Investigating Metabolomic Biomarkers of Hypoxic Ischaemic Encephalopathy

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Declaration

This is to certify that the work I am submitting is my own and has not been submitted for another degree, either at University College Cork or elsewhere. All external references and sources are clearly acknowledged and identified within the contents. I have read and understood the regulations of University College Cork concerning plagiarism.

Signed: _____ Date: _____

Dedication

To

Joe and Rita Hyland

Eithne, Bill and Brian Denihan

Acknowledgements

When I arrived at University College Cork in 2011 to embark on a PhD, I was extremely privileged to meet Dr Deirdre Murray and Prof Geraldine Boylan whose passion for neonatal research was infectious and their work clearly outstanding. With their support I was primed to devote the next four years to a challenging and meaningful PhD project. When, the following year, I met Dr Jennifer Kirwan, who shared her love and passion for metabolomics, I knew our project was in safe hands.

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Abstract

Background

An early objective biomarker to predict the severity of hypoxic ischaemic encephalopathy (HIE) and identify infants suitable for intervention remains elusive. Our knowledge of the full metabolic response to perinatal asphyxia, and its sequelae, HIE is also incomplete. The ability to identify metabolite alterations and perturbed biochemical pathways in HIE may identify early biomarkers of injury and suggest new treatment targets. Investigations have begun using metabolomics to study the compliment (metabolome) of low molecular weight biochemicals (metabolites), with early evidence suggesting extensive metabolic derangement post hypoxic ischaemic insult. However, these early studies have varied with regard to analytical method, biospecimen, animal model and study cohort. To date no study has reported validated findings. To progress a metabolomic biomarker toward clinical utility, discovery work must be followed by study and cohort validation in a well phenotyped cohort of infants with consistently biobanked samples, and ideally with long-term neurodevelopmental follow up. Furthermore, to translate to practical use it is imperative that potential biomarkers are robust to preanalytical variables.

This thesis aims to progress our knowledge of metabolomic markers of HIE through a pipeline of biomarker development, completing the discovery and technical study validation phase by employing a novel untargeted mass spectrometry metabolomic method. Furthermore the potential of proposed metabolomic markers to predict neurodevelopmental outcome will be assessed. I have also aimed to improve technical validity by investigating the effect of haemolysis on cord blood metabolites.

Methodology

The primary study cohort was recruited between September 2009 and June 2011 in a single maternity hospital. Term infants with perinatal asphyxia were identified and all had umbilical cord blood (UCB) drawn and biobanked within three hours of birth. HIE was defined by a Sarnat score at 24hours and confirmed by continuous multichannel-EEG monitoring. Infant neurodevelopment was assessed at 36-42 months using the Bayley Scales of Infant and Toddler Development Ed. III (BSID-III). Simultaneously a matched control population of healthy term

infants, with identically biobanked UCB samples, was recruited over the same period as part of an ongoing birth cohort study.

Untargeted metabolomic analysis of UCB from these infants was performed using direct injection FT-ICR mass spectrometry (DI FT-ICR MS). Both polar and non-polar (lipid) features were detected in positive and negative ion mode, to achieve global characterisation of the metabolome. For each reproducibly detected metabolic feature, mean fold differences were calculated between two study arms, HIE *vs.* matched controls (ΔHIE) and perinatal asphyxia *vs*. matched controls (ΔPA). Putative metabolite annotations and lipid classes were assigned and pathway analysis was performed.

Retrospective metabolomic data from the perinatal asphyxia/HIE cohort was used to evaluate the potential ability of two previously proposed metabolite models to predict HIE, by correlating with three year neurodevelopmental outcome. The models were assessed for clinical utility and compared to standard biochemical and clinical tools currently in use.

Retrospective data from the healthy control cohort enabled a preliminary investigation into the effect of visual haemolysis on metabolite concentrations. This was followed by an untargeted DI FT-ICR MS metabolomic analysis into the effect of haemolysis on the UCB metabolome. Similarly biobanked, paired infant UCB serum samples from healthy term infants, one haemolysed (*in vitro*) and one non-haemolysed were chosen. The same untargeted workflow to that used in the biomarker discovery study was employed, to enable a comparison between the studies.

Results

Untargeted metabolomic analysis: Thirty enrolled infants were diagnosed with HIE, including 17 mild, 8 moderate, and 5 severe cases. Forty enrolled infants were defined as perinatal asphyxia, having no neurological signs of HIE. 100 putatively annotated polar metabolic features were significantly different between the study groups ($p<0.05$), 29 remained significantly altered post false discovery correction. Pathway analysis revealed that ΔHIE was associated with a 50% and 75% perturbation of tryptophan and pyrimidine metabolism respectively, alongside alterations in amino acid pathways. Altered metabolites include; melatonin which increased 1.9 fold (1.4, 2.8 95%CI) in HIE; leucine, kynurenine, urea, and methoxytyrosine which differentiated between infant groups (ΔPA *vs.* ΔHIE); and tryptophan, D-erythrose-phosphate, indolelactate and N-acetylneuraminate all significantly different across grades of HIE severity.

Significant metabolite alterations were detected from six putatively identified lipid classes including fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids and prenol lipids. Fatty acyl compounds were predominantly altered (>1.5 mean fold change) in the ΔHIE comparison including fatty amide, fatty esters and polyunsaturated fatty acids compounds. Three metabolic features displayed a significant difference between comparison groups (ΔPA *vs.* ΔHIE), a glycerophospholipid (p=0.02), fatty acyl (p=0.05) and glycerolipid (p=0.05), while glycerophospholipids were significantly altered across severity grades of HIE.

*Outcome prediction:*Thirty six (model A) and thirty one (model B) infants had both UCB metabolomic analysis and neurodevelopmental outcome at 36-42 months available. Both metabolite model scores significantly correlated with outcome R=0.429 p=0.009 (model A) and R=0.549 p=0.001 (model B) respectively. Model B demonstrates the potential to predict both severe outcome (AUROC of 0.915, p=0.004) and intact survival (AUROC of 0.800, p=0.006). Neither model score correlated with infant performance in the individual BSID-III subscales (cognitive, language or motor).

*The effect of haemolysis:*The preliminarily investigation into the effect of visual haemolysis on cord metabolite concentrations identified 43 metabolites significantly altered including, lipid compounds (acylcarnitines, glycerophospholipids and sphingolipids) carbohydrates, tricarboxylic acid cycle intermediates and amino acids. Comprehensive untargeted assessment revealed on average 5% of polar and 1.5% of non-polar features were altered between paired

haemolysed and clean samples, including putatively annotated carbohydrates, amino acids, sterol and prenol lipids. However unsupervised multivariate analysis concluded that the preanalytical variability introduced by haemolysis was negligible compared with the inherent biological inter-individual variability.

Conclusion

This research has employed untargeted metabolomics to identify potential early cord blood biomarkers of HIE which may help direct treatment and has performed the technical validation of previously proposed metabolite markers. It has also detected early lipid alterations and perturbed metabolic pathways specific to HIE which could provide key insights into its pathogenesis. Furthermore, I have shown that metabolite models measured at birth can outperform other standard biochemical markers for the prediction of neurodevelopmental outcome at three years. Finally I report that the use of haemolysed samples in biomarker discovery is feasible, however haemolysis must be controlled for in the interpretation of results. This research has progressed metabolomic markers of HIE toward clinical utility. Future work will focus on the validation of these findings in a larger independent neonatal cohort recruited as part of the overall BIHIVE study.

Chapter 1

1 Introduction

1.1 Background to thesis

Despite remarkable improvements in obstetric and neonatal care, hypoxic ischaemic encephalopathy (HIE) remains a persistent worldwide problem occurring in approximately 2 of the 20 per 1000 infants born with perinatal asphyxia. Surviving children are at risk of lifelong neurological impairment and represent a large global burden of disease (1). The recent introduction of therapeutic hypothermia has further improved survival rates and critically, reduced neurological disability and improved neurocognitive outcomes (2, 3). However, for hypothermia to be effective, clinicians must diagnose HIE and commence treatment within 6 hours of birth. Yet, the biochemical and neurological assessment tools currently used to select infants suitable for therapy are lacking, resulting in some infants being missed. A diagnostic biomarker of HIE, which is rapid, robust, non-user dependent and can be measured at birth is urgently needed.

The encephalopathy triggered by a hypoxic ischaemic insult is largely metabolic, though the underlying mechanisms are not fully understood. A biochemical cascade led by a switch to anaerobic metabolism occurs including mitochondrial impairment, generation of reactive oxygen species and the accumulation of Ca^{2+}, glutamate and excitatory amino acids(4). To discover novel biomarkers of this early injury we need a greater understanding of the complex interactions of individual metabolic pathways.

Metabolomics, the systematic study of low molecular weight biochemicals (metabolites), can provide a snapshot of human metabolism which reflects the body's health at a point in time. Metabolomic studies in perinatal asphyxia and HIE have largely focused on the metabolic response to hypoxia in animal models, proposing alterations in tricarboxylic acid (TCA) cycle intermediates, branched chain amino acids and cell membrane constituents(5, 6). More recently, studies in neonatal cohorts have expanded upon these findings(7, 8), however their targeted analytical approach has limited the investigation to well-known metabolites perhaps missing subtle pathway differences. To date no study in this field has conducted an untargeted and comprehensive mass spectrometry analysis of the biochemical derangements caused by HIE and none have assessed the usefulness of proposed early markers to predict long-term outcome. Furthermore, to translate a metabolomic biomarker to the clinic, multifaceted

validation is required to eliminate false discoveries. Technical validation using alternate analytical techniques and validation in independent neonatal cohorts are required and finally, the effects of preanalytical variation on metabolomic biomarker alterations cannot be ignored.

1.1.1 Aims and objectives

Aim 1: Identify novel metabolomic markers of perinatal asphyxia and HIE through untargeted metabolomic analysis.

- Apply an untargeted metabolomic workflow to analyse the cord blood metabolome of a human neonatal cohort.
- Investigate the metabolic response to perinatal asphyxia and HIE through examination of metabolic pathways and lipid classes.
- Identify novel metabolomic markers with the potential to predict HIE.

Aim 2: Technical validation of previously identified cord blood metabolomic markers and their potential to predict long-term outcome.

- Validate metabolite markers previously proposed in the neonatal cohort under investigation, using an alternate analytical method.
- Correlate previously proposed metabolite models with three year neurodevelopmental outcome.
- Compare the ability of these models to predict neurodevelopmental outcome with current standard biochemical and clinical markers.

Aim 3: Examine the robustness of metabolite markers to haemolysis.

- Conduct a preliminary study to assess whether haemolysis may interfere with cord blood metabolite measurements.
- Apply an untargeted metabolomic workflow to examine global metabolic alteration to determine the feasibility of using haemolysed UCB as a biomarker discovery specimen.
- Assess individual metabolite alterations caused by haemolysis to ensure biomarker validity.

1.1.2 Scope

This focus of this thesis is to investigate the ability of metabolomic biomarkers detected in umbilical cord blood to predict HIE. This thesis is composed of eight chapters including an introductory chapter, background chapter, five results chapters and a concluding chapter.

*Chapter 1*introduces the clinical problem of HIE under investigation and highlights the gap in knowledge in the area of metabolomic biomarkers of this injury. It also outlines the aims and scope of the thesis, including its contribution to the research field.

Chapter 2 details all relevant background to the work described in this thesis. It provides an overview of HIE both clinically and biochemically outlining the current diagnostic assessment tools. It includes an introduction to metabolomics, the experimental strategies and most common analytical platforms available to use. A literature review of metabolomic studies in perinatal asphyxia and HIE outlines all animal and neonatal research conducted to date, as well as, the special considerations for conducting research in this field. Finally, the issue of preanalytical variation in metabolomic studies is presented focusing specifically on haemolysis.

*Chapter 3*describes polar (hydrophillic) metabolite alterations in the cord blood metabolome of infants with perinatal asphyxia and HIE versus their respective matched healthy controls, using an untargeted metabolomic method. It illustrates alterations in both novel and previously identified compounds and provides a comprehensive analysis of potential metabolic pathway perturbations.

Chapter 4 describes alterations in lipid (hydrophobic) metabolite classes for the same cohort using untargeted direct injection mass spectrometry. The extraction, analysis and annotation methods vary from chapter 3 due to the complexity of lipid analysis and high variability associated with human biological data. I identify potential lipid classes altered as a result of HIE, which warrant further investigation.

Chapter 5 presents two metabolite models proposed previously by our group through targeted and semi-targeted metabolomic investigations. These models, composed of individual endogenous metabolites measured at birth have the potential to be early biomarkers of HIE.

This chapter examines their potential to predict long-term outcome of infants with HIE who have undergone neurodevelopmental assessment at three years of age, and compares them to current standard clinical measurements.

*Chapter 6*is focused on the potential difficulties, which may arise in the metabolomic analysis of umbilical cord blood acquired from neonates at delivery, due to the physiologically high haematocrit and consequently increased rate of haemolysis. I examined the hypothesis that haemolysis will alter metabolite concentration levels measured in umbilical cord blood. Using previous analysis of metabolomics and clinical data, I described those metabolites potentially affected by hemolysis.

Chapter 7 undertakes a comprehensive analysis of how the umbilical cord metabolome is affected by haemolysis. I determined whether a haemolysed sample should be used in metabolomic biomarker discovery studies. This analysis provides detailed analytical information on all metabolic features altered by haemolysis and lists putatively annotated metabolites particularly vulnerable to interference, which may need to be excluded from practical use as a biomarker of disease.

Chapter 8 presents a summary of the main finding arising from the thesis. It outlines the implications of these findings for the clinical research field, describes the strengths and limitations of the work, summarises the contribution this work makes and discusses future directions of this research.

1.1.3 Contribution

This thesis makes a significant contribution the area of metabolomic investigations in perinatal asphyxia and neonatal HIE, by progressing metabolomic markers of HIE through the pipeline of biomarker discovery and evaluating their longterm and practical utility.

Publications arising from thesis;

Denihan NM, Boylan GB, Murray DM. *Metabolomic Profiling in Perinatal Asphyxia: A Promising New Field.*BioMed research international 2015, 9.

Denihan NM, Walsh BH, Reinke SN, Sykes BD, Mandal R, Wishart DS, Broadhurst DI, Boylan GB, Murray DM.*The effect of haemolysis on the metabolomic profile of umbilical cord blood.* Clinical Biochemistry 2015 48: 534-537

Denihan NM. *Who is the cool kid? Biomarkers to help treat newborn brain injury.* The Boolean, June 2014, 13-17.

Denihan NM, Looney A, Boylan GB, Walsh BH, Murray DM. *Normative levels of Interleukin 16 in umbilical cord blood*. Clinical Biochemistry 2013 46: 1857-1859.

For further information of the dissemination of this research seeAppendix D

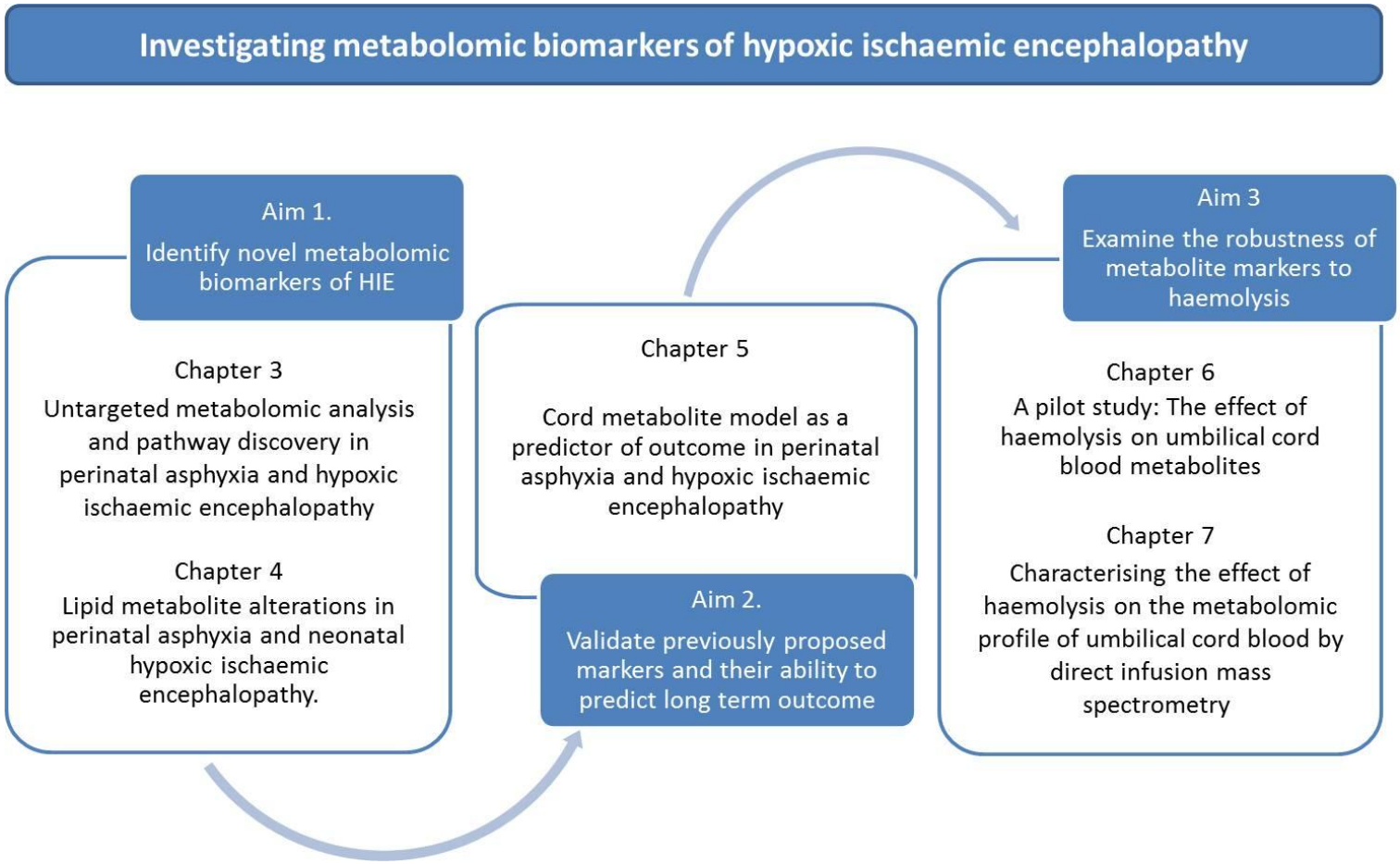

Figure 1.1Thesis overview containing the study aims and corresponding chapter..

Chapter 2

2 Background

2.1 Hypoxic ischaemic encephalopathy

2.1.1 Overview

Hypoxic ischaemic encephalopathy (HIE) is the most common cause of brain injury among full term neonates, with an incidence of 1.6 per 1000 term live births(1) and estimates as high as 26 per 1000 in the developing world (9). HIE arises from perinatal asphyxia, a persistent worldwide problem occurring in 20 per 1000 deliveries, whereby placental gas exchange to the foetus is compromised with failure of the initiation of respiratory gas exchange at delivery(10). A range of maternal, obstetric and neonatal conditions predispose the foetus and newborn to asphyxia therefore the timing of the insult varies, occurring antenatally, intra-partum or post-partum(11, 12).

The diminished oxygen supply associated with perinatal asphyxia causes hypoxemia and hypercarbia of the blood which, alongside glucose deprivation results in a derangement of aerobic metabolism. Blood flow may also be interrupted causing ischaemia which ultimately compromises cardiac and vascular functions, impairing the removal of respiratory and metabolic by-products. Depressed myocardial function will further restrict blood flow and oxygen, worsening the hypoxic-ischemic effect. It is these two intertwining pathogenic mechanisms, hypoxia and ischaemia which deprive the perinatal brain of oxygen, causing newborn HIE.

