groups as shown in Figures 13 and 14 (females and males respectively). VIAVC was used to identify important metabolites in each individual comparison. A new PCA model was then constructed for each comparison using only the optimal subset of metabolites as identified by VIAVC (Figures 15 and 16). The optimal subset of metabolites for each comparison is listed in Table 3 along with the p-value and percent difference of those metabolites. The AUC that corresponds to a multivariate ROC model made with the optimal subset of variables from each group is also listed in the table.

Along with the PCA models, hierarchical clustering and a heatmap of each comparison are provided (Figures 15 and 16).

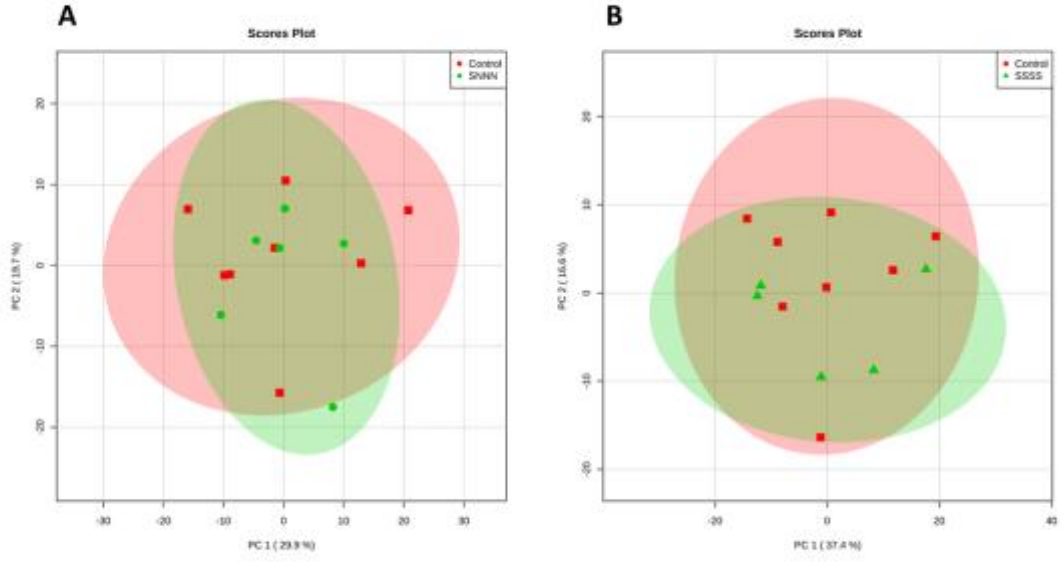


Figure 15. PCA scores using all variables for female F4-SNNN (A) and F4-SSSS (B). Plots show components 1 (x-axis) and 2 (y-axis) with the percentage of the variance explained by each component labelled on the axis. Ovals indicate the 95% confidence interval.

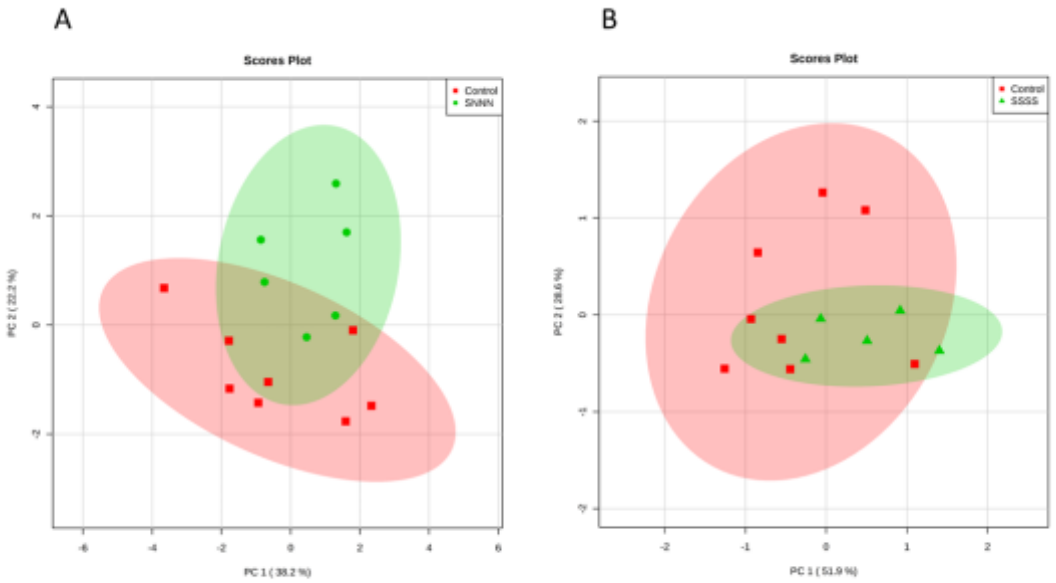


Figure 16. PCA scores using all variables for male F4-SNNN (A) and F4-SSSS (B). Plots show components 1 (x-axis) and 2 (y-axis) with the percentage of the variance explained by each component labelled on the axis. Ovals indicate the 95% confidence interval

