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BIO615 - Literature Review

CURRENT METABOLOMIC STRATEGIES FOR THE DISCOVERY OF BIOMARKERS FOR FATIGUE

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List of Abbreviations

ADF	Australian Defence Force
ANOVA	Analysis of Variance
APCI	Atmospheric Pressure Chemical Ionisation
AUC	Area Under the Curve
BAC	Blood Alcohol Content
C	Carbon
CI	Chemical Ionisation
CSF	Cerebrospinal Fluid
ECF	Extra Cellular Fluid
ED	Electrical Detection
EI	Electron Impact
ESI	Electrospray Ionisation
FIA	Flow Injection Analysis
FE	Forced Exercise
FQM	Feature Quantification Matrix
FDR	False Discovery Rate
GC	Gas Chromatography
HCA	Hierarchical Clustering Analysis
HILIC	Hydrophilic Interaction Chromatography
HPLC	High Performance Liquid Chromatograph
LC	Liquid Chromatography
MS	Mass Spectrometry
m/z	Mass-to-charge
MRS	Magnetic Resonance Spectroscopy
N	Nitrogen
NMR	Nuclear Magnetic Resonance
O	Oxygen
O-PLS-DA	Orthogonal Partial Least Square Discriminant Analysis

PCA	Principal Component Analysis
PLS	Partial Least Square
PLS-DA	Partial Least Square Discriminant Analysis
ROC	Receiver Operating Characteristic
SF	Sleep Fragmentation
SI	Sleep Interruption
SOM	Self-Organising Maps
SR	Sleep Restriction
TOF	Time of Flight
TSD	Total Sleep Deprivation
UHPLC	Ultra-High Performance Liquid Chromatography

Abstract

The effects of fatigue on cognition has been extensively studied however the biochemical mechanisms are still not well understood. With the advancements of the various "-omics" sciences, understanding the biochemical effects of fatigue has become an increasingly popular area of research. Of all the "-omics" platforms, metabolomics has risen to show great applicability in many applications of biomedical research, particularly with biomarker discovery. This paper has reviewed the current metabolomic strategies for biomarker discovery and developments in fatigue biomarker research. This highlighted gaps in the literature; studies in this field are dominated by analysis on male subjects therefore there is a lack of studies that investigate the effects of fatigue on females. There is also a lack of research on the analysis of urine with NMR spectroscopy, both of which show great potential for the development of biomarkers. This instigated the development of a study to address the gaps in the literature to enhance the knowledge based surrounding the metabolomic effects of sleep deprivation on cognitive function.

1. Introduction

The reliance of 24 hour operations in a number of industries such as health care, defence and transportation has led to fatigue as a result of insufficient sleep being one of the most widespread concerns in modern day society[1]. It is well noted that the quantity and quality of sleep are key aspects in the maintenance of healthy physical and cognitive function[2,3]. Without adequate sleep, cognitive performance indicators such as focus, reaction times and alertness can be severely impaired[2,3]. This can increase the risk of physical injury, leading to devastating consequences in workplaces and on the road. A recent study by Welehan et al[4]., concluded that sleep deprivation negatively impacted the technical skill performance of surgeons. They reported a decline of up to 32% in the performance of surgeons in simulated surgeries, which is concerning as it can have serious clinical implications for patient safety[4]. Similarly, the Australian Defence Force (ADF) also stated that fatigue was one of the leading contributors to serious accidents that occurred amongst their officers[5]. This can lead to a diminished efficiency of operations and can put the safety of personnel and civilians at jeopardy.

Australia's large size and underdeveloped rail system has resulted in a heavy reliance on road transport, consequently fatigue and road safety is a particularly strong concern[6]. Studies have shown that 20% of all traffic accidents can be attributed to impaired cognitive performance of the driver due to fatigue[7]. Alarmingly, alongside drink driving and speeding, reduced cognitive performance as a result of fatigue has been reported as one of the largest contributors to deaths on Australian roads[8]. Fatigue-related performance impairment has been compared to that of impairments experienced when under the influence of various blood alcohol content's (BAC), with fatigue induced by 28 hours of sleep deprivation equating to a BAC of 0.1%[9]. Unlike BAC though, there is no universal method of accurately quantifying therefore it is difficult to regulate or monitor.

The demand for a method of predicting impaired cognitive performance as a way of assessing 'fitness for duty', both as a roadside screening tool and in the workplace, could be addressed via metabolomics. The premise of metabolomics is based on the assumption that a stimulus – in this instance fatigue as a result of insufficient sleep – will disturb normal metabolism and induce a measurable change in the metabolic signature of an individual[10]. By comparing cohorts of healthy samples with fatigued samples, discriminatory metabolites that act as a quantitative "biomarker" of fatigue can be identified. These biomarkers can then be used to understand and monitor the biochemical pathways associated with fatigue, in order to predict impaired cognitive performance as a result of fatigue.

This paper will review the current metabolomic strategies for biomarker development and their applications to studies of fatigue as a result of insufficient sleep, mainly including the terminology of metabolomics,

metabolomics workflow, current developments and limitations. It is also important to note that fatigue can manifest both mentally and physically, therefore, there are many definitions of fatigue in the literature. Physical fatigue is likened to muscle fatigue, whereas mental fatigue is synonymous with impaired mental performance[1]. This review will adopt Lal and Craig's[1] definition of fatigue as a "transitory period between wake and sleep and if uninterrupted, can lead to sleep", and therefore all further mention of fatigue will refer to mental fatigue. For the purpose of this review, all subcategories of sleep impairment such as "sleep deprivation", "sleep restriction" and "sleep interruption" will be included in the definition of insufficient sleep.

2. Why metabolomics for fatigue biomarker discovery?

The decoding of the human genome in the 1990's revolutionised the way biomedical research was conducted[10,11]. Rather than focusing on the structure and function of a particular gene, transcript, protein or metabolite, research was directed at mapping the simultaneous changes of all molecular entities and reintegrating them to elucidate how they interact in the context of a living system[10,11]. From this emerged the field of systems biology[10,12], which is defined as the "integrated system of genetics, protein, metabolite, cellular and pathway events that are in flux and interdependent"[13]. The study of each of the platforms developed into their own independent fields and introduced the various "omics" sciences; genomics, transcriptomics, proteomics and metabolomics[10,12,14]. Each of these platforms are explored in many areas of biomedical research, such as drug discovery, disease diagnosis and biomarker discovery[15–17]. Metabolomics appears to be the platform of choice in the field of biomarker studies, because even though metabolomics sits at the bottom of the systems biology hierarchy, it has a number of advantages over the other "-omics" platforms[10,17].

Firstly, information on what may happen in an organism can be gathered from genomics, transcriptomics and proteomics, however it is not always conclusive[15]. With genomics the possibility of a phenotype may be predicted, however, mutations at the genome level don't always translate to the phenotype and they don't account for environmental or epigenetic influences[15,18]. The post-translational modification of proteins and differential splicing of transcripts results in the proteome and transcriptome not being a true reflection of the current biological state, making proteomics and transcriptomics much more complex to work with[10,17]. Metabolomics is the only platform that can provide a definitive answer as it can accurately capture a "snapshot" of the current state of an organism[10,11]. This is very useful in biomarker research because it is assumed that various stimuli, such as fatigue, will disrupt normal functioning of biochemical pathways, resulting in a characteristic "metabolic fingerprint" as the outcome[10,19]. Studies have shown that the metabolic phenotype of healthy individuals are consistent[20], which allows for the discrimination of a fatigued metabolic phenotype from a healthy one with confidence in the validity of the comparison.

Secondly, metabolites are the terminal molecules of all the processes in the central dogma of molecular biology, therefore making the metabolome the most proximal system to the phenotype[10,21]. Metabolites are small enough to quickly and easily distribute all around the body and are present in all biofluids, unlike nucleic acids or large proteins which are not normally present due to their size[10,22,23].Their presence in biofluids, such as urine, plasma and saliva, also allows for the easy transition from metabolomic studies to use in clinical practice[10,22,24–26], as they can be collected easily with minimal sample preparation unlike other biological samples such as tissues which needs extensive preparation prior to analysis[10,27]. There are also fewer numbers of metabolites in comparison to the vast numbers of genes, transcripts and proteins[10,24,25], increasing the likelihood of detecting changes as a result of fatigue.

Finally, a metabolic response to stimuli are prompt and will occur within seconds[28], in contrast to proteins which may take days to show a response or even no response at all in the instance of nucleic acids.[22] Consequently, the metabolome will indicate the current biological state, which is important in the investigation of biomarkers as the response to stimuli can be measured directly[17]. The highly dynamic nature of the metabolome and its sensitivity to a multitude of endogenous and exogenous factors make the metabolome unique in its ability to capture even the most subtle of changes[10,19]. Even if molecular changes within the other "-omics" platforms are not observed, changes in metabolites indicative of fatigue can be present in biofluids long before physical symptoms present[14]. Although there are clear advantages in the use of metabolomics for biomarker discovery, there are still limitations that need to be addressed, which will be discussed later. These limitations are directly related to the various steps in the metabolomics workflow, such as with the consistency of sample collection and preparation, choice of analytical platform and data analysis, and can have detrimental impact on the accuracy and validity of the results.

3. Terminology

Put simply, the field of metabolomics investigates all metabolites (identification and quantification) present in a biological system[10,14]. There are many technical definitions, which are often incorrectly used interchangeably as they differ greatly with respects to the analytical approach[14,22,29,30]. These approaches consist of two groups and are categorised according to whether the experimental design will allow for the investigation of a specific metabolite (targeted metabolomics) or the analysis of as many metabolites as possible (untargeted metabolomics)[10,16,17].

There are many reoccurring definitions that appear in the literature[14,31], including: (1) metabolite target analysis, (2) metabolite profiling, (3) metabolic fingerprinting, (4) metabonomics, (5) metabolomics, (6) metabolic footprinting.

1. **Metabolomics** is the most widely used definition[10,22,27,31–33]. It is defined as the unbiased global identification and quantification of all metabolites in a biological system[10,31,32,34]. With this approach, the metabolic phenotype of the biological system under investigation, or "metabotype" can be ascertained[10,33].
2. **Metabonomics** is a method that was pioneered in 1999 by Professor Jeremy Nicholson[19]. It is defined as the quantitative measurement of the metabolic response of a biological system to pathophysiological changes, genetic modification or external stimuli, such as drugs or toxins[10,19,31,35,36]. There is a lot of debate on what the exact differences between metabolomics and metabonomics are, however there is growing unanimity in that metabolomics focuses on endogenous, cellular metabolism[14,29,31]. Metabonomics on the other hand extends the definition to include information gathered as a result of disturbances of metabolism by exogenous influences such as diet or disease[19,27,31,36].
3. **Metabolic fingerprinting** involves screening for differences in the metabolic "signatures" of a biological sample and a larger sample population[10,14,31,35]. Once the differences in the signatures are detected, the metabolites and their biochemical pathways can be determined to understand their biological relevance[10,14]. This method requires minimal sample preparation and is considered a "semi-quantitative" analysis as the identification and quantification of each individual metabolite is not required[10,28,30].
4. **Metabolite target analysis** is a method that is used to quantitively identify a target metabolite, or a small group of metabolites that are related to a specific biochemical pathway, in a biological sample[10,14,32,35]. This method relies on extensive sample preparation and separation to isolate the metabolite in question prior to detection on highly sensitive equipment[31].
5. **Metabolite profiling** is very similar to metabolite target analysis, however instead of looking at a few metabolites, it aims to analyse a larger number of metabolites that are related through their metabolic pathways[10,14,30,31].

When investigating for novel biomarkers, it can be futile to adopt targeted approaches as they only provide a very narrow study window. Rather than looking at a small number of specific metabolites, it would be more efficient to simultaneously map a large number of metabolites[10,13]. This opens up opportunities to investigate biochemical pathways that may have previously been unexplored and maximises the likelihood of detecting metabolites that are up or downregulated during different physiological states, such as when one is fatigued[10,37]. This is supported by Schrimpe-Rutledge et al.[38], who described untargeted methods as "hypothesis generating" approaches that are ideal for the discovery of new metabolites, whereas targeted methods are "hypothesis driven" and more applicable for validating the discoveries of untargeted approaches.

As this paper aims to review the current methods for developing biomarkers for fatigue, it will therefore focus on the metabolomics workflow of an untargeted metabolomics experiment, but will later discuss the findings of all metabolomic experiments that have investigated metabolites of fatigue.

4. Metabolomics workflow

As mentioned earlier, the principle of using metabolomics in the search for a fatigue biomarker is based on the assumption that a stimulus, such as sleep deprivation, will disturb normal physiological metabolism and induce a measurable change in the metabolome[10,19]. To achieve this aim, the design of the metabolomics experiment must be carefully thought-out and executed to guarantee results that are reproducible[10]. This is done by selecting an analytical technique that will provide the most coverage of the metabolome and then analysing and interpreting the data with appropriate chemometrics and bioinformatics[10,11,14,16,18]. The fundamental goal is to identify metabolites that can discriminate between fatigue and non-fatigued samples that can undergo validation studies to assess their potential as a biomarker[10,17].

At the moment, there is no sole analytical method that can achieve a complete assessment of the entire metabolome[10,11,31]. Due to the fact that metabolites are highly heterogeneous in their chemical structures, properties and concentrations, the process of analysis always has a degree of bias[10,17]. This is a result of the metabolite selection, and occurs all the way from extraction through to detection[10,17]. To reduce the effects of bias, certain measures in the collection, handling, extracting, storing and preparation of samples must be employed[34,39]. Furthermore, appropriate methods of obtaining, normalising and interpreting of data are required to report accurate findings[10,32]. The optimisation of sample preparation protocols have recently gained a lot of interest and, in combination with developments in analytical approaches, the coverage of the metabolome has grown greatly[10,25,37]. Hence, to ensure that metabolites are optimally collected, extracted, detected, identified and analysed there are different steps that must be adhered to in a metabolomic experiment[10,40,41]. These steps, also known as the metabolomics workflow, comprise of six key steps: (1) study design, (2) sample collection and preparation, (3) metabolites separation and detection, (4) data mining and extraction, (5) data analysis and interpretation and (6) biomarker validation [10,40,41]**,** that will be described below.

4.1 Study Design

Due to the nature of the metabolome being highly sensitive to various endogenous (i.e., age and sex) and exogenous (i.e. diet, lifestyle, medication) factors, careful study design is crucial to the success of a metabolomics study investigating fatigue biomarkers[10,42]. Each of these factors have the potential of biasing the results of a study by introducing variance and complicating the interpretation of experimental data[10,42]. The type of study subjects (i.e. males vs females, humans vs rodents), the cohort size and the number of

controls are some crucial factors take into consideration to ensure statistical significance when associating metabolites to a phenotype[10,42]. Data on the subjects pertaining to their medical history and current medication, a dietary log and environmental exposure should also be collected[10,42]. To minimise the effects of dietary variation, investigators often provide the test subjects with strict diets or fasting schedules during study periods[10,42]. The consistency of sampling procedures, time between sample preparation, processing and storage also needs to be carefully reviewed as some analytes can be at risk of degradation (i.e. bacterial growth or on-going biochemical reactions) [10,42].

4.2 Sample Collection and Preparation

By definition, the aim of an untargeted metabolomics study is to assess all metabolites in a biological sample, therefore an ideal experiment would have no sample preparation prior to analysis to minimise the risk of contaminating the sample with foreign metabolites[10,17,31]. However, this is not practicable and appropriate sample collection, handling and preparation measures must be enforced to ensure a consistent and unbiased comparisons of metabolites between samples[10,17,31]. In fact, rather than compromising the metabolome, contamination risks are actually reduced through optimised sample handling and storage protocols that protect the sample from the appearance of unrelated metabolites (e.g. bacteria)[24] or degradation due to ongoing biochemical reactions[37]. As mentioned previously, metabolomic analysis can be achieved with a number of different biological samples[10]. In human fatigue metabolite studies, a large majority were conducted on biofluids such as urine, plasma and serum[25,43–53]. Plasma has the advantage of providing an instantaneous snapshot of the current metabolic state of all anabolic and catabolic processes occurring in the organism at the time of collection[54]. Urine, on the other hand, only enables the investigator to assess the metabolites that are excreted as a by-product of catabolic processes[54]. The advantages of using urine in metabolomic studies however, is that it can be obtained easily, in large amounts and with non-invasive procedures, which is especially useful when repeated sampling is required[10,54] . It also has the added benefit of less sample preparation as it contains minimal proteins[55]. Blood samples on the other hand must be pre-processed to remove all large proteins as they can mask smaller metabolites during analysis[55].