HIEis a complex dynamic injury and not a static one. Timing and duration of hypoxia-ischaemia influences injury severity, causing the injury to either evolve or in less severe cases, resolve. As a result neonates who develop HIE present with varying clinical signs. The severity of encephalopathy arising from hypoxia-ischaemia is generally classed as mild, moderate or severe and most commonly defined using the Sarnat scoring system (13), discussed in Section 2.3.2. Clinically, infants with mild HIE typically demonstrate irritability and hypertonia after delivery, and the electroencephalogram (EEG) may be normal or show mild abnormalities(14), symptoms which resolve over the first days of life. On examination infants with moderate HIE are lethargic after delivery, with hypotonia and decreased or absent primitive reflexes and may require mechanical ventilator support. The initial EEG may be moderately or severely abnormal but has the potential to improve (15). Though symptoms may

improve, the neurological exam on discharge may be variable. In severe HIE the infant presents as stuporous after delivery, with diminished response to external stimuli, absent primitive reflexes and will most likely require mechanical ventilator support. Severe HIE increases the risk of multi-organ dysfunction, seizures and the likelihood of death being the outcome.

Biochemically HIE is an evolving injury and the culmination of three distinct phases, (i) the primary hypoxic-ischaemic insult, (ii) a latent recovery phase and (iii) a secondary energy failure (see Figure 2.1). A biochemical cascade is initiated by the hypoxic and/or ischemic insult which primarily causes a switch to anaerobic metabolism, excitotoxicity and the accumulation of reactive metabolites. All of which result in cellular energy depletion and necrosis. Following resuscitation of the infant reperfusion ensues and brings with it a transient or 'latent' phase recovery of oxidative metabolism however inflammation and apoptotic cascades take hold. This is followed approximately 6-48 hours later by a secondary deterioration and failure of oxidative metabolism. This 'secondary' phase is characterised by a depletion in ATP and phosphocreatine (16), the formation of free radicals and excitotoxins, and activation of proteases and caspases which lead ultimately to permanent neuronal cell death(17, 18).

The outcome for infants with HIE is highly variable and depends upon the severity of encephalopathy. HIE can result in neonatal death while survivors are at risk of significant morbidities, discussed in Section 2.3.3. Infants with moderate to severe encephalopathy are more likely to develop disabling long-term morbidities such as cerebral palsy, visual impairment, hearing loss, severe cognitive and motor disabilities, or epilepsy(19). Traditionally, infants who sustained a mild encephalopathy were thought to develop normally(20), however, recent studies suggest they are also at risk of mild yet permanent neurological morbidities such as memory deficits, or social and behavioural difficulties(21).

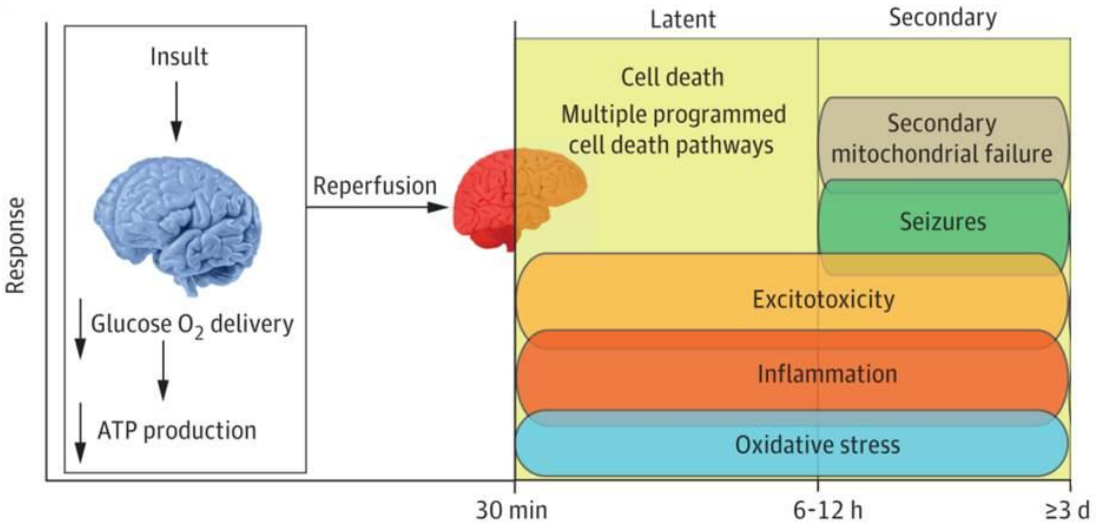

Figure 2.1 Schematic overview of the pathophysiological features of HIE, adapted from Douglas-Escobar and Weiss 2015 (22).

2.1.2 Current state of the art

Therapeutic hypothermia, whereby the infant's core body temperature is lowered to between 33-34 °C for up to 72 hours (17) is currently the only widely accepted treatment for neonatal HIE. The advent of theraputic hypothermia has for the first time, allowed us to intervene and alter the course of this disease with improved rates of mortality and intact survival (2, 23-25). It has arisen from the key insight that the primary hypoxic ischaemic insult precipitates a complex biochemical cascade, which makes a partial or latent recovery before leading to secondary neuronal damage, discussed in more detail in Section 2.2.2 and 2.2.3. It is during the latent phase that therapeutic hypothermia has the potential to intervene and alter the course of the biochemical cascade and attenuate the delayed energy failure. To date two methods, total body cooling and head cooling, have been trialled in large multicentre randomised controlled studies (26-29), and subsequent meta-analysis has deemed both modalities efficacious with significant improvement in neurodevelopmental outcome at 18-24 months in those with moderate and severe HIE (3, 30).

Though the exact mechanism of action is poorly understood, hypothermia is thought to attenuate the inflammation and cell death cascade associated with secondary energy failure and resultant damage to the brain. The latent phase presents the optimal window of

opportunity to commence cooling, prior to the irreversible secondary deterioration (31). Therefore, to be effective hypothermia must be initiated within the first 6 hours of birth, before secondary energy failure has commenced (32). By studying the injury in foetal sheep, Gunn et al. have showed the effectiveness of therapeutic hypothermia decreases when initiation is delayed. For example, early commencement of head cooling prevented the secondary cytotoxic edema and improved electroencephalographic recovery (33). Only partial neuroprotection was evident when cooling was delayed to just before secondary injury and no protection was evident once secondary injury and seizures were established (34, 35). Inclusion criteria in the large randomised control trials stated that hypothermia must be initiated within 6 hours of birth (23, 25, 26). Early cooling of asphyxiated newborns before 3 hours of age, has shown significantly improved motor outcomes(32). The consensus is now that 'time is brain', and cooling should be initiated as early as possible in eligible infants and maintained until resolution of the secondary phase of injury (32, 36). Unfortunately the timing of the primary insult is often difficult to estimate in asphyxiated neonates and some may have suffered a hypoxic ischaemic injury prior to delivery. This leaves a short window of opportunity available for treatment, making our ability to quickly and correctly diagnose HIE of paramount importance.

Currently several assessment tools are used to diagnose HIE in the delivery room including; low Apgar scores, high cord blood lactate and low cord blood pH. However, these tools are only useful at extremes having a combined positive predictive value of 25% (37). Clinical assessment using the Sarnat Score (discussed in Section 2.3.2.)is the most accurate however it requires repeated assessments and is most accurate at 24 hours, missing the narrow 6 hour treatment window. These markers suffer from inherent deficiencies to detect all infants suitable for treatment. The degree of EEG abnormality is the most efficient and reliable tool available to diagnose HIE and predict its severity. The EEG evolution over the first 24 hours of life can also strongly predict long-term outcome (15, 38). However, EEG requires highly specialised and skilled interpreters round the clock, which is rarely available in the acute setting, drastically limiting its use.

Over the past decade, the search for useful biomarkers to accurately diagnose perinatal asphyxia and HIE has become a growing area of interest. Many attempts have been made to identify reliable biochemical and haematological markers for identifying newborn infants at

risk. Previous markers such as S100B, IL-6, glial fibillary acidic protein (GFAP), neuron specific enolase (NSE), ubiquitin carboxy-terminal hydrolase L1 (UCHL-1), and phosphorylated axonal neurofilament subunit H (pNf-H) have shown some promise in small cohorts of infants with HIE (39-43). Despite the potential of many promising markers, few studies have been validated and none have led to clinical advances, or commercially viable bedside tests (44-46).

2.2 Biochemical pathophysiology of HIE

2.2.1 Normal carbohydrate and energy metabolism

Metabolism, derived from the word metabolē meaning 'change' describes the life-sustaining chemical reactions within the cells of a living organism. These enzyme mediated reactions are essential for human growth and reproduction, enabling cells to maintain their structure and respond to their environment. It also refers to the chemical reactions integral to digestion and the transport of substances between cells. At its core, metabolism involves the breakdown of organic matter to produce energy (catabolism) and construction of metabolites and other biochemicals (anabolism), for example cellular components.

Oxygen and glucose are the principle driving forces of energy metabolism in the newborn brain. It is supported almost exclusively by mitochondrial oxidative metabolism which generates adenosine triphosphate (ATP), the cell's primary energy modulator. ATP is the driving force for biochemical reactions and physiological processes in the cell and therefore its homeostasis is essential for neuronal viability. Under normal conditions a method of facilitated diffusion allows glucose to enter the brain. This diffusion is mediated by three glucose transport proteins; GLUT1 (55 kDA) allowing glucose to cross the blood brain barrier, GLUT2 (45k DA) allowing it to cross glial membranes and GLUT3 facilitating glucose transport across neuronal membranes. Compared to the adult brain, the immature brain holds relatively low levels of these proteins, limiting glucose transport and the subsequent cerebral metabolic rate for glucose.

Upon entering the brain, glucose is phosphorylated to the critical metabolite glucose-6-phosphate, which will progress down one of three paths (see Figure 2.2); (i) glycolysis and energy production, (ii) glycogen synthesis which stores the carbohydrate or (iii) the pentose monophosphate shunt associated with the synthesis of lipids. Primarily glucose-6-phosphate enters the glycolytic pathway and is converted first to fructose-6-phosphate and then to fructose-1,6-diphosphate by the rate limiting enzyme phosphofructokinase. This enzyme's activity is constrained by ATP, phosphocreatine (PCr) and low pH. Pyruvate is the major product of glycolysis and under aerobic conditions it enters the mitochondrion and is converted to acetyl-CoA via the complex pyruvate dehydrogenase. This vital complex is regulated by the

ATP/ADP ratio, in other words an increase in the ratio (energy) inhibits activity of the complex and a decrease in the ratio promotes its activity.

Mitochondrial acetyl-CoA then enters the tricarboxylic acid (TCA) cycle and is ultimately oxidised to carbon dioxide. The conversion of isocitrate to alpha-ketoglutarate is the primary rate limiting step in this cycle, regulated by the enzyme isocitrate dehydrogenase which is also governed by the ATP/ADP ratio and responds to the cells need for energy. The TCA cycle functions to produce electrons or reducing equivalents (NADH, $FADH_2$), which are converted to energy via oxidative phosphorylation. These electron donors, NADH and $FADH_2$, enter the electron transport chain where electron carrier proteins embedded in the inner mitochondrial membrane move electrons from a donor (via oxidation) to the terminal electron acceptor oxygen (reduced to water) via a series of redox reactions. These reactions release energy, creating a proton (H^+) gradient in the intermembrane space. Through chemiosmosis protons then enter the ATP synthase channel where they are phosphorylated by ADP and Pi, releasing ATP into the mitochondrial matrix.

The ATP generated by the TCA cycle and oxidative phosphorylation is transported from the mitochondria, by a specific carrier to be utilised in the developing brain. Here it has two vital functions; (i) the transport and regulation of ions and neurotransmitters for impulse transmission (e.g. cytosolic calcium and extracellular glutamate) and (ii) the synthetic production of structural and functional proteins, neurotransmitters and membrane lipids.

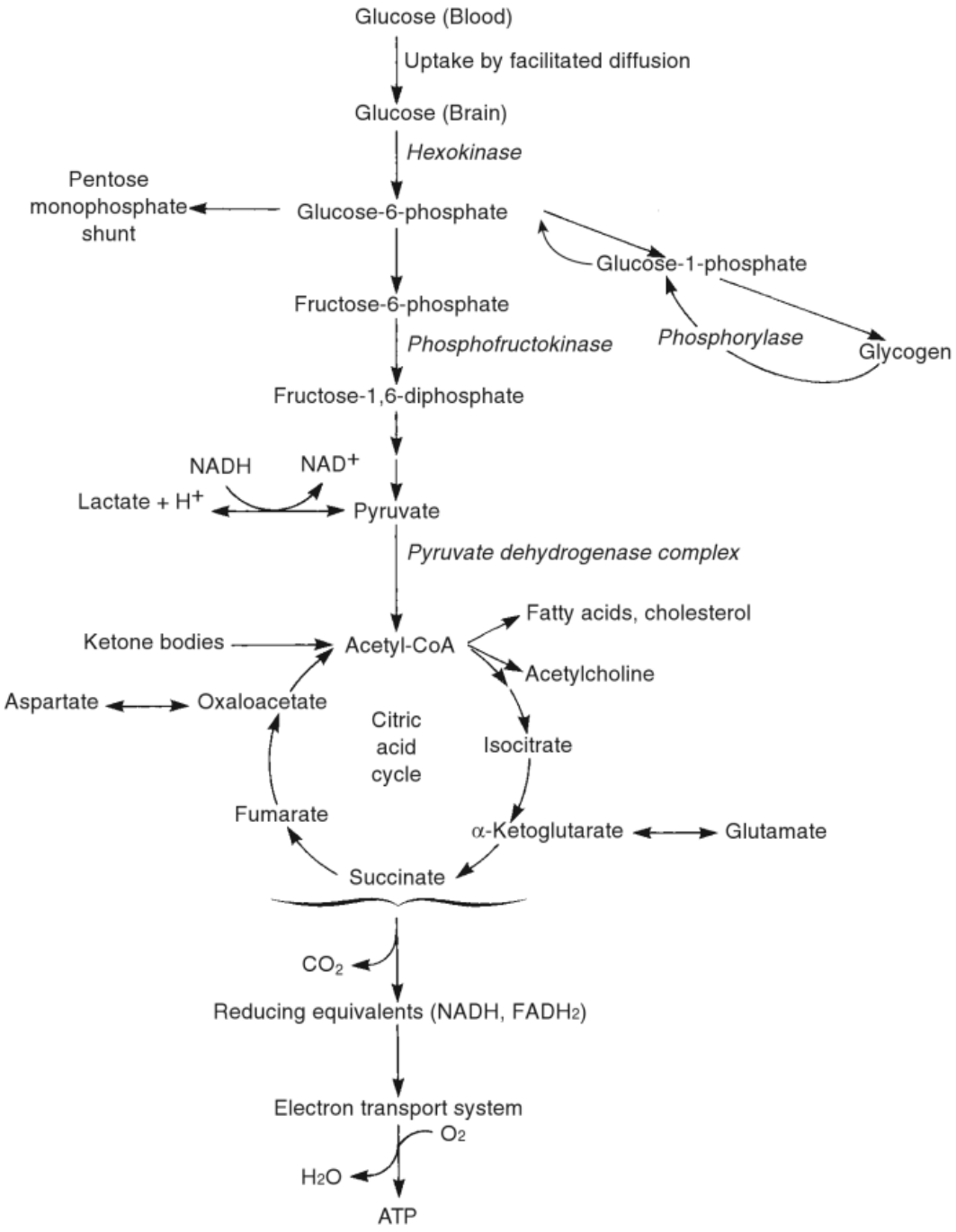

Figure 2.2 Features of carbohydrate and energy metabolism in the brain. Reproduced from Volpe 2001 (47).

2.2.2 Hypoxia-ischaemia – The energy deficit

Hypoxia and ischaemia are the two major mechanisms which deprive the neonatal brain of oxygen and glucose, the principle driving forces of carbohydrate and energy metabolism. This energy deficit will in turn alter cellular homeostasis, with neuronal and glial cells being particularly vulnerable to ischaemia. Current understanding of the injury indicates that hypoxia-ischaemia triggers a dynamic biochemical cascade of deleterious events (48).

Working in tandem, hypoxia and ischaemia quickly diminish the supply of high energy phosphate metabolites such as ATP. Inadequacies in oxygen availability inhibit normal function of the TCA cycle and electron transport chain. This causes a shift from aerobic metabolism which produces 38 ATP to the less efficient anaerobic metabolism producing only 2 ATP per mol of glucose consumed. Cells are forced to rely on glycolysis as the sole source of energy production. As an initial adaptive response, cyclic adenosine monophospate (cAMP) levels increase, up to 13 fold several minutes after ischaemia, tostimulate glycolysis via the activation of phosphofructokinase (49). To meet the demand for glucose, cAMP enhances glycogenolysis by activating phosphorylase which converts brain glycogen stores back to glucose. The glucose transporters GLUT1 and GLUT3 become upregulated and the net uptake of glucose from the blood to the brain increases. Unfortunately the impaired cerebral blood flow limits glucose supply and its utilisation becomes greater than its influx.

Lactate and its associated hydrogen ions (H^+) begin to accumulate while stores of high energy phosphates in the form of phosphocreatine (PCr) decline in an effort to preserve ATP formation (50). Animal models of ischaemia, produced for example by bilateral carotid ligation, have depicted declining levels of high energy phosphates and pH after several minutes of ischaemia(16). While studies in newborn infants with a hypoxic-ischaemic injury detected similar decreases in high energy phosphates and elevations in lactate using proton (^1H)magnetic resonance (MR) spectroscopy(31, 51). Tissue acidosis caused by the accumulation of lactate and H^+, is worsened by the impaired cerebral circulation which hampers the clearance of metabolic and respiratory by-products, in particular NADH, FADH, lactate and pyruvate. It also diminishes the ability of the bicarbonate buffering system to restore pH balance. Progressive tissue acidosis leads directly to metabolic acidosis whereby cellular injury and necrosis ensues (52).

Despite compensatory mechanisms, energy demands cannot be met and ATP levels being to fall. Using MR spectroscopy, altered brain energy stores in the form of the PCr/P_i ratio (P_i, free inorganic phosphate) were demonstrated in the neonatal dog during severe hypoxemia (53). The deficit in ATP production leads to the disruption of closely interrelated mechanisms. A decrease of 50% in PCr/P_i occurs at a critical point whereby oxidative phosphorylation is inhibited, and relates to brain lipid peroxidation and Na^+, $K^+$and ATPase activity (54). The sodium/potassium (Na^+/K^+) ATP dependant ion pumps, which function to maintain cellular homeostasis, begin to fail, hampering the export of Na^+ from the cell in exchange for K^+. In turn, intracellular levels of K^+begin to deplete while Na^+ and chloride (Cl^-) accumulate. Water can then enter the cell by osmosis causing cytotoxic edema or cell swelling(55), which may trigger cell death by necrosis.

This disturbance of ionic homeostasis leads to membrane depolarisation, whereby the negative internal charge of the cell becomes positive. Voltage dependent Ca^{++} channels begin to open, and neurotransmitter activation of specific post-synaptic glutamate receptors(NMDA and AMPA), as well as the g-protein linked metabotropic glutamate receptors (mGluR) occurs, encouraging excessive Ca^{++}entry(56). Since normal function of the ATP dependant sodium/calcium (Na^+/Ca^+) pump is disrupted there is also an accumulation of intracellular Ca^{++} by its impaired removal. This increased Ca^{++} triggers a deluge of events including the formation of toxic free radicals and the activation of enzymes involved in cell cycle control (protein kinase, MAP kinase, ERK1 and ERK2)(57). Extracellular glutamate concentration begins to increase due to the impaired energy dependant reuptake and its exaggerated release caused by depolarisation. A cycle of toxic events ensues, hinged upon the overproduction of calcium ions, membrane depolarisation and neurotransmitter activation, combined with mitochondrial damage propagated by free radicals. Though many neurons do not die during this primary phase of HI injury, a cascade of pathological processes is sparked which leads to further loss of neurons hours to days later (48).

2.2.3 Injury progression

This primary insult is followed by restoration of blood flow and oxygen to the ischaemic tissues, termed reperfusion (see Figure 2.3). During reperfusion pH returns to normal and aerobic metabolism should commence re-oxygenating the cells(58). A 'latent' or early recovery phase ensues, characterised by the transient restoration of cerebral oxidative metabolism and high energy phosphate levels return to normal (31, 33). However, alterations that occur to energy metabolism in the brain during reperfusion are not clear and it may be responsible for additional postasphyxial damage to organs and cells. The cause is multifactorial involving inflammatory reactions, an imbalance between excitatory and inhibitory neurotransmitter systems and the release of reactive oxygen metabolites.

