Developing metabolomics for a systems biology approach to understand Parkinson's disease

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Chapter 1

Introduction

Chapter 1

1

Systems biology in human health

Healthcare is constantly reaching new heights in the treatment of acute and chronic illnesses. These developments come in several forms; they can be economical[1], political[2], technological[3] or scientific. With this ongoing progression, human longevity is lengthened with each generation that passes[4]. Mortality rates have decreased due to improved healthcare, understanding of diseases and therapeutic treatment options. For example, causal factors have been associated with diseases such as smoking with lung cancer[5,6], high fat diet with cardiovascular disease[7] and alcohol with liver disease[8]. Moreover, landmark therapeutic treatments that vastly reduce mortality can be seen in the discovery of antibiotics, introduction of vaccines, and insulin treatment for diabetes. However, the reduced mortality rate comes at a cost with an increased morbidity rate. It has been estimated that the population of people in Europe aged 65 years and over will increase from 90.5 million in 2019 to 129.8 million in 2050 (shown in Figure 1.1)[9]. Unfortunately, the human body is limited due to time-dependent physiological changes, i.e. ageing. These changes occur during the maturation processes, both genetically and metabolically[10]. Many of the age-related illnesses are neurodegenerative diseases that result in the loss of function or homeostasis within the brain. Neurological disorders have been identified by the World Health Organization (WHO)[11] as a public health challenge and can manifest over a broad demographic in the form of conditions such as depression, schizophrenia, addiction and epilepsy. However, there are specific conditions that are associated with aging; these are neurodegenerative diseases including Parkinson's, Huntington's and Alzheimer's disease. As mentioned above, these diseases are becoming more prevalent as human longevity increases which in turn has created a surge in the demand for improved disease interpretation, comprehensive diagnostic procedures and available treatment options[11]. Systems biology is a useful tool that has the ability to produce an encyclopedic evaluation of neurological disorders and diseases.

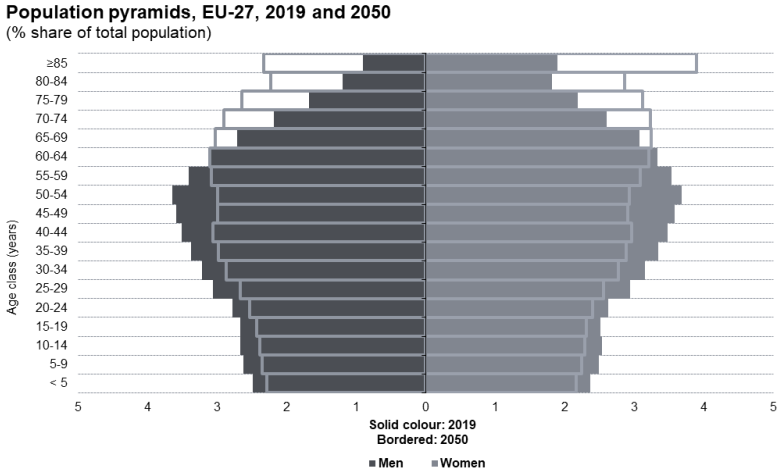

Figure 1.1. presents the aging demographic of the population in Europe across 27 nations between the year 2019 (shown in solid colours) and 2050 (shown in bordered colours). We see men in dark grey on the left and women in light grey on the right. This figure has been adapted from Eurostat[9]. [accessed on 24/11/2020]

Systems biology is a mathematical and computational research field that involves the construction of models to analyse and evaluate biological systems by integration of experimental and computational data[12,13]. In recent years, there has been an incline in the application of systems biology due to the technological growth in the "omics" research fields[14] and modelling approaches. Systems biology has the ability to detail biological systems with a breadth and depth that would otherwise be challenging, if not impossible, for the human mind[15]. It achieves this by not only investigating a single gene, protein or metabolite, but by assessing and evaluating the system holistically[16]. Models are usually constructed using data from genomics, transcriptomics, proteomics and, more recently, metabolomics. One modelling approach that is useful for improving the understanding of specific illnesses is genome-scale constraint-based modelling which uses genetic information to predict human metabolism (Step E, Figure 1.2). Constraint-based modelling is an approach that requires absolute quantitative metabolomics data to identify the physicochemical and biochemical bounds that exist within a biological system, identifying the steady-state metabolic fluxes[17]. After integration of omics data, the model can be used to predict metabolic exchange fluxes which provides insight into

the biological function[18]. Several models have been created using this principle, with the main modelling approach demonstrated by *Recon3D*[19] which contains organ-specific data from several scientific disciplines. After the network is constructed, disease-specific models can be established. Examples of this have modelled human metabolism and gut microbiota in the virtual metabolic human (available online at VMH.life). After construction of these disease-specific models, they can be utilised for biomarker discovery, therapeutic treatment strategy identification and drug repurposing[19]. The general workflow of this thesis has been visually represented in Figure 1.2. We are going to focus on the metabolomics developments and application along with systems biology approaches to improve our understanding of the disease state and potentially identify new therapies of neurological disorders with a specific focus on Parkinson's disease (Step F, Figure 1.2).