The collection of various samples require different considerations, especially biofluids such as urine and blood that naturally have diurnal variation[10,23,56]. Diurnal variation refers to the cyclic variations that occur according to the time of the day as a result of circadian rhythm, or our “sleep-wake” cycle[56]. This needs to be taken into consideration when collecting samples for fatigue biomarker discovery to ensure that any variation observed in metabolites is due to sleep deprivation and not as a result of normal circadian rhythm. A number of studies were also conducted on tissues such as liver and brain tissue, however these are almost exclusively done on rodents[57–62]. The following will highlight the collection methods of samples collected for fatigue metabolomic studies.

4.2.1 Urine

The importance of urine preparation and storage conditions was highlighted in a study that showed significant changes in the metabolome of raw urine due to bacterial contamination[63]. It was concluded that to prevent bacterial growth, preservatives such as sodium azide must be added to the samples[63]. There are a number of concerns when using sodium azide, however, namely that it can have a potential impact on unknown metabolites and that it is a toxic compound[54]. Safer options, such as filtration systems, are also available to prevent bacterial contamination. A recent study comparing the efficiency of 0.2-um filtration with sodium azide concluded that filtration was in fact a far more superior method for preventing bacterial growth[63].

In addition to this, some studies believe that quenching metabolic processes in urine at low temperatures is a necessary step to stop all chemical activity in the sample. Van de Merbel et al.[64], reported that when urine was kept at ambient temperatures, metabolites that were easily oxidised were prone to depletion. They also observed that conversion of metabolites, such as cytidine into uridine, continued at a rapid rate after sample collection. This, however, is contraindicated in a study by Dunn et al.[65], who compared the stability of urine samples that were immediately frozen at -80°C with samples that were stored at 4°C for 24 hours before being frozen at -80°. Interestingly, they reported no statistical significance in the metabolic profiles of the samples under the two different storage conditions, indicating that quenching may be an unnecessary step in the preparation and storage of urine.

4.2.2 Plasma

The sampling and storage conditions of plasma is similar to urine in respects to storage temperatures, however it does require additional steps such as addition of anticoagulants and protein precipitation[27,55]. There are a number of anticoagulants that are used, such as EDTA, heparin and citrate, and are chosen according to downstream analytical processes. When using NMR, the use of heparin is recommended as EDTA and citrate are known to produce interfering signals in the spectra[27]. Protein precipitation is crucial in the preparation of plasma for metabolomic analysis due to the high number of large proteins that can obscure small metabolites. Of all the different organic solvents, acids and salts that can be used, acetonitrile, trichloroacetic acid and zine sulfate were found to be the most effective protein precipitants[66].

4.2.3 Tissue

Unlike urine, there is no debate on whether quenching is a necessary step in the processing of tissue samples. When collecting tissue samples, the sampling procedure can initiate a “stress” response in their metabolites, therefore samples must immediately be quenched to halt the metabolism in that tissue[10,67]. The most common method of quenching is “flash freezing” with liquid nitrogen or a method called “freeze clamping”, which involves the use of containers that are of a low temperature[10,67]. Acid treatments for the precipitation of

proteins, such as perchloric acid and nitric acid, can also be used but must be carefully controlled as metabolites can become unstable at extreme pH levels[32].

4.3 Separation and Detection

There are many analytical methods that are used in metabolomic studies, each having their own advantages and disadvantages, particularly when it comes to sensitivity, reproducibility and cost effectiveness[10,25]. Therefore, when choosing an analytical platform, one must consider sample types, available resources and the purpose of the analysis. According to the literature, the two most commonly used analytical approaches are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy[10,13,16,28,36,40]. The pioneering metabolomics experiments were NMR spectroscopy based[19], however, in more recent years there has been a switch to opting for MS-based approaches over NMR[17]. As neither of these methods alone can provide a truly comprehensive assessment of the entire metabolome, it is not uncommon to see studies incorporating the use of both of these techniques in parallel or one preceding the other. Other spectroscopy methods such as Raman or infrared can also be used, however these are much less common[31].

4.3.1 NMR Spectroscopy

This technique is based on the interaction of atomic nuclei with radio-waves in the presence of a powerful magnet which allows for the identification of an atom and its relative location within a molecule[10,68–70]. When an atomic nuclei in a low energy state is exposed to radiofrequency pulses, under the influence of a strong magnetic field, the energy is absorbed and the atomic nuclei transitions to a high energy state[10,68–70]. Once the radiofrequency pulse is removed, the atomic nuclei returns to its original low energy state as it loses the energy that it had absorbed[10,68–70]. The change in energy states will produce a pattern of resonance peaks, called a spectral output or NMR spectrum, which are unique to each molecule[10,19]. To obtain the NMR spectrum of an entire sample, the spectra of all metabolites must be simultaneously evaluated and then superimposed[10,19].

There are many advantages to using NMR spectroscopy in metabolomics, namely that it is a highly reproducible technique (typically >98%)[25] that can quantitatively analyse metabolites in a sample[10]. It involves minimal sample preparation because unlike MS approaches, separation of molecules prior to analysis is not required, allowing for the integrity of the sample to be preserved[71]. Furthermore, NMR structural information obtained from the NMR spectrum can be used to identify unknown metabolites, which is especially useful when investigating novel biomarkers[71]. Additionally, NMR is non-discriminative in that it can detect all compounds that have a resonating atomic nuclei, most commonly hydrogen (^{1}H-NMR) however ^{13}C, ^{15}N and ^{17}O can also be used[10,72]. C-NMR spectroscopy provides greater spectral variety in comparison to ^{1}H-NMR, however it is not as commonly used as ^{1}H-NMR as it has limited sensitivity due to the naturally lower abundance of ^{13}C nuclei (1.1%) compared to ^{1}H nuclei (99.9%)[10].

There are a number of downsides to using NMR in metabolomic research. Firstly, the overall sensitivity of NMR in comparison to MS-based approaches is much lower, which can result in important metabolites being overlooked[68–70]. Larger sample volumes are required as the ability of NMR to detect metabolites of a low abundance is weak, often requiring much more expensive instrumentation to do so[27,36,68]. Another disadvantage of using H-NMR is that it is susceptible to spectral interference from hydrogen molecules present in water[73]. This can make the interpretation of biofluids that contain a high concentration of water, such as urine, very difficult. It can also be difficult to interpret the spectra of biofluids due to the natural variation in metabolite concentrations. Potential biomarkers that are present in trace amounts can be missed during analysis, as the presence of molecules in very high concentrations can often obscure molecules that are present in lower concentrations[73].

Magnetic resonance spectroscopy, or MRS, is a technique that couples the foundations of NMR with MRI, to allow the quantification of metabolites within tissue samples[73]. It is completely non-invasive and does not require any pre-collection of samples, hence it can be used to analyse metabolites *in vivo*[74]. This has successfully been used in a number of fatigue metabolite studies investigating the effects of sleep deprivation on metabolites of the brain[57,58]. As with NMR, there are a number of limitations to MRS. Magnetic susceptibility of adjacent tissues, such as bone next to brain tissue, can be a major issue as the difference in susceptibility can cause interference with the MRS analysis[74]. Availability of instrumentation can be quite limited as it is very expensive and requires specialised knowledge to operate and interpret the spectra[74].

4.3.2 Mass Spectrometry

Mass Spectrometry is a highly sensitive and specific analytical tool that is used to measure the mass-to-charge (m/z) of molecules in a sample[10,14,28,36]. The high sensitivity of mass spectrometry enables a greater coverage of the metabolome as it can detect metabolites in the nM to pM range[10,14,28,36]. In order to do so, however, these molecules must first be ionised and converted to gas-phase ions[10,14,28,36]. These ions are then sorted according to both their charge and their mass[10,14,28,36]. These separated ions then reach a detector in order of increasing mass, as the lighter ions travel quicker, and are measured and displayed as a mass spectrum[10,14,28,36]. The mass spectrum depicts the mass-to-charge ratios of each ion as a peak plotted against their relative abundance, or intensity. Hence, each mass spectrum will be a unique pattern of peaks specific to the molecule, which is why it is so useful in the identification of metabolites[10,14,28,36]..

Typically, mass spectroscopy is used to identify metabolites of a known structure, by comparing the mass spectrum obtained from the analysis of a sample with those in a standardised library or database[75,76]. Mass spectra can often provide overwhelmingly large data sets of high complexity, so to reduce this MS is commonly coupled with various chromatographic techniques, such as liquid chromatography (LC) or gas chromatography

(GC)[75,76]. This combination of chromatographic separation prior to mass spectrometry analysis hugely impacts the resolution, sensitivity and selectivity of mass spectrometry instruments and can even allow for the identification of isobaric metabolites that are of the same mass but different molecular constituents[10,75,76]. Chromatographic separation also provide additional information on various physiochemical properties by measuring the retention time of the metabolites and offer greater separation of mixtures that are complex in nature[10,75,76].

The physiochemical properties of metabolites, along with the source and mechanism of ionisation all determine the efficiency of ionisation. There are many ionisation mechanisms that have been used in MS based metabolomics, all of which were selected according to what chromatographic separation was used prior[10]. GC-MS is often paired with electron-impact (EI) or chemical ionisation (CI) mechanisms. EI ionisation is very sensitive and powerful mechanism that creates unique fragmentation patterns, however this often results in the molecular ion being lost from the spectrum[10,77]. This, in combination with its limited mass detection range, can limit the capacity of maximum metabolome coverage and hinders metabolite identification[10,77]. In contrast to this, CI mechanisms are much less energetic, resulting in ions that are more stable and the molecular ion present in the spectrum[10,77]. LC-MS on the other hand is commonly combined with electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI). APCI is often preferred as it not as affected by the ionisation suppression and enhancement effects that is observed in ESI mechanisms[10,77]. The advantages and limitations of each of these ionisation mechanisms highlights the importance of study design to achieve the best experimental outcomes.

There are clear advantages to using mass spectrometry in metabolomics, however MS does not come without its downsides. The most obvious drawback is the need for extensive sample preparation prior to analysis, which can change the metabolic composition of a sample and therefore create a data set that is not truly reflective of the original sample metabolome[10,22,24]. Unlike NMR spectroscopy, which can quickly identify and quantify metabolites, quantification isn't a feature of mass spectrometry and processing times can be quite long (hours vs minutes when using NMR)[10,24]. Furthermore, even though NMR has very high instrumentation costs, the cost per sample of MS analysis is considerably higher than NMR[10,75].

4.4 Data Pre-Processing

Data pre-processing is a crucial step in the metabolomics workflow and can significantly impact the statistical analyses and final interpretation of data[10,78]. Due to the nature of the untargeted metabolomics studies, the volume of raw data obtained is astronomical and often complicated by experimental drifts in the instrumentation[10,78]. This means that all spectra, whether it be obtained by NMR or MS, must be corrected for any variation that appears as a result of experimental variables to reduce any possible bias[10,38]. There are many

steps that are applied, namely: (1) baseline correction, (2) feature detection, (3) peak detection, (4) spectral alignment, (5) feature normalisation and (6) deconvolution[78].

1. **Baseline Correction** is used to eradicate differences in samples that are due to experimental or instrumental variation and to eliminate any low frequency artefacts. This is often paired with high-frequency filters to remove any electronic noise generated by the instrumentation[78].
2. **Feature Detection** is used to quantitively identify the features present on a spectrum[78]. MS based approaches tend to quantify metabolites by using a 'peak-base' method to detect peaks across a spectrum and combine their areas[79–81]. This is used in combination with spectral alignment, either before or after detection[78]. With NMR based approaches on the other hand, 'binning-based' methods are more widely used to detect peaks of interest in complex samples[78]. However, in comparison to peak-based methods, they perform quite poorly[82]. This is especially true when there is substantial misalignment in the spectra or when different metabolites have overlapping peaks[82]. Peak overlap makes interpretation of spectra very difficult, and spectral 'deconvolution' methods have been implemented to address this problem[83,84]
3. **Peak Detection** is achieved by using filters to smoothen the spectra and detection thresholds to identify individual peaks[85–87]. Parameters such as peak heights, areas and signal to noise ratios are all subject to these thresholds[87]. When studies involve large sample numbers, frequency filters such as consensus peak signal filters can also be applied to only select peaks that are present in a low frequency[78].
4. **Spectral Alignment** is one of the most crucial pre-processing steps in metabolomics, particularly with studies that involve multiple samples[78]. When looking at the spectra of multiple samples, non-linear shifts can significantly affect the position of peaks that belong to the same metabolite[78]. In MS-based studies, peak shifts along the retention time axis are observed when there is a change in the stationary phase of a chromatographic column[88]. Whereas in NMR-based studies, peak shifts are caused by discrepancies in the samples chemical environment, such as differences in pH, and are observed on the ppm axis[89]. Spectral alignment methods fall into two categories, warping and segmenting[78]. Warping methods of spectral alignment maximise the correlation between multiple spectra by elongating or shortening segments of the spectra along the ppm (NMR) or retention time (MS) axes[78,90,91]. On the other hand, spectral segmenting methods align multiple spectra by applying a constant shift to all of the peaks[78,90,91]. This is achieved by either aligning the entire spectra or segmenting the spectra into smaller sections and aligning each section independently[78,90,91].
5. **Feature Normalisation** is a key step in removing experimental biases to leave only biologically relevant discriminatory features[78]. This is especially important when investigating complex biological samples such as blood and urine, where the use of internal standards is difficult and differences in metabolite

concentrations between samples are large[78]. To address this there are two commonly used methods, the use of total spectral area (area under the curve) and the incorporation of endogenous metabolites, such as creatinine in urine[78,89,92,93].

6. **Deconvolution** as mentioned earlier, one of the key pitfalls of metabolite quantification using NMR or MS is the overlap of peaks belonging to different metabolites[78]. This method allows the investigator to identify an individual metabolite from a group of overlapping peaks by fitting the sample spectra to a template[78,94,95]. The key downside of this method is that they are unable to identify peaks belonging to metabolites that have not been characterised before because there would not be a reference spectra to fit the sample with[78].

4.5 Data Analysis

Once the raw data is pre-processed, the resulting prepared data is statistically analysed to obtain group clusters (for example, healthy versus fatigued) to identify the discriminatory characteristics of the samples' subsequent metabolic signatures[10,78]. The most effective way to easily observe discriminative patterns between groups is by using chemometric methods such as univariate and multivariate statistics[78,96]. Following pre-processing, the metabolomics data is often presented as a table called a feature quantification matrix, or FQM[10,78]. The samples are presented in the rows and the metabolic features are presented in the columns, with each features relating to the concentration of a particular metabolite. The input metabolomic features, for data analysis is dependent on the upstream analytical and pre-process techniques[10,78]:

- Spectral peak areas: For both MS and NMR metabolomics data, spectral peak areas is one of the most commonly used metabolomic feature. These peaks are used to identify the metabolite that it corresponds to provide a biologically relevant meaning to the results[78].
- Metabolite concentrations: Metabolites are identified to obtained concentrations to be used to data analysis. This feature is useful to not only provide biological meaning, but also minimises redundancy of peak areas when one metabolite is represented by a number of peaks[78].
- Spectral bin areas: This feature is more common in NMR studies and is often used in addition to peak areas and metabolite concentrations. This is achieved by segmenting the spectra into evenly spaced segments that are later recombined to obtain the matching bin area. To prevent issues such as peaks falling into two neighbouring bins, some methods have adopted algorithms that divide bins unevenly. These algorithms don't come without their downsides, they often include features that are not informative, such as areas with no peaks, and don't perform well with spectra that is significantly misaligned[78].