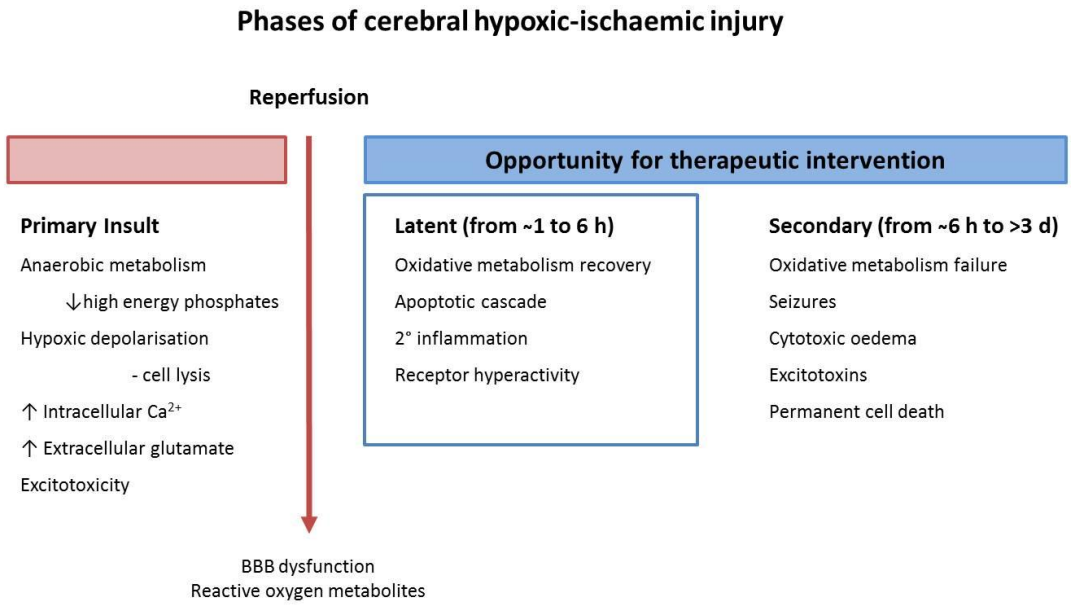

Figure 2.3 Diagram illustrating the relationship between the phases of the hypoxic ischaemic injury.

Both neonatal and animal studies have documented a secondary energy failure or neuronal damage that occurs several hours after the primary insult (16, 31, 51). Using phosphorous (^{31}P) MR spectroscopy to examine the behaviour of oxidative phosphorylation metabolites, Lorek et al. demonstrated that between 6 - 48 hours post resuscitation a gradual decline in [PCr]/[Pi] and ATP occurred, without intracellular acidosis. Moreover the severity of this secondary

energy failure was directly related to that of the primary insult (16). A similar pattern of primary insult, latent phase recovery and secondary energy failure was seen post HI which closely correlated to the severity of neuronal injury evident in rat and foetal sheep models (59, 60). The maintenance of systemic pH during the secondary phase, despite cerebral lactate increasing again, indicates alternate mitochondrial mechanisms to the primary insult. Potentially alternate methods of proton extrusion are at play or there is heterogeneous damage of mitochondria which is unable to disrupt pH (58).

The exact mechanisms underlying the progression to secondary energy failure are unknown however it goes beyond glucose and energy metabolism. It is thought mechanisms of secondary injury are centred on the vulnerability of mitochondria to damage following the primary insult including; oxidative stress and free radical accumulation, inflammatory factors, primarily the cytotoxic action of delayed microglial cells; excitotoxicity, the extracellular build-up of excitatory amino acids and glutamate; and ultimately cell death by apoptosis (61). Some of the biochemical effects of hypoxia and ischaemia, for example, ionic pump failure, neurotransmitter redistribution and membrane alterations can also predispose the brain to seizure activity. Seizures may in turn contribute to delayed neuronal loss (48). The severity of secondary energy failure correlates with neurological deficits and injury. Emerging targets for early intervention and neuroprotection have been focused on the inhibition of these potentially destructive molecular pathways.

2.3 Current assessment tools

Infants who experience asphyxia severe enough to adversely affect brain homeostasis will exhibit signs and symptoms of neurological dysfunction in the neonatal period (10). This is the clinical entity of Hypoxic-Ischaemic Encephalopathy. We currently use a number of tools, each with varying degrees of accuracy and usefulness, to help diagnose or assess the severity of this state and to attempt to establish prognosis.

2.3.1 Clinical and biochemical

Apgar score

The Apgar score was first proposed as a method to evaluate the newborn infant in 1953, by Dr Virginia Apgar then Professor of Anaesthesiology at Columbia University (62). The purpose of the Apgar score was 'to establish a simple and clear classification of newborn infants which can be used to compare the results of obstetric practises, types of maternal pain relief and the results of resuscitations' (63). Dr Apgar proposed five clinical signs; heart rate, respiratory effort, reflex irritability, muscle tone and colour, all of which could be quickly and easily evaluated by staff in the delivery room. The score is the sum of the five aspects, whereby each is assigned a grading of 0, 1 or 2 depending on absence or presence, one minute after the infant's birth.

The Apgar score is now almost universally assigned in the delivery room however, more recently its use has come under scrutiny for going beyond the scope of its initial remit. Large cohort studies have demonstrated a strong correlation between the Apgar score at 5 min of age and neonatal mortality, yet its sensitivity and positive predictive value (PPV) for death and cerebral palsy in survivors is low (64, 65). Often felt to be useful at extremes, one in five infants with an Apgar score of 0 at 10 minutes will survive to school age without a moderate or severe disability (66). The Apgar score is also inherently subjective, with the exception of heart rate the clinical signs are all assessed by visual inspection of the infant. A study by O'Donnell et al. found large inter-observer variability between scores (67).

The Apgar score was never intended to be a measure of perinatal asphyxia or outcome, as Apgar herself noted, 'while we believe the score is useful, it has many limitations... nor will it

predict neonatal death or survival in individual infants' (68). A low Apgar score is nonspecific and may occur in the absence of global hypoxic-ischaemic brain injury, however a consensus statement from the American Congress of Obstetricians and Gynaecologists (ACOG) notes that in combination with biochemical, neuroimaging, physiological markers and timing, an Apgar score of < 5 at five and ten minutes can be useful in determining the likelihood that an acute peripartum or intrapartum event may have contributed to neonatal encephalopathy (69). More recently, efforts have focused on creating an expanded or combined score by combining the conventional Apgar score with several resuscitation parameters (70). A low 5 minute combined Apgar score could diagnose perinatal asphyxia (sensitivity 97% and specificity 99%), and independently predict the occurrence of HIE, however it could not predict the severity of HIE or adverse neurological outcome (70).

Acid-base balance

Foetal asphyxia has been defined as 'a condition of impaired blood gas exchange leading to progressive hypoxemia and hypercapnia with a significant metabolic acidosis' (71). Foetal acidosis is the accumulation of acid in tissues leading to a low blood pH (acidaemia). It is commonly used as a marker of foetal oxygenation and distress. The oxygen deficit in foetal or perinatal asphyxia necessitates a switch from the metabolism of carbohydrates to anaerobic metabolism which produces lactic acid causing metabolic acidosis. Foetal asphyxia can also cause respiratory acidosis characterised by accumulating carbon dioxide, arising from compression of the cord, decreased foetal cardiac output or insufficient placental perfusion (72).

After delivery, a segment of the umbilical cord is double clamped and blood from the section of the cord between the clamps can be sampled for acidosis, usually measured as pH or base deficit. Mild acidosis occurs in most labours and is directly related to the length of labour (73), however, more severe acidosis is associated with newborn morbidity (71, 74). However infants born with perinatal asphyxia may not always demonstrate acidaemia in umbilical artery blood at birth, and recent studies have shown that severe acidaemia is not a prerequisite for those who suffer a subsequent brain injury. Ruth and Raivio described umbilical artery metabolic acidosis as a poor predictor of perinatal brain damage, with a PPV of 8% (75). In a large prospective cohort study Dennis et al. did not find any statistically significant associations

between acidosis and developmental outcome. In fact, infants who were most severely acidotic at birth (pH less ≤ 7.04) were more likely to be disability free as children, and those with low Apgarscores but normal pH were found to be most at risk of impairment (76). Several retrospective studies have also reported that about half of all infants born asphyxiated with resultant encephalopathy were born without severe acidosis (77). More recently Murray et al. examined pH in a clearly defined population of infants with HIE and found that pH was unable to differentiate between those with mild and moderate 7.05 (6.95- 7.14) or severe 7.01 (6.83-7.05) HIE (78).

In response to the evidence that in the absence of metabolic acidosis the infant may be at greater risk of encephalopathy or impaired outcome, Hermansen has introduced the concept of 'beneficial acidaemia' whereby the failure of an asphyxiated infant to establish acidaemia may place the infant at increased risk for brain injury (77). Both human and animal studies have illustrated the newborn infant's ability to use lactic acid as an alternate fuel source for brain metabolism (79). Animal studies have also shown that lactic acidaemia may increase cerebral blood flow, potentially reducing the risk of cerebral ischaemia (80). Finally, the Bohr Effect promotes the shift of the oxygen dissociation curve in response to a falling pH. This decreases oxygen's affinity for haemoglobin and promotes its beneficial unloading into the surrounding tissue.

Similar to the Apgar score, foetal acidosis should not be used in isolation to diagnose perinatal asphyxia or subsequent HIE, but in combination with other clinical markers. A foetal umbilical artery pH of < 7.0 or base deficit ≥ 12 mmol/L can alert clinicians to a potentially damaging peripartum or intrapartum event (69). A pH or base deficit measurement cannot indicate the aetiology of injury by itself, only the degree of acidosis at that specific time point. More importantly, the absence of metabolic acidosis does not exclude a diagnosis of perinatal asphyxia or HIE.

Lactate

Lactic acid is the primary end product produced by the fermentation of pyruvate to yield 2 ATP during anaerobic metabolism. Therefore, it is directly related to tissue hypoxia and the ensuing metabolic acidosis, and may be a more precise measurement of foetal acidosis than indirect measures like pH or base deficit. Studies have shown strong correlations between lactate and

pH/base deficit in UCB samples and postnatal infant samples (81-83). More recently, Bowler and Beckmann demonstrated that a foetal scalp lactate measurement has a high sensitivity but low specificity in predicting foetal umbilical blood pH (84).

Much like pH and base deficit, there is varying evidence as to whether lactate can identify those with perinatal asphyxia or HIE. A lactate level of 8 mmol/L has been suggested to indicated intrapartum asphyxia, based solely on its correlation with a base deficit of 12 mmol/L (82). In asphyxiated neonates, da Silva et al. reported an association between plasma lactate concentrations of >9 mmol/l and moderate or severe encephalopathy with a sensitivity of 84 % and specificity of 67 % (81). However the authors mention that between their established upper (>9 mmol/l) and lower thresholds (<5 mmol/l) 'neonatal evolution was unpredictable as a number of infants tolerated severe asphyxia without central nervous system damage, while others developed serious cerebral injury'. A study by Kruger found that foetal scalp blood lactate was a superior predictor of moderate to severe HIE compared to pH, and suggested a lactate concentration of > 4.8 mmol/L as suitable cut-off limit to indicate foetal asphyxia (85).

Though Murray et al. described a raised serum lactate in all infants with HIE, the degree of lactic acidosis in the first 30 minutes of life did not predict the severity of the ensuing encephalopathy. On the other hand, the prolonged time to normalisation of serum lactate was related in particular, to the electrographic seizure burden (78). This is in agreement with similar serial lactate measurements by Shah et al. (86). Proton magnetic resonance spectroscopy has also shown cerebral lactate to be persistently raised, up to one month after birth in infants with HIE and an abnormal neurodevelopmental outcome (87). They hypothesised that an abnormality in cerebral energy metabolism, perhaps due to by dysfunctional mitochondria, brought about the prolonged elevation of lactate. The raised lactate levels may also be attributed to delayed lactate clearance, which may indicate hepatic dysfunction, a systemic effect of severe HIE.

The severity of lactacidaemia may reflect the degree of foetal hypoxic-ischaemia, but a single lactate measurement gives no definitive information regarding the duration of asphyxia, the foetal response to hypoxia, or risk of an ensuing encephalopathy.

2.3.2 Early neurological outcome measures

The Sarnat Score

The Sarnat score was developed in 1976 by Harvey and Margaret Sarnat, as a method of classifying neurological dysfunction in infants. The Sarnat scoring system classifies infants as having either mild (Sarnat I), moderate (Sarnat II) or severe (Sarnat III) encephalopathy, and is derived from original observations the authors made by recording clinical and EEG data from 21 infants with HIE (13). The clinical neurological sequelae assessed by the Sarnat score are thought to be better predictors of outcome than Apgar scores and blood gases (88).

Sarnat and Sarnat took account of the fact that HIE is an evolving encephalopathy, and that the neurological symptoms may worsen and/or recover over the first days of life. In practise the infant may shift from one stage to another illustrating the continuum of the injury. In their original investigation all 21 infants received neurological examinations at regular intervals, over the first 6 days of life, and subsequently on alternate days until death or discharge. An EEG recording was performed at 24 hours and then every 3-5 days until discharge (13).

Robertson and Finer suggested the prognostic values of the stage of encephalopathy is greatest when evaluated over a seven day period, and Amiel-Tison and Ellison found the evaluation of the infant on day 7 to be most useful (89). This outlines the importance of assessing the infants repeatedly over the first few days, to ensure the injury severity is properly categorised.

One drawback with the Sarnat score is that it requires access to EEG. To this day, in the majority of neonatal intensive care units, a neonatologist may not have ready access to multi-channel EEG in the acute setting. To overcome this issue, the Sarnat score has been modified to exclude EEG and in some instances the score's clinical parameters were also simplified (88, 90). Levene's modified Sarnat score, Table 2.1, has been widely adopted by clinicians and researchers worldwide, and is now considered a robust tool for the grading of encephalopathy and indicating prognosis. For example, Murray et al examined 51 neonates with HIE and found that Levene's modification of the Sarnat score at 24 hours was strongly correlated to outcome (r=0.64, p=0.001) (91). Interestingly, they found of the infants graded moderate HIE, half had a normal outcome and half an abnormal outcome at 2 years, highlighting the difficulty in predicting outcome or accurately defining moderate encephalopathy.

In the absence of EEG, careful neurological assessment remains the best clinical method of assigning prognosis in neonatal HIE and is useful for directing clinical care pathways regarding long-term follow up. However, an accurate clinical grade of encephalopathy takes over 24 hours to assign and this may be hampered by the use of sedatives or anti-epileptic medications, which make clinical assessment of HIE severity more difficult (14). Therefore the optimum time for utilising clinical assessment is beyond the window for the initiation of therapeutic hypothermia and may also be too late for alternate neuroprotective therapies. In addition therapeutic hypothermia in itself will reduce the reliability of clinical assessment (92).

Table 2.1 The Modified Levene Classification– distinguishing features of the three clinical stages of postanoxic encephalopathy in the full-term newborn infant.

Grade 1 (mild)	Grade 2 (moderate)	Grade 3 (severe)
· Irritable 'Hyperalert'	· Lethargic	· Comatose
· Mild hypotonia	· Marked abnormalities of tone	· Severe hypotonia
· Poor Sucking	· Requires tube feeding	· Failure to maintain spontaneous respiration

Electroencephalography (EEG)

Electroencephalogram (EEG) is a method of physiological monitoring which measures the electrical activity in the brain. Brain cells communicate via electrical impulses which can be detected using electrodes attached to the scalp and amplified and displayed using an EEG machine.

Continuous multichannel EEG is an important tool for the early assessment of HIE(93)and detection of electrical seizures(94). Previous studies have found a strong correlation between the grade of HIE as defined by EEG and infant outcome (13, 15, 95).Grading of HIE using multichannel EEG is based on visual analysis of the background activity. The background activity is usually classified by interpreting features such as amplitude and symmetry, continuity of the signal, frequency and seizures, transients and sleep-wake states (96, 97).

Increasingly one or two channel amplitude integrated EEG (aEEG) is used in the NICU because it allows for quicker and easier application (two electrodes) and its perceived ease of interpretation. For said reasons, aEEG was used as an inclusion criteria measure for the TOBY randomised control trial for therapeutic hypothermia (23). Though very useful in the hands of experienced aEEG users(98, 99), an international survey of neonatologists has shown that many are not confident in their ability to interpret the aEEG (100).

Unlike clinical evaluation, continuous EEG allows the user to monitor the early evolution of the encephalopathy, not just a specific time point. For example, Murray et al. have found the worst EEG grade of HIE to be apparent as early as six hours after birth (15). This real time measurement of cerebral function is relatively easy to implement at the cot-side. Unfortunately

neonatal EEG is complex and difficult to interpret. It requires neurophysiologists with the required neonatal experience or a clinician with expertise in EEG to be available around the clock to interpret the recordings. This resource is scarce, meaning the application of EEG is restricted to a minority of neonatal units. Furthermore there is still no single standardised classification system available to grade neonatal HIE (97).

Despite these drawbacks, EEG remains the gold standard for accurately determining HIE grade. The EEG background consistently correlates with the clinical grade of HIE and long-term outcome (15, 93, 101). A prerequisite being that the EEG must be recorded and analysed at multiple time points over the first 48 hours of life, and ideally in a continuous fashion. The classification system widely used by this research group is given in Table 2.2. It has been published and validated in a large cohort of neonatal HIE with long-term follow up, and found to be highly accurate (14, 15, 37, 102).

Table 2.2 Classification of EEG Background Activity(15).

Grade	Description of features
Normal	Continuous background pattern with normal physiologic features such as anterior slow waves
Normal/mild abnormality	Continuous background pattern with slightly abnormal activity (e.g., mild asymmetry, mild voltage depression, or poorly defined SWC)
Moderate abnormality	Discontinuous activity with IBI of <10 s, no clear SWC, or clear asymmetry or asynchrony
Major abnormality	Major abnormalities Discontinuous activity with IBI of 10–60s, severe attenuation of background patterns, or no SWC
Inactive EEG findings	Background activity voltage of <10 μV or severe discontinuity with IBI of >60 s

IBI: inter-burst interval; SWC: sleep wake cycle;

2.3.3 Long-term outcome in HIE

HIE can lead to significant lifelong morbidity which represents a large global burden of disease. An ideal biomarker measured at birth would provide prognostic clinical information not just for the immediate neonatal period, in terms of identifying and predicting severity of HIE, but also

for the longer term neuropathological and neurodevelopmental outcome of the child. A recent systematic review estimated that in 2010, 1.15 million babies suffered from HIE resulting in 287,000 deaths, 233,000 infants surviving with moderate/severe disability and 181,000 living with mild impairment (1). Several long-term neurological morbidities are associated with HIE. For example approximately 10-13% of infants with moderate-severe encephalopathy develop cerebral palsy, with dyskinetic cerebral palsy and spastic quadriplegia being the most common subtypes (103). HIE may also disrupt sensory functions causing visual impairment (104) or hearing loss (105), or result in long-term seizure disorders such as epilepsy (103). Learning difficulties may also be present with or without motor or sensory dysfunction. Long-term follow up of infants with an encephalopathy has revealed; reduced school readiness including a drop in attention scores and increased irritability at five years old (106), memory and executive function impairments at 7 years (107) and lower working memory, reading accuracy and comprehension scores between 8-11 years (108). Learning impairment, neuropsychological deficits and behavioural difficulties are seen in over 40% of surviving children at school age (109).

A metabolic biomarker which could predict long-term outcome for infants post HIE would provide powerful clinical utility for guiding clinical management, informing parents and long-term resource planning. It could determine response to treatment and allow early referral to medical, educational or support services to maximise each child's potential. For infants with a predicted severe neurological outcome such knowledge may guide implementation of experimental therapies or redirection of care.

To accurately assess our ability to predict long-term outcome in infants with HIE standardised methods are required. Subtle deficits in learning and memory may not become apparent for many years, but provide a significant societal burden following neonatal HIE. Therefore, long years of recruitment need to be followed by years of careful follow up to progress this rapidly evolving field. Retaining participants for long-term follow up studies can be difficult and continued communication with parents is essential (110).