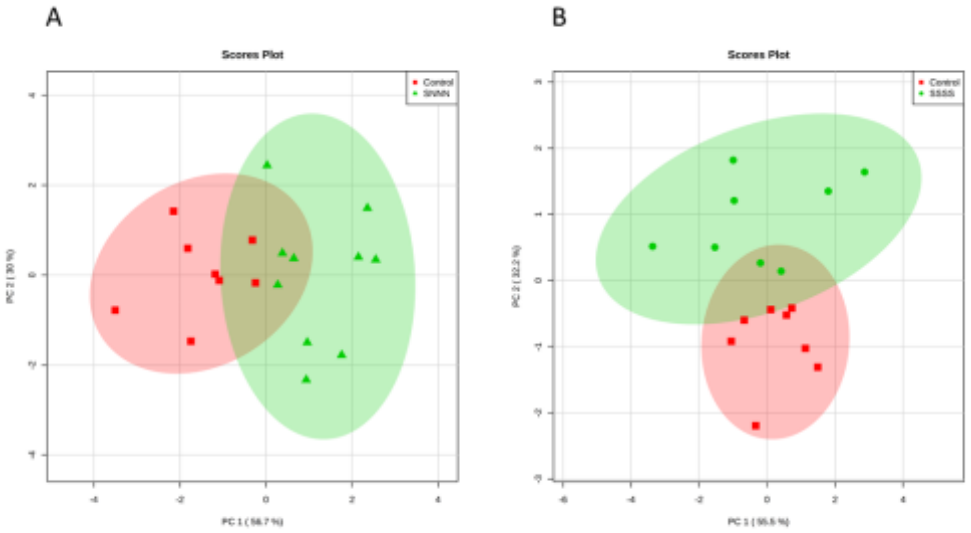


Figure 17. PCA scores using the optimal subset of variables for female F4-SNNN (A) and F4-SSSS (B). Plots show components 1 (x-axis) and 2 (y-axis) with the percentage of the variance explained by each component labelled on the axis. Ovals indicate the 95% confidence interval

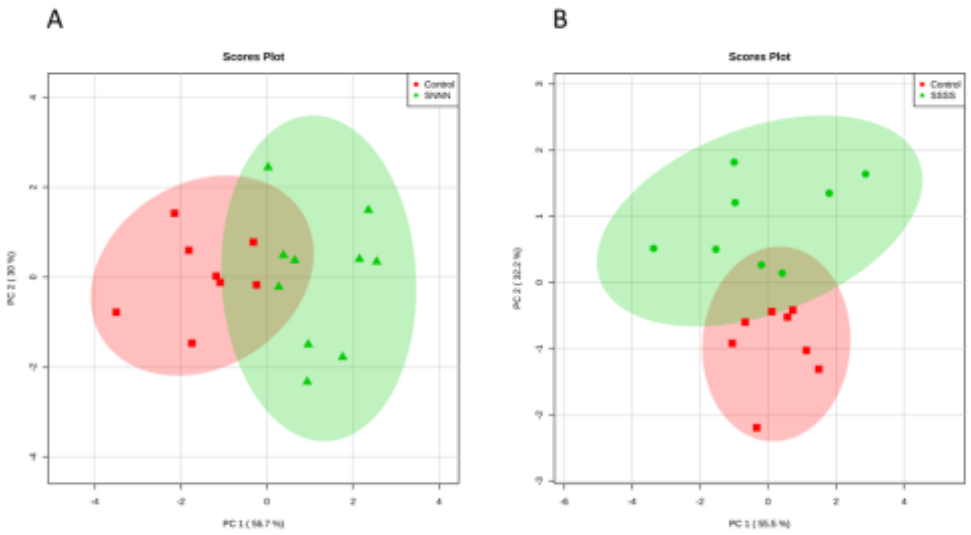


Figure 18. PCA scores using the optimal subset of variables for male F4-SNNN (A) and F4-SSSS (B). Plots show components 1 (x-axis) and 2 (y-axis) with the percentage of the variance explained by each component labelled on the axis. Ovals indicate the 95% confidence interval.