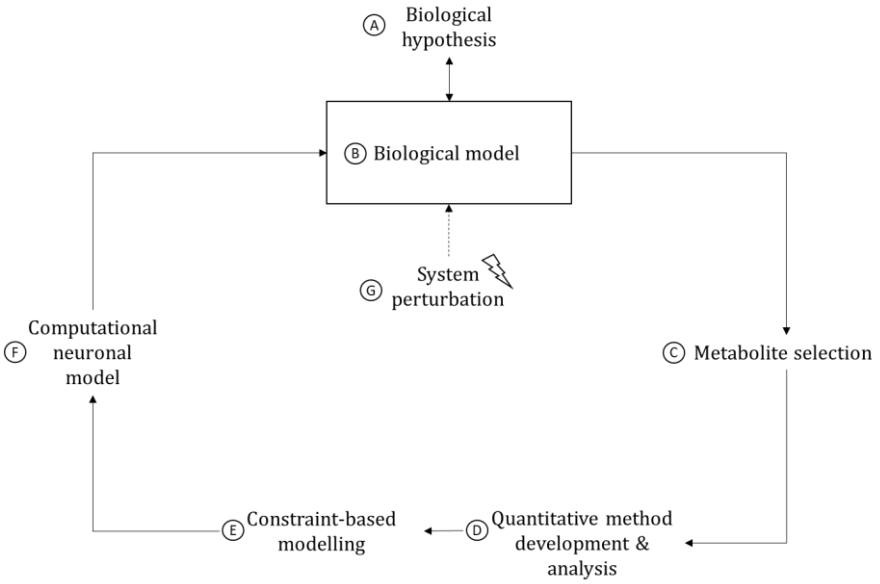

Figure 1.2. A schematic workflow of the systems biology approach with the use of metabolically constraint-based modelling. This figure highlights the workflow that is used throughout this thesis.

Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative condition which leads to a loss of fine motor movements, creating symptoms such as tremor, bradykinesia, postural instability and rigidity. In addition to these symptoms, PD patients also experience non-motor symptoms such as depression, memory loss and sleep disturbances[20]. Parkinsonism and PD are often confused to be the same. However, Parksinonism is the group term for neurological conditions which present movement disorders. A range of these conditions manifest with similar symptoms. Clinical determination of PD is only possible by post-mortem examination of the neural tissue, proving PD to be diagnostically challenging to physicians and specialists alike. Currently there are 6.9 million people in the world suffering from PD. This number is set to increase to 14.2 million by 2040, highlighting the importance for further research in the future[21]. There has been extensive research into PD for many years, though this has not translated into drug therapies on the market that are able to cure the condition. Symptomatic treatments are available such as levodopa, dopamine agonists, COMT inhibitors and anticholinergic agents, but these treatments do little to halt the progression of PD. Additionally, drugs such as dopamine agonists (i.e. ropinirole) can have undesirable adverse effects causing impulsive control disorders, such as gambling, binge eating and hypersexuality[22].

Parkinson's disease is caused by the loss of dopaminergic neurons within the substantia nigra par compacta (SNpc); this area of dopaminergic neurons is allocated the term A9[23]. The cause of PD is still unknown, but all associated causes lead to the depletion of the neurotransmitter dopamine which produces the distinctive motor symptoms that have been described above. Parkinson's disease has also been associated with mitochondrial dysfunction which is said to be present in approximately 10-20% of all PD patients[24]. Currently, several genes have been linked to PD; PINK1[25], Parkin[26], LRRK2[27], SNCA[28], DJ-1[29], ATP13A2 and GBA[30,31]. Here, we hypothesise (Step A, Figure 1.2) that the analysis of PD samples using metabolomics methods which target central carbon and energy metabolism will identify a disease phenotype. With this, we believe that we can identify biomarkers and create disease assays that can distinguish between the different genetic mutations that affect PD. This has opened new possibilities to understand the cause of PD and can identify new potential pathways for therapeutic targeting.