There are many neurodevelopmental outcome measurement tools (107). One such tool widely used to assess cognitive function in high risk infants is The Bayley Scales of Infant and Toddler Development Edition III (BSID-III) (111). Composed of a series of administered tasks, it is a

standardised developmental test used primarily to assess the motor (fine and gross), language (receptive and expressive), and cognitive development of infants and toddlers ages 0-3 years. Raw scores of successfully completed tasks are converted to scaled and composite scores, to measure child's performance through comparison with normative values for their age. A mean (± standard deviation (SD)) score of 100±15 is deemed normal on the Cognitive, Language and Motor subscale composite scores, while scores less than 85 (1 SD below the mean) are an indicator of neurodevelopmental impairment. Large clinical trials of therapeutic hypothermia have used the BSID-III to assess infant outcome at approximately 18 months of age (23, 27). However, performance on developmental assessments can be influenced by general motor dysfunction. This can be formally evaluated in the toddler period by structured physical examination such as the Gross Motor Function Classification Score or Dubowitz score (112, 113).

2.4 Metabolomics

2.4.1 Metabolites, metabolomes and metabolomics

Metabolomics can be defined as the comprehensive study of the metabolome, a collection of all low molecular weight biochemicals or 'metabolites' present in an organism(114).

Metabolites are a direct signature of biochemical activity, serving as substrates, intermediates and products of enzyme mediated cellular metabolism and thus are closely correlated with phenotype (115). They represent the final end products of gene expression, yet are also the building blocks for all biological structures, for example, proteins (amino acids), genes and transcripts (nucleotides) and cell membranes. Metabolites have low molecular weights ranging from 1 (proton) to 2000 Da for lipids and though predominantly organic molecules (amino and fatty acids, carbohydrates, vitamins, and lipids), inorganic metabolites such as iron have an equally important role to play (116). The physiochemical properties including; molecular weight, hydrophobicity or hydrophilicity, pH and boiling point can differ between metabolic compounds. For example, hydrophilic metabolites are low molecular weight polar compounds such as amino acids, while hydrophobic metabolites are higher molecular weight non-polar compounds such as lipids. This diversity necessitates the need for several analytical strategies, including different analytical platforms, in order to analyse the complete complement of metabolites in a biological system. Yet, it also aids the classification of metabolites, which can be based upon polarity (polar or non-polar), molecular weight, chemical structure and presence in a metabolic pathway (117).

The metabolome has been described as the quantitative complement of metabolites in a biological system(118). The seminal work of both Kacser and Burns, and Heinrich and Rapoport, recognised that metabolomes could not be understood in isolation and instead indeed to be treated as systems of interacting components (119, 120). The human metabolome consists of a network of interacting metabolites, which vary in size and complexity depending on the sample type analysed. Metabolomes are also interconnected providing 'cross talk', for example that of blood and urine. Currently it is estimated that several thousand metabolites comprise the human metabolome however this may be an under-estimate as many lipids, drug metabolites and pollutants go undefined(117, 121). Metabolites within the human metabolome are derived

from enzyme catalysed chemical reactions central to metabolism. These include catabolism, the breakdown of products to produce energy, and anabolism; the construction or production of chemicals. The metabolome offers the unique potential to examine the mechanistic biochemistry which relates to the cellular phenotype (115).

The aim of metabolomics is to observe and quantify the full complement of metabolites in a cell or system, creating a biochemical profile (122). Developments in analytic platforms for example, mass spectrometry (MS) allow the rapid and simultaneously measurement of thousands of metabolites. Metabolomics is particularly powerful when used in combination with theory, bioinformatics and computational statistics, as a systems biology approach(123).However, many detected metabolites have yet to be included in metabolite databases and repositories, indicating the breath of cellular metabolism still unknown (124). The field of metabolomics is expanding rapidly, as too are its applications in for example toxicology testing, nutritional analysis, plant and microbial analysis and clinical metabolomics.

Metabolomics enables the measurement and examination of endogenous compounds and with high-throughput capacity increasing the coverage. Regarding clinical utility, one important application of this is the identification and determination of 'biomarkers' or risk factors that change as an indicator of disease or injury. Another is the acquisition of a greater understanding into the pathophysiology of disease onset and progression. Metabolomics and the measurement of endogenous biochemicals have already been translated successfully to the clinical environment particularly in the field of paediatrics. For example, using mass spectrometry newborn babies are screened for inborn errors of metabolism related to defects in 30 enzymes that catalyse the metabolism of carbohydrates, amino acids, fatty acids, nucleic acids and the urea cycle (125-127).

2.4.2 Experimental strategies

In the investigation of metabolite markers two metabolomic approaches are usually taken, an untargeted hypothesis generating approach or a targeted analysis of a defined set of metabolites specific to a disease or mechanism, see Figure 2.4(122). Ultimately the experimental strategy with respect to sample preparation and analytical instrumentation depends upon the number and chemical composition of metabolites to be studied.

Untargeted metabolomics or 'metabolite fingerprinting' aims to simultaneously detect as many metabolites as possible (hundreds or thousands) in a given biological sample(128). This method is hypothesis generating and no *a priori* knowledge of the metabolites of interest is needed. It acts as a screening tool to investigate whether metabolite features can discriminate between samples of different biological status without bias. Metabolite fingerprinting requires highly sensitive analytical platforms capable of rapid and high throughput peak detection from biological samples. MS is one such platform, whereby peak can be referred to as either metabolite or mass feature and corresponds to a detected ion with a unique mass to charge ratio. Generally sample preparation is simple and chromatographic separation is absent to ensure the global analysis of metabolites is possible. Quantification and metabolic identification are generally not employed. Untargeted metabolomic analysis produces very large and complex datasets, it is impossible to inspect these manually therefore researchers rely on metabolomic software to refine the data. Metabolite identification remains a challenge though can be achieved to some extent through in silico library and database searches. However untargeted metabolomics has wide applicability in answering biological questions, given its sensitivity, high throughput and minimal sample requirements, for example it has the potential to provide insight into biological mechanisms that change as a function of health and disease (124). In particular it has facilitated the characterisation of unknown pathways and been instrumental in the identification metabolomic pathways altered by disease that represent novel drug targets 'therapeutic metabolomics' (129).

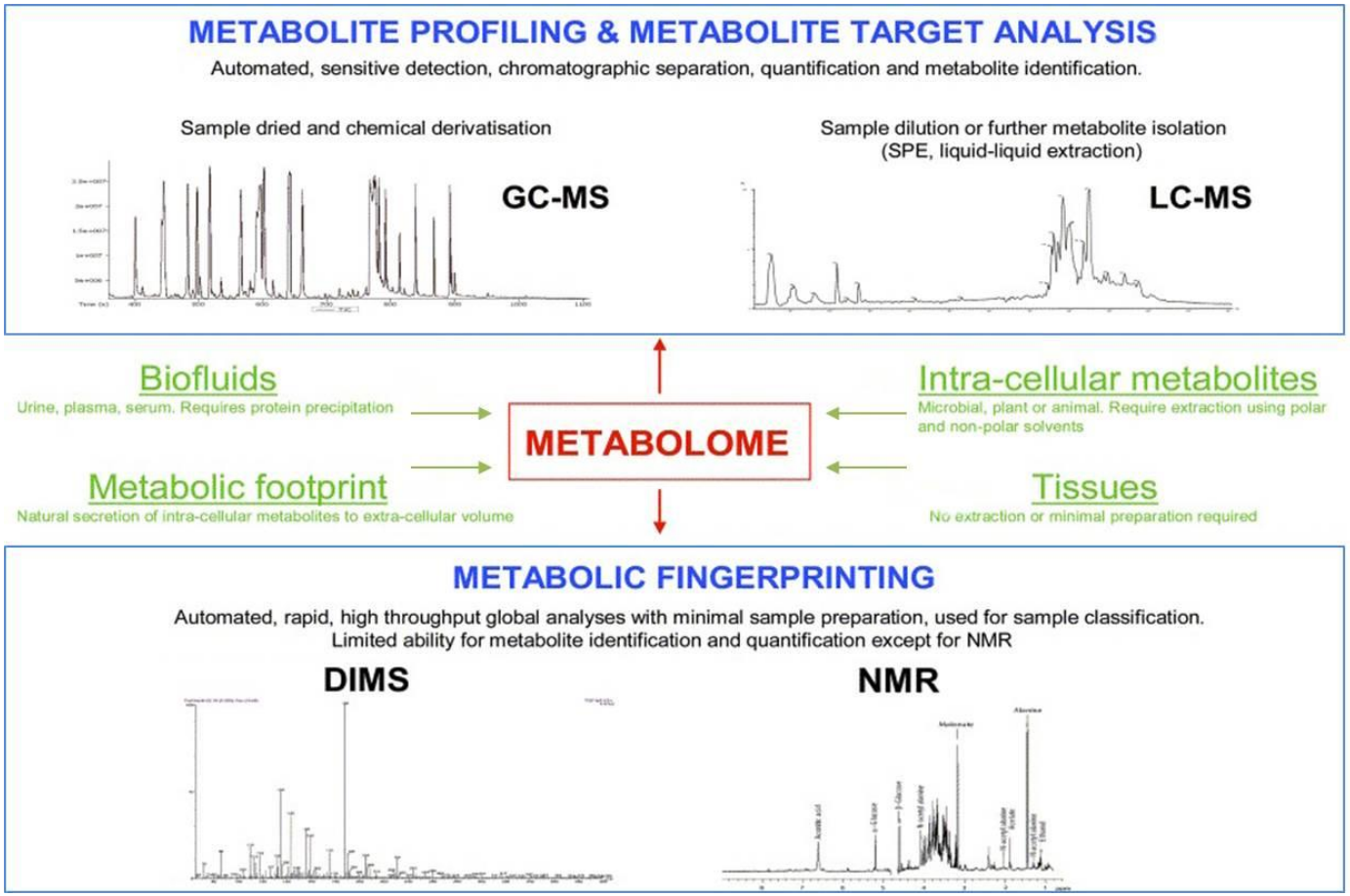

Figure 2.4Summary of the different metabolomics-based strategies, adapted from Dunn and Ellis 2005 (130).

Targeted metabolomics refers to the measurement of a specified number (usually < 20) of pre-defined metabolites (131). This approach is hypothesis driven and usually focuses on the investigation of particular metabolic pathways. Sample preparation is more extensive and chromatographic separation is generally used to isolate the candidate markers prior to detection by sensitive tandem MS (MS/MS) or ultra violet (UV). Isotopic internal standards for the compounds of interest are needed for their absolute identification and quantification with high specificity, precision and accuracy.

Semi-targeted methods have recently been developed to quantify or semi-quantify the concentration of a greater number of metabolites (≤400) with higher specificity, precision and accuracy then for untargeted studies(122). One such method is the Biocrates Absolute-IDQ targeted metabolomic technology (BIOCRATES, Life Science AG, Innsbruck, Austria)(131, 132). This validated assay incorporates metabolite standards to allow for the simultaneous detection and quantification of metabolites in a high-throughput manner. Metabolites from varying metabolic pathways and chemical classes are included to provide wide coverage of the metabolome. This strategy can rapidly apply chemical identities and concentration values which facilitates the translation of results to practical applications.

Both untargeted and targeted metabolomics are useful methods in metabolic biomarker investigations which need to progress through a pipeline of (1) biomarker discovery, (2) study validation, and (3) cohort validation (122) before they can reach clinical utility (see Figure 2.5). Typically biomarker discovery might begin with metabolite fingerprinting (untargeted) to define unbiased pathway or mechanistic alterations between case-control groups. The large number of detected metabolites makes this method susceptible to reduced precision and accuracy, increasing the potential of random chance discovery even when statistical corrections are applied. Therefore, careful validation is needed to ensure findings are reproducible and the candidate markers can progress. Study validation or technical validation can be achieved through metabolite profiling (semi-targeted) whereby a select number(<100) of pre-defined metabolites are identified (128) or detected using an alternate metabolomics method. Finally, cohort validation (targeted), which focuses on a short list of valid and reproducible candidate markers is performed using a larger, independent patient cohort. A targeted metabolomic approach is employed for the qualitative and quantitative analysis of candidate markers.

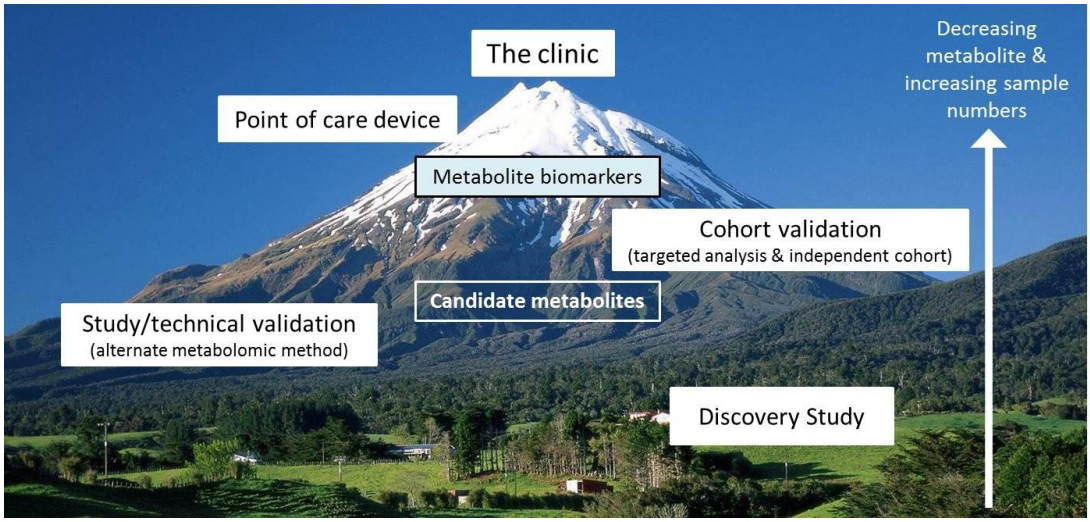

Figure 2.5The journey of a metabolite biomarker discovery study. A pipeline of biomarker discovery, study/technical validation and cohort validation, before translation to the clinic.

2.4.3 Analytical platforms

Mass spectrometry (MS), often coupled to chromatography and Nuclear Magnetic Resonance (NMR) spectroscopy are the most widely applied analytical platforms in metabolomics today, with their range extending from metabolite profiling and target analysis to metabolic fingerprinting, see Figure 2.4(130). Many analytical technologies exist therefore it is important to remember that each offers specific advantages depending on the research question. The diversity of metabolites in terms of concentration and physiochemical properties means that no individual platform is appropriate for all investigations. Furthermore, no single platform can offer precise, accurate identification and quantification of all metabolites of interest.

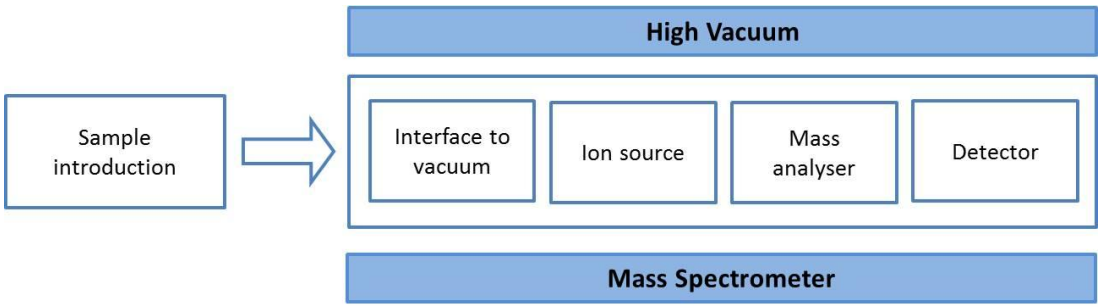

Figure 2.6 Schematic workflow of a mass spectrometer.

Mass spectrometry

A mass spectrometer is designed to separate gas phase ions according to their mass to charge ratio (*m/z*) value. Its four main components are the ion source, mass analyser, detector and data collection module (computer), see Figure 2.6, in addition to a vacuum system, tools to introduce the sample and tools to produce the gas phase.MS operates by forming ions (either positively or negatively charged) at the ion source from the sample of interest. The mass analyser then separates the ions based on their mass to charge state using an electrical or magnetic field. This operates under high vacuum, moving the ions along to the detector. Importantly, the high vacuum reduces the occurrence of ion-ion or ion-molecule collisions which impinge mass resolution, mass accuracy and instrument sensitivity. The data collection, module produces a spectrum of mass to charge ratio values and the corresponding intensity of the detected ion.

MS is typically used in the analysis of gaseous or liquid biological samples therefore an important requirement is that the ionisation process must not destruct the biomolecules. For this purpose the soft ionisation techniques, Matric Assisted Laser Desorption Ionisation (MALDI) and Electrospray Ionisation (ESI) were developed, allowing biochemical ionisation at either high vacuum (MALDI) or atmospheric (ESI) pressure respectively.

A mass spectrometer is set to scan mass ranges of 20 to 2000 Da; the typical range of interest for metabolomic researchers. Its main advantage is its high sensitivity providing the ability to simultaneously detect hundreds to thousands of metabolites per sample. The resultant mass spectrum can be used for sample classification and if the instrument is of high mass accuracy

and resolution, chemical identification of metabolites is possible. However, automated identification of molecules remains difficult and the identification of all detected features is unattainable.

Direct injection - mass spectrometry (DI-MS)

The high complexity of metabolomes demands for MS techniques to produce high mass accuracy to ensure fit for purpose mass separation of the majority of metabolites detected, particularly in untargeted metabolite investigations. The sample is directly introduced into the mass spectrometer's ionisation source, negating the need for chromatographic or electrophoretic separation prior to detection. DI-MS facilitates the analysis of a large number of samples as process times are generally <1 min, increasing inter-sample reproducibility.

DI-MS generally utilises ESI to allow robust interfacing of the liquid sample and mass spectrometer. As an ionisation source, ESI uses electrical energy to create droplets of either positively or negatively charged ions which are subsequently evaporated, passed into the MS and mass analysed (133).Only compounds that can ionise by the addition (ESI(+)) or removal (ESI(-)) of a proton or ionic species can be detected. Wider metabolome coverage can be achieved when both polar and non-polar metabolites are ionised in both positive and negative mode. ESI will fail in the presence of high salt, acid or base concentrations therefore composition of the sample solution is important. Automated nano-ESI systems (Advion Biosciences Inc.), a chip containing an array of nano-electrospray nozzles, have been created to facilitate high throughput fingerprinting (134, 135).

As mentioned, untargeted metabolomic investigations require high mass accuracy and resolution. Mass resolution defines the ability to distinguish two peaks with similar *m/z*values and is calculated by dividing the *m/z*value of the peak by the Full Width Height Maximum (FWHM), in other words the full width of the peak at half the maximum peak intensity. Higher mass resolutions produce narrower peak widths and hence the ability to separately detect metabolites of similar but not identical accurate mass. Mass accuracy defines the error of the determined mass of a metabolite compared to the theoretical mass, therefore high mass accuracy = low mass error. Mass error is the difference between the theoretical *m/z* and the observed *m/z*in a spectrum, normalised to the *m/z*.

Achieving high mass accuracy and resolution is challenging, particularly in the absence of prior chromatographic separation, as peaks with differences in monoiostopic masses <0.025 Da can overlap. To overcome this problem mass analysers such as Fourier Transform Ion Cyclotron Resonance (FT-ICR) are used (136). Detector plates record the charge of proximal groups of ions as a function of time and Fourier Transform is used to deconvolute signals into frequencies which are proportional to the *m/z*value of the ions. Though expensive, FT-ICR offers very high mass resolution (>1,000,000) and currently, the highest available mass accuracy (<1ppm external calibration, <0.1 internal calibration) and low detection limits in the attomole to femtomole range (137). This allows all possible metabolite peaks to be resolved and their empirical formula to be accurately calculated, making DIFT-ICRMS a promising tool for metabolite fingerprinting investigations(138).

As with all metabolomic platforms DI-MS has a number of limitations. Definitive metabolite identification is limited as chemical isomers, or metabolites with the same exact mass but different chemical structure, such as the sterioisomers glucose and fructose, cannot be distinguished and will be detected as a single *m/z*. Ion suppression is a concern when using ESI, as all sample components are introduced simultaneously into the ionisation source. Both signal suppression and enhancement can occur, affecting the sensitivity (micromolar limits of detection) and impeding any meaningful data analysis (128). As well, the sample physically interacts with the instrument therefore the response can change over a short to medium period of time. To avoid this drift, an identical quality control sample can be injected throughout each batch and using correction algorithms the inter- and intra- batch variation can be adjusted for (135).