4.5.1 Univariate Analysis

Univariate methods are common statistical approaches used to analyse each metabolomic feature independently[10,78]. There many methods available and are selected according to the statistical properties of the distribution of metabolomic features and whether they fit assumptions of normality[78,97]. For example, when comparing the differences between two groups, parametric tests such as Students t-test or ANOVA can be used if the data obtained is normally distributed. If normality of the data cannot be assumed, then non-parametric tests such as Mann-Whitney U test or Kruskal-Wallis analyses can be used instead[78,97].

Additionally, when selecting a suitable statistical test for the analysis of metabolomic data, attention must be drawn to the issue that arises with testing multiple features at the same time[78,97]. As most untargeted metabolomics studies simultaneously analyse numerous metabolomic features, there is a high probability of reporting a false metabolomic correlation (i.e., a false positive) just by chance[78,97]. To address this, various correction methods, such as the Bonferroni approach, have been developed to prevent false positives, but this is often done at the expense of increasing the probability of discarding true metabolomic correlations (i.e., false negatives) [78,97]. Rather than looking at minimising the probability of at least one false positive, there are other approaches available that are less conservative and focus on reducing the false-discovery rates (FDR) of metabolomic features[78,97]. These FDR-based methods work by minimizing the expected amount of false positives within the total number of reported positives, which is a much more applicable approach for untargeted metabolomics studies that investigate a large number of metabolites[78,97].

Univariate statistical approaches are easy to conduct and interpret, however the main draw back with this method is that they don't account for any potential interactions between the metabolic features[78,97]. This can be unfeasible for metabolomics studies as the entire nature of metabolomics is based on the correlations between features from the same metabolite and the interactions of metabolites belonging to the same biochemical pathway[78,97]. Studies have also shown that solely using univariate methods can increase the chance of falsely reporting positive or negative results[78,97]. This is because they also do not take into consideration the effects of confounding variables such as gender or age[78,97]. For these reasons, multivariate analytical techniques are often the analytical platform of choice for metabolomic studies[78,97].

4.5.2 Multivariate Analysis

Unlike univariate statistical methods, multivariate analytical approaches simultaneously account for all of the metabolomic features of a sample and therefore can detect patterns of relationship between the features[78,97]. These pattern-recognition approaches comprise of two main categories: unsupervised and supervised[78,97]. The main difference between these two techniques is that provision of knowledge on the sample class[78,97]. Unsupervised methods discriminate between the metabolic composition of samples without any prior

knowledge of the class or type of the samples. In supervised methods however, clusters are generated with the use of sample labels[78,97]. These labels are used to classify a metabolomic feature, or combination of features, that is related to a particular phenotype.

4.5.2.1 Unsupervised Methods

Unsupervised approaches are commonly used to condense and simplify the complicated data that arises from metabolomic studies[78]. They are particularly useful for quickly identifying any outliers and similarities or differences between samples (in this case, fatigue-related patterns). The most commonly used unsupervised approach in the metabolomics community is principal component analysis, or PCA[98,99]. This method involves reducing the dimensionality of metabolomics data by identifying patterns in a large data set and transforming it into a smaller one that is still representative of the information of the original data set[98,99]. By reducing the number of variables in a data set, the accuracy of the analysis is also reduced. This is a commonly accepted trade-off for the simplicity of visualising and interpreting the smaller data sets, or 'principal components'[98,99]. PCA can be applied to identify hidden biases or sample outliers, it is also frequently used to assess the quality of data[98,99]. A number of studies investigated how technical variation can impact the analysis of metabolic phenotypes and did so by using PCA analysis[98,99].

Hierarchical clustering analysis (HCA) and self-organising maps (SOMs), are two other unsupervised methods that are frequently used in metabolomics[78,100]. These methods are especially appropriate for the detection of non-linear trends in data that can't easily be covered with PCA[78,100]. SOMs are commonly used to envisage metabolic phenotypes and patterns whilst giving priority to metabolites that are of interest based on their likeness in a form of a heat map[78,100]. HCA on the other hand is a visualisation tool that provides hierarchical clustering information as a dendrogram[78,100].

4.5.2.2 Supervised Methods

Supervised approaches are a useful way of identifying metabolic patterns that are associated with the phenotype of interest while reducing the influence of other variances[78]. The most commonly used supervised method in metabolomics is partial least square (PLS)[101]. In contrast to PCA, PLS components maximise the covariance between the variable of interest and the rest of the dataset[101]. This allows PLS components to portray how much a particular feature contributes to the discrimination of various sample cohorts[101]. The downside of PLS is that it can be easily influenced by metabolic features that don't correlate with the phenotype of interest[101]. To address this, orthogonal PLS (O-PLS) was developed, which categorises the data variance into two groups: a first group which correlates with the phenotype of interest and a second, orthogonal group, which does not correlate [101,102]. Metabolites are commonly classified by fitting PLS-DA and O-PLS-DA, the discriminant versions of these analyses[101,102]. There has been extensive comparisons of the

performance of both PLS and O-PLS models, yet there is no consensus on which is the superior method. In recent years, however, the metabolomics community has shifted to using O-PLS models over PLS models[101,102].

4.6 Metabolite Identification and Spectral Databases

Once the discriminatory metabolic signatures have been determined, the metabolites must then be identified by comparing experimental spectra with spectral databases[10]. There are a number of databases available for metabolite identification, however the most commonly used databases are the Human Metabolome Data (HMDB)[12], METLIN[103], SetupX[104] and the Kyoto Encyclopedia of genes and genomes (KEGG)[105]. These databases can provide a range of metabolomics information; specific to certain species (HMDB), to chemical structure and properties (METLIN) and even information about experimental workflows (SetupX)[10]. After the metabolite is identified, their biological significance needs to be determined with pathway analysis which can be done with the use of pathway databases such as KEGG[10]. Next, novel biomarkers are identified using supervised analysis models as they have the ability to summarise the quantification of multiple metabolites[78]. To transition from research to clinical applications, the validity and efficiency of the final classification models must be evaluated[78]. This is achieved via performance assessment and model validation[78].

4.7 Performance Assessment

Performance assessment analyses how well the real metabolomic outcome matches the outcome that was predicted by the model[78]. This is achieved by a number of corresponding measures, namely: predictive accuracy (percentage of subjects that were correctly classified), sensitivity (percentage of correctly classified true positives) and specificity (percentage of correctly classified true negatives)[78]. With a set limit for the decision boundary, the performance of the classifier can be assessed by these three measures[78]. This can introduce bias to the analysis as the choice of decision boundary and outcome prevalence can have an influence on the performance measures[102]. This can be avoided with the use of a receiver operating characteristic (ROC) curve, which is why it the most used method of performance assessment in metabolomics[102]. To predict how well a classification model will perform when fitted to other samples, a validation step is required when designing a classification model[78]. This is especially important when working with small sample cohorts to prevent overfitting of the model[78]. This can be achieved with permutation testing, which aims to determine the performance of a prediction model by calculating the probability of observing an equal or better performance purely by chance[106]. This is accomplished by estimating the 'area under the curve' (AUC) or null distribution of the performance measures under the assumption that the sample cohorts display no differences[106]. This is performed by randomly swapping the sample cohorts multiple times and determining the statistics under each swapped dataset[106]. To be considered statistically significant, the performance measures of the sample model must lie outside the confidence internals of the estimated null distributions (e.g., 95 or 99%)[106].

5. Potential fatigue biomarkers revealed using metabolomics

As mentioned previously, the metabolome is a highly dynamic system that has fluctuating rhythms of metabolites[10]. This is especially true for fatigue metabolites as the regulation of the sleep-wake cycle is a text book example of circadian rhythms in the metabolome[107]. This can be both an advantage and a challenge for developing fatigue biomarkers. The following section will discuss some of the key findings of total sleep deprivation (TSD) and sleep restriction (SR) metabolomics studies in both human and rodent models, which will also be summarised in table 1. It will also highlight the studies that are focusing on fatigue metabolites alone, or both fatigue and circadian metabolites.

5.1 Human studies

When compared to control cohorts, differences in the overall metabolome of sleep deprived urine and plasma samples have been observed. Giskeødegård et al.[43], reported 16 discriminatory metabolites (8 increased, 8 decreased) in the urinary metabolome of male subjects after 24 hours of TSD. Similarly, Davies et al.[108], subjected male volunteers to 24 hours TSD and reported an increase in 27 of the 171 serum metabolites identified. They also reported that more than half of the identified metabolites retained their circadian rhythmicity, however they were all reduced in amplitude as a result of forced wakefulness. Of the 27 metabolites that increased, the majority consisted of species of carnitines and glycerophospholipids. In a different study[49], however, these same metabolite species were reported to have decreased in the plasma metabolome of females after TSD. The contraindication of these findings highlights the importance of including both sexes in studies investigating sleep related metabolic variations. Even though there is evidence of confounding variation due to sex, male only subjects dominate metabolomic fatigue biomarker studies and there is a serious gap in the literature investigating female subjects. Although SD studies have delivered many insights to the metabolic effects of insufficient sleep, it is also important to investigate the effects of sleep restriction as well. This is because SR will reveal metabolites that are more realistic in a natural scenario, which can sometimes not correspond with what's reported from SD studies. An good example of this is with acylcarnitine levels, which has been shown to decrease with SR[89] but increase with SD[43]. There is a gap in the literature of studies that investigate metabolites under both sleep conditions in parallel, which is a potential subject area that could be addressed in future studies.

As mentioned previously, when deciding on sample types and analytical platforms, urine and NMR have been highlighted as being some of the most popular choices. They have been readily used in a number of biomedical research areas, but have especially shown great potential for biomarker discovery. In light of this, of all the metabolomic studies investigating biomarkers of fatigue, only one analysed urine with NMR[43]. This is surprising, given the number of advantages both urine and NMR have for biomarker research.

5.2 Rodent studies

Due to the similarity of the genomes of rodents and humans and the ability to strictly control confounding factors, rodents have been readily used in a number of metabolomic studies investigating biomarkers of fatigue[45,59–62,109–112]. This lead to a shift in the prevalence of sample types collected and analysed. The investigation of metabolites within tissue samples is rarely conducted in human studies, however this is not the case in rodent studies. Approximately half of all the studies that investigated the metabolic fingerprint of fatigue analysed brain and liver tissue. Unlike in human studies, serum was not as widely investigated and urine was not featured at all. A summary of these studies are displayed below in Table 1.

It is important to note that although rodents and humans share genetic similarities, they still have huge differences in their physiology[107]. Firstly, rodents are naturally nocturnal, which immediately will show a huge difference in the metabolic rhythms when compared to humans[107]. Studies have also shown that rodents have a metabolic rate that is seven times higher than that of humans and can express different metabolic enzymes[107]. These differences in rodents and humans may bias experimental results, therefore highlighting the necessity to carefully consider the choice of study subjects.

Table 1. Summary of the findings of metabolomic studies investigating biomarkers of fatigue

Metabolite	Cohort	Sample	Study	Method	Biological Significance	Reference
↑ Taurine, formate, citrate, 3-indoxyl sulfate, carnitine, 3-hydroxyisobutyrate, TMAO, acetate (increased); ↓ Dimethylamine, 4-DTA, creatinine, ascorbate, 2-hydroxyisobutyrate, allantoin, 4-DEA, 4-hydroxyphenylacetate	n = 15 (15 M)	Urine	24h TSD	H-NMR	**3-indoxyl sulfate** – metabolite of tryptophan (involved in sleep/wake), **formate** – intermediate carboxylic acid for many processes, **carnitine** – fatty acid metabolism, **taurine** – sleep/wake and depression	**Giskeødegård et al., 2015**[43]
Prostaglandin PGE2	N = 15 (11 M)	Urine	88h TSD	PGE-M assay	**PGs** – mediators of inflammation and pain	**Haack et al., 2009**[46]
Propionylcarnitines, phosphatidylcholines, sphingomyelins	N=16 (8M)	Plasma	5 x 5h SR	MS	ATP binding cassette transporters in lipid homeostasis, phospholipid metabolic process, plasma lipoprotein remodelling, sphingolipid metabolism	**Depner et al., 2020**[47]
↑ Aconitic acid, uridine; ↓ Phosphoric acid, proline	n = 13 (9M)	Plasma	40h TSD	HILIC-LC-MS	Mainly related to glycolysis and Krebs cycle pathways	**Grant et al., 2019**[48]
↑ melatonin, tetradecenoylcarnitine, taurine, methionine, threonine; ↓ histidine, glutamate, glutamine, lysine, citrulline, carnitine, SDMA, LysoPC a c18:0, 6 x glycerophospholipids, 7 x phosphatidylcholines	N=12 (5M)	Plasma	40h TSD	UPLC + assay	**Melatonin** – sleep/wake regulator, **histidine** – precursor to histamine, which plays a major role in arousal, **glutamate** –excitatory neurotransmitter and precursor to GABA, which plays a role in sleep-promoting systems, **glutamine** – antioxidant, removes excess nitrogen/ammonia, **SDMA** – negatively associated with nitric oxide production. Nitric oxide regulates sleep/wake state	**Honma et al., 2019**[49]
↓ ATP, Glutathione, Homocysteine, GSH	N = 19 (10 M)	Plasma	40h TSD	HPLC - ED	**GSH** – plasma antioxidant	**Trivedi et al., 2017**[50]

↑ Phosphatidylcholines and triacylglycerides; ↓ Choline plasmalogens	N = 20 (20M)	Plasma	40h TSD	HPLC-MS	**Choline plasmalogen** – important for membrane structure, **phosphatidylcholines** and **triacylglycerides** – modulate lipid metabolism	**Chua et al., 2015**[51]
↑ acylcarnitine C14:1, C18:1, C18:2	N =16 (9 M)	Plasma	4h SR	Assay + FIA/Ms/MS	**Acylcarnitine** - vital for energy homeostasis	**Van den Berd et al., 2015**[113]
↑ Tryptophan, serotonin, taurine, 8 x acylcarnitines, 13 x glycerophospholipids, 3 x sphingolipids	n = 12 (12 M)	Plasma	24h TSD	LC-MS	**Serotonin** – sleep/wake**, tryptophan** – vital for the formation of serotonin	**Davies et al., 2014**[108]
↑ 12 amino acids, fatty acids, bile, steroid hormone, tricarboxylic acid; ↓ Glucose, monosaccharides, gluconate, 5C alcohols	N = 11 (6 M)	Plasma	8 x 5.5h SR	UHPLC/MS/MS + GC/MS	Amino acid and peptide metabolism, carbohydrate and energy metabolism, xenobiotics, cofactors and vitamin, nucleoside metabolism	**Bell et al., 2013**[52]
Plasma (35) – 14 increased/13 decreased; **Saliva (27) –** 8 Increased/18 decreased	N = 10 (10M)	Plasma + Saliva	40h TSD	LC-MS + GC-MS	**3 – hydroxybutyrate** (increased in both samples) – energy carrier and mediator of normal cognitive function	**Dallmann et al., 2012**[53]
↑ GGHPPPP and ESPSLIA	N = 19 (19 M)	Saliva	48h TSD	LC-MS	GGHPPPP and ESPLIA - are part of the Fatigue Biomarker Index (FBI) of saliva	**Michael et al., 2013**[114]
↓ LDL; ↑ Phosphatidylcholines, phosphatidyl ethanolamides, triglycerides and cholesterol esters	N = 14 (14M)	Serum	5 x 4h SR	NMR + UPLC-qTOF-MS	Lipid metabolism	**Aho et al., 2016**[44]
↓ Oxalic acid and diacylglycerol 36:3; ↑ phospholipids	N = 20 (10 rats/10 human, 5M)	Serum	5 x 4h SR	LC/GC-qTOF-MS	**Oxalic acid** – glycolate metabolism, **diacylglycerol** – lipid messenger	**Weljie et al., 2015**[45]
↑ CSF Orexin	N = 13	CSF	5 x 4h SR	Assay + HPLC-ED	**Orexin** - involved in sleep/wake cycle	**Olsson et al., 2018**[115]
↑ Glycine; ↓ Myo-inositol	N = 17 (17 M)	Brain tissue	40h TSD	H-MRS	Both metabolites are linked to the **mGluR5-Homer1a-IP_3 signalling cascade** – sleep/wake regulation	**Holst et al., 2017**[57]
↓ N-acetyl-aspartate (NAA) and Choline containing compounds (Cho)	N = 18 (18 F)	Brain tissue	40h TSD	H-MRS	**NAA** – brain energy metabolism, **Cho** – essential component of cell membranes and precursor for acetylcholine (neurotransmitter)	**Urrila et al., 2006**[58]
Phosphatidylcholines (PC), lysophosphatidylcholines (LPC), triacylglycerols (TAG), diacylglycerols (DAG), sphingomyelins (SM) and amino acids	N = 16 rats	Serum + Brain tissue	96h TSD + 10 x 4h SR	HPLC-QqQ-MS + GC-MS	**Pathways involved** – Arginine and proline metabolism/Aminoacyl-tRNA biosynthesis/Alanine, aspartate and glutamate metabolism/Purine metabolism/Pantothenate and CoA biosynthesis/Glyoxylate and dicarboxylate metabolism/Nitrogen metabolism/Ascorbate and aldarate metabolism/Glycolysis or gluconeogenesis	**Yoon et al., 2019**[116]
↑ Acetyl coenzyme A, 3β-hydroxybutyric acid, acylcarnitines	N = ? mice	Liver tissue	6h SR	CE-TOF-MS + LC-TOF-MS	**Acetyl-CoA** – vital for Krebs cycle – energy production, **3β-hydroxybutyric acid** – energy carrier and mediator of normal cognitive function	**Shigiyama et al., 2018**[60]
↑ NAP, NADP, N-methylnicotinamide, nicotinamide riboside, histidine, glutamine, adenine, adenosine, AMP, guanosine, glutamine, AMP, glutamine, NAD, methionine, SAH, SAM, methionine sulfoxide; ↓ 2-Pyr, 4-Pyr, serine, aspartate, ATP, urea, xanthine, xanthosine, urea, aconitate, citrate, isocitrate	N = 10 rats	Liver tissue	5 x 4h SR	UPLC-MS	**Pathways involved** – Nicotinate and nicotinamide metabolism/Purine metabolism/Ammonia recycling/Urea cycle/TCA cycle/Methionine metabolism	**Sengupta et al., 2018**[61]