Metabolomics

Metabolomics is the study of the molecular phenotype of biological organisms; it is defined as the "the comprehensive study of the metabolome, the repertoire of biochemicals (or small molecules) present in cells, tissues, and body fluids" by Beger *et al.* [32]. The small molecules are usually those below 1.5 kDa including but not limited to amino acids, nucleic acids, organic acids, small peptides (dipeptides, tripeptides), sugars, fatty acids, hormones, minerals and vitamins. Our understanding of physiological function, disease states and therapeutic target sites has been derived from genomics, transcriptomics and proteomics. Metabolomics provides an alternative yet complementary technique with the other omics approaches. With techniques such as genomics, we have the ability to understand the potential of the biological system, however, metabolomics provides us with the ability to assess the functional status of the system [33].

The diverse physiochemical properties of metabolites within the human metabolome and differing matrices can provide challenges for analytical chemistry[34] from an identification and quantitation perspective. Within metabolomics, there are four main techniques used to quantify metabolites; these are Near-infrared spectroscopy (NIRS), ultraviolet–visible spectroscopy (UV-VIS), nuclear magnetic resonance (NMR) and mass spectrometry (MS)[35]. The most commonly used techniques are MS and NMR. Both techniques are used to identify and quantify metabolites in biological samples, with NMR providing better quantitative results with high reproducibility. Additionally, NMR has the ability to elucidate the structure of metabolites, aiding identification of isomers, and analyse samples that are challenging to ionise in MS or require derivatization[36]. However, another issue with the human metabolome is that some metabolites exist at very low concentrations – sub-nanoMolar. This is where MS emerges in superiority as it provides sensitivity when measuring low abundant metabolites in a quantitative manner. Moreover, MS is also better than NMR at identifying compounds in complex mixtures.

The metabolome reflects the combination of biological and environmental factors. The overall governance of the metabolome is directly influenced by the genome and, in turn, the transcriptome and proteome. The genome is influenced by factors such as genetic mutations, age, sex and ethnicity. Several diseases are associated with genetic risk factors across a broad range of illnesses, such as cancer, cardiovascular

disease and neurological disorders. All of these diseases manifest with an altered metabolome that are being studied extensively within the metabolomics field[37-39]. The second major influence on the metabolome is the exposome, i.e. the impact of environmental factors on a biological system[40,41]. Common factors include: diet, gut microbiota, employment, drugs, exercise, geographical location, pollutants, cosmetics, smoking and alcohol consumption. The study of the exposome is complex and challenging due to the multifactorial effects on the metabolome. Researchers are faced with the underlying genetics plus these environmental factors experienced by the host over a lifetime. To truly understand the human metabolome, the interaction between both genetic and environmental factors must be considered. An example of these environmental effects includes the microbiota in the gut that has been associated with motor deficits and neuroinflammation in models relevant to PD[42]. Pollutants and toxins can also influence the human disease state as seen in PD that can be induced by exposure to the naturally occuring pesticide rotenone[43] or the illicit drug by-product MPTP[44]. The impact of pesticide rotenone on the human metabolome[45] will also be discussed further in **chapter 2** where we use it as a chemical perturbation to mimic mitochondrial dysfunction (Step G, Figure 1.2). In this thesis, we quantitatively capture the broad metabolome, which requires the appropriate selection of metabolites that holistically capture the genome-exposome interaction.

Metabolite selection

The human metabolome is vast in size and new metabolites are being identified each year. As it currently stands, databases such as the Human Metabolome Database (HMDB 4.0) have identified 114,100 metabolites using a combination of measurements, expectations and predictions. Each metabolite has its own biological role, metabolic pathway(s), transport mechanism and physiological concentration[46]. Metabolic pathways contain metabolites in an intricate and dynamic system that is constantly adapting to the physiological demands. Within the human body, metabolites range from core metabolites, such as amino acids, to TCA cycle metabolites that control energy production within the mitochondria, to metabolites that exist only to facilitate the intermediate stages of a pathway. Other metabolites are present in their metabolised form awaiting excretion via the liver and kidney,

such as sulphated dopamine. Despite these differences in role and function, the majority of the metabolites are essential to support and sustain life.