For the untargeted metabolomic analysis of the umbilical cord blood, it was important to choose an analytical platform which could achieve high coverage of the metabolome therefore high mass accuracy and high sensitivity was required. Therefore DI nESI FT-ICR MS was the instrument chosen to acquire data for this thesis.

Other popular platforms used in metabolomic applications.

Gas chromatography - mass spectrometry (GC-MS)

Gas chromatography (GC)is a mature analytical technique, being applied in combination with MS for over 50 years. Its two main advantages are its high sensitivity with typical detection limits in the pmol or nmol range, and high resolving power. Its main metabolomic applications are in studying plant, clinical and microbial metabolomes. Coverage of the metabolome depends on volatility of the sample components, as GC-MS operates by vaporising the sample into a gas before injecting it through a capillary column located in an oven (≤ 350°C). Therefore, metabolites suitable for GC-MS are volatile, low molecular weight compounds with low boiling points, to allow vaporisation. The sample is transported though a column whose inner walls are coated with the stationary phase via an inert carrier gas (mobile phase). High chromatographic resolution can be achieved by varying the polarity of columns, or increasing column temperature.

Endogenous metabolites generally have low volatility and requires enhancement by chemical derivatisation to be detected. The resultant chromatograms are complex, containing peaks from hundreds of metabolites and multiple derivatisation products. Quantification is performed by either external calibration, which is labour intensive, or by examining the response ratio (peak area of metabolite/peak area of standard). Identification of metabolites can be achieved manually by inspecting the fragmentation pattern and ions, or by computationally matching the retention time and mass spectrum of the sample peak with those of a known pure compound. Unfortunately, these are not available for a large number of metabolites, even in commercially available repositories though the field is continually building its mass spectral libraries.

Liquid chromatography - mass spectrometry (LC-MS)

The advent of atmospheric pressure ionisation techniques such as ESI,have helped introduce liquid chromatography (LC) into routine use in metabolomics. LC separates the metabolites to be measured based on differential partitioning between a liquid mobile and solid (or liquid) stationary phase. The mobile phase (containing the sample) passes thorough a column packed with particles upon which the stationary phase is present. Traditionally LC produced wide peak widths and poor chromatographic resolution. It differs from GC-MS by having lower analysis temperatures therefore volatile samples are not required and sample preparation is simpler.

In 2004, ultra-performance liquid chromatography (UPLC) was introduced whose narrow peak widths could rival that of GC. UPLC achieved higher resolution and sensitivity by increasing flow rates, pressure and producing smaller diameter column packing, reverse phase C_8 and C_{18}columns being the most widely used. These columns operate using a solvent system of high polarity gradually incorporating an organic solvent, suitable for the separation of non-polar (lipids) molecules but biased against polar compounds (amino acids, sugars). To analyse polar molecules hydrophilic interaction chromatography (HILIC) has been employed, which uses a hydrated silica column and mixed organic/aqueous stationary phase. Whichever strategy used, hundreds of features can be detected.

The metabolites separated by the LC are ionised by atmospheric pressure ionisation which allow robust interfacing of the liquid sample and mass spectrometer and passed into the MS and mass analysed (133). Similar to GC-MS, quantification can be achieved by external calibration or comparing the response ratio. The higher throughput the method, the less potential there is for quantification. Metabolite identification is difficult and time intensive. Unlike EI, ESI does not produce fragmented molecular ions and mass spectral libraries for comparison are limited. Generally an accurate mass measurements or tandem MS analysis (MS/MS) is needed. Applications of LC-MS currently extend to plant, food and microbial metabolomics while clinically it may be used a screening tool or in biomarker discovery.

Nuclear magnetic resonance (NMR) spectroscopy

For decades NMR spectroscopy has been the analytical tool used mostly by chemists and structural biologists. Its use has expanded into clinical based metabolomics coining the term metabonomics (139). ^{1}H-NMR is most commonly used in clinical studies as most common metabolites contain hydrogen atoms. NMR functions by applying a strong magnetic field and radio frequency pulses, so that nuclei of atoms absorb and re-emit electromagnetic radiation. Nuclei with an odd atomic number (^{1}H) or odd mass number (^{13}C) have ‘nuclear spin’ and will spin in the presence of a magnetic field. The alignment of nuclear spins can ‘flip’ from a low energy, spin aligned state to a higher energy spin opposed state. The sample is positioned in the magnetic field and excited via pulsations of radio frequency. When the pulsation is relaxed the excited nuclei will emit its absorbed radiation (energy). This radiation is detected and is

used to output an NMR spectrum, which contains peaks at different frequencies or ppm values. The spectrum is based on a reference proton from a reference compound solution, for example tetramethylsilane (TMS) in solution set at 1ppm. The spectrum is measured in chemical shifts, which is the difference in ppm between the resonance frequency of the observed proton and the reference proton, and can vary in length, for example 0 – 10 ppm for ^{1}H and 0 – 250 ppm for ^{13}C. Each compound will produce a splitting pattern, which arises from spin coupling, and the chemical shift will depend on the shielding of other electrons orbiting the nucleus. As well as the chemical shift and splitting pattern, each peak will display different intensities, which depend on the number of identical nuclei. These intensities allow for compound quantification.

NMR has several properties which lend it to applications in clinical and pharmaceutical metabolomics (139).

- Non-destructive analysis: A single bio-fluid sample can endure several rounds of analysis.
- Non-selective and non-biased: Metabolite extraction is universal and does not depend on the metabolites polarity, volatility or acidity, meaning it will detect any metabolite within the detection limit.
- Rapid and high throughput: The introduction of higher magnetic fields and automated injection from 96 well plates has shortened analysis times.
- Increased sensitivity: Modern cryogenic probes increase sensitivity and allow smaller sample volumes (approx. 2 µl) to be analysed (140, 141). This is particularly important for paediatric studies where bio-fluid volume is limited.

The limitations of NMR include its low sensitivity, compared to mass spectrometry techniques (130). Furthermore NMR can only quantify high concentration metabolites due to its detection limits.

2.4.4 Untargeted metabolomic workflow

Figure 2.7shows a typical methodological pipeline of an untargeted metabolomic study, similar to that used in this thesis. The workflow starts with generation and processing of spectral data to generate sample metabolic information (i.e. metabolic features). These features are processed to obtain a final set of robust and reproducible features. Finally bioinformatics can be applied, enabling the statistical and biochemical analysis of the data to ultimately extract meaningful biological knowledge from the analytical data. A brief overview of each step in the workflow applied in this thesis will be described herein, more detailed information is provided in the Methodology sections 3.3, 4.3 and 7.3 and Appendix G.

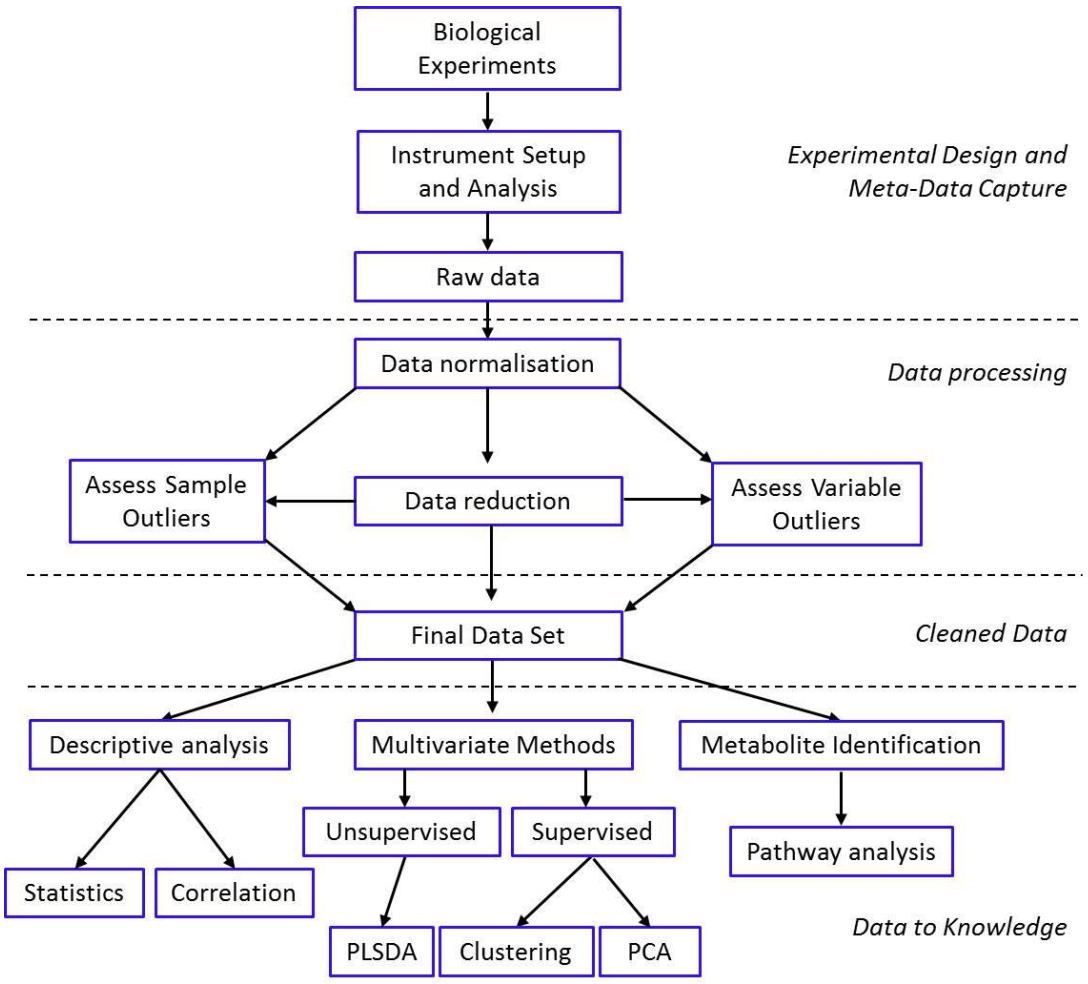

Figure 2.7 Analysisworkflow in an untargeted metabolomic study, modified from Dunn 2011(122).

Sample preparation and data acquisition

Sample preparation is extremely important to ensure metabolic activity remains quenched and that the profile measured is representative of the metabolome at the time of sampling. Typically metabolites need to be extracted from the sample, a process which separates metabolites from matrix species into a solvent suitable for analysis. Extraction methods are dependent on sample type, experimental strategy and analytical instrument to be used. Biofluids require an initial protein precipitation (removal) step to ensure only small molecules are isolated from the sample with maximum recovery. If both polar and non-polar (lipid) metabolites are of interest a bi-phasic solvent extraction system (142), which uses organic and aqueous solvents to extract hydrophobic and hydrophilic compounds respectively, is the most appropriate extraction method. Analysis throughput largely dependent on the analytical platform and its automation, DI nESI FT-ICR MS was the instrument chosen to acquire data for this thesis, see Appendix G.3.

Data Processing

Metabolomic data acquired on analytical instruments or 'raw data' is complex, therefore pre-processing is needed to reduce file size and ensure that individual metabolites or features are identified consistently, as the same metabolites or features in all samples analysed. Pre-processing aims to accurately identify features from the mass spectrum, until a final set of robust and reproducible features is obtained. Spectral processing generally includes baseline correction, noise filtering, feature detection, feature alignment, normalisation and deconvolution (143), but depends on the analytical instrument used. Pre-processed data is considered 'clean data' and arranged in a feature intensity matrix (for DI-MS) suitable for subsequent analysis.

Prior to statistical analysis of clean data further data processing is required including; data normalisation, scaling or transformation(144), the imputation of missing values (145) and the detection and removal of outliers. Signal correction may also be performed to reduce the effects of bias in the dataset, for example instrument drift (135).

Data analysis

Bioinformatics is a management information system for molecular biology, defined as 'conceptualising biology in terms of molecules and applying "informatics techniques", derived

from disciplines such as applied maths, computer science and statistics, to understand and organise the information associated with these molecules on a large scale'(146). It allows for high-throughput analysis and interpretation of metabolomic data, including statistical analysis, metabolite identification and pathway visualisation.

Statistical analysis

Once robust and reproducible metabolic features are obtained univariate and multivariate statistical methods can be applied, to examine the structure of the metabolomic dataset and, the relationship between metabolic features and phenotypic data associated with the samples. The choice of univariate method will depend on the statistical properties of the feature distribution (147). Large datasets, particularly from untargeted mass spectrometry studies, are susceptible to a large number of erroneous significantly changing peaks, or high false discovery rate. A popular false discovery correction method applied to metabolomic data is the Benjamini-Hochberg correction, which creates an adjusted *p*-value based on the number of calculations to minimise the false discovery rate (148).

To visualise large metabolomic datasets in a comprehensible manner, multivariate analysis methods are applied, which take into account all the metabolic features simultaneously. Multivariate analysis is either supervised or unsupervised; both are described as pattern recognition methods used to detect patterns that correlate with biological variables. Unsupervised methods detect patterns within the data without knowledge of sample class or phenotype, principal component analysis (PCA) being the most common technique in metabolomics (149), and is used throughout this thesis. PCA visualises the variation in a dataset by generating an axis of linearly uncorrelated variables (principal component) based on the linear transformation of metabolic features (143). The first principal component (PC1) contains the maximal variance and each subsequent component contains the maximal variance remaining.

Supervised multivariate methods are used to identify features or metabolic patterns that are more associated with the phenotype of interest, while down weighting other sources of variance. Supervised methods are the basis for building classifiers or models that attempt to describe the observed data based on metabolomic features (144). The partial least squares (PLS) method is universally used in metabolomics, as either a regression analysis or a binary

classifier (150). The latter, partial least squares discriminate analysis (PLSDA) is used in this thesis. PLS components maximise the covariance between the variable of interest and the metabolomic data, producing feature coefficients (loadings) which represent a measure of how much a feature contributes to the separation of different sample groups(150, 151).

Metabolite identification and pathway analysis

Identification of metabolites from metabolic features is essential in providing biological meaning to the data. Furthermore, metabolites interact via networks or 'pathways', a series of reactions which converts a set of substrates into a set of products (152). Biological databases for example the Human Metabolome Database (HMDB), the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and Lipid Metabolites and Pathway Strategy (LIPID MAPS) database (117, 153, 154), contain vast amounts of detailed metabolite and metabolic pathway information.

To determine the identity of an interesting feature and ascertain its biochemical pathway involvement, the accurate mass of the compound is searched for in extensive metabolite databases. A database match presents only a putative 'level 2' identification as defined by The Metabolomics Standards Initiative (155, 156), therefore it must be confirmed by comparing retention time (chromatography) and MS/MS data of a pure compound to that from the feature of interest. Absolute identification represents a bottleneck in untargeted metabolomic studies; it is time consuming, expensive and highly labour intensive, with unknown features requiring *de novo* characterisation.

2.5 Metabolomics in a neonatal HIE cohort

2.5.1 Study population

A major limitation in neonatal biomarker studies is the difficulty of recruiting a sufficient and appropriate study population. Disease prevalence of HIE is low (0.3%) making it difficult to collect representative high quality samples in adequate numbers suitable for metabolomic biomarker discovery. Therefore, even in large maternity hospitals with 5-10,000 deliveries per year and with full recruitment, only 10-20 cases of moderate-severe HIE and 20 cases of mild HIE per annum would be expected. In a study that recruited 256 newborns from three separate

hospitals, only 11 were cases of severe asphyxia (8 HIE and 3 perinatal death) compared to 216 healthy controls (16 excluded) (157). Human neonatal biomarker studies clearly require standardised recruitment and clear case definition across multiple centres.

To ensure a biomarker is in fact detecting hypoxic-ischaemic injury we must rule out the many other causes of a neurologically depressed neonate at birth. Infants with metabolic encephalopathies, sepsis, genetic abnormalities or neuronal migration disorders can all present with low Apgar scores and poor tone in the first days of life (158, 159). To progress biomarker research we need to surpass the "default diagnosis" of neonatal encephalopathy (158), and carefully define our population using clear recruitment criteria.

Standardised recruitment poses its own challenges; firstly the clinical grading of HIE can vary between centres. To standardise the grade of HIE, EEG and amplitude integrated EEG monitoring as early as possible after birth has proved very useful (97, 160). A definitive diagnosis of HIE is often not available in the immediate postnatal period, and so the opportunity to collect early bio-samples may have passed. One solution is to collect samples from all infants "at risk" of HIE, using a study inclusion criteria encompassing broad signs of birth asphyxia. For example, ≥ 36 weeks gestational age, Apgar score ≤ 6 at 5 minutes, cord blood pH < 7.1 or, requiring intubation or CPR at birth (161). Inevitably infants with perinatal asphyxia but without HIE will be recruited, this must be favoured over omitting infants who progress to display more severe clinical signs later in the postnatal period.

2.5.2 Sample and data collection

The highly unpredictable nature of HIE requires a regimented and multidisciplinary approach to identify participants and collect samples 24 hours a day, 365 days of the year. The acute clinical setting demands the participation of medical, nursing, midwifery and research staff from both neonatal and maternity units. A biomarker suitable for early diagnosis of HIE must be detectable soon after birth, therefore the specimens used in the discovery phase should be collected, processed and biobanked from every 'at risk' infant, within 3 hours of birth. This short window may mean the reliance on labour ward staff to identify study candidates, draw a sample and notify the research team immediately after delivery. Informed parental consent

can be sought after delivery when clinically appropriate, due to the inability to identify cases prior to birth. Control infants can be simultaneously recruited by consenting antenatally.

The demographic variability of the HIE population will itself account for metabolomic differences, for example, gestational age creates a distinct metabolomic profile (162), as does mode of delivery (163) and in utero growth (164). Other possible confounding factors include gender, ethnicity, maternal BMI and the length of time the blood sample has spent in the freezer. Matching cases 1:1 with controls is vital to achieve adequate power with smaller numbers (147), while incorporating demographic, physiological and lifestyle factors into a matched cohort will help to eliminate their potential discriminatory bias on the metabolomic model (165).

As discussed, high quality bio-specimens are essential for metabolomic investigations to ensure the sample is a representative snapshot of the metabolome at the time of collection (122). Several different types of biological fluids and tissues can be analysed, each giving a unique snapshot of biochemical changes and complex interactions in a particular metabolome, depending on the physiological or environmental perturbation. In maternal-foetal metabolomic investigations a range of bio-specimens have already been employed including; plasma, urine, amniotic fluid, placental tissue, vaginal secretions and cord blood(166). It is important to note that no single specimen will reflect the total metabolomic disturbance of an organism; therefore one must be carefully chosen based on availability, suitability to achieve the study aims, and level of invasiveness.

For infants with perinatal asphyxia and HIE, postnatal specimen collection is difficult and can easily be confounded by progression of disease, postnatal interventions or timing of collection. The average 3.5 kg infant will have only 280 ml of blood. Ethical guidelines limit research sampling to 1% of circulating blood volume per 24 hours, giving an upper limit of 3 ml for postnatal sampling, or approximately 1-1.3 ml of serum or plasma. This limited availability of blood for metabolomic analysis leaves little room for error.

The metabolomic analysis of umbilical cord blood (UCB)provides a potent source of early metabolic information offering the unique potential to identify the severity of cellular hypoxia and resulting metabolic damage. The umbilical cord consist of the umbilical vein containing

blood directed from the placenta to the foetus and the umbilical arteries containing blood of the foetus returning to the placenta. A ^{1}H-NMR based metabolic profiling study comparing blood from both reported no noticeable difference in the metabolomic profile of the two, however there were significant differences for some individual metabolites (167).