↑ L-tryptophan, myristoylcarnitine and palmitoylcarnitine; ↓ Adenosine monophosphate, hypoxanthine, L-glutamate, L-aspartate, L-methionine and glycerophosphocholine	N = 48 rats	Brain tissue	15 x SF (30s on/90s off FE)	LC-MS	Alanine, aspartate and glutamate metabolism pathway common pathway	**Yoon et al., 2019**[59]
↑ Kynurenic acid (KYNA) – only in males	N = 208 rats	Brain tissue + serum	6h SR	HPLC-ED	KYNA – mediates healthy cognitive function	**Baratta et al., 2018**[62]
↑ Uric acid, allantoic acid, Vaccenyl carnitine, palmitoylcarnitine, 2-hexadecenoyl-carnitine, 4,8-dimethylnonanoyl carnitine; ↓ Retinal, retinol, retinoic acid, retinol acetate, docosapentaenoic acid, valine, choline	N = 20 mice	Serum	14 x SI	LC-MS	**Choline** – precursor of acetylcholine which mediates cognitive function, **valine** – precursor of glutamate which mediates normal cognitive function, **retinoids** – antioxidant, protects cognitive function, **carnitines** – involved in energy supply	**Feng et al., 2015**[117]
↑ glutamate, homovanillic acid, lactate, pyruvate, tryptophan, uridine, D-gluconate, N-acetyl-beta-alanine, N-acetylglutamine, orotate, succinate/methylmalonate	N = 19 mice	Brain ECF	6h SR	UHPLC-MS	**Lactate** and **pyruvate** – involved in glycolytic and astrocytic functions, **orotate** and **uridine** - de novo pyrimidine pathway	**Bourdon et al., 2018**[110]
↑ 5-hydroxyindoleceticacid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA)	n = ? rats	Brain ECF	6h SR	HPLC-MS	**5-HIAA** – metabolite of serotonin, **DOPAC + HVA** – metabolites of dopamine	**Zant et al., 2011**[111]
↑ 5-hydroxytriptamine (5-HT)	N = ? rats	Brain ECF	24h TSD	LCMS-ED	**5-hydroxytriptamine** – also known as serotonin, key mediator of sleep/wake cycles	**Lopez-Rodriguez et al., 2003**[112]

6. Current limitations of metabolomics

Even with all the technical advancements and numerous successful experiments, the field of metabolomics persists with a number of challenges. The main concerns and their potential resolutions will be discussed further below.

6.1 Massive amounts of complex data

The complex nature of the metabolome is very advantageous for biomarker research, however this is also one of the main limitations of metabolomics[10]. Data analysis and interpretation can be very challenging due to the large numbers of metabolites with varying physiochemical properties[28]. This is further complicated by the various dynamics of certain metabolites, such as those that have circadian rhythms[53]. Unlike genes and proteins, that are comprised of combinations of the same foundations of four nucleotides and 21 amino acids respectively, metabolites can comprise of a variety of different compounds, such as amino acids, lipids, organic acids and carbohydrates[67]. Finally, the variety of concentrations in the metabolome has been estimated to range from pM to mM[10]. As such, the analysis of trace metabolites in the presence of metabolites of larger quantities is very difficult[10]. This, in combination with the enormous amounts of data produced by metabolomics experiments remains to be one of the key factors in the difficulty of transitioning experimental biomarkers to use in clinical applications[24].

6.2 Metabolite identification

Metabolomics rely on the availability of reference databases in order to identify the metabolites that are detected[78]. In theory, this is an excellent approach however it's practical application can be limited because unlike the human genome, the human metabolome has not been fully determined[12]. This challenge initiated the launch of the Human Metabolome Project (HMP), which in turn facilitated the development of the most complete database of human metabolic information, HMDB[12]. This database is a collective of all information from books, journal articles and other databases[12]. It currently contains structural, physiochemical and spectral data for almost 8000 metabolites, yet it still is not enough for the comprehensive identification of the entire metabolome[12]. In light of this, metabolomics is a highly dynamic field and is rapidly evolving, so it is expected that more complete databases are attainable in the coming years.

6.3 Inter and Intra-Individuality

The metabolome is a highly sensitive system to both endogenous (i.e. age and sex) and exogenous (i.e. diet and medication) factors[29,36,118]. These factors can all contribute to the overall metabolome, making it difficult to interpret data. It can be hard to determine what metabolic differences between sample cohorts are due to a target phenotype, such as fatigue, or if they are due to these confounding factors. The effects that these confounding factors have on the metabolome must therefore be "subtracted" from the study to avoid this kind of bias[29,36,118]. Methods of minimising the impacts of confounding variances have been developed to be implemented during the pre-processing stage of the metabolomic workflow[78].

In addition to this, biomarkers of fatigue are identified by comparing the metabolic signatures of a fatigued sample with a "normal" metabolic signature from a control group[10,56,119,120]. The susceptibility of the metabolome to confounding factors make it very difficult to define what a "normal" metabolic signature should look like[10,56,119,120]. In actual fact, numerous studies have shown that the urinary metabolome of healthy volunteers showed great inter- and intra- variability purely due to differences in cultural backgrounds, age and dietary intake[120]. This is further supported by the findings of Saude et al.[56], who compared the urinary metabolites of humans and guinea pigs. The strain, age, gender, diet and environment were all identical in the guinea pig cohort, however these confounding variables were not controlled in the human cohort[56]. Interestingly, after analysing the urinary metabolites of both groups, they reported that both the controlled and uncontrolled cohorts consisted of similar coefficients of variability[56]. This shows that prior to any comparison with fatigued groups, the range of inter- and intra- metabolic variability of the control group must first be defined.

6.4 Reproducibility

The sensitivity and dynamic nature of the metabolome, in combination with the enormous amounts of data, necessitates consistency and reproducibility in metabolomics experiments[10,121]. Even with the advancements

of various aspects of the metabolomics workflow, differences are still observed in the analysis of the same sample at different laboratories[10,24]. As more attention has been drawn towards endogenous, exogenous and experimental confounding factors, the importance of standardisation and careful study design has also come to light[63,92,121–123]. At each step of the metabolomics workflow, there is the potential for bias, which if introduced, can have a serious impact on the final metabolic profile obtained[24]. This, therefore, highlights the importunacy of having strict control over experimental procedures as well as identifying and removing any sources of inconsistency. It is also crucial to collect information on confounding factors such as age, sex, diet and medication and keep in mind when analysing and interpreting the experimental data[24,120,122].

7. Conclusions

This paper has reviewed the potential for the use of metabolomic strategies in the search for a biomarker of fatigue. By doing so, the best experimental approaches and gaps in the current knowledgebase have been identified. It has been shown that there is a lack of studies that investigate urine samples, female subjects and use NMR approaches. This has guided the development of a study that aims to address all three of these gaps to add to the current understandings of the metabolomic effects of fatigue on cognitive function.

8. Experimental Design

As previously described there is a gap in the literature of: (1) studies that investigate fatigue biomarkers in urine, (2) with both male and female subjects using (3) NMR spectroscopy. This research will addressed this gap in the literature by aiming to identifying single or multiple metabolites that may be indicators of fatigue in urine samples using an untargeted NMR spectroscopy approach. This study aims to achieve the following objectives:

- Observing, identifying and categorising metabolites and relative metabolite concentrations detected in urine using NMR spectroscopy
- Calculating the prevalence of each metabolite and relative metabolite concentrations across the entire data set
- Performing statistical analyses to assess whether these metabolites and relative metabolite concentrations occurred at a level greater than what was expected by chance
- Recognising variations between metabolites and metabolite concentrations across urine samples due to whether where they were subjected or not subjected to sleep deprivation

8.1 Hypotheses

H_1: That with NMR based metabolomic analysis of urine it is possible to identify single or multiple metabolites that will discriminate between a fatigued cohort and a control cohort

H_0: That with NMR based metabolomic analysis of urine it is not possible to identify single or multiple metabolites that will discriminate between a fatigued cohort and a control cohort

8.2 Methodology

This study period will run over the course of three consecutive days (1700-1700). The outline of the study period in brief is:

- Period 1 (normalisation) – sleep from 11pm-7am, controlled diet, no urine collected
- Period 2 (baseline) – sleep from 11pm-7am, controlled diet, urine collected
- Period 3 (fatigue) – no sleep for the duration of 24 hours, controlled diet, urine collected

Approximately 10-15 healthy volunteer participants, both male and female, will be recruited. Participants will be non-smokers, have no known metabolic or sleep disorders and not be using any medication that can affect sleep quality. Standardised meals will be provided and participants will be instructed to consume these meals during specific time frames to reduce diet related variation. Participants will be instructed to not consume any caffeinated or alcoholic beverages for the entirety of the study period (72 hours). Participants will be asked to keep a sleep log and not engage in any strenuous physical activity during the study period.

The participants will self-collect mid-stream urine samples (during period 2 and period 3) every time they pass urine and note the times that they were collected. Each urine sample will then be immediately stored at -20°C. During the normalisation and baseline periods, the participants will be requested to sleep from 11pm-7am. During the fatigue period the participants will asked not to sleep for the duration of the 24 hours. If the participants fall asleep, they will be asked to notify the research when they did so and for how long. Depending on compliance, the researcher will determine whether to include or exclude these samples from the study.

Samples will then be prepared and analysed in accordance to Beckonert et al., procedure recommendations for the metabolomic profiling of urine using NMR spectroscopy. NMR spectra will be corrected for all variation that may have been caused by experimental variables (peak normalisation, peak alignment, baseline correction etc). After pre-processing, the data will be statistically analysed to obtain group clustering using various pattern recognition methods. Following this, NMR spectral information will be searched against databases to identify the metabolites that enabled group clustering. Once identified, biochemical pathway analysis will be conducted to evaluate biological relevance of how these metabolites are being produced when fatigued.

9. References

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INVESTIGATING THE METABOLIC FINGERPRINT OF FATIGUE IN URINE USING NMR SPECTROSCOPY AND MASS SPECTOMETRY

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Abstract

The National Road Safety Action Plan concluded that "fatigue is four times more likely to contribute to impairment than drugs or alcohol", however, unlike drugs and alcohol, there is currently no universal method of objectively testing for fatigue. This demand for a method of assessing fatigue-induced cognitive impairment as a way of determining a driver's 'fitness for operating a vehicle', however, may be addressed via metabolomics. Here, this study explored the metabolic response to sleep deprivation in the urinary metabolome of 9 individuals (6 female, 3 male) who were subjected to 24 hours of continual wakefulness using Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry (MS). With NMR, an untargeted discovery approach, to highlight new metabolic pathways that may be impaired during sleep deprivation, as well as a targeted approach using a panel of 50 metabolites quantified by a special extraction algorithm was conducted. MS was used to investigate the effects of sleep deprivation on a panel of 19 bioactive metabolites from the Tryptophan pathway (consisting of amino acids, kynurenines and neurotransmitters). Analysis of the untargeted NMR spectral data showed a strong influence of urinary dilution on the metabolite profiles. The utilization of PQN normalisation to account for dilution revealed spectral differences that were not associated with fatigue. Further integrated multivariate statistical analysis of the targeted NMR and MS metabolites highlighted three metabolites (acetone, nicotinic acid and picolinic acid) which appeared to present in higher concentrations and four metabolites (dopamine, valine, citric acid and hydroxyindole acetic acid) in lower concentrations within the fatigue cohort. Of these seven metabolites, only acetone ($p = 7.82E-05$), dopamine ($p = 0.026544$) and hydroxyindole acetic acid ($p = 0.002662$) were univariately significant ($\alpha = 0.05$). Whilst trying to control for diet related variables, a new confounding variable was introduced – fasting. It was determined that acetone was not significant due to fatigue, rather due to the participants fasting over the fatigue period. Further univariate comparisons of the four significant metabolites also showed no statistically significant differences between males and females. The results of this study indicate that the urinary metabolome may be useful for identifying discriminatory biomarkers of fatigue that can be used in a forensic context for both males and females, however further investigation is required. Future studies should incorporate a larger number of participants, alternate normalisation methods to correct for dilution effects and minimize the confounding effects of fasting and urine dilution.

Key Words: Fatigue, Biomarker, Metabolomics, Sleep Deprivation, NMR Spectroscopy, Mass Spectrometry, Statistical Analysis

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List of Abbreviations

AUROC	Area Under the Receiver Operating Characteristic Curve
FID	Free Induction Decays
GC	Gas Chromatography
LC	Liquid Chromatography
LOO-CV	Leave-one-out Cross Validation
LTR	Long Term Reference

MS	Mass Spectrometry
NMR	Nuclear Magnetic Resonance
O-PLS-DA	Orthogonal-Projection to Latent Structure-Discriminant Analysis
PC	Principal Component
PCA	Principal Component Analysis
PQN	Probabilistic Quotient Normalisation
RPM	Revolutions Per Minute
TP	Tryptophan Pathway
TSD	Total Sleep Deprivation
TSP	Trimethylsilylpropanoic acid
UCr	Urinary Creatinine

1. Introduction

It is well noted that the quantity and quality of sleep are key aspects in the maintenance of healthy physical and cognitive function. Without adequate sleep, cognitive performance indicators such as focus, reaction times and alertness can be severely impaired with sleep-related misperception increasing the risk of physical injury, leading to devastating consequences on the road. The National Road Safety Action Plan concluded that "fatigue is four times more likely to contribute to impairment than drugs or alcohol"[1], however unlike drugs and alcohol, there is currently no universal method of objectively testing for fatigue. This makes it difficult to regulate and put legal procedures in place to act as a deterrent to impaired drivers or ban repeated offenders. Out of an estimated $27 billion, fatigue-related road accidents have been thought to cost the Australian economy $3 billion per year[2]. The Road Safety Commission of Western Australia has reported that 20-30% of all serious injuries and fatal crashes on Australian roads are attributed to fatigue[3]. This figure, however, is thought to be underrepresented due to the lack of currently available methods to accurately quantify fatigue. The methods of fatigue detection that are available presently involve highly technical electroencephalography assessments or psychomotor vigilance tasks to assess behavioural responses[4]. Both methods are useful in a scientific setting, however they are not viable in roadside environments as they can be difficult to interpret, require highly specialized equipment and are time consuming[4]. This demand for a method of assessing fatigue-induced cognitive impairment as a way of determining a driver's 'fitness for operating a vehicle', however, may be addressed via metabolomics.