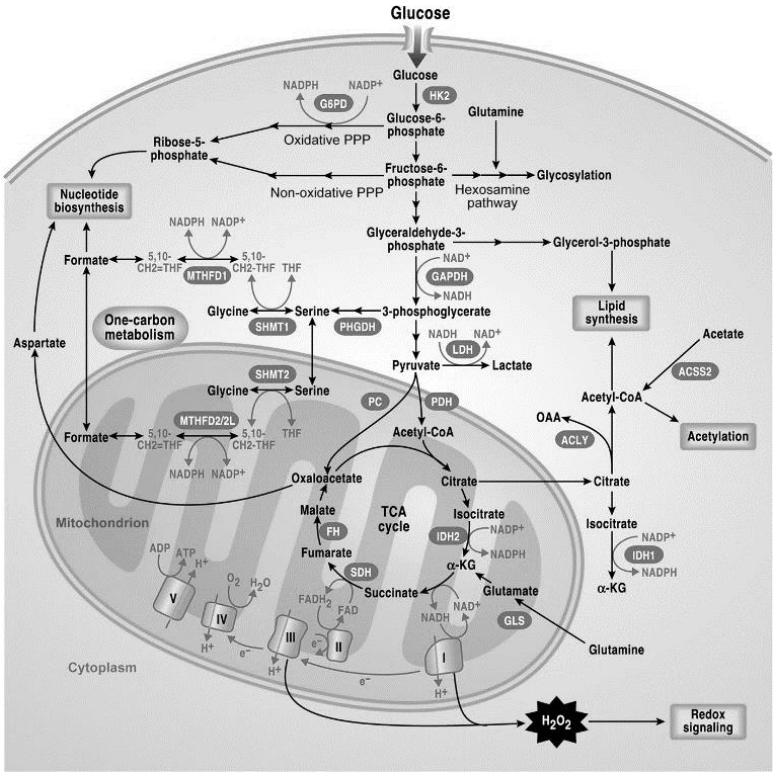

Figure 1.3. Metabolites associated with the energy processes within the cell. Glycolysis, the Krebs cycle and intermediate metabolites as well as amino acids involved in the energy process are detailed. Reprinted DeBerardinis and Chandel (2016)[37] © The Authors, some rights reserved; exclusive licensee AAAS. Distributed under a CC BY-NC 4.0 License..

Neurological disorders, as with all diseases, each have their own characteristic metabolic profile which can further vary in relation to an individual. These disease phenotypes respond to the chemical cues created by the metabolite levels in the corresponding pathways and functions. For this reason, the relevant metabolic targets must be specifically chosen for study (Step C, Figure 1.2). Burte *et al.* (2017) identified several metabolites associated with PD that are involved in the energy metabolism and Krebs cycle, which corresponds to PD being associated with

mitochondrial function[24]; thus, metabolite selection must cover the energy metabolism. One of the main energy processes that occurs within the mitochondria is the electron-transport chain which involves a range of organic acids within the Krebs cycle, such as succinate and malate (Figure 1.3). Another important metabolic pathway is energy generation by glycolysis. This metabolite can enter the Krebs cycle in the form of oxaloacetate produced by pyruvate carboxylase from pruvate or acetyl-CoA following conversion with pyruvate dehydrogenase. Pyruvate also has a role in anaerobic respiration where it can be converted into lactate for a simple energy supply. Other metabolites associated with the energy metabolism include amino acids, such as aspartic acid, glycine, serine, glutamine and glutamate, and other common metabolites, such as acylcarnitines[39] and *N*-acetylated amino acids[39,47]. These metabolites are all investigated within this thesis, specifically in **chapter 2**, **chapter 3** and **chapter 5**.

Neurological disorders can be profiled using metabolites that are more specific to neurons. For example, neurotransmitters such as dopamine, GABA, serotonin, epinephrine, norepinephrine and glutamate have roles in the maintenance of homeostasis within neurons but also in the communication network across the neuronal cells. The brain is composed of several types of neuronal cells such as neurons and glial cells (oligodendrocytes, astrocytes, ependymal cells and microglia). The glial cells are distributed throughout the brain and are difficult to distinguish by metabolic profile. To date, the number of identified neurotransmitter molecules is over 100. The neurotransmitters are physicochemically diverse and exist in the form of amino acids (glutamate, D-serine and aspartic acid), monoamines, purines, neuropeptides (*N*-acetylaspartic acid) and others. Neurotransmitters such as GABA, glutamate and dopamine are key in the functionality of the substantia nigra and they exist in a complicated balance[23]. Deciphering the function of these neurotransmitters can help improve the understanding of PD and other neurodegenerative diseases. In this thesis, key neurochemicals and neurotransmitters are investigated in **chapter 4** and **chapter 5**.