Though UCB is not routinely stored, it is suited to biomarker discovery as large volumes (20 ml) can be non-invasively extracted allowing for whole blood, serum and plasma to be collected. A mixed arterial/venous cord sample simplifies collection and user friendly testing in the acute setting. Ideally, cord blood will be drawn from the placenta within 20 minutes of birth and immediately stored at 4°C before processing to quench enzymatic activity and ensure subsequent biological observations are accurate. To be suitable for metabolomic analysis, samples must be processed in a sterile environment; all work surfaces must be decontaminated and sterile consumables (e.g. pipette tips) are recommended. Samples should be biobanked in microaliquot volumes that are sufficient for one metabolomic experiment, eliminating the need for freeze thaw cycles (122). Ideally biobank freezers should be housed in a facility containing temperature control, ventilation and generator backup power. Monitoring freezers 24 hours a day using internal temperature probes with the ability to produce log sheets and reports will verify -80°C is maintained, and hence no metabolic degradation has occurred.

2.6 The emerging role of metabolomics in PA and HIE

The metabolic response to hypoxia-ischaemia remains unclear, as does its role in alleviating or attenuating the injury sustained. Traditionally investigations have relied on a reductionist approach, which focused on small number or individual metabolites in isolation, when large scale disruption of multiple pathways is likely to occur. An integrative systems biology approach could help us understand the behaviour of all biological components in HIE, particularly their mechanistic interactions.

Therapeutic hypothermia and the ongoing search for additional neuroprotective therapies has heightened the need for both early diagnosis of HIE and a better understanding of its initial pathophysiological mechanisms. Current markers used to assess the severity of the injury in routine clinical practice are unreliable and do not accurately predict long-term outcome (37).

There is an urgent need for a quick, reproducible, non-invasive and user independent biological marker to quantify HIE severity and likely prognosis (46).

Metabolomics has the unique potential to elucidate disease mechanisms in perinatal asphyxia and neonatal HIE by describing qualitatively or quantitatively, the rapid alteration and interaction of metabolic pathways in response to the hypoxic environment and ultimately depict an infant's phenotype (168). A unique metabolomic fingerprint of hypoxia-ischaemia or metabolic biomarkers could be used to indicate injury severity, predict response to treatment and potential neurodevelopmental outcome.

2.6.1 Early work in the field

Huang et al. (1999) began this search by examining the early urinary ratio of lactate to creatinine in asphyxiated neonates, using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. They defined a lactate to creatinine ratio value of 0.64 or higher, within 6 hours of birth, to be highly specific (100%) and sensitive (94%) for predicting the development of HIE (n=16). Furthermore, the mean ratio was significantly higher in infants who had an adverse outcome at one year of life (161). A urinary metabolomic profile of infants with clinical evidence of severe asphyxia has also been described using gas chromatography-mass spectrometry (GC-MS), which linked four energy and four oxidative stress metabolites to good and poor infant outcome respectively (157). While this initial work proved very promising, the severity of injury, timing of collection and outcome of the infants were poorly defined.

2.6.2 Animal studies

Several studies have employed animal models of asphyxia/hypoxia to study metabolomic alterations and a number of putative metabolomic fingerprints have been proposed. The accumulation and delayed recovery of TCA cycle intermediates (for example, fumarate, succinate, malate and alpha keto-glutarate) have been described in a range of models (5, 6, 169, 170). These energy metabolites are a product of the shift toward anaerobic conditions, illustrated by the accumulation of lactate (87, 161, 171), and mitochondrial dysfunction (172)

leading to disturbance of the TCA cycle. Due to reduced ATP availability, these metabolites cannot enter the respiratory chain for energy production and have been suggested as possible cofactors in lactate formation (5).

Altered amino acids profiles where shown in a number of animal studies (169, 173, 174). Amino acid ratios of alanine to branched-chain amino acids (BCAA) and of glycine to BCAA,in combination with the energy metabolites succinate and propionyl-L-carnitine, were shown to highly correlate (R^2 =0.96) with the duration of hypoxia in a piglet model (5). BCAA act as alternate energy sources for the brain and muscles (174, 175). Their marked presence in metabolomic studies has been confirmed also in asphyxiated neonates (176) and may illustrate their mobilisation under condition of reduced ATP production. However valine a BCAA known to increase during excitotoxicity, has been described as contributing to hypoxia-ischaemia facilitating the return of nitrogen to astrocytes (177).

Disturbance of the cell membrane, perhaps through the release of oxygen free radicals which cause membrane lipid perioxidation and membrane dysfunction, has been illustrated by the marked elevation of arachidonic acid, a constituent of the phospholipid bilayer (6). Metabolomic examination of neuronal tissue revealed three differentiating metabolites which participate in glycerophospholipid metabolism (CDP-choline, choline and acetylcholine) (178), also a component of cell membranes. Lastly, urinary metabolites, which are reflective of kidney function have displayed potential in predicting death in newborn piglets (172), reflecting the systemic organ failure caused by severe hypoxia.

Animal models have made important contributions to the field of neonatal medicine. Most notably, translational research of this nature provided the evidence base to administer therapeutic hypothermia to neonates with HIE, which is now standard of care (179). Clinically a hypoxic-ischaemic insult is unpredictable, usually occurring in the perinatal period but the exact timing and duration is often difficult to estimate. Animal models provide the ability to control the timing of the injury, allowing for the timed detection of metabolite alteration and elucidation of disease mechanisms. Genetic and environmental variability can be tightly regulated ensuring the phenotype is representative of the injury, unlike the dynamic inter and intra individual variability in humans which demands large sample numbers to statistically

control (165). They also overcome ethical issues for example, using a non-treatment or normothermia control group.

Despite these obvious advantages, animal models may not capture the complex organ interactions and multiple co-morbidities, especially neurodevelopmental, associated with HIE. The majority of studies in animal models have investigated the effect of a single insult (180). Many models exist but there is no consensus on which best mimic the pathophysiological and clinical condition (181). As a result, animal studies have varied in terms of species, outcome measures, method of asphyxial injury, bio-specimen sampled and metabolomic approach, presented in Table 2.3.

Apart from Beckstrom et al. (6), animal models in this field have generally assessed neonatal but not the clinically occurring in utero or perinatal asphyxia. Newborn piglet models have induced systemic hypoxia by lowering the fraction of inspired oxygen (FiO_2) to between 0.06 – 0.13, however, this fails to inflict the clinically concurrent ischaemia associated with HIE. A rodent model by Vannucci has classically combined unilateral carotid artery ligation with 8% oxygen to mimic perinatal asphyxia (182). This model was used by Liu et al, to assess brain tissue metabolites in postnatal day 7 mice (177), which were originally considered to be equivalent to a 32-36 week gestation human infant (183). Choosing an optimal animal model whose brain is equivalent to that of a term newborn infant remains contentious (184).

In the aforementioned studies, animals are usually sacrificed soon after recovery for histological examinations; no definitive diagnosis of HIE is provided and the extent of the brain injury is not assessed. Combining imaging and outcome studies with animal models is becoming increasingly important to assess the phenotype of newborn injury. Larger animals can be recovered post injury and their physiological and neurobehavioral responses assessed, for example, magnetic resonance imaging (MRI) and EEG can identify injury severity and seizure activity (185, 186). Yet, subtle long-term deficits in memory or processing speed remain difficult to measure.

Table 2.3 Summaryof metabolomic studies in animal models of hypoxia/asphyxia.

Author, year	Ref	Studypopulation	Specimen	Analytical platform	Metabolite alterations	Key finding
Fanos, 2014	(187)	Newborn piglets (n=40)	Urine	^{1}H-NMR	15 metabolites	· Resuscitation with 21% O_2 improves cellular recovery
Arduini, 2014	(171)	Piglets12-36 hours old (n=10)	Retinal & choroid tissue	UPLC-MS/MS	Lactate	· ↓mitochondrial metabolites, anaerobic shift
Murgia, 2013	(173)	Newborn piglets (n=40)	Urine	^{1}H-NMR	n-phenylacetylglycine, alanine, acetoacetate, dimethylamine methanol, glucose, sarcosine& succinate,	· Re-oxygenation speed alters metabolite profile
Solberg, 2013	(178)	Piglets, 12-36 hours old (n=10)	Retinal tissue	UPLC-QTOFMS UPLC-MS/MS	CDP-choline, acetylcholine & choline	· Phospholipid synthesis involvement and neuroprotective metabolites
Skappak, 2013	(169)	Piglets, 1-3 days old (n=32)	Urine	^{1}H-NMR	1-methylmicotinamide,amino acids, energy metabolites& betain	· Urinary metabolites identify hypoxia
Liu, 2013	(177)	Mice, PN day 7 (n=50)	Brain tissue	^{1}H-NMR	Energy metabolites, ATP-ADP, glutamate, phosphocholine, lactate, glucose &valine	· Hypothermia treatment alters metabolites
Beckstrom, 2011	(6)	Macaques (n=25)	Plasma	2D GC/MS	10 metabolites	· TCA cycle metabolites altered and cell membrane disturbance

Solberg, 2010	(5)	Piglets, 12-36 hours old (n=33)	Plasma	LC-MS/MSFIA–MS/MS	Carnitines&Alanine/BCAA, glycine/BCAA (correlation R^2 =0.96)	· ↓ TCA cycle intermediates & delayed recovery with 100% O_2 resuscitation
Atzori, 2010	(172)	Piglets, 1-4 days (n=40)	Urine	^{1}H-NMR	Creatinine, hydroxyisobutyric acid, methylguanidine,urea &malonate	· Prediction of death
Van Cappellen, 2001	(174)	Foetal lambs (n=23)	CSF	^{1}H-NMR	Amino acids, BCAA, lactic acid & hypoxanthine	· Prolonged mild hypoxia leads to energy degradation

Ref: reference; PN: postnatal;^{1}H-NMR: proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy; UPLC-MS: ultra performance liquid chromatography – quadrupole time of flight mass spectrometry; UPLC-MS/MS: ultra performance liquid chromatography – tandem mass spectrometry; GC/MS: Two dimensional gas chromatography – mass spectrometry; LC-MS/MS: liquid chromatography – tandem mass spectrometry; FIA–MS/MS: Flow injection analysis – tandem mass spectrometry; BCAA: branched chain aminoacids; CSF: cerebrospinal fluid.(174)

Metabolites generally reflect the genetic divergence between species. However, distinct concentration differences were evident when human metabolomes of the pre-frontal cortex, primary visual cortex, cerebellar cortex, kidney cortex and thigh skeletal muscle, were compared to those of chimpanzees, macaque monkeys and mice (188). This study reported that the metabolites of human pre frontal cortex undergo a fourfold divergence from their expected evolutionary lineage, indicating the advanced human metabolome may not be directly comparable to those of animals.

Finally, ethical considerations in animal research to 'replace, reduce and refine' have resulted in study cohorts being relatively small making it difficult to extrapolate meaningful results. For example, Murgia et al. assessed the association between metabolite changes and recovery time post hypoxia in 10 piglet subjects but commented on the distinct basal inter-individual metabolomic differences among subjects (173).

Though animal experiments are providing valuable information in this developing field, the varying cerebral maturation and metabolism of species mean that no one model reflects the human condition (189). Many early metabolomic investigations have failed to comply with the minimum reporting standards for metabolomic analysis which aim to enable the interrogation, replication and comparison of data (155). This is impinging the translation of animal findings to the human scenario.To progress metabolomic animal experimentation toward clinical benifit, parallel validation studies in cohorts of human neonates are essential (190).

In summary, a number of putative metabolite fingerprints persist throughout the literature discussed. Metabolomic data thus far is consistent with the perturbation of energy metabolism and mitochondrial injury in hypoxia-ischaemia. In particular both human and animal work has described the alteration of TCA cycle metabolites (5-7, 157, 169, 170), amino acids (5, 7, 8, 169, 173) and constituents of the cell membrane (6, 8, 178). These metabolic changes correlate with the previous understanding of injury mechanisms, evidence of strong biological plausibility.

2.6.3 Metabolomic profiling in neonates

Despite these challenges, some progress in neonatal studies has been made, see Table 2.4. The biochemical derangement in UCB after a neonatal hypoxic-ischaemic injury has recently been reported. A quantitative DI-MS/MS and LC-MS/MS approach showed significant alterations between groups (HIE vs matched control, perinatal asphyxia vs matched control) in 29 serum metabolites from three distinct classes; amino acids, acylcarnitines and glycerophospholipids (8). Energy production depends on the conversion of amino acids to glucose and ketone bodies, or their role as precursor metabolites to the TCA cycle (175). Disruption of the TCA cycle has been shown to increase its intermediates (5), which may account for the elevated precursor amino acid concentrations. When functioning normally, acylcarnitines transport long chain fatty acids across the mitochondria via a carnitine shuttle. Acylcarnitine levels were increased in neonates with both perinatal asphyxia and HIE relative to matched controls, suggesting a defect in fatty acid oxidation, resulting in their accumulation and release into circulation (191). The metabolite profile of these infants could differentiate between HIE and controls with an AUC of 0.92 and demonstrated the potential to distinguish between injury severity. While a metabolite model combination of just five metabolites (decenoyl-L-carnitine, 3,5-tetradecadiencarnitine, PC ae C38:0, phenylalanine and proline) could discriminate HIE from other outcomes.

A 1D ^{1}H-NMR spectroscopy method was used to specifically characterise primary energy metabolites, finding significant changes in 18 UCB metabolites from infants with either perinatal asphyxia or HIE (7). A metabolic profile of severe HIE was illustrated by the mean fold increase of succinate and glycerol and decrease of ketones; acetone and 3-hydroxybutyrate. Ketones act as an alternate energy source for the infant brain and in severe HIE, ketone depletion suggests that the magnitude or duration of hypoxia-ischaemia has depleted these stores and reduced critical ATP generation (192, 193). Mild hypoxia has previously shown no elevation in 3-hydroxybutyrate in contrast with severe hypoxia (174). The presence of acetoacetate and succinate post hypoxia has been previously reported in animals (173). Acetoacetate is integral to ketogenesis whereby it is decarboxylated to acetone or reduced to 3-hydroxybutyrate, which in turn can be transformed to acetyl-CoA for energy production and hence the release of succinate. Many animal models have described the alteration of succinate but not its association with HIE severity. Succinate was found to increase 8-fold in severe HIE

compared to 2-fold in mild/moderate HIE, suggesting it plays a more integral role in disease mechanisms. It potentially promotes a shift to anaerobic metabolism by stabilising hypoxia-inducible factor 1α (HIF1 α), an important component in the hypoxic stress response.

Finally Longini et al. reported alterations in urine metabolites from six infants with perinatal asphyxia compared to eight controls, also using ^{1}H-NMR spectroscopy to measure metabolites (194). This study confirmed significant alterations in lactate and glucose, as well as reporting alterations in trimethylamine N-oxide (TMAO), threonine and 3-hydroxyisovalerate. Multivariate models built with the NMR data detected a metabolomic profile which could distinguish asphyxia from control infants however these results must be interpreted with caution due to the very low sample size.

Whilst these early results are promising and are supported by animal data, they have yet to be validated in additional prospective neonatal cohorts. This is the next challenge in order to progress the field towards clinically useful metabolomic biomarkers.

Table 2.4 Summaryof metabolomic studies in human neonates with perinatal asphyxia and/or HIE.

Author, year	Ref.	Study population	Specimen	Analytical platform	Metabolite alteration	Key finding
Longini, 2015	(194)	6 PA *vs.* 8 control	Urine	^{1}H-NMR	lactate, TMAO, glucose & threonine + 3-OH isovalerate	· Urinary metabolite profile can differentiate asphyxia from controls
Reinke, 2013	(7)	34 PA *vs.* matched control 25 HIE *vs.*matched control	UCB	^{1}H-NMR	Organic acids, amino acids, urea cycle metabolites, ketones & phospholipids	· Severe HIE significantly alters ketones, succinate and glycerol · A four metabolite model classifies severe HIE with AUC of 0.95
Walsh, 2012	(8)	40 PA *vs.*matched control 31 HIE *vs.*matched control	UCB	DI-MS/MS LC-MS/MS	Acylcarnitines, glycerophospholipids &amino acids	· A five metabolite model classified HIE infants with AUC of 0.92
Chu, 2006	(157)	13 good-outcome *vs.* control 11 poor-outcome *vs.*control	Urine	GC-MS	3-OH-3-methylglutarate, 2-OH-glutarate, Glutarate, methylmalonate, 3-OH-butyrate & orotate, 2-oxo-glutarate ethylmalonate	· Energy and oxidative stress metabolites associated with good and poor outcome respectively
Huang, 1999	(161)	58 control*vs.*26 PA *vs.* 16 HIE	Urine	^{1}H-NMR	Ratio of lactate to creatinine	· Ratio value ≥ 0.64 predicts HIE development with specificity (100%) and sensitivity (94%)

Ref: reference; PN: postnatal;^{1}H-NMR: proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy; GC/MS: Gas chromatography – mass spectrometry; LC-MS/MS: liquid chromatography – tandem mass spectrometry; DI–MS/MS: Direct injection– tandem mass spectrometry; OH: hydroxy.

2.7 Preanalytical variationin metabolomics

High quality bio-specimens are essential for metabolomic investigations to ensure the sample is a representative snapshot of the metabolome at the time of collection (122). Metabolomes are highly dynamic, operating with high metabolic fluxes, measured in units of seconds, compared to minutes or hours for proteins and transcriptomes. This makes metabolites ideal biomarkers due to their immediate response to endogenous and exogenous perturbation however invariably they are more susceptible to preanalytical variation.Valid data is fundamental in metabolomics to enable its reliable interpretation and validation, particularly in biomarker discovery.

This knowledge coupled with the potential applications for metabolomics has boosted recent interest into the effects of preanalytical variation on metabolites(195). Studies have begun to systematically examine the effects of collection, processing and storage variables on metabolite measurements, for example; blood collection tubes(196, 197), freeze thaw cycles(197, 198), temperature (before and during processing)(197-199), buffy layer contamination and clotting time (199), long and short term stability (time to processing and time in freezer)(197, 199, 200) and haemolysis(197, 199). All of these studies report that preanalytical variations will impact upon metabolite measurements, and need to be controlled. However some variation is unavoidable therefore individual researchers need a thorough understanding of the effects of the most pertinent variation in their own investigations.

To limit preanalytical variation prospective planning and the inclusion of quality assurance (QA) systems are necessary (199), particularly when large epidemiological biobanks are collected over long periods of time (201). QA measures or 'planned process activities' can be embedded into standard operating procedures (SOP), an invaluble tool for the metabolomic researcher which ensures that each sample is biobanked and analysed in an identical manner (202-204). This level of QA will minimise the variability introducedbetween staff and across sites. SOPs should provide step by step procedures for sample handling, processing and curation with adequate detail to ensure meticulous and consistent execution(122, 199).

For the purposes of this thesis, samples were collected using standardised biobanking SOPs which detail collection times, temperature and consumables (E.3.1). Furthermore, samples

were stored in microaliquot volumes suitable for an individual metabolomic experiment, eliminating the need for freeze thaw cycles, and cases were matched with controls for length of time in freezer to limit variation unrelated to the subject of study. Despite these stringent measures, the sampling of umbilical cord blood was error prone due to the unpredictable time of birth, complications during delivery, physiologically high haemocrit levels (205) at birth, and variation in clinical staff collecting UCB samples. As a result haemolysis is a common preanalytical error that occurs when umbilical cord blood is sampled in the delivery room.

2.7.1 Haemolysis

Haemolysis commonly occurs during the collection of blood products and describes the reputing of erythrocytes (red blood cells) and release of their contents into the surrounding fluid.It can occur *in vivo*as a clinical marker of disease, for example haemolytic anaemias, haemolytic uraemia, sickle cell anaemia and rhesus incompatibility, but more commonly *in vitro* due to specimen collection errors. Several *in vitro* errors have been documented for example, difficult collection or handling, incorrect needle size, unnecessary mixing, under fill of the sample tube and excessive force, can all result in the breakdown of red blood cells which contaminate the surrounding serum or plasma (206, 207).

Haemolysis can influence and interfere with metabolites, through the release of erythrocyte contents, ultimately causing spurious analyte measurements (207). Depending on the ratio of analyte between the erythrocyte and serum/plasma dilution effects or false concentration increases can occur(208). The erythrocytes may also release enzymes which catalyse further metabolic reactions *ex vivo,*increasing metabolite consumption or production. Several studies have examined the influence of haemolysis on routine biochemical teststypically 20-30 analytes measured individually, and described the extent of chemical alterations (209-212).Guidelines advising when not to report a result based on haemoglobin concentration are routinely used in clinical laboratories to guide practice(213).In clinical laboratories, 3.3% of specimens reaching the laboratory are haemolysed, while haemolysis accounts for the highest percentage (60%) of rejected specimens (206, 214).