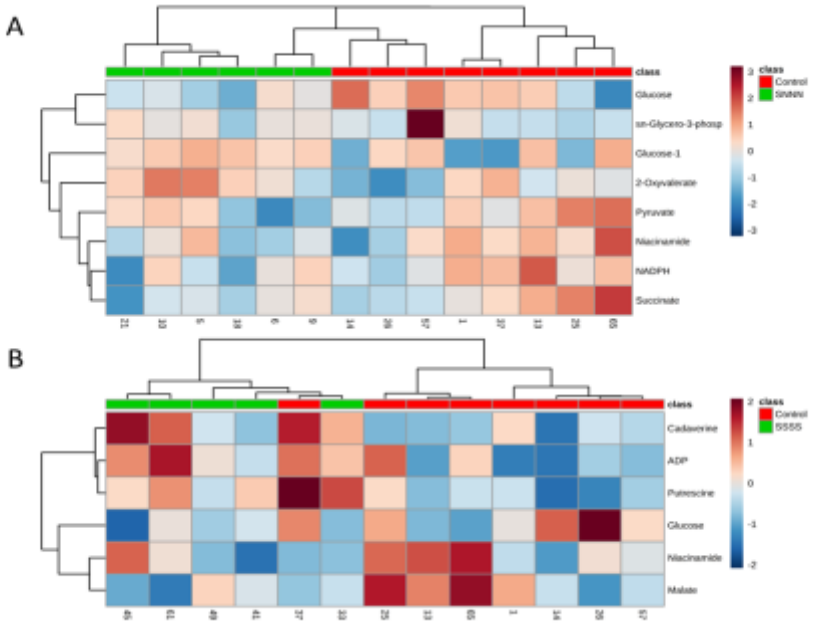


Figure 19. Heat maps for female (A) F4-SNNN multigenerationally stressed lineage versus controls and (B) F4-SSSS transgenerationally stressed lineage versus controls. The X- and Y-axis show the class and the metabolite identity, respectively. These heat maps visually indicate either up- or down-regulation of the metabolites presented in Table 3. The dendrogram at the top of each heatmap illustrates the results of the unsupervised hierarchical clustering analysis.

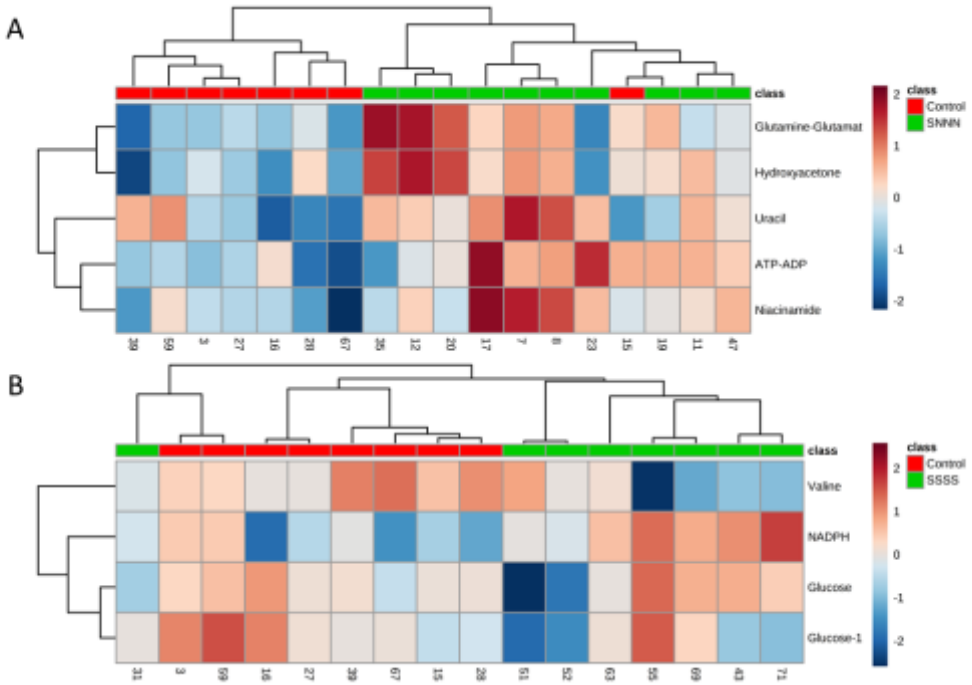


Figure 20. Heat maps for male (A) F4-SNNN multigenerationally stressed lineage versus controls and (B) F4-SSSS transgenerationally stressed lineage versus controls. The X- and Y-axis show the class and the metabolite identity, respectively. These heat maps visually indicate either up- or down-regulation of the metabolites presented in Table 3. The dendrogram at the top of each heatmap illustrates the results of the unsupervised hierarchical clustering analysis.

Table 3. Display of the percent differences and p-values of metabolites that were determined to be significant in each comparison group. Each group listed is compared to the respective controls for each sex. A positive percent difference indicates an upregulation of the metabolite in the experimental group.