Metabolomics (also termed metabolic profiling, fingerprinting or phenotyping) is the study of small molecules (<2kDa) to characterise a system's response to genetic and environmental stimuli[5–7]. Along with proteomics, transcriptomics and genomics, metabolomics is one of the core pillars in the field of system's medicine, a scientific discipline that studies the simultaneous changes of molecular entities whilst reintegrating them to understand how they interact in the context of a living system[5,8,9]. Systems medicine is structured around the central dogma of molecular biology, therefore they are all interrelated and have some impact on the final phenotype, however as metabolites are the terminal molecules of all the processes that occur, it makes them the most proximal system to the phenotype[5,10].This is highly advantageous for biomarker studies as any stimulus that occurs will affect the metabolome and is likely to produce a measurable phenotypic expression. This is not the case in other systems of biology such as the genome, which has been used for predicting phenotypes, however changes at the genome level don't always present in the expressed phenotype[11,12].

Another advantage to metabolomics is that unlike other biological systems which may take days to show a response to stimuli, a metabolic response is prompt and often occurs within seconds[6,13]. The sensitive and dynamic nature of the metabolome allows it to capture even the most subtle of changes, so even if molecular

changes within other "-omics" platforms are not yet observed, changes may be present in the metabolome[5,14]. The metabolome can provide a true "snapshot" of the current state of a biological system[5,15]. This is favourable as it allows the investigator to be able to characterise systemic changes in metabolite profiles, or "metabolic fingerprints"[5], and phenotype individuals according to their reaction to an internal or external stimulus, such as for sleep deprivation induced fatigue[14].

In the field of system's medicine, commonly investigated biofluids include blood-derived analyte matrices (e.g. plasma and serum) and urine, with the latter often being preferred due to the ability to collect large amounts with non-invasive methods[5,16]. This is beneficial as it can then allow for an easy transition from the research laboratory to use as a forensic investigative tool. The metabolites found in urine comprise of endogenous water-soluble (produced by the body) and exogenous (e.g. from food or drug) metabolites which are the end products of countless metabolic interactions[4,5,17]. The analysis of urine will provide an indication of the events that have occurred previously, whereas blood, even though its more invasive, can show the interactions that were occurring at the time of sampling[4,17].

There are a number of analytical platforms that are used in metabolomics, however the two most common are nuclear magnetic resonance spectroscopy (NMR), and mass spectrometry (MS) coupled to a chromatographic separation method such as liquid or gas chromatography (LC and GC, respectively)[4,5,17–20]. Each of these methods have their own advantages and limitations; NMR is useful as it is a quantitative[5], non-destructive method that requires minimal sample preparation and therefore has negligible sample bias[21]. It is non-selective and provides structural information that can be used to identify unknown compounds[21]. For these reasons, NMR is often used for untargeted metabolic profiling methods to generate thousands of chemical variables that reflect internal metabolic process of an individual at a specific point in time. The downfalls of NMR, however, are its lack of sensitivity[22–24], therefore there is the potential of overlooking significant metabolites, and that it is susceptible to various matrix effects such as ionic strength and pH, which can lead to spectral interference including small chemical shift variations[25].

MS, on the other hand, is an extremely sensitive and specific technique that provides high resolution analysis of metabolites[5,9,13,19]. When studies wish to adopt a targeted approach and focus on a particular metabolic pathway, rather than explore everything that could be in the sample, often they perform a specific mass spectrometry assay. A number of studies[26–33] exploring the effects of sleep deprivation on the metabolome have specifically investigated metabolites of the tryptophan pathway (TP). Tryptophan is an essential amino acid which is heavily involved with the synthesis of a number of bioactive compounds that are involved in sleep regulation, such as serotonin and melatonin[34]. The pitfalls of MS is that it relies on extensive sample

preparation prior to analysis, which can introduce sample bias as the resulting metabolic fingerprint is taken from a modified extract of the original sample[5,6]. Another limitation of MS is that processing times are very long and can take hours[5,35], unlike NMR which only takes a few minutes, and cost-per-sample is higher for analysis[5,36].

A limitation of investigating the metabolome is that it is a highly sensitive system to both endogenous and exogenous confounding factors, such as sex and diet, respectively[19,37,38]. These factors can contribute to the overall metabonome and make it difficult to interpret data as it can be hard to determine whether a metabolic fingerprint under investigation is a result of the stimulus, such as sleep deprivation, or due to these confounding factors. The confounding effects of sex on the plasma metabolome following total sleep deprivation (TSD) was reported in a number of studies[27,28]. Davies et al.,[28] reported an increase in the concentrations of 27 metabolites, mainly phospholipids and carnitines, in males after TSD. These findings conflict with results reported by a comparable study[27] that investigated the effects of TSD on the plasma metabolome of females, as they reported a decrease in these same metabolites after TSD. This discrepancy in the findings of these studies highlights the importance of looking at the effect of fatigue on the metabolome of both males and females as sex seems to be a confounding factor when looking at these specific metabolites. In addition to this, these studies used a targeted approach to analyse specific metabolites that they expected to see a change in after sleep depriving their participants, which biases their approach due to their narrow search parameters.

When exploring the global systemic changes in response to sleep deprivation, an untargeted approach can provide the opportunity to investigate all changes and potentially highlight biochemical pathways that were previously overlooked. This was successfully demonstrated in previous studies[4,26] that investigated the effects of TSD of the urinary metabolome using NMR spectroscopy. Giskeødegård et al.,[26] investigated the urinary metabolome of sleep deprived males after 24 hours of TSD and reported 8 discriminatory metabolites that had increased, and 8 that had decreased. This showed great promise for the development for a biomarker panel for fatigue however it cannot be assumed that these same metabolites will also show similar effects in a female cohort as sex proved to be a confounding factor in studies that investigated the effects of TSD plasma metabolome[27]. Similarly, DelRaso et al.,[4] investigated the effects of 36 hours of TSD on the urinary metabolome using NMR spectroscopy on a cohort of 23 individuals that included 4 females. Interestingly, they did not detect any confounding variables due to sex, however they did report diet related confounding variables as they reported that over half of their significant metabolites were associated with energy metabolism and nutritional status[4].

To address this gap in the literature for a universal biomarker of fatigue, this study was conducted with the aim to identify and validate peripheral biomarkers of sleep deprived fatigue in urine in both males and females. Due to the variability in the strengths NMR compared to MS, this study included both platforms in an effort to obtain a wider coverage of the urinary metabolome. An untargeted NMR approach was performed with the aim to discover new metabolic pathways that may be affected during sleep deprivation. In parallel to the NMR , a targeted panel of 19 bioactive metabolites of the tryptophan pathway consisting of amino acid, kynurenines and neurotransmitters were investigated using MS (Figure 1). This panel was selected as each of the metabolites have a role sleep regulation and, as discussed beforehand, were reported in previous sleep deprivation studies[26–33].

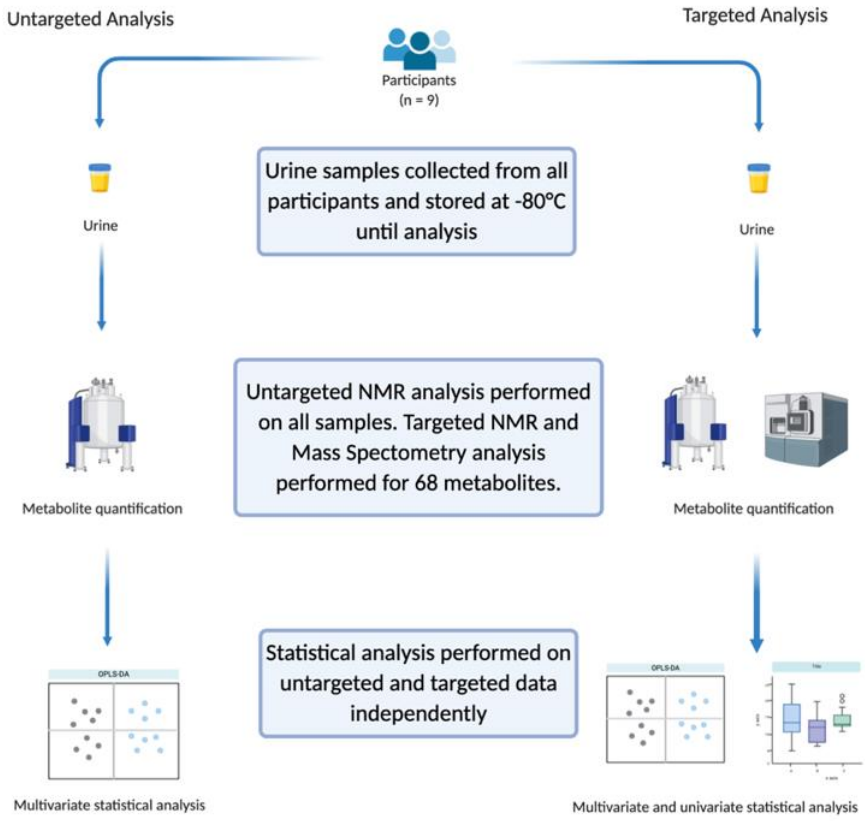

Figure 1. Graphical summary of study workflow including metabolite analysis of urine collected from 9 participants.

2. Methods

2.1 Study participants and sample collection.

The study was approved by the Human Research Ethics Committee of Murdoch University (Approval 2020/133) and conducted in accordance with the Declaration of Helsinki. Written informed consent was provided by all participants prior to partaking in the study. Participants were screened via questionnaires to determine eligibility for the study. Study exclusion criteria comprised the presence of existing metabolic or sleep related medical disorders (i.e. diabetes or insomnia), intake of prescription medication or supplements

that may affect sleep quality (i.e. melatonin or dextroamphetamines), and recreational drug use including tobacco smoking and heavy alcohol consumption (no more than 10 standard drinks a week). All study participants were considered free of these exclusion criteria based on the results of their questionnaires, however no further diagnostic testing was conducted to verify this. Participants were informed that they were permitted to withdraw at any point during the study and that all identifying data were encrypted and treated as confidential to ensure anonymity.

A total of nine healthy male (n = 3, age 26.1 ± 2.7 years (mean ± SD)) and female (n = 6, age 23.9 ± 0.95 years (mean ± SD)) volunteers were enrolled in the study, which took place in their home environments. The study was conducted in longitudinal fashion and involved three consecutive overnight periods: during the normalisation (night 1) and baseline (night 2) period the participants were requested to obtain a normal night's sleep (8h sleep opportunity 23:00-7:00), whereas during the third period (night 3) the participants were requested to stay awake, experiencing 24 hours of continual wakefulness (total sleep deprivation).

Participants were requested to go about their day as they would normally; however they were requested to abstain from caffeine and alcohol consumption, or engage in any strenuous physical activity for the entirety of the study period (three 24 hour cycles). Strenuous physical activity was defined as any activity that can't be completed whilst talking. Midstream urine samples were self-collected by the participants during the baseline and fatigue periods at 3 hour intervals and immediately stored at -20°C. Diet was controlled for, study participants were allowed to uptake meals that were identical in caloric and nutritious content within allocated time frames, as highlighted in Figure 2. At the end of the study period, all samples were collected and stored at -80°C prior to analysis.

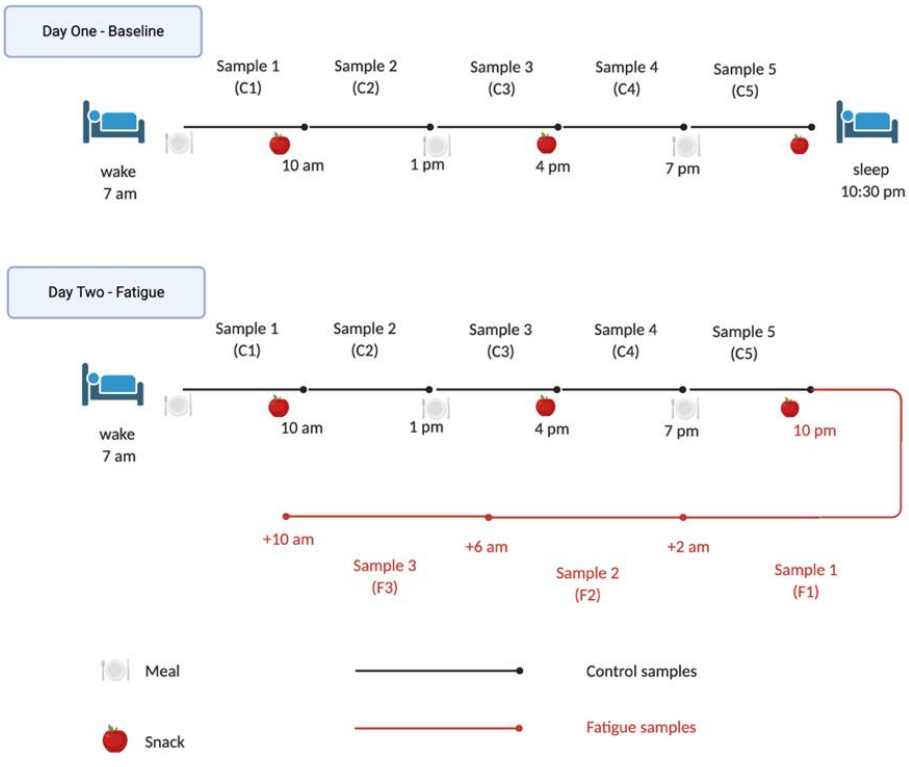

Figure 2. Timeline of sample collection and meal times.

2.1 NMR Spectroscopy

2.1.1 NMR data acquisition.

Laboratory procedure for sample preparation was adapted from Dona *et al.*[39] Urine samples were thawed at room temperature, and centrifuged at 13000 RPM for 15 minutes at 4°C. A volume of 585µL of the supernatant was aliquoted in to a microcentrifuge tube and mixed with 65µL of buffer (1.5M KH_2PO_4, 2mM NaN_3, 0.1% TSP, pH 7.4). 600µL of this mixture was then transferred to NMR tubes (5 mm diameter). A standard one dimension proton NMR (^{1}H NMR) and 2D J-resolved NMR spectroscopy experiments were performed for each sample using a Bruker Avance 600 MHz spectrometer (Bruker Biospin GmbH, Germany) operated at 300K. The spectrometer was equipped with a refrigerated (5°C) autosampler (Bruker Samplejet) and the analytical run was automated in high-throughput fashion using the software ICON NMR and TopSpin (V 3.6.2, Bruker Biospin). The data acquired from NMR underwent two processing pipelines. The first resulted in a metabolic profile, whilst the second utilised a Bruker proprietary algorithm to quantify 50 metabolites from the spectra.

2.1.2 NMR profiling data pre-processing.

Free induction decays (FIDs) were zero-filled to double the number of data points, multiplied by an exponential line broadening function (exp 0.2) and Fourier transformed to obtain one dimensional NMR spectra. Spectra were zero order phase and baseline corrected. The chemical shift axis was calibrated to trimethylsilylpropanoic acid (TSP), giving rise to a singlet resonating at zero ppm. Spectral areas bearing no biologically relevant information were excised, including $\delta < 0$ ppm, δ 4.5-5.2 ppm (residual water peak) and $\delta > 9$ ppm. To account for individual urine dilution effects, e.g. due to differential uptake of water, spectra were normalised using probabilistic quotient normalisation (PQN)[40].

2.1.3 NMR IVDR targeted quantification data pre-processing.

Absolute concentrations of 50 metabolites were obtained via an automated peak fitting routine using the standard 1D ^{1}H NMR spectra available within the Bruker AVANCE iVDR experiment suite (B.I. QUANT-UR 1.1 RUO, Bruker Biospin). Metabolites were normalised to creatinine to account for individual urine concentrations.