The advent of metabolomics has enabled the simultaneous detection of hundreds if not thousands of analytes in a given sample. For the majority of these metabolites, their response to haemolysis is unknown. Early investigative studies into the effect of haemolysis in metabolomics have begun. Only two previous papers have been published using varied analytical methods and specimen types. Kamalge et al. examined the effect of haemolysis on plasma metabolites using four mass spectrometry coupled analytical methods, gas chromatography (GC-MS), liquid chromatography (LC-MS/MS), solid phase extraction (SPE-LC-MS/MS) and ultra-high performance liquid chromatography (UHPLC-MS/MS), and reported that up to 30% of metabolites were altered (199). Yin et al. also studied the response of plasma metabolites to haemolysis using UPLC-MS, and found alterations in 18% of detected metabolic features (197). Both of these studies recommended avoiding the use of haemolysed samples in metabolomic investigations.

Due to the scarcity of literature, excluding haemolysed samples from metabolomic investigations may not be justified. To date, no study has examined the effect of haemolysis on umbilical cord blood or serum metabolites, or used an untargeted direct injection (DI-MS) analytical approach.

Though the prevalence of haemolysis in biobanked umbilical cord blood is unknown, anecdotally we believe it is higher than the 3% reaching clinical laboratories, due to physiologically high haematocrit levels found in fetal circulation at birth (haematocrit range 42-65%, haemoglobin range 14-21.5 g/dL (at >37 weeks gestation)). Neonatal samples are expensive and difficult to collect, particularly those from infants with rare conditions.In addition the physical condition of the placenta and umbilical cord will affect the ease of sample extraction. Therefore those infants most of interest in perinatal research (preterm, growth restricted or chronic placental dysfunction) may be most at risk of haemolysis affecting their sample quality. Excluding all haemolysed samples will limit cohort diversity and potentially affect study feasibility.It is imperative to examine the effects of haemolysis on cord blood metabolites in more detail to; (i) Assess whether the metabolomic profile of cord blood serum is altered by haemolysis, (ii) Define a list of metabolic features which may be unreliable to measure in haemolysed cord blood and (iii) Ensure potential metabolic biomarkers are robust to haemolysis. We hope that thisnovel information will be an invaluable resource to the metabolomic community.

Chapter 3

3 Untargeted metabolomic analysis and pathway discovery in perinatal asphyxia and hypoxic ischaemic encephalopathy

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3.1 Abstract

Background

The full metabolic response to perinatal asphyxia (PA) and its sequelae hypoxic ischaemic encephalopathy (HIE) remains unclear. The ability to identify potential biochemical pathways perturbed in HIE may suggest early biomarkers of injury and new treatment targets. This study examines the early metabolic response to PA and HIE using untargeted metabolomics in a carefully defined population.

Methodology

The degree of encephalopathy was defined using continuous multichannel-EEG at 24hours. Infants were stratified by disease severity; 30 HIE (17 mild, 8 moderate, 5 severe), 41 perinatal asphyxia and 71 matched controls. Untargeted metabolomic analysis of umbilical cord blood was performed using direct injection FT-ICR mass spectrometry in positive and negative ion mode. For each reproducibly detected metabolic feature, mean fold differences were calculated between two study groups, HIE vs. controls (ΔHIE) and PA vs. controls (ΔPA). Putative metabolite annotations were assigned and pathway analysis was performed.

Results

100 putatively annotated metabolic features were significantly different between the study groups ($p<0.05$), 29 post false discovery correction ($q<0.05$). Pathway analysis revealed ΔHIE was associated with a 50% and 75% perturbation of tryptophan and pyrimidine metabolism respectively, alongside alterations in amino acid pathways. Altered metabolites include; melatonin which increased 1.9 fold (1.4, 2.8 95%CI) in HIE; leucine, kynurenine, urea, and methoxytyrosine which differentiated between infant groups (ΔPA *vs.* ΔHIE); and tryptophan, D-erythrose-phosphate, indolelactate and N-acetylneuraminate all significantly different across grades of HIE severity.

Conclusion

This study has identified potential biomarkers which measured at birth may help direct treatment and has identified perturbed metabolic pathways specific to HIE.

3.2 Introduction

Hypoxic ischaemic encephalopathy (HIE) remains a persistent worldwide problem occurring in approximately 2 per 1000 term neonates (215, 216). Surviving children are at risk of lifelong neurological impairment and represent a large global burden of disease (1). The recent introduction of therapeutic hypothermia has improved survival rates and critically, reduced neurological disability and improved neurocognitive outcomes (2, 3). Now a robust and non-user dependent method to equivocally diagnose HIE and rapidly guide this therapy is needed.

Current understanding of the encephalopathy which stems from perinatal asphyxia, suggests the primary injury response is metabolic (4). However the full extent of metabolic change which occurs in response to HIE remains unclear, as does its role in alleviating or attenuating the injury sustained. To discover novel biomarkers of this early injury we need a better understanding of the full metabolic response to perinatal asphyxia, and resultant HIE. An investigation of the complex interactions of individual metabolic pathways may yield new insight into the processes involved and potential options for therapeutic intervention.

Our group previously reported metabolic alterations in a population of infants with carefully defined perinatal asphyxia and HIE, using both a targeted and semi-targeted metabolomic approach (7, 8). The current study aims touse untargeted metabolomicsto more extensively examine the cord blood metabolome's response to injury, allowing us to achieve two objectives; (i) identify key biochemical pathways perturbed in HIE, which may help elucidate new treatment targets and, (ii) select disease specific biomarkers with the potential to characterise an infant's phenotype. The ability to identify at-risk infants at an early stage may improve our ability to provide targeted neuroprotection and hence, improve long-term outcome.

3.3 Methodology

3.3.1 Study population

Ethical approval was granted by the Research Ethics Committee of the Cork Teaching Hospitals. Recruitment took place from May 2009 to June 2011 in a single maternity hospital with 9000 deliveries per annum. Case infants were > 36 weeks gestation and met one or more of the following criteria for perinatal asphyxia at delivery (161); umbilical cord pH < 7.1, 5 minute Apgar score ≤ 6, the need for intubation or cardiopulmonary resuscitation. UCB was collected from the placental cord within minutes of birth, and samples were processed and stored within 3 hours of delivery. Following resuscitation parents were approached and written informed consent was obtained. All cases had demographic and clinical details collected prospectively. Cases were divided into those that had clinical or biochemical signs of perinatal asphyxia at birth, but recovered quickly with no clinical signs of encephalopathy (PA group) and those who developed an evolving clinical and electrographic encephalopathy consistent with hypoxic-ischaemic encephalopathy (HIE group). The grade of HIE (mild, moderate, or severe) was based on continuous multichannel EEG monitoring at 24h of life.

Multichannel video-EEG monitoring the current best standard in injury severity grading (97) was commenced shortly after delivery for all cases (HIE and perinatal asphyxia) and continued until the background normalized or electrographic seizures had ceased for 24 hours. Clinical grade of HIE was assigned using the modified Sarnat score at 24 hours. EEG was visually analysed by an experienced neonatal neurophysiologist (GB) and EEG grade was assigned at 24 hours. EEG grading of HIE in this cohort has been previously described (217) using a validated modified grading system (14, 15) to assign a HIE grade of mild, moderate, or severe.

During the same time period a healthy control cohort was recruited as part of an ongoing birth cohort study, the Cork BASELINE Birth Cohort Study (218). Infants were matched case-control one-to-one based on the following clinical variables; gender, gestational age, method of delivery, birth weight and centile, as well as sample storage time at -80°C.

More detailed information on recruitment and EEG analysis is provided inAppendix E.1.

Sample collection and storage

UCB was drawn from cases and controls and stored within 3 hours of birth. UCB was collected within 20 min of placental delivery and placed in an additive free serum tube. After 30 min clotting time it was centrifuged at 2400 x g for 10 min. The serum was removed, placed in a clean spin tube and centrifuged at 3000 x g for 10 min, all at 4°C. Aliquots of clean serum were placed in microtubes and stored at -80 °C until metabolomic analysis.

3.3.2 Metabolomic analysis of UCB serum with DI FT-ICR MS

Sample preparation

A modified Bligh-Dyer biphasic extraction method was used to extract polar metabolites from UCB serum (142, 219). Extractions were conducted on ice to minimise metabolic activity. Methanol was added to frozen serum samples followed by chloroform and water to achieve a ratio of 2:2:1.8. The extraction was centrifuged at 1800 g for 15 min at 4 °C to allow separation of the upper polar phase consisting mainly of methanol/water with hydrophilic metabolites. Aliquots of polar phase were dried down in a centrifugal evaporator (Thermo Savant, Holbrook, NY) and stored at -80 °C until re-suspension for MS analysis.

DI FT-ICR MS analysis and signal processing

For maximum coverage of the human metabolome, the polar fractions from each sample were analysed in both positive and negative ion mode mass spectrometry. Metabolomic analyses used a hybrid linear ion trap/Fourier transform ion cyclotron resonance 7-T FT-ICR mass spectrometer (LTQ FT Ultra, Thermo Fisher Scientific, Bremen, Germany) with a chip-based direct infusion nanoelectrospray ionisation (nESI) assembly (Triversa, Advion Biosciences, Ithaca, NY). To increase metabolome coverage and maintain mass accuracy, spectra were collected using the 'SIM stitching' method (220). All data processing was conducted within Matlab software (version R2013, MathWorks, Natick, MA, USA) using custom written software except where stated. Duplicate replicate analyses of each sample were taken and filtered into a single peak list via a three step filtering process to enhance spectral reliability (221).

For each analytical batch, quality control (QC) samples representative of the infant population being studied were included as a quality assurance measure. QC samples were injected multiple times at the beginning of each batch for equilibration. Then injected every fifth sample

and at the end of the batch to assess and correct the precision and reproducibility of individual mass spectral features (222, 223). To reduce inter and intra batch variation the Quality Control-Robust Spline Correction (QC-RSC) batch correction algorithm (135) was applied, in conjunction with three spectral cleaning algorithms, to remove unreliable, inconsistent or unreproducible features. For statistical analysis, the final intensity matrices were normalised using the probable quotient normalisation (PQN) method (224) and logarithm (log_{10}) transformed to reduce technical variation.

More detailed sample preparation and DI FT-ICR MS are provided inAppendix G.

3.3.3 Statistical analysis

All statistical analyses were performed using MatLab (version R2013, MathWorks, Natick, MA, USA). Statistical comparisons of clinical patient data between cases and matched controls were performed using Student's *t*-test or Mann Whitney U test as required.

Univariate analysis of metabolomic data

The relative standard deviation (RSD) of each reproducibly detected mass feature was calculated for the QC samples (RSD^{QC}). Mass features were included for further analysis only if the RSD^{QC}< 20%, a measure of analysis precision (165). Normality assumptions for each class were tested using the Lilliefor's test. Comparisons between both HIE and matched controls (ΔHIE) and perinatal asphyxia (no encephalopathy) and matched controls (ΔPA) are reported. Alterations in each mass feature were calculated using a paired Student's t-test for all matched case-control pairs with a Benjamini-Hochberg false discovery correction for multiple testing based on an assumed false discovery rate of 5% (148). This yielded an adjusted p value, termed q value, for each variable. Any pair of samples with one or more missing values for that mass feature was excluded. For each mass feature, mean fold change differences were calculated using log_{10} transformed data for ΔHIE and ΔPA and reported with 95% confidence intervals. Using the PQN data, the differences in effect size between the arms of the study (ΔHIE vs ΔPA) were tested using a 2 sample t-test and associated p-values reported. To determine if metabolites differed across the grade of injury, a one way analysis of variance (ANOVA) with Tukey's post hoc analysis was performed on the mean fold differences of the matched case-control pairs. Analysis was focussed on those peaks for which putative annotations could be determined.

Multivariate analysis of metabolomic data

Unsupervised principal component analysis (PCA) was used as an unbiased method to visualise overall metabolic differences between groups.All of the reproducible mass features for a given comparison were included. Multivariate analysis was conducted using PLS toolbox v. 7.9 (Eigenvector Research Inc, Manson, WA, USA) within Matlab. Prior to PCA, missing values were imputed using k-nearest neighbour and the data was autoscaled(145). Traditionally, autoscaling has been regarded as emphasising noise peaks in mass spectrometry. However, as we applied a

cleaning step following batch correction using the QC-RSC algorithm(135), only robust and reproducible mass spectral features were retained, enabling the use of autoscaling. Its advantage is that the weighting of each feature in the model is then independent of its variance.

Supervised multivariate predictive modelsusing Partial Least Squares Discriminant Analysis (PLS-DA) were constructed using PLS toolbox, with internal cross validation. The optimal number of latent variables (LV) in each model was selected using a stratified ten-fold cross validation (venetian blinds) approach anddetermined by the minimum classification error. The associated squared correlation coefficient (R^2) and averaged correlation coefficient (Q^2) statistics were calculated as a measure of "goodness-of-fit" and "goodness-of-prediction" respectively.Model utility was determined using the area under receiver operating characteristic curve (AUROC), sensitivity and specificity.

Next, to test the performance of the PLS-DA models and assess the statistical significance of the model's predictive power, permutation testing was performed and another PLS-DA model was built. Internal cross validation was used to calculate a "permuted" classification error rate and *p*-value. To further improve upon these models by eliminating noise from the data, a forward selection of variables was performed. This comprised of calculating the absolute Variable Importance of Projection (VIP) for each mass feature and ranking them in descending order. Starting with the feature with the highest VIP, multiple new models were then constructed by consecutively adding each feature back into the model, until a minimum classification error was achieved. This results in a model comprised of only features which are mainly responsible for the discrimination between classes (HIE *vs.* non-HIE).

3.3.4 Putative metabolite annotation and pathway analysis

Annotation was performed by applying the Metabolite Identification Package (MI-Pack) workflow described previously (225). A single peak search was conducted using the HMDB and KEGG database to putatively annotate metabolites and biochemical pathways (117, 154). 'Transformation mapping' was also performed to increase confidence in metabolite annotations (225). A chain of linked metabolites in a single pathway would be deemed to have

a higher level of confidence in the identification of each of the individual metabolites than alternative annotations, which placed each metabolite in a different pathway.

It is important to remember that several metabolites can be detected with the same accurate mass, likewise, multiple spectral features with different accurate masses can define a single metabolite, as different positive ($[M+H]^+$) and negative ($[M-H]^-$) adducts. This approach where metabolic features were identified by putative annotation based on accurate mass equates to 'level 2' identification as defined by The Metabolomics Standards Initiative (155, 156).

A more detailed description of putative annotation and pathway analysis is provided in Appendix G.5.

3.4 Results

3.4.1 Study population

One hundred newborn infants were recruited to the study (Figure 3.1). Twenty nine were excluded for the following reasons; 5 UCB serum unavailable, 16 had no EEG recordings, 5 had missing clinical data and 3 were subsequently given an alternate diagnosis. Seventy one infants from the 'at-risk' study population were suitable for analysis. Thirty enrolled infants were diagnosed with HIE, including 17 mild, 8 moderate, and 5 severe cases. Forty-one enrolled infants were asphyxiated at birth but did not present clinical or neurological signs of HIE and thus their condition is referred to as perinatal asphyxia (PA). In parallel, seventy one matched control infants were recruited. Demographic and clinical data are presented in Table 3.1.

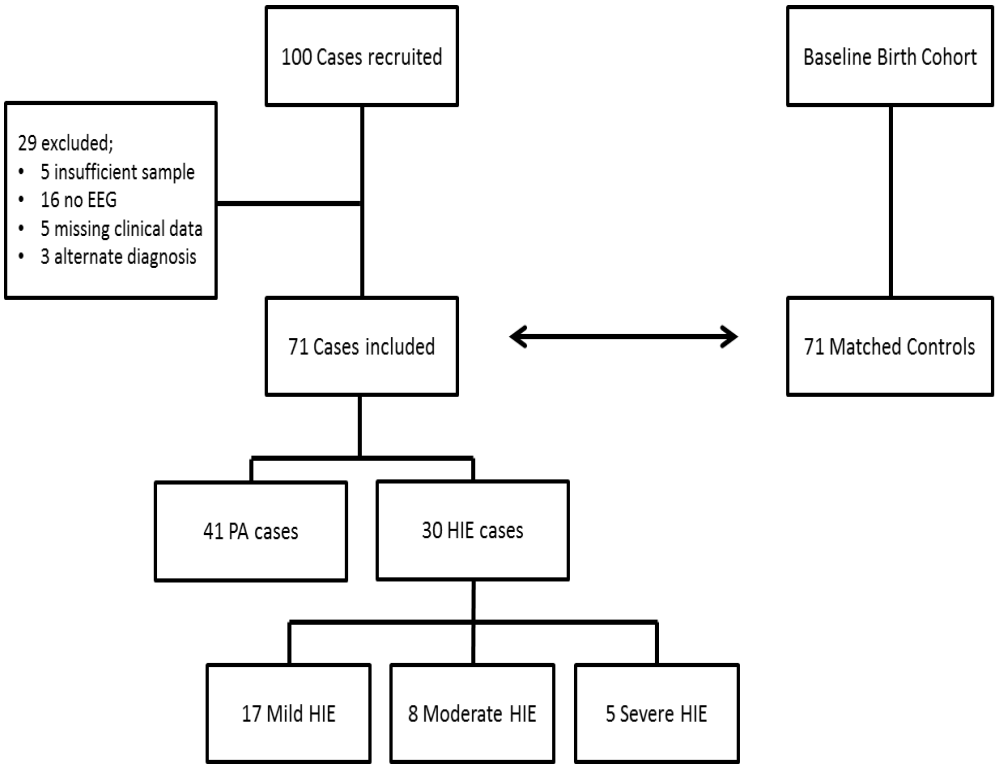


Figure 3.1 Flow diagram detailing the enrolment of study infants. Grades of HIE have been confirmed by EEG.

Table 3.1 Clinical and demographic data of the study population.

	HIE (n = 30)	**Control HIE** (n = 30)	***p*-value**	**PA** (n = 41)	**Control PA** (n = 41)	***p*-value**
Gestational Age (weeks)	40.3 (39.5, 41.3)	40.3 (39.9, 41.1)	0.705	40.6 (39.6, 41.1)	40.5 (40.1, 41.2)	0.741
Gender (Male/Female)	23/7	21/9		26/15	25/16	
Birth Weight (g)	3571.3 (585)	3524.7 (449.8)	0.73	3692.9 (573.5)	3683.2 (461.8)	0.933
Weight Centile (%)	43 (15.5, 76.5)	48 (19.8, 83.2)	0.716	60 (33.3, 83.8)	61.8 (30.3, 85.3)	0.702
Method of Delivery						
SVD	6 (20%)	7 (23.3%)		14 (34%)	15 (37%)	
Instrumental	13 (43.3%)	13 (43.3%)		21 (51%)	21 (51%)	
Emergency LSCS	10 (33.3%)	11 (37%)		5 (12%)	4 (10%)	
Elective LSCS	-	-		1 (2%)	1 (2%)	
1st pH	6.98 (6.9, 7.1)	7.26 (7.2, 7.3)	<0.001	7.0 (7.0, 7.1)	7.2 (7.2, 7.3)	<0.001
Apgar score 1 min	2 (1,5)	9 (9,9)	<0.001	5 (3,7)	9 (9,9)	<0.001
Apgar score 5 min	5 (3,7)	10 (9,10)	<0.001	8 (6,9)	10 (9,10)	<0.001
Maternal Ethnicity						
Caucasian	25 (83%)	29 (97%)		39 (95%)	41 (100%)	
Indian/Pakistani	3 (10%)	1 (3%)		-	-	
African	2 (7%)	-		-	-	
Asian	-	-		2 (5%)	-	
Maternal Age (years)	27.9 (5.1)	31.2 (4.7)	0.013	30.8 (6.5)	31 (4.8)	0.851
Maternal BMI (Kg/m^2)	28 (23.5, 31)	32 (30, 33)	0.194	31 (26.5, 35.5)	31 (28, 34)	0.352

Values are mean (SD), median (interquartile range), or n (%).