Sex	Lineage	Metabolite	p-value	% Difference	AUC
Female	SNNN	sn-Glycero-3-phosphocholine	<0.001	-29.5	
		Succinate	<0.001	-26.2	
		Pyruvate	<0.001	-10.8	
		Niacinamide	<0.001	-3.3	0.875
		NADPH	<0.001	-4.1	
		Glucose	<0.001	-20.1	
		2-Oxovalerate	<0.001	7.4	
	SSSS	Cadaverine	<0.001	6.8	
		Glucose	<0.001	-5.3	
		ATP	<0.001	33.3	0.875

		Malate	<0.001	-15.4	
		Putrescine	<0.001	6.7	
		Niacinamide	<0.001	-7.7	
Male	SNNN	Hydroxyacetone	<0.001	16.5	
		Niacinamde	<0.001	18.3	
		ATP	<0.001	16.4	0.883
		Glutamine	<0.001	15.9	
		Uracil	<0.001	13.7	
	SSSS	Glucose	<0.001	-6.3	
		Valine	<0.001	-12.2	0.889
		NADPH	<0.001	11.2	

Inspection of the VIAVC PCA graphs shows that in males and females, there is separation of groups in the SNNN lineages. However, in the SSSS lineage, the females show very little separation, while the males show similar separation to the SNNN group. This indicates a sex difference in how repeated ancestral stress affects the offspring, and suggests that there may be some resiliency being built up in the female offspring of repeated ancestral stress, but not the males [180]. The hierarchical clustering dendrograms pictured at the top of the heatmaps also indicate that the VIAVC metabolites are able to separate the controls from experimental cases. While this separation is not

perfect, it still illustrates that differences do exist in the metabolic profiles of liver tissues of subjects in this experiment.

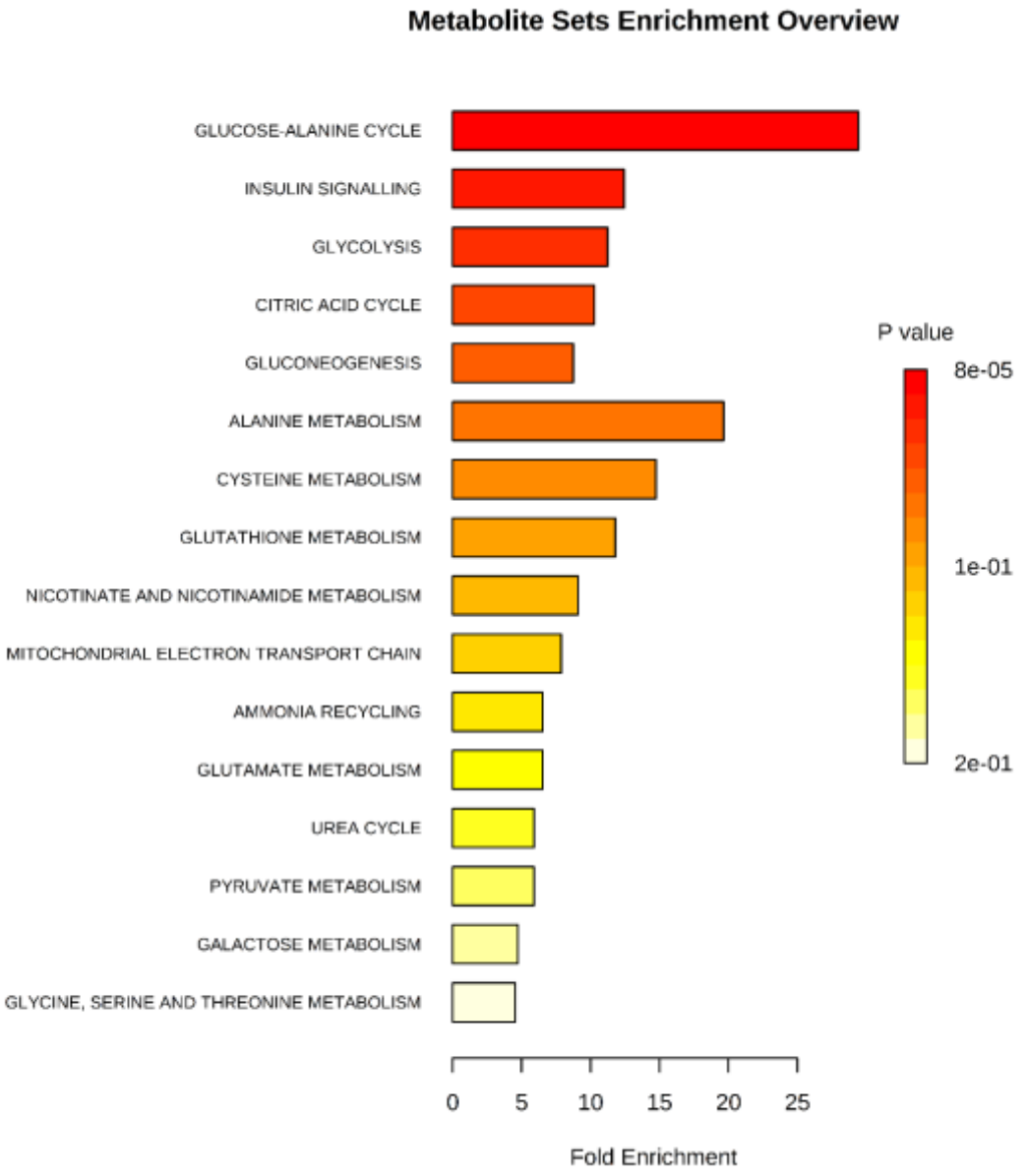


Figure 21. Summary plot for the over representation analysis of metabolic pathways associated with the complete list of metabolites shown in Table 3 for the Female SNNN group. The Pp-values for the metabolic pathways are color coded with dark red being h