Biological samples in metabolomics

The metabolome can be measured from a range of biological fluids (matrices) that are extracted from human subjects; for example, blood serum and plasma[48], urine[45], faeces[49], sweat[50], tissue[48], semen[51] and breast milk[52]. The most commonly used

biological matrix is blood plasma due to the safe and simple extraction procedures that exist. Furthermore, the majority of the metabolites that are excreted from cells and organs are transported through the blood, providing a broad overview of the metabolome. However, not all metabolites are excreted into the blood or pass the blood brain barrier; for example, tissue-specific metabolites and those excreted by the gut microbiota[53]. Additionally, some metabolites exist in their modified forms, such as sulphated dopamine, or experience degradation, which provide analytical challenges.

In addition to human models, another common approach to study disease is animal modelling. One of the main strengths of animal models is the reduction of exposome influences which allows the researcher to focus on the genetic influence on the metabolome whilst providing a full organism with functional organs[54]. Additionally, animal studies have fewer ethical considerations compared to studies that are designed in humans. Animal models unfortunately suffer from the fact that they do not fully represent the human physiological system and there are clear differences within the metabolome. Despite this, they provide a useful tool for scientific research.

In vitro cell culture work is similar to animal models by which they also minimise the exposome influence. In addition, it requires the least amount of ethical considerations. There are a range of cell lines available for the study of diseases; specific cell lines can be investigated according to the disease of interest, from oncology, endocrinology to neurology. Cell culture approaches allow the researcher to investigate single cell lines or use a co-culture in the attempt to make them more physiologically relevant. Cell cultures can also be 2-dimensional or 3-dimensional, with the latter said to provide a more physiologically realistic environment[55]. However, cell culture has limitations which include existing in an artificial environment (cell culture media), variation in performance between scientists and simple cell lines not sufficiently representing complex organs. One approach to get closer to physiologically relevant cell lines is the use of induced pluripotent stem cell-derived cell lines which carry the genetics of an individual[56]. A summary of the pros and cons of each study sample type is listed in Table 1.1.

In this thesis, we utilise cell culture and human urine in **chapter 2**, cell culture in **chapter 3**, animal models in **chapter 4**, and induced pluripotent stem cell-derived

neurons in **chapter 5** to demonstrate the need to match the most appropriate biological matrix with the hypothesis (Step B, figure 1.2).

Human	Animal	Cell line	iPSC
Examples	Examples	Examples	Examples
• Plasma • Serum • Urine • CSF • Faeces • Breast milk	• Primate • Canine • Rodent	• HEK292 (kidney) • SUIT-2 (pancreas) • PC-12 (adrenal) • HeLa (cervix)	• Dopaminergic neurons • Hepatocytes • Endothelial cells
Strengths	Strengths	Strengths	Strengths
• Multi-organ system • Organism of interest • Verbal evaluation allowed • Realistic exposome environment	• Multi-organ organism • Reflects human physiology closely • Suitable for early-phase research • Controlled exposome	• No ethical considerations • Transfection possible • Inexpensive • Ease of use • Availability	• Patient specific • More physiologically realistic • Co-culture possible
Weaknesses	Weaknesses	Weaknesses	Weaknesses
• More ethical considerations • Exposome influence • Information complexity	• More ethical considerations • Animal welfare • Human genetic difference	• Less physiological relevance • Artificial growth environment	• Artificial growth environment • Cell age is young • Time-consuming • More expertise required • Expensive

Table 1.1. Examples of common study sample type that can be used in metabolomics for the study of diseases. The strengths and weaknesses associated with each sample type are also detailed.

Metabolomics analysis

There are two main approaches used in metabolomics; untargeted and targeted. Untargeted is the global overview of the metabolism without a specific class or pathway of metabolites being identified – chemical unknowns[57]. Using this approach provides a large amount of data that captures as much of the metabolome as possible. After this, the data can be compared to identify patterns in the human metabolism or disease state. However, as it currently stands, we are only able to identify < 2% of peak identified using the untargeted mass MS workflow[58]. Additionally, the metabolites presented by untargeted metabolomics are not absolute quantitative values, thus, they cannot be integrated into constraint-based metabolic models in

systems biology. However, untargeted metabolism is highly desirable in exploratory research to identify new metabolites and pathways associated with illnesses. Within this thesis, we focus on the application of targeted metabolomics using absolute quantitative values.

Targeted metabolomics

Targeted metabolomics is where a specific metabolic pathway or class of metabolites are selected prior to analysis and the methods are optimised around the desired candidates to ensure accurate qualitative and quantitative results. An ideal instrumental setup for targeted quantitative analysis of abundant metabolites, such as amino acids, sugars and organic acids, from a biological matrix that is high in volume would be NMR[36]. However, as mentioned previously, to truly understand the metabolome, you need to delve deeper using a range of biological matrices (some of which are low in volume/material-limited) with low concentrations of metabolites, thus LC-MS/MS using a triple quadrupole (QqQ) MS becomes the gold standard. One limitation of QqQ MS, is the lack of mass resolution, reducing the specificity and qualitative performance. There is where Quadrupole Time-of-Flight MS (qToF) MS provides a solution for this problem to provide high-resolution accurate mass data and improve the identification of metabolites at the expense of losing sensitivity compared to QqQ MS.