2.2 Mass Spectrometry

A targeted mass spectrometry screen of fourteen bioactive metabolites, including amino acids, kynurenines and neurotransmitters was completed as reported previously[41]. In short, a Biomek i5 sample processing robotic system was used to extract metabolites using a sample volume of 50µL of urine, mixed with 20µL of SIL internal standards, and 250µL of 2mM ammonium formate in methanol. The samples were then transferred to a Phenomenex PHREE™ solid phase extraction plate to remove phospholipid compounds (Phenomenex, NSW, Aus.), and were washed with 150µL of 2mM ammonium formate in methanol. A SpeedVac vacuum concentrator (Thermo Fisher, Massachusetts, USA) was used to dry the eluent collection plates. Prior to LC-MS analysis, the dried extracts were re-suspended in 100µL of 0.1% formic acid in water.

LC-MS analysis was completed using a Waters Acquity UPLC®(Waters Corp., Milford, MA, USA) together with a Waters Xevo TQ-S MS (Waters Corp., Wilmslow, UK). The TargetLynx package in Mass Lynx v4.2 (Waters Corp., Milford, MA, USA) was used for peak identification and signal quantification by means of peak integration.

2.3 Statistical analyses.

The final NMR data matrix comprised of 25859 spectral variables that showed a high degree of multicollinearity, primarily due to the fact that a single peak is composed of multi variables, secondary due to the co-dependency of metabolites (two or more metabolites may play a role in the same metabolic pathway). Therefore, statistical analysis using univariate measures is inappropriate and data were interrogated using multivariate projection methods principal component analysis (PCA) and orthogonal-projection to latent structure (O-PLS). PCA is an unsupervised data analysis method that creates a new coordinate space (each PCA dimension if termed principal components) that summarised the systemic data variation of the original variables. This method effectively compresses the NMR data and allows data interpretation in only a few dimensions. Supervised analysis was conducted with O-PLS. This method is comparable to PCA, however, it models the outcome variable (fatigue) by relating it to the NMR data using a principal components space. To avoid data overfitting, the optimal number of components was determined in a leave-one-out cross validation (LOO-CV) framework).

The final targeted metabolite data set comprised of 50 NMR variables and 18 MS variables. Univariate statistical group comparison was performed for each metabolite using the non-parametric and two-sided Kruskal Wallis rank sum test[42]. The significant metabolites were further investigated via Mann-Whitney U test to determine if there differences in how males and females were affected by sleep deprivation. The level of significance was fixed at α = 0.005 for both tests. p values were not adjusted for the number of performed tests as the condition of statistical independency was violated due to strong pathway connectivity's of the assayed tryptophan metabolites. An effect size measure using differences in mean, however, was included to quantify the degree of differences in individual metabolites between the control and fatigue groups. All statistical analyses were performed using R (V 4.0.3) in RStudio (V 3.6.1), NMR data pre-processing and multivariate statistical analyses were performed using the metabom8 library (V 0.2) available at https://github.com/tkimhofer/ (accessed 11 Dec 2020).

3 Results

To ascertain the effects of sleep deprivation on the urinary metabolome, analysis was focused on identifying discriminatory metabolites using an untargeted ^{1}H NMR discovery profile approach (full resolution spectra and 50 NMR-derived metabolites fitted using peak integration), in combination with the measurement of 18 tryptophan pathway-related metabolites using a targeted MS approach.

3.1 ^{1}H NMR Urine Discovery Profile

3.1.1 Principal Component Analysis (PCA)

Unsupervised analysis was conducted on 139 NMR spectra via principal component analysis (Figure 3), to obtain an unbiased view of the clustering of samples and to identify any outliers. Firstly, the long-term reference (LTR) samples -a pooled quality control sample that undergoes repeat preparation using the same method as the study samples and run at regular intervals throughout the NMR data acquisition workflow - were tightly clustered, indicating high reproducibility of the experiments. The first principal component (16% of total data variation) describes an evenly spread sample distribution, with a trend of fatigue and control samples having positive and negative scores, respectively. The second principal component (8.1% of total data variation) describes a clustering trend of two subgroups where the majority of samples, which are represented in the first subgroup, having positive scores whereas the smaller subgroup having negative scores.

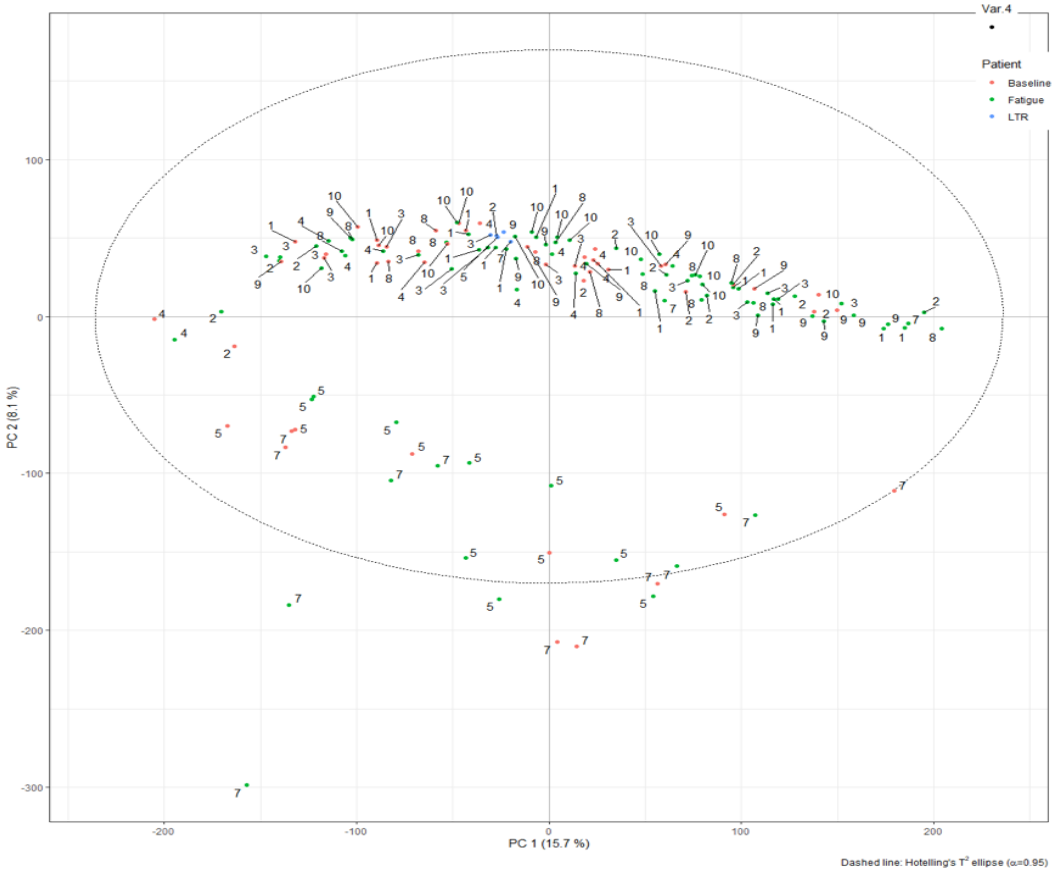

Figure 3. Principal component analysis scores plot displaying untargeted NMR spectral data

To ascertain the NMR features that were driving the observed scores distribution in PC1 and PC2, PCA loadings were interrogated. The PC1 loadings, as shown in Figure 4., highlight a number of significant peaks in the negative scores, namely in the 7-8 ppm spectral region. In contrast, the PC2 loadings, depicted in Figure 5., show minimal significant peaks apart from the broad peak observed at 5.75ppm.

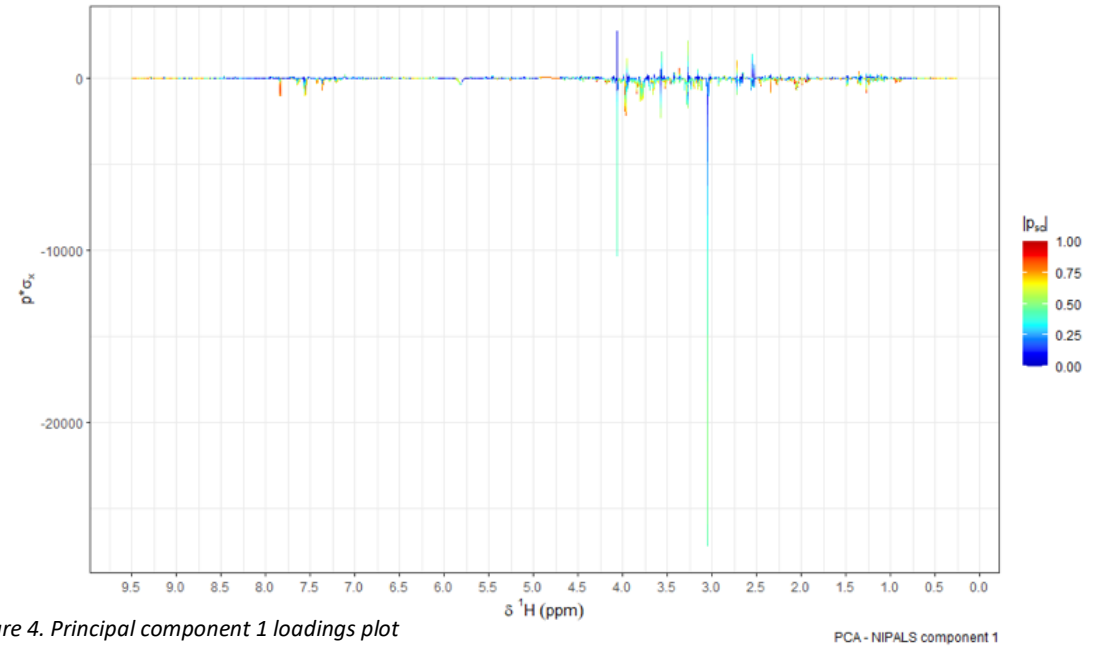

Figure 4. Principal component 1 loadings plot

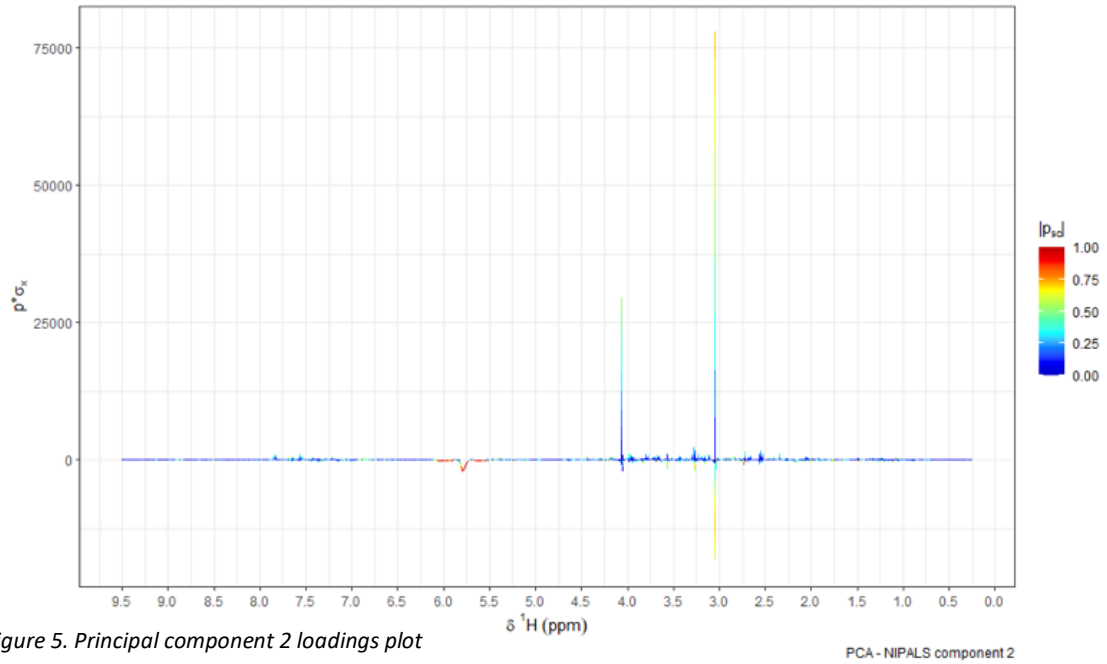

Figure 5. Principal component 2 loadings plot

3.1.2 Supervised analysis – Orthogonal-Projection to Latent Structure-Discriminant Analysis

An orthogonal-projection to latent structure-discriminant analysis (O-PLS-DA) was conducted modelling NMR spectra from fatigue vs control samples, and is displayed in Figure 6. There were two components, a predictive component describing the differences between sample class and an orthogonal component describing non-class driven data differences. The primary purpose of the O-PLS-DA was to determine whether there was a set of metabolites able to discriminate between the control and fatigue sample subsets. The O-PLS-DA model scores shown in Figure X indicate a strong systemic difference in the control urine samples in comparison to the sleep deprived urine samples and classified the two subsets with an AUROC = 0.99 (R^2X = 0.07, R^2Y = 0.98, Q^2 = 0.9) and a leave-one-out- (LOO-CV) cross validated AUROC = 0.90. The high cross-validated AUROC, close to 1, indicates that the model has not overfitted the data and that this model is likely to achieve similar discriminatory power in comparable data sets. There is a clear trend in the predictive component scores, with fatigue samples having positive predictive scores and control samples have on average negative predictive scores.

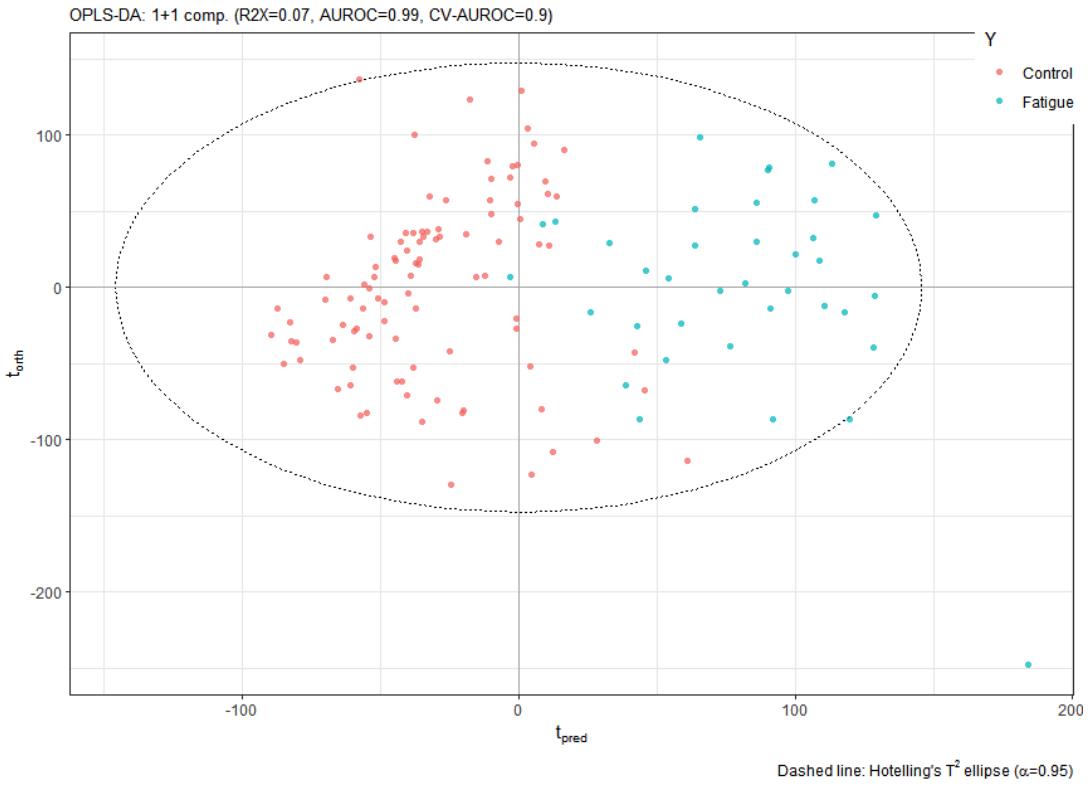

Figure 6. Orthogonal-Projected on Latent Structures-Discriminant Analysis Scores Plot (Q^2 = 0.9, R^2X = 0.07, R^2Y = 0.98, AUROC = 0.99, CV-AUROC = 0.90)

The O-PLS-DA loadings plot (Figure 7) was produced to ascertain the NMR features that were driving the variance in the model. The loadings plot indicates the spectral areas that are contributing the most to the

variance in the data set, colouring them by significance, with red being the most significant. There were very few regions in the spectral data that were significant, mainly the 0-0.5 ppm, 1.5-2.5 and 8.5-9.5 ppm regions.