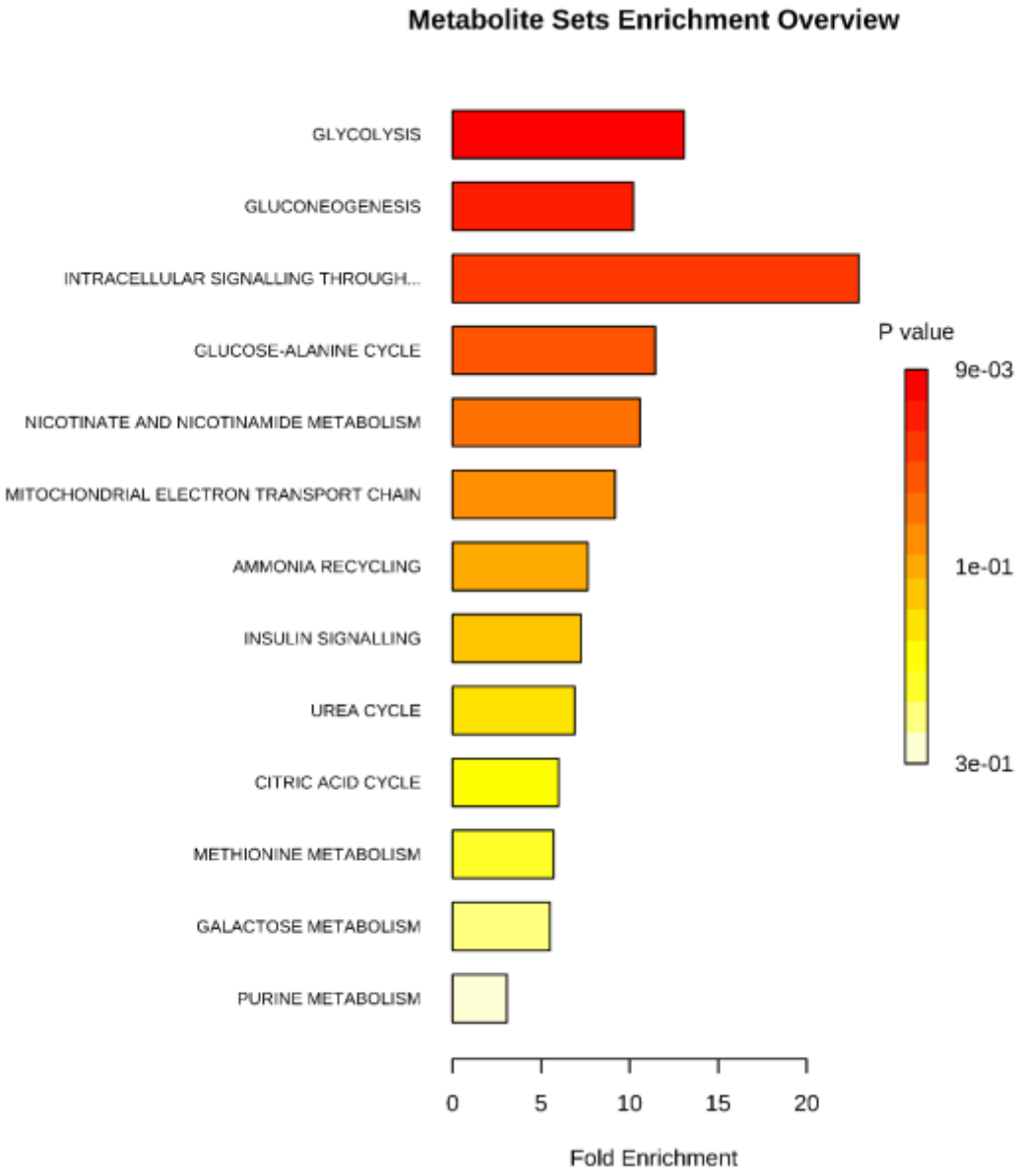


Figure 22. Summary plot for the over representation analysis of metabolic pathways associated with the complete list of metabolites shown in Table 3 for the Female SSSS group. The Pp-values for the metabolic pathways are color coded with dark red being highly significant and white being the least.