One of the main challenges with QqQ mass spectrometry is distinguishing metabolites with non-unique masses. One way this is addressed is the hyphenation with a separation science such as gas chromatography (GC), liquid chromatography (LC), supercritical fluid chromatography (SPF) or capillary electrophoresis (CE). With the utilisation of a separation science hyphenated to MS, it reduces the risk of mass interference from isomeric compounds, such as amino acids isoleucine and leucine. In this thesis, we focus on the use of LC-MS/MS. The most common system setup for metabolomics methods is LC hyphenated to MS.

A major issue with quantitative workflows using RPLC-MS/MS is matrix effect. This occurs during the electrospray ionisation (ESI), prior to MS detection. Matrix effect (Figure 1.4) is the result of several metabolites, salts or proteins eluting simultaneously, which can either lead to ion suppression (reduced charging of target analyte) or ion enhancement (increased charging of target analyte)[59]. Furthermore,

negative ionisation is more susceptible to ion suppression when compared with positive ionisation. This unpredictable ionisation variable introduces challenges when it comes to accurate quantitation and reproducible analysis of the target analyte as the matrix changes (inter-sample). Moreover, the ion suppression can be so severe that the analyte of interest is suppressed below the detection level. One approach to characterise matrix effect is the use of isotopically labelled internal standard pairs for analytes of interest. For example, alanine-D_3 could be injected simultaneously as an analyte pair with alanine to characterise matrix effect. However, the cost of isotopically labelled metabolites is expensive and not all metabolites have an available isotopically labelled form. Therefore, analytical scientists tend to extend their internal standards to cover more than the analyte pair. Methods have been developed that use 10 internal standards to characterise matrix effect for 70 metabolites, however, this yields poor quantitative quality assurance due to the difference in elution time and physiochemical properties. The utilisation of the internal standard pair also does not reduce ion suppression, therefore, the issue of suppression below the limit of detection (LOD) still exists. Moreover, the issue of detection sensitivity is not addressed when it comes to the quantitation of material-limited samples.

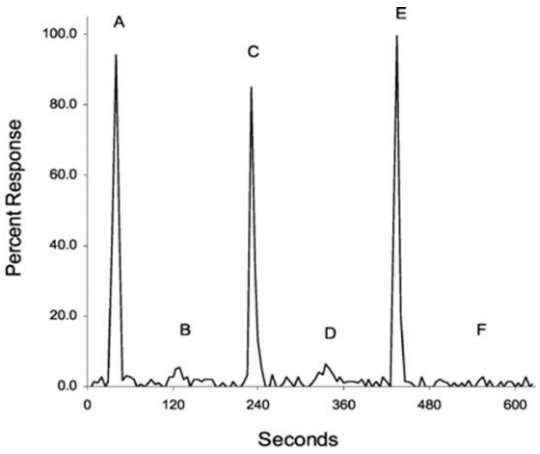

Figure 1.4. Signal response comparisons (m/z 195) for caffeine added to serum extracts prepared by solid-phase extraction, solvent extraction, and protein precipitation. (A, C, and E), 1 mg/L caffeine solution. (B), solid-phase extract with 1 mg/L caffeine added. (D), methylene chloride extract with 1 mg/L caffeine added. (F), serum protein precipitation extract with 1 mg/L caffeine added. Figure extracted from Annesley et al (2003)[59] and produced following the copyright permission from Oxford University Press Journals for personal thesis non-commercial use.

Material-limited samples

The use of modern techniques to assess the metabolome function in a more realistic biological environment compared to 2D cell culture has been increasing in recent years. Approaches such as 3D cell culture[55,60], human cell transplantation into animal models[61] and microdialysate[62,63] are becoming increasingly common. This has improved our physiological representation of the metabolome and enabled dynamic sampling in a high-throughput manner. However, this has led to a reduction in sample volume and reduced metabolite concentrations. Several approaches exist to increase sensitivity such as sheathless CE-MS[64] and nanoLC-ESI-MS[65] but these approaches are limited in their coverage or quantitative profile. To evaluate the metabolome of PD samples and other neurological disorders, sensitive quantitative analysis method are required that can still capture the broad metabolome[66]. In

chapter 3, we use chemical derivatization as an approach to increase sensitivity of material-limited sample whilst maintaining the quantitative coverage.