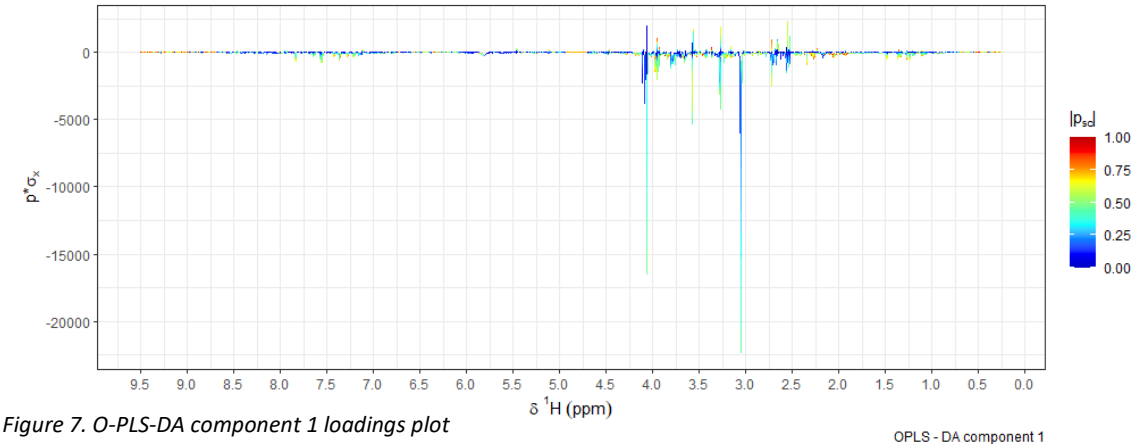

Figure 7. O-PLS-DA component 1 loadings plot

3.2 Targeted NMR and Mass Spectrometry Metabolites

A total of 68 targeted metabolites were analysed by ^{1}H NMR spectrometry (50 metabolites) and Mass Spectrometry (18 metabolites). The quantified metabolites were then normalized to creatinine dividing the concentrations of each metabolite by the concentration of creatinine. Following normalisation, the samples were filtered to only include those metabolites that were quantified in at least 80% of all samples, resulting in a final targeted panel of 33 metabolites. Once filtered, the small metabolite concentrations of the remaining panel were compared using multivariate statistical analyses.

3.2.1 Principal Component Analysis

Firstly, unsupervised principal component analysis was performed to ascertain the data quality for each analytical platform independently. The total data variation in the NMR PCA model, as shown in Figure 8A. was 81.5%, with the first principal component contributing to 72.6% of the total data variation due to the two outliers observed away from the main cluster. This was similarly reflected in the MS PCA model, depicted in Figure 8B., as it also had two outliers influencing a large first principal component contribution to the total data variation by 87.5%, contributing greatly to the total data variation of 95%. As class separation was difficult to observe due to a high degree of overlap as a result of the two outliers, these outliers were removed prior to further analysis.

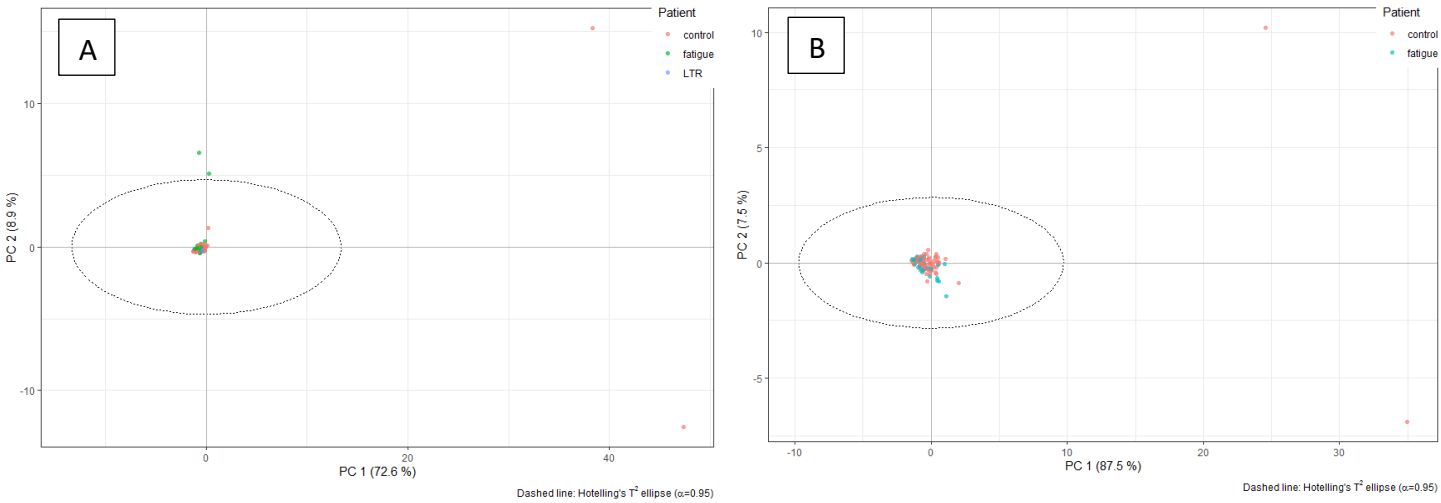

Figure 8. ***A).*** *Principal component analysis of targeted NMR data scores plot;* ***B)*** *Principal component analysis of targeted MS data scores plot*

3.2.2 Supervised analysis – Orthogonal-Projection to Latent Structure-Discriminant Analysis

Following the unsupervised PCA analysis and removal of outliers from the dataset, integrated supervised analysis was conducted. The quantified NMR and MS metabolites were combined and orthogonal-projection to latent structure-discriminant analysis performed to identify metabolites that may be able to discriminate between the fatigue and control samples. The O-PLS-DA model scores (Figure 9) show a systemic difference between the two sub sets, classifying them with an AUROC = 0.96 ($R^2X = 0.1$, $R^2Y = 0.95$, $Q^2 = 0.78$) and a leave-one-out cross validated AUROC = 0.79. There is a trend in the predictive component, with fatigue samples having more positive scores and control samples have negative scores, however there is a degree of overlap.

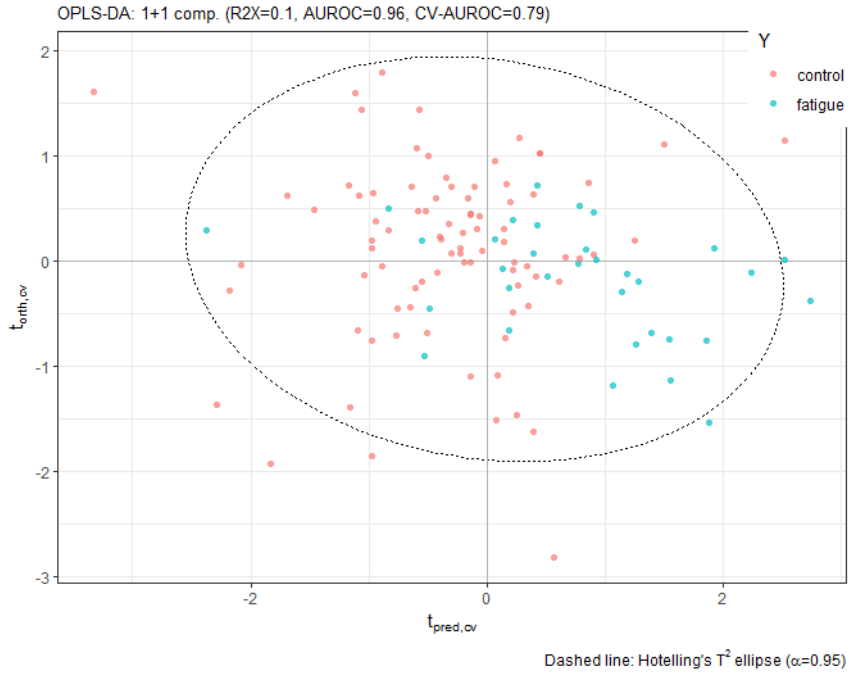

Figure 9. O-PLS-DA scores plot of integrated NMR and MS data (AUROC = 0.96 ($R^2X = 0.1$, $R^2Y = 0.95$, $Q^2 = 0.78$) and a leave-one-out cross validated AUROC = 0.79)

When interrogating the O-PLS-DA loadings plot (Figure 10) to ascertain the metabolites that were driving the variance in the OPLS-DA model, a number of key metabolites were observed. Acetone, nicotinic acid, and picolinic acid appeared to be present higher in the fatigue samples as they had the highest positive scores in the predictive component, with Acetone having the highest score. On the other hand, dopamine, valine, citric acid and X5. hydroxyindole acetic acid were lower in the fatigue samples as they had the highest negative scores in the predictive component. Of these four metabolites, dopamine had the highest predictive score, however it was only marginally different to the other metabolites, unlike acetone which was substantially larger.

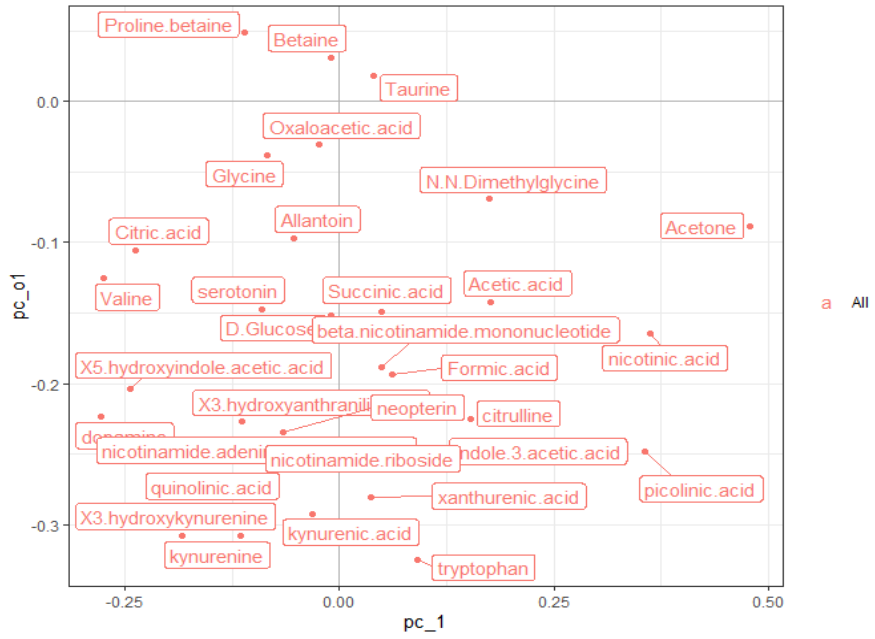

Figure 10. O-PLS-DA of integrated NMR and MS data loadings plot

3.2.3 Univariate analysis – Kruskal-Wallis test

Once discriminatory metabolites are identified it is important to investigate how significant they are to the model to avoid making conclusions based on results that only reflect a small number of samples. From the panel of 33 metabolites, 4 were univariately significant ($p < 0.05$, post-Kruskal Wallis test) between the control and fatigue groups (Table 1). Higher concentrations of acetone ($p = 7.82E\text{-}05$) were observed in the fatigue group, whilst lower concentrations of 5-hydroxindole acetic acid ($p = 0.002662$), dopamine ($p = 0.026544$) and oxaloacetic acid ($p = 0.038506$) were observed in the fatigue group (Table 1).

Table 1. p value table of 32 targeted metabolites. Univariate analysis was completed using Kruskal-Wallis non-parametric testing.

Metabolite	p value	Effect size	Mean (Control)	Mean (Fatigue)
Acetone	7.82E-05	0.501807	0.002057	0.004421
5-hydroxyindole acetic acid	0.002662	-0.38663	984.6654	551.491
dopamine	0.026544	-0.28546	23.66416	17.49988
Oxaloacetic acid	0.038506	-0.20958	0.007962	0.006665
picolinic acid	0.062862	0.239386	12.13387	16.67021
nicotinamide adenine ribonucleotide	0.081713	-0.22403	10.21481	8.202
Valine	0.087064	-0.21996	0.003773	0.002992
Citric acid	0.119095	-0.20054	0.326498	0.245905
quinolinic acid	0.13125	-0.19422	1068.701	937.0796
N,N-Dimethylglycine	0.138844	0.188799	0.005692	0.007016
kynurenic acid	0.214071	-0.15989	156.373	132.4545
Allantoin	0.265209	-0.12647	0.010842	0.007572
xanthurenic acid	0.273499	-0.14092	67.38154	57.26515
serotonin	0.315481	-0.12918	22.2659	16.7031
3-hydroxyanthranilic acid	0.322297	-0.12737	62.31237	49.72624
3-hydroxykynurenine	0.325741	-0.12647	21.17543	15.78371
kynurenine	0.403538	-0.1075	48.14143	39.07643
tryptophan	0.407501	0.106594	969.7178	1081.398
neopterin	0.532145	0.080397	8.368238	7.970101
Proline betaine	0.539124	0.074074	0.027499	0.028076
Succinic acid	0.586703	-0.0682	0.006534	0.005485
beta-nicotinamide mononucleotide	0.689075	-0.05149	214.7979	207.5467
D-Glucose	0.699603	0.047877	0.026739	0.029099
indole-3-acetic acid	0.715101	-0.04697	745.5473	818.0668
nicotinamide riboside	0.725608	-0.04517	15.83013	14.21837
Taurine	0.745784	-0.03162	0.043509	0.038592
nicotinic acid	0.84968	-0.02439	34.8675	43.16017
Glycine	0.866207	0.02168	0.169021	0.177123
Formic acid	0.868177	-0.02123	0.013435	0.012827
Acetic acid	0.899179	0.01626	0.008777	0.008443
citrulline	0.916144	-0.01355	84.86919	92.66394
Betaine	0.98593	0.002258	0.012896	0.012502

Time trajectory plots (Figure 11) and boxplots (Figure 12) were then created for the four significant metabolites, centred to 0, to interrogate the changes of the metabolites across various time points and to observe inter-subject trends. From the time trajectory plots, it was observed that both acetone and dopamine showed large fluctuations when comparing the participants to each other, however looking at them as a whole at the box plots showed minimal fluctuations, particularly with 5-hydroxyindole acetic acid and dopamine. Although 5-hydroxyindole acetic acid was determined to be significant, this was driven by participant 3 having an extremely high concentration in C2, skewing the results. Oxaloacetic acid was also highlighted as a significant metabolite, however when further investigated it was observed that this metabolite was only detected in 6 of the 9 participants and also being outlier driven due to participant 2's first fatigue sample.