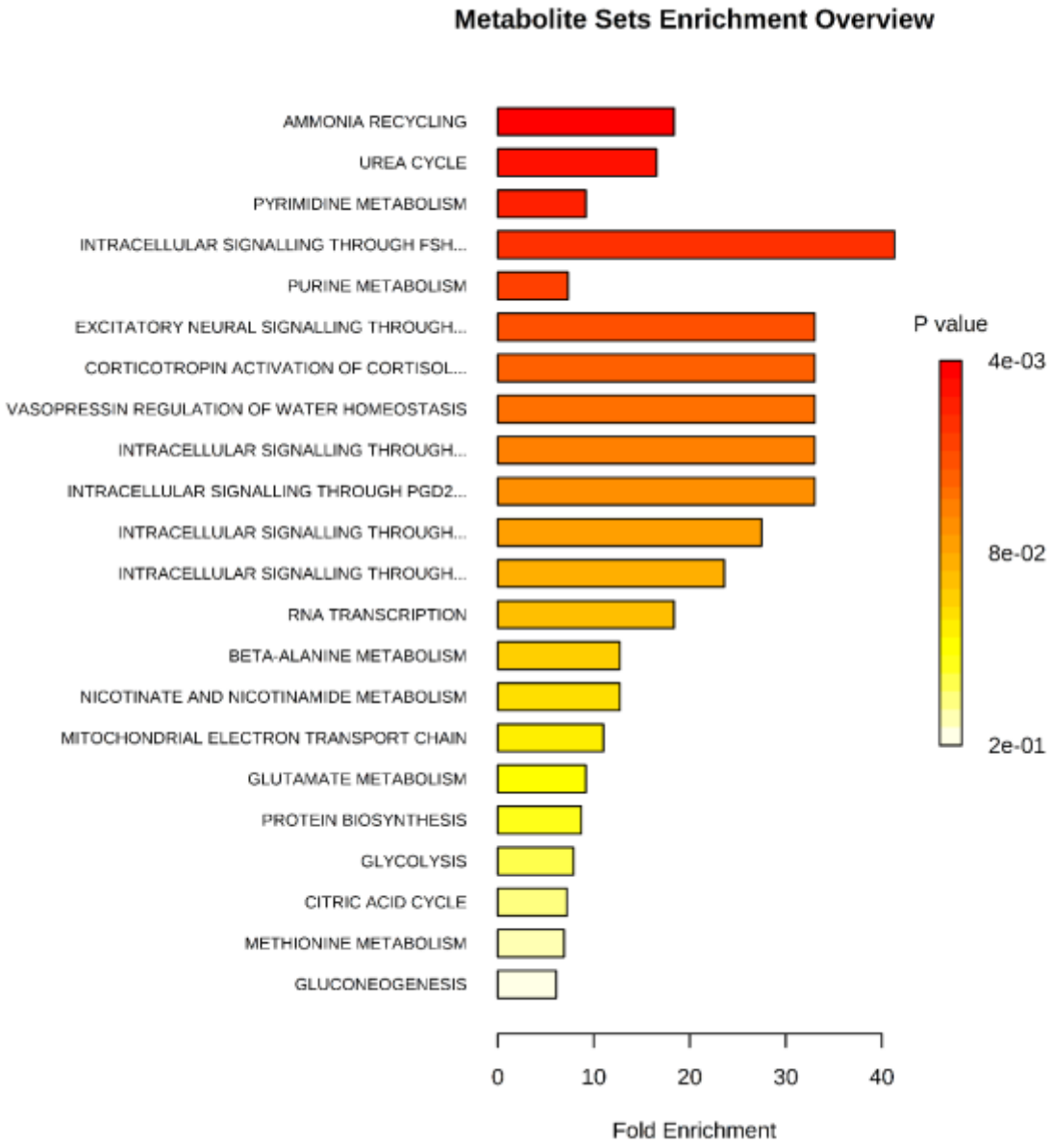


Figure 23. Summary plot for the over representation analysis of metabolic pathways associated with the complete list of metabolites shown in Table 3 for the Male SNNN group. The p-values for the metabolic pathways are color coded with dark red being highly significant and white being the least significant.