Chemical derivatization

Above, we have discussed the main analytical methods using LC-MS for quantitative metabolomics and the need for improved quantitation and sensitivity. However, limitations exist that reduce the quantitation and detection. The main issues have been summarised here:

- Chromatography robustness
- Ionisation characteristics
- Metabolite physiochemical properties – suited to different separation sciences
- Metabolite stability
- Matrix effect
- Detection limit

One method that can be used to solve the issues listed above is chemical derivatization. This approach is our method of choice for accurate quantitation throughout this thesis.

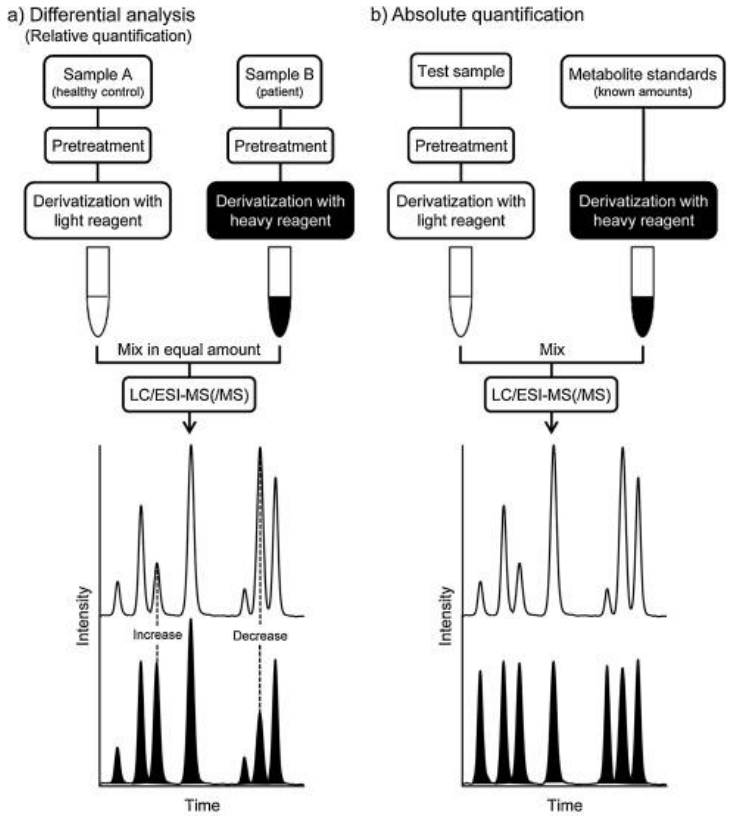

Fig 1.5. The schematic workflow used with ICD derivatization for qualitative differential analysis (a: left workflow) and absolute quantitative (b: right workflow). Figure extracted and modified from Higashi et al. (2016)[67] and produced following the copyright permission from Elsevier Journals for personal thesis non-commercial use.

Chemical derivatization is the process of adding a chemical group to the analyte to enhance the separation and detection within LC-MS. By changing the physiochemical properties of the analyte of interest, characteristics such as volatility, hydrophobicity, detectability, stability and polarity can be adjusted. GC-MS commonly uses chemical derivatization by alkylation and silylation, to improve the volatility and retention for metabolites such as amino acids and organic acids[68]. Additionally, aliphatic metabolites have no UV or fluorescence properties – thus pose challenges with LC-UV[69]. For this reason, derivatization can be used, introducing aromatic groups which have a UV absorbance. Three common reagents used for UV detection are benzoyl chloride[69], dansyl chloride[70], phenacyl bromide[71,72], and AccQ-tag[73,74]. Additionally,

metabolites such as catecholamines (i.e. dopamine), have phenol groups which are highly vulnerable to degradation outside of the cell[75], presenting difficulties in the analytical community. Reagents such as benzoyl chloride have the ability to derivatize these functional groups thus stabilising the metabolite for analysis[76,77]. Therefore, to stabilise, detect and quantify metabolites relevant to PD in a simple and cost-effect method, we were required to develop and apply this approach through this thesis (Step D, Figure 1.2).