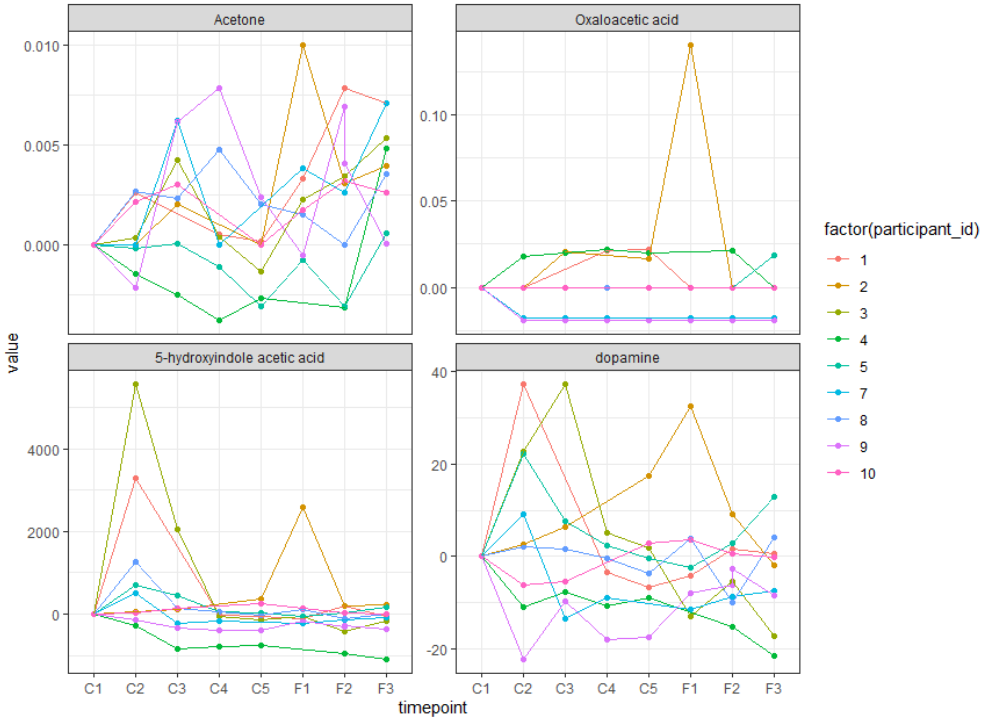

Figure 11. Time trajectory plots of the four univariately significant metabolites (acetone, oxaloacetic acid, hydroxyindol acetic acid and dopamine) to observe their changes over time

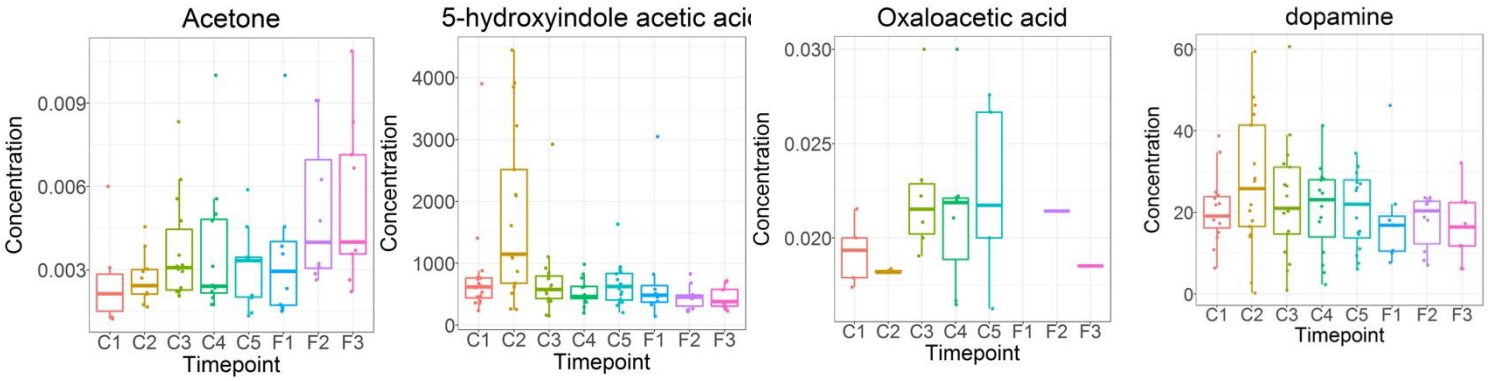

Figure 12. Boxplots of the four univariately significant metabolites (acetone, oxaloacetic acid, hydroxyindol acetic acid and dopamine) to observe their changes over time

The concentration of these metabolites were then investigated to observe their changes over time (Figure 14). Interestingly, as the day progressed, the concentration of metabolites appeared to fluctuate however when the most fatigued time point, F3, is compared with the first control time point, C1, there isn't a significant difference in concentrations. The exception to this is Alanine, which decreased and Formic Acid which increased in concentration as time progressed.

3.2.4 Mann-Whitney U Test

The four significant metabolite concentrations were integrated and univariate analysis was conducted via Mann-Whitney U test, to observe whether there were differences between males and females, and are displayed in Table 2. The test was conducted with a two tailed hypothesis and set a significance threshold of $\alpha = 0.05$. There was no statistically significant difference between the male and female concentrations for acetone (p = 0.65994), 5-hydroxyindole acetic acid (p = 0.24604), dopamine (0.27134) or oxaloacetic acid (0.96012).

Table 2. p value table of the differences between males and females in the four significant metabolites. Univariate analysis was completed using Mann-Whitney U testing.

Metabolite	z value	p value
Acetone	0.43615	0.65994
5-hydroxyindole acetic acid	1.16194	0.24604
dopamine	1.10061	0.27134
Oxaloacetic acid	0.0477	0.96012

4 Discussion

4.1 Effect of Sleep Deprivation on ^{1}H NMR Spectra of Urine – Untargeted

This study aimed to identify urinary metabolites able to discriminate between a rested and fatigued state using untargeted and targeted metabolomic approaches. PCA is an unsupervised method which describes the metabolites that are showing the highest degree of variation in the model[43]. O-PLS-DA, on the other hand, will describe the metabolites that are related to the outcome (fatigue)[43]. Following the analysis of the PCA and OPLS-DA models, there was evidence of confounding effects on the variation observed in the model due to dilution effects, rather than sleep deprivation.

Firstly, the spectral areas of significance highlighted by the first principal component loadings plot included peaks that belong to metabolites that are produced as a result of paracetamol metabolism (δ 7-8 ppm[44]). During the study period, participants were requested to not consume any medication, caffeine or alcohol,

however this was not was feasible for participant ID 7, who had consumed 1000mg of paracetamol on day 2. The presence of high concentrations of paracetamol in samples of participant ID 7 explain the PCA scores distribution, with participant ID 7 forming a scores subcluster in PC2, separated from other individuals, driving the majority of the variance in the PC2 component. Samples of Participant ID 5 also showed outlier tendencies in the PCA scores as the spectra showed broad background signals, most likely due to the presence of proteins in the sample (blood contamination due to menstruation). These samples were not excluded from statistical analysis due to the small number of study participants (n = 9), however future studies may choose to exclude outlier samples like these as the contributed variance is unrelated to the study investigation and can introduce confounding variables.

Secondly, the second principal component's loadings plot shows minimal positional peak shifts (indicated by the peak distortions with zero-crossing in the loadings line plot), apart from the broad peak observed at 5.75ppm. This peak belongs to urea, a metabolic end-product of amino acid turnover and the most abundant metabolite found in urine of healthy individuals[45]. Urea exchanges protons with water, so the urea peak is not quantitative and should be excluded for statistical analysis. Future studies should remove the spectral area which contains the urea peak and re-model the data. There was further evidence of dilution effects in the loadings of the OPLS-DA plot, which showed significant peaks in the 0-0.5 ppm, 1.5-2.5 and 8.5-9.5 ppm regions. These spectral regions are dominated by detector background noise (a normal phenomenon), and are usually insignificant in metabolomic studies as there are no peaks here that belong to metabolites. These regions being assigned high model important (red coloured in the loadings line plot), indicate that the background noise in the spectra is quite dynamic and is showing lots of variation between the samples, which is a characteristic signature of variation introduced with the normalisation process (low intensity/highly diluted samples are upscaled and high intensity spectra/highly concentrated samples are downscaled). Samples that were collected early in the morning were highly concentrated and showed high spectral peak intensities, however samples that were collected later in the day were very dilute and the resulting NMR spectra showed very low peak intensities. To account for this, Probabilistic Quotient Normalisation (PQN) was performed to scale all spectra to common statistical reference (median spectrum), and in doing so, the background noise in the spectra were scaled as well. Therefore, when investigating the PCA plot, the areas of significance were areas that should have only been baseline noise, due to all the differences that occurred when normalising the data to correct for dilution. Future studies may consider controlling participant's uptake of water to control for sample dilution to avoid this issue.

Dilution sensitivity is further supported in the 1.5-2.5ppm spectral area, which once zoomed in shows peaks affected by spectral shift (Figure 13), which are characterised by a blue starting colour at baseline level and transitioning to red towards the peak apex. Spectral shift is a commonly observed feature in urine NMR analysis and occurs as a result of minimal differences in pH and ionic strength across samples[46]. The pH of healthy human urine can range from 5.5-6.5[25], hence a pH buffer was added to the samples before NMR analysis, therefore in this instance the spectral shift observed is more likely a result of differences in ionic strength. In urine, ions such as Na^+, Mg^{2+}, K^+ and Cl^- are present in concentrations that are much higher than other compounds[47]. These ions can interact with metabolites in the urine and cause them to shift in their spectral regions, as observed in Figure 9. Differences in dilution will greatly impact the ionic strength in samples, as the more concentrated a sample is the greater the ionic strength due to the higher concentration of ions present.

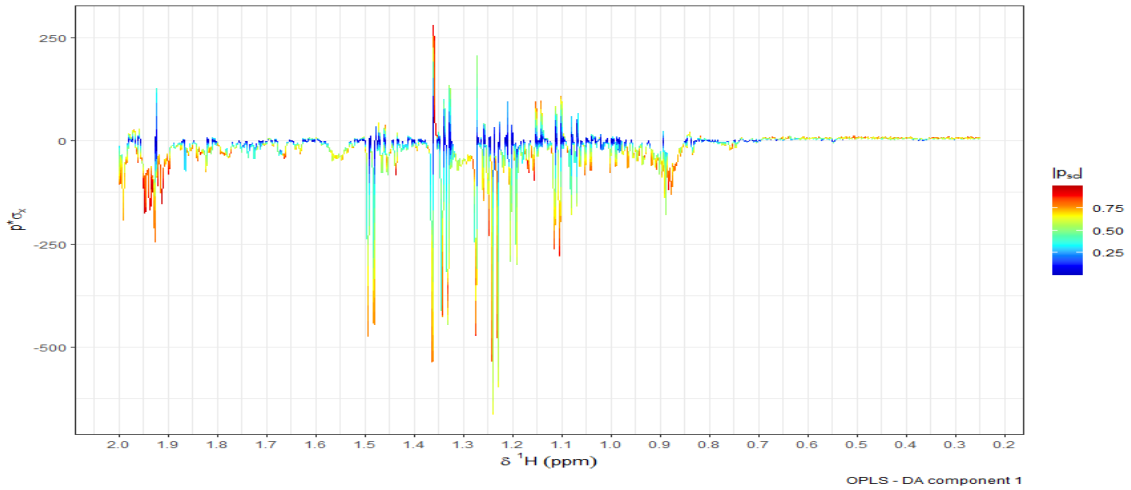

Figure 13. OPLS-DA loadings zoomed in 1.5-2.5 spectral region

Due to the normalisation effects and contribution to variance in the multivariate models, the spectra were unsuitable for metabolite assignment and quantification. To avoid this, future studies may adopt a number of strategies. First, increasing the number of data acquisition scans during NMR analysis will significantly improve the signal-to-noise ratio of spectra from diluted samples. Each standard 1D spectrum represents the summation of 32 FID's ("number of scans"), therefore it is recommended to increase the number of scans when working with samples where a large range in dilution is expected. Secondly, to reduce dilution related variation, the spectra may be normalised using urinary creatinine (UCr) concentrations rather than PQN. This is a commonly used method in clinical biochemistry to normalise analytes for quantification[48]. Creatinine is used as it is a waste product of metabolism and is continuously excreted in urine at a relatively constant rate, therefore the concentration of creatinine in the sample indicates the concentration of the urine[48].

4.2 Effect of Sleep Deprivation on targeted NMR and Mass Spectrometry Metabolites

Due to the effects of dilution that was observed when normalizing the untargeted NMR data with PQN, the targeted metabolites that were quantified were immediately normalised to creatinine to control for this variation. The final metabolite panel comprised of all 18 MS metabolites, which can be attributed to the sensitivity of mass spectrometry. NMR spectroscopy on the other hand is not as sensitive, hence why it was only able to quantify 15 of the 50 metabolites that were under investigation. This, however, does not mean that the remaining 35 metabolites were not present in the urine samples, rather that they may have been present in concentrations lower than the detection limit of the NMR (< 0.01 mM). The PCA models generated from both the NMR and MS metabolites both appeared to be very outlier driven, resulting in very high variance scores. To increase the strength of further supervised analysis models, these outliers were identified, removed and a strong OPLS-DA was performed showing excellent model generalization capacity (indicated by the high cross validated AUROC of 0.90). The statistical significance of this model, however, requires validation as the study cohort size was fairly small (n = 9). The OPLS loadings plot highlight that urinary acetone, nicotinic acid and picolinic acid were elevated in fatigue samples, whereas dopamine, valine, citric acid and 5-hydroxyindoleacetic acid was lower in the fatigue cohort. Of these 7 discriminating metabolites, however, only acetone, dopamine and hydroxyindole acetic acid were found to be statistically significant across both study groups using univariate testing by the Kruskal-Wallis non-parametric method.

Acetone is a ketone body that is produced during lipolysis, the metabolism of lipid triglycerides[49]. When the body has exhausted all available glucose, such as during exercise or fasting, it will undergo lipolysis to convert lipids for its energy source[49]. In an effort to control diet related variables, the participants were requested not to eat any food from 9:30pm till 8:00am the following morning during the fatigue period. Whilst doing so, however, a different confounding variable was introduced – fasting. Previous studies[50] have demonstrated that exhaled acetone concentrations in the breath underlie significant circadian rhythms, being increased overnight while sleeping, and therefore being in a fasted state, and decreased during the day when they were allowed to eat normally. It is evident that the high concentrations of acetone in the fatigue cohort is not primarily an indication of sleep deprivation, but rather a marker of the participants being in a fasted state.

Dopamine is another metabolite that is regulated by circadian rhythm and plays a major role in the sleep-wake cycle by reducing the effects of norepinephrine[51]. Norepinephrine is a neurotransmitter which stimulates the release of melatonin, the key sleep inducing hormone[51]. Dopamine is therefore associated with wakefulness, hence why it is targeted by drugs that increase feelings of alertness, such as amphetamines, which work by increasing dopamine levels in the brain[52]. The decreased urine dopamine concentrations observed in the fatigue cohort in the present study reflects the sleep regulatory effects of dopamine, however in a similar

urine NMR metabolomics study by DelRaso et al[4], dopamine was found as not being significantly associated to sleep deprivation. The difference in results may be attributed to the analytical platform used, as this study quantified dopamine using Mass Spectrometry which is a far more sensitive and specific technique than NMR, the platform used by DelRaso et al[4].

5-hydroxyindoleacetic acid is one of the main metabolites of serotonin, another key neurotransmitter involved in the sleep-wake cycle[53]. Serotonin promotes wakefulness by inhibiting rapid eye movement (REM) sleep to bring the sleep cycle to an end[54]. As serotonin is produced via the metabolism of tryptophan, an essential amino acid that can be present in high concentrations in a number of different foods[54]. The decrease of 5-hydroxindoleacetic acid in urine in the fatigue cohort observed in this study is assumed to reflect alterations to serotonin metabolism during sleep deprivation and not as a result of diet as all participants were given the same meals at the same time across the entire study period. As mentioned beforehand, there has been conflicting results from studies investigating fatigue biomarkers within the plasma metabolome as males and females have showed different responses to sleep deprivation. With this in mind, further univariate statistics were performed on the four significant metabolites highlighted above to investigate sex differences. The results of the Mann-Whitney U test highlighted no differences of statistical significance between the effects of sleep deprivation on males and females. This is a very promising result, highlighting the potential that the urinary metabolome has for the discovery of a universal biomarker of fatigue.

5 Conclusion

The objective of this study was to employ both a discovery metabolomics approach using NMR and targeted MS assay, to gain new knowledge on the effects of sleep deprivation on the urinary metabolome. The results of this study indicate that the urinary metabolome may be useful for identifying discriminatory biomarkers of fatigue that can be used in a forensic context for both males and females, however further investigation is required. Future studies should incorporate a larger number of participants, alternate normalisation methods to correct for dilution effects and minimize the confounding effects of fasting and urine dilution. As tryptophan is a precursor for the synthesis of key sleep modulators such as serotonin and melatonin, future studies could also investigate the effects of dietary tryptophan on sleep deprivation and whether it has an confounding influence on potential biomarkers.

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