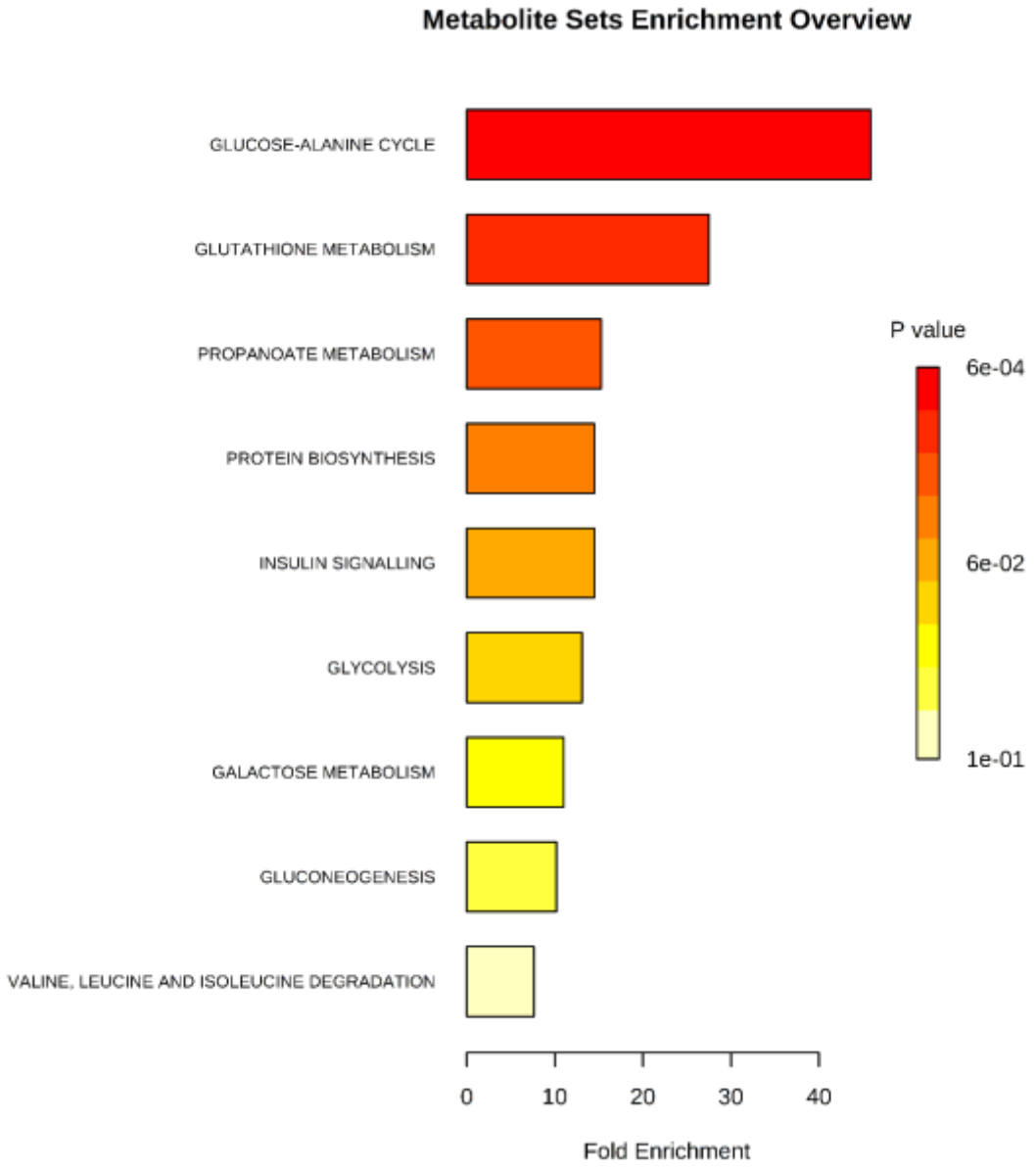


Figure 24. Summary plot for the over representation analysis of metabolic pathways associated with the complete list of metabolites shown in Table 3 for the Male SSSS group. The p-values for the metabolic pathways are color coded with dark red being highly significant and white being the least significant.

While the specific metabolites that were identified in each group are different, many are related by biochemical pathways related to cellular energy management. This is supported by the MSEA analysis for all comparisons returning glycolysis and

gluconeogenesis as pathways that were impacted by the ancestral stress. Glycolysis occurs in the cytosol of the cell and converts glucose into pyruvate, which then enters the citric acid cycle in the mitochondria. Gluconeogenesis is a process whereby non-carbohydrate substrates, such as proteins and fats, are converted to glucose. Both of these processes prepare molecules in the body for energy production.

Of the identified metabolites, succinate, pyruvate, malate, and glucose are all involved in normal energy production through the citric acid cycle [181]. Reduction of all of these metabolites across the various groups could indicate a reduction or depletion of energy derived via the citric acid cycle. Conversely, hydroxyacetone and 2-oxovalerate are both ketone bodies that originate from the metabolism of fats for energy use [182]. Increases of both of these metabolites suggest a greater a reliance on alternative energy sources as a result of and altered stress response induced by ancestral stress.

Niacinamide is a component of NADPH, which acts as a reducing agent in energy producing reactions within the cell [183]. Both Niacinamide and NADPH were altered in this study. ATP, the primary energy currency of cells, was also altered in two of the groups. Taken together, these results indicate that ancestral stress impacts the energy metabolism in multiple generations of offspring. This is most likely mediated through aberrant cortisol levels resulting from a dysfunctional stress response.

Increased stress has consequences for the immune system. Excessive activation of the stress response lowers immune function and increases the likelihood of contracting disease or growing bacterial infections [184]. There was a moderate increase in the levels of putrescine and cadaverine in the female SSSS group. These two metabolites have been linked to the presence of unhealthy bacterial colonies in the organism and suggest that these subjects were less protected against infection [185].