Aim and scope of thesis

With our hypothesis that a mitochondrial phenotype can be identified in a subset of Parkinson's disease (PD) patients using metabolomics, we aim to develop and improve quantitative metabolomics methods using a targeted MS workflow and integrate the obtained data into constraint-based metabolic models for the study of PD. The association of PD with mitochondrial dysfunction and energy imbalance will create an identifiable metabolic phenotype. To achieve this, we must first target the appropriate metabolites associated with the central carbon and energy metabolism as well as the neurochemical communication and homeostasis. With this selection, we form the backbone of this thesis from which we can derive further understanding into the functional roles of the metabolism associated with PD. We pursue the need for the ability to detect and quantify metabolites with a method that has maximized coverage of all relevant targets and optimised quantitation. Thus, we focus on the development and application of targeted LC-MS/MS workflow, utilising chemical derivatization to achieve this.

In **chapter 2**, we aim to create an absolute quantitative method to study the energy and central carbon metabolism using a single separation and analysis technique. Chemical derivatization is a technique that can be employed to achieve this goal**.** This derivatization technique described simultaneously labels carboxylic acids, thiols and amines using the reagent dimethylaminophenacyl bromide (DmPABr) in a high-throughput, reliable single RPLC-MS/MS analysis with a 10-minute acquisition time using only positive ionization mode. Few published methods can target carboxylic acids and amines simultaneously – both of which form a large proportion of the human metabolome. In addition, quantitation is further enhanced by isotope-coded derivatization (ICD), which uses internal standards derivatized with an isotopically labelled reagent (DmPABr-D_6). Sixty-four central carbon and energy-related metabolites were detected and quantified from human urine and SUIT-2 cells, including amino acids, *N*-acetylated amino acids, metabolites from the Krebs cycle and pyruvate metabolism, acylcarnitines and medium-/long-chain fatty acids.

In **chapter 3**, the derivatization method described in **chapter 2** is applied to material-limited cell samples. Sensitivity is a common hindrance when faced with low sample concentrations. Previous studies have attempted to overcome this issue in the form of costly microscale separation such as CE and micro/nano-LC coupled to

mass spectrometers with low-diameter ionization emitter sources. By employing chemical derivatization, it is possible to improve chromatographic separation and enhance MS ionization. Favourable, sensitive and specific fragmentation is also achievable. Our novel method applies RPLC-MS/MS analysis to HepG2 cells, ranging from 250 cells to 1×10^5 cells, after fast and accessible derivatization DmPABr. The primary amine, secondary amine, thiol and carboxyl submetabolome are labelled, and we also utilize ICD as done previously. Thirty-seven metabolites were detected and quantified in a sub-10,000 HepG2 cells extract, with an additional 11 metabolites detected below LLOQ.

We discovered a lack of absolute quantitative metabolite reference values in relation to the mammalian brain whilst trying to study neurological disorders. Therefore, in **chapter 4**, we pursued the quantitation of neurochemicals across 25 regions of the rat brain. However, as highlighted previously, analytical methods have their pros and cons. Here, we utilised the benzoyl chloride derivatization technique as it has the ability to stabilise vulnerable catecholamines and capture neuroactive metabolites. To achieve this, we optimised LLE extraction and followed it with the derivatization LC-MS/MS technique. After the analysis, we obtained a comprehensive profile of 43 metabolites including important neurotransmitters such as dopamine, epinephrine, norepinephrine, GABA and serotonin. Additionally, we covered the urea cycle, and polyamine and tyrosine metabolism extensively. The brain regions investigated range from the frontal lobe to the brain stem, covering regions such as the orbitofrontal cortex, cerebral cortex, ventromedial prefrontal cortex and subcortical structure. After generation of the absolute quantitative reference values, we believe this data can be integrated into metabolic models, thus improving our understanding of the mammalian brain.

In **chapter 5**, utilising induced pluripotent stem cell (iPSC)-derived dopaminergic neurons, we conducted a multi-omics data investigation to understand the functionality and potentially identify vulnerabilities in Parkinson's disease. We then integrated the multi-omics data into a genome scale constraint-based reconstruction and analysis model that focused on the metabolism. With the focus being heavily dependent on absolute quantitative metabolomics, applying AccQ-tag derivatization with RPLC-MS. AccQ-Tag was used to capture the biogenic amine and neurochemical profile. Additionally, we used GC-MS to quantify sugars. With these values, the

Recon3D reconstruction of the generic human metabolome was used to generate stoichiometrically and flux consistent constraint-based model of dopaminergic neuron metabolism. The metabolism was constrained using manual literature curation, transcriptomics, and the metabolomics input. With this, we constructed the *iNESC2DN* model that can be used for biomarker discovery, therapeutic treatment strategy identification and drug repurposing[19].

Finally, we conclude this thesis with **chapter 6**, where we revisit the content of our work and address the future prospective of quantitative metabolomics in the application to human diseases and, specifically, neurological disorders. We also discuss the creation of disease-specific constraint-based models.

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