4.3 Conclusion

Although no coherent pattern of specific metabolites was consistently affected in all the experimental groups, the metabolic functions of the important metabolites were related. The results support the theories that prenatal stress alters the stress response of the offspring, and that this altered response will effect metabolism. Importantly, this study illustrates that cumulative and remote ancestral stress can have metabolic repercussions multiple generations removed from the stressful event.

These results may also suggest that social isolation is less stressful than the forced swim task and restraint. The results from the urine study in Chapter 3 were more robust than those obtained analyzing the liver. This may be because the liver metabolome is less impacted by the ancestral stressor; however, there is the possibility that the stressors applied in the Chapter 3 study were more intense than those applied to the current study, and thus elicited a stronger response. This theory cannot be tested with the present data as the information was collected from two different sample types. Further research could investigate if differential metabolic responses result from different stress regimes.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSIONS

5.1 Summary

The objective of this thesis was to investigate the metabolic alterations caused by ancestral transgenerational prenatal stress (TPS) and ancestral multigenerational prenatal stress (MPS). As far as we are aware, this work is the first to study the metabolic effects in the F4 generation offspring of TPS and MPS maternally stressed lineages in a rat cohort. In Chapter 1, the general theory of metabolomics and how it translates to a measure of phenotypic expression was discussed. As the majority of the work described in this thesis involved the development and implementation of metabolomics experiments at the University of Lethbridge, Chapter 2 discussed NMR theory as it applies to metabolomics, and it outlined the workflow and procedures followed when performing NMR metabolomics, and provided a summary review of representative studies that applied NMR metabolomics to study the brain. In Chapter 3, ^{1}H NMR of urine samples from TPS and MPS F4 male offspring were studied to investigate the metabolic changes that occur as a result of ancestral stress. Chapter 4 also described ^{1}H NMR, this time to analyze the metabolome of liver homogenates from TPS and MPS F4 male and female offspring. Combined, these studies offer a method for monitoring the long term impact that ancestral stress can have on metabolism.

5.2 Ancestral Stress Alters Metabolism

The effects of ancestral stress on offspring have been well documented. While a large amount of research has focussed on cognitive and behavioural effects, as well as reprogramming of the stress response, this study focussed on the metabolic changes that accompany these other alterations. As the stress response utilizes signalling hormones and releases cortisol, a major metabolism regulator, it is intuitive that metabolic differences will be present. Indeed, this is what was observed. Of the metabolic alterations that were

detected, all significant changes were directly linked to processes that the literature indicates are either involved in or affected by the stress response. This supports the view that metabolism plays an integral role in body function and can serve as a sensitive measure and biomarker of phenotype.

5.3 Limitations and Considerations for Future Research

The present series of studies were limited to the analysis of the F4 offspring in a controlled breeding lineage. Analysis of all previous generations would be valuable in showing how the effects of stress either increase or plateau with time. It would also be interesting to analyse future generations to see if or when effects begin to diminish. For the urine study presented in Chapter 3, ideally one would analyse more than one urine sample per subject in order to get a more accurate measure of the individual metabotypes, as well as to mitigate any abnormal variation that may have been present at the time of collection. As the rats were sacrificed when the urine was collected, the collection method would need to be altered to obtain multiple samples. For the liver study in Chapter 4, multiple samples could have been prepared from each liver. This would have helped to quantify the amount of variation present in the samples due to experimental error. Additionally, it would have been valuable to analyze the epigenome in order to confirm, rather than infer, that epigenomic mechanisms were heavily involved in the transfer of the TPS phenotype. Similar work has been performed in earlier studies [126, 131, 142, 148, 149, 150, 162].

The stress applied to the rats differed in the two studies presented, ranging from restraint and forced swimming to social isolation. It would be worthwhile to design experiments that involved the same analysis of the different stress procedures to determine what, if any, differences exist in the metabolic response to different stressors.

Future studies should be designed with metabolomics in mind and have multiple sample types available for each subject. A weakness of the work presented in this thesis is that the urine study and the liver study used different subjects. Ideally, we would have been able to have liver and urine from the same animals which would facilitate correlating tissue abnormalities with urinary markers. If urine, blood, CSF, and multiple tissues were available for every subject, multiple correlations could be made and more information would be available to move forward with for biomarker studies.

5.4 Conclusions

The results of this work indicate that ancestral stress alters the phenotype of offspring for up to four generations after the stressor, and that this change in phenotype can be detected in the metabolic profiles of urine and liver homogenate. Further generations may be also affected, which remains to be determined. The metabolic changes point to altered host-microbiome interactions, dysregulated monoamine signalling, altered immune function, and altered energy metabolism. All these functions suggested by the metabolic markers have previously been shown to be affected by prenatal stress. Additionally, the results from the study in Chapter 3 were correlated with behavioural outcomes, showing that the metabolic markers can be linked to other phenotypic measures. The net results of this study validate the potential of using metabolomics for studies of health and disease. It also highlights the potential that metabolomics has to serve as a technique to discover and monitor novel bio-markers.

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