3.4.2 Univariate analysis

The DI FT-ICR MS analysis of umbilical cord blood serum yielded a total number of 1276 and 1737 polar mass spectral features in nano-electrospray ionisation (nESI) positive (+) and negative (-) mode respectively. Following spectral cleaning to remove unreliable, inconsistent or unreproducible features (>20% RSD^{QC}), 1042 nESI(+) and 1191 nESI(-) metabolic features remained and were subject to analysis. The high mass accuracy of DI FT-ICR MS enables mass features to be compared and assigned to known metabolites of a similar mass (<1.25ppm error) which are endogenous to the human metabolome and listed in the HMDB and KEGG databases. A putative metabolite name was assigned to 17% (179) of masses detected in nESI(+) mode and 9% (109) in nESI(-) mode in total. It is important to note that metabolite identifications discussed should be viewed as preliminary identifications until fully validated using chemical standards.

To select the most important spectral features associated with perinatal asphyxia and HIE a fold change comparison, of two separate groups, perinatal asphyxia *vs.* matched controls (ΔPA) and HIE *vs.* matched controls (ΔHIE) was performed. In total 571 (55%) nESI(+) metabolic features were significantly altered (p<0.05); 526 ΔPA and 360 ΔHIE while 315 of these features were common to both groups. For nESI(-), 688 (58%) metabolic features were significantly altered providing a breakdown of: 593 ΔPA, 486 ΔHIE, and 391 features being present in both groups. Of the 571 nESI(+) and 688 nESI(-) cord blood metabolic features altered in these infants (Figure3.2), 63 and 37 were putatively annotated yielding 70 and 44 uniquely detected empirical formulae respectively. Assessment of the QC measurements for each putatively annotated metabolic feature confirmed excellent technical reproducibility for features with a median RSD^{QC} of 11% nESI(+) and 9% nESI(-) (223). See Appendix H, Table H.1for details on individual features.

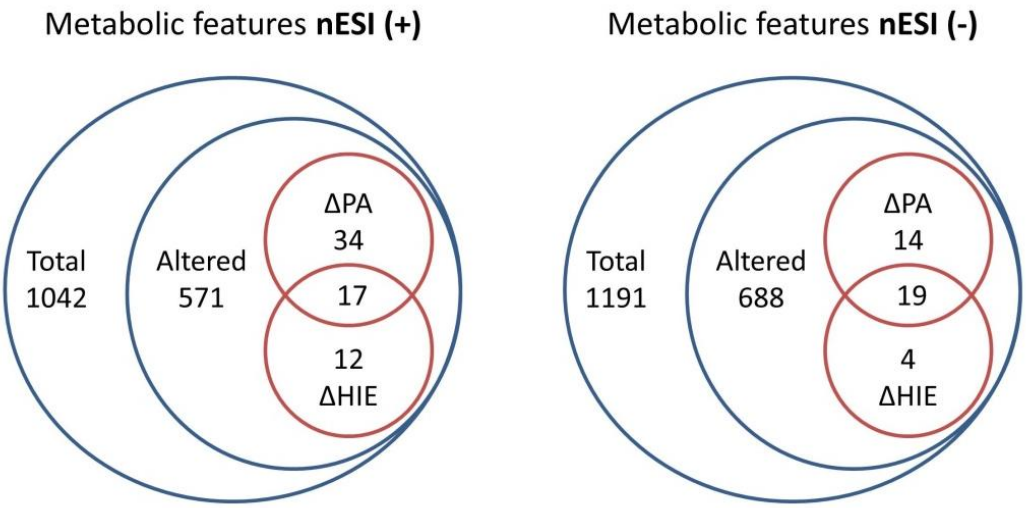


Figure3.2 Summary of the number of mass features detected and significantly altered in nESI(+) and nESI(-) respectively. Red Venn diagrams show the distribution of putatively annotated metabolic features, of those significantly altered for each comparison groups; perinatal asphyxia *vs.* matched controls (ΔPA) and HIE *vs.* matched controls (ΔHIE).

Using a critical q-value of 0.05, 29 putatively annotated metabolic features were significantly altered post false discovery correction in the ΔHIE and/or ΔPA comparisons with respect to their matched controls (Table 3.2). A derangement of simple carbohydrate compounds is known to occur post a hypoxia-ischaemia event, caused by the stark energy deficit. Herein, alcohol derived sugar compounds, for example, sorbitol (m/z 205.06822) displayed the most extreme fold changes as high as five fold in ΔPA and ΔHIE.

Significant elevations were detected in amino acids and acylcarnitines, compound classes previously found altered in these infants (7, 8). Putatively annotated phenylalanine (m/z 204.04209) was significantly altered in both comparison groups, while glutamate (m/z 148.06041) and leucine (m/z 154.08382) were increased in ΔHIE, although unlike the ΔPA group, this was not significant. Interestingly when effect size was examined, leucine was significantly altered (p = 0.03) between the comparison groups ΔPA *vs.* ΔHIE. Acetylcarnitine (m/z 226.10498) and Isobutyryl-carnitine (m/z 232.15433) increased significantly in both groups with fold changes (95%CI) of 1.6 (1.3, 2.1) and 1.4 (1.2, 1.7) in ΔHIE respectively. Finally

acetone (m/z 94.9904) was significantly raised in ΔPA and glycerol (m/z 115.03656) significantly raised in both injury groups, increasing 1.4 (1.2, 1.6 95%CI) fold in ΔHIE ($p<0.001$).

Significant depletions in features linked to pyrimidine metabolism and steroid metabolism were also detected. Uridine (m/z 243.06225) was highly significant, increasing 1.5 fold (1.3, 1.7 95%CI) ΔPA and 1.4 fold (1.2, 1.7 95%CI) inΔHIE. While uridine-triphosphate (UTP) (m/z 524.92947) and dUTP (m/z 502.94334), were significantly decreased in both groups, UTP having a fold change of 0.7 (0.6, 0.8 95%CI) in ΔHIE. Steroid metabolites dehydroepiandrosterone sulfate (m/z 367.15846), diphosphomevalonate (m/z 330.9958) and hydroxyandrost-ene-dione (m/z 325.17765) were significantly depleted in both comparison groups. Two other features belonging to this class of metabolites were significantly altered although they did not pass false discovery correction including hydroxypregn-en-one sulfate (m/z 395.18974) in both ΔHIE and ΔPA, and methoxy-hydroxyphenylethyleneglycol (m/z 225.03456) in ΔPA alone.

Table 3.2Putatively annotated metabolic features significantly altered post false discovery correction in the ΔHIE and/or ΔPA comparison groups. Values in bold indicate features that have passed the defined level of significance (q<0.05).

m/z	Putative annotation	Perinatal asphyxia *vs.* matched controls (ΔPA)			HIE *vs.* matched controls (ΔHIE)			ΔPA *vs.* ΔHIE
		p-value	*q*-value	mean fold (95%CI)	*p*-value	*q*-value	mean fold 95%CI	*p*-value
94.99040 (-)	Acetone OR Propanal	**0.002**	**0.041**	1.2 (1.1,1.3)	0.064	1.121	1.2 (>1,1.4)	0.21
115.03656 (+)	Glycerol	**<0.001**	**0.004**	1.3 (1.2,1.5)	**<0.001**	**0.025**	1.4 (1.2,1.6)	0.28
125.00092 (-)	2-Methylpropanoate OR Acetoin OR Ethylphosphate (>3)	**<0.001**	**<0.001**	1.4 (1.2,1.5)	**0.020**	0.469	1.3 (1.1,1.7)	0.34
127.01656 (-)	Aromatic aldehyde OR 2,3-Butanediol	**0.001**	**0.024**	1.2 (1.1,1.4)	**0.002**	0.089	1.3 (1.1,1.5)	0.14
132.07674 (+)	3-Guanidinopropanoate OR Creatine	**0.001**	**0.028**	1.3 (1.1,1.5)	**0.006**	0.224	1.3 (1.1,1.6)	0.16
141.01582 (+)	Methylmalonate OR Succinate OR Threonolactone (>3)	**<0.001**	**0.001**	1.3 (1.2,1.4)	**0.001**	0.068	1.4 (1.2,1.7)	0.09
148.06041 (+)	Glutamate OR L-4-Hydroxyglutamate semialdehyde OR O-Acetyl-L-serine (>3)	**0.002**	**0.045**	1.6 (1.2,2.1)	0.745	6.517	1.1 (0.7,1.5)	0.50
154.08382 (+)	6-Aminohexanoate OR L-Isoleucine OR L-Leucine (>3)	**0.001**	**0.023**	1.5 (1.2,1.9)	0.919	7.178	1 (0.8,1.3)	**0.03**
159.02771 (+)	Hypoxanthine	**<0.001**	**0.010**	1.5 (1.2,1.8)	0.222	3.121	1.2 (0.9,1.5)	0.60
167.03144 (+)	3-Hexenedioic acid OR 3-Methylglutaconic acid OR Methylitaconate (>3)	**0.001**	**0.022**	1.3 (1.1,1.6)	**0.034**	0.810	1.2 (>1,1.5)	0.92
171.02741 (-)	2-Deoxyribonic acid OR D-Ribose OR D-Xylose (>3)	**<0.001**	**<0.001**	1.7 (1.5,2)	**<0.001**	**0.010**	1.8 (1.4,2.2)	0.15

199.97885 (-)	3-Methylsulfinylpropyl isothiocyanate OR Acetylcysteine	**<0.001**	**0.005**	0.9 (0.9,0.9)	**0.044**	0.855	0.9 (0.9,<1)	0.23
204.04209 (+)	4-Hydroxy-1-(3-pyridinyl)-1-butanone OR L-Phenylalanine OR 3-Pyridinebutanoic acid (>3)	**0.001**	**0.041**	1.3 (1.1,1.5)	**0.013**	0.400	1.3 (1.1,1.6)	0.28
205.06822 (+)	D-Sorbitol OR Galactitol OR Mannitol (>3)	**<0.001**	**0.012**	2.4 (1.5,3.6)	0.073	1.402	1.7 (0.9,3.2)	0.58
209.06662 (-)	Sedoheptulose OR Tetrahydro-beta-carboline	**0.001**	**0.015**	1.3 (1.1,1.5)	**0.008**	0.252	1.3 (1.1,1.6)	0.21
226.10498 (+)	L-Acetylcarnitine	**0.003**	0.077	1.4 (1.1,1.8)	**<0.001**	**0.031**	1.6 (1.3,2.1)	0.31
232.15433 (+)	Isobutyryl-L-carnitine OR O-Butanoylcarnitine	**0.001**	**0.018**	1.5 (1.2,1.8)	**0.001**	**0.041**	1.4 (1.2,1.7)	0.91
243.06225 (-)	Pseudouridine OR Uridine	**<0.001**	**<0.001**	1.5 (1.3,1.7)	**<0.001**	**0.018**	1.4 (1.2,1.7)	0.69
255.13569 (+)	(R)-3-Hydroxydodecanoic acid OR 12-Hydroxydodecanoic acid OR Hydroxydodecanoic acid	**<0.001**	**0.001**	1.5 (1.3,1.7)	0.142	2.318	1.1 (1,1.4)	0.31
269.08776 (-)	Melatonin	**<0.001**	**0.003**	1.7 (1.3,2.1)	**0.001**	**0.039**	1.9 (1.4,2.8)	0.14
325.17765 (+)	11beta-Hydroxyandrost-4-ene-3,17-dione OR 19-Oxotestosterone OR Hexadecanedioic acid (>3)	**<0.001**	**0.013**	1.3 (1.1,1.5)	0.057	1.192	1.2 (>1,1.4)	0.88
329.04344 (-)	Cyclic de-hypoxanthine futalosine	**<0.001**	**0.002**	1.6 (1.3,2)	**0.018**	0.434	1.4 (1.1,1.9)	0.96
330.9958 (+)	(R)-5-Diphosphomevalonate OR (R)-Mevalonic acid-5-pyrophosphate	**<0.001**	**0.002**	0.8 (0.7,0.9)	**0.005**	0.210	0.8 (0.7,0.9)	0.53
331.04028 (+)	(+)-Catechin OR (-)-Epicatechin OR cis-3,4-Leucopelargonidin	**<0.001**	**0.001**	1.8 (1.4,2.3)	**<0.001**	**0.025**	1.6 (1.3,2.1)	0.94
367.15846 (-)	Dehydroepiandrosterone sulfate OR Epitestosterone sulfate OR Testosterone sulfate	**0.001**	**0.024**	0.8 (0.6,0.9)	**0.006**	0.193	0.7 (0.6,0.9)	0.58

373.02921 (+)	sn-glycero-3-Phospho-1-inositol	**0.001**	**0.042**	1.3 (1.1,1.6)	**0.001**	0.053	1.3 (1.1,1.5)	0.72
471.03289 (-)	dIDP	**0.002**	**0.047**	0.9 (0.8,0.9)	**0.028**	0.596	0.9 (0.8,1)	0.71
502.94334 (-)	dUTP	**<0.001**	**<0.001**	0.8 (0.7,0.9)	**0.003**	0.113	0.8 (0.7,0.9)	0.79
524.92947 (+)	UTP	**<0.001**	**0.001**	0.6 (0.5,0.8)	**<0.001**	**0.007**	0.7 (0.6,0.8)	0.87

(>3); indicates more than three possible putative annotations.

>1 or <1 indicates a confidence interval of greater or less than 1, whose decimal points have been rounded.

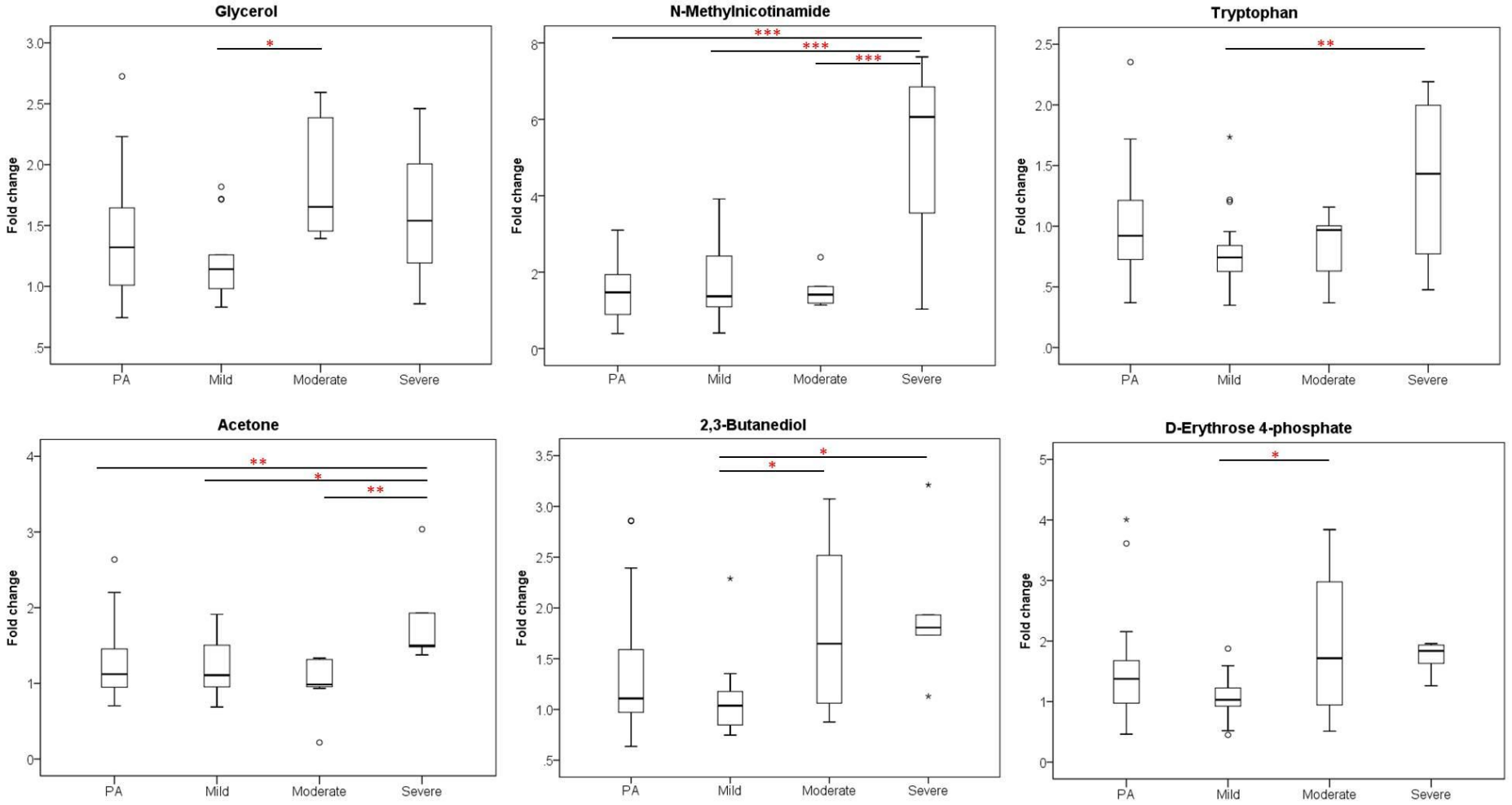
The effect size (ΔPA *vs.* ΔHIE) of the significantly altered features was examined. Apart from leucine, putatively annotated features which could discriminate between infant groups include kynurenine (m/z 209.09203, $p<0.01$), urea (m/z 83.02159 $p=0.02$), methylhistidine (m/z 170.09237, $p=0.04$) and methoxytyrosine (m/z 232.05918, $p=0.04$). Significant features without a putative annotation were ascribed an empirical formula(e) and are listed in Appendix I, Table I.1.

To assess whether metabolites could be used to predict HIE severity, the mean fold change differences across the grades of HIE (compared to their matched controls; ΔMild, ΔModerate and ΔSevere) and ΔPA were analysed. This identified 16 putatively annotated metabolites which were differentially altered across the grades of HIE. Due to reduced group size for this analysis, only three metabolites passed false discovery correction. These three metabolites were butanediol (m/z 127.01656, $q=0.037$), methylnicotinamide (m/z 137.07092, $q=0.001$) and acetone ($q=0.037$). The full list of 16 metabolites found to be altered across HIE grade is listed in Table 3.3 and boxplots for nine metabolites are provided in Figure 3.3. Of these, three metabolites were related through their participation in tryptophan metabolism; tryptophan (m/z 227.0791, $p=0.048$), indolelactate (m/z 204.06656, $p=0.033$) and kynurenine (p=0.024), while D-Erythrose-4-phosphate an intermediate of tryptophan biosynthesis was also significant (m/z 201.01594, $p=0.029$). Post hoc analysis revealed tryptophan and indolelactate could differentiate between mild *vs.* severe, while kynurenine could differentiate between mild *vs.* PA. Of note succinate (m/z 141.01582, $p=0.011$), glycerol ($p=0.042$) and acetone were significantly altered across injury severity. We have previously detected alterations in these 3 metabolites in severe HIE using NMR analysis in the same HIE cohort (7).

Table 3.3 Putatively metabolic features significantly across the perinatal asphyxia and HIE severity groups (ΔPA vs ΔMild vs ΔModerate vs ΔSevere). Values in bold indicate features that have passed the defined level of significance (p<0.05).

m/z	Putative annotation	ΔPA vs ΔMild vs ΔModerate vs ΔSevere	
		***p*-value**	***q*-value**
94.9904 (-)	Acetone OR Propanal	**0.004**	**0.037**
115.03656 (+)	Glycerol	**0.042**	0.241
125.00092 (-)	2-Methylpropanoate OR Acetoin OR Ethylphosphate (>3)	**0.03**	0.176
127.01656 (-)	2,3-Butanediol OR Aromatic aldehyde	**0.004**	**0.037**
137.07092 (+)	N-Methylnicotinamide	**<0.001**	**0.001**
141.01582 (+)	Succinate OR Methylmalonate OR Threonolactone (>3)	**0.011**	0.186
148.00386 (+)	Taurine	**0.050**	0.241
201.01594 (+)	D-Erythrose 4-phosphate OR (2R,3S)-2,3-Dimethylmalate OR 2(R)-Hydroxyadipic acid (>3)	**0.029**	0.231
204.06656 (-)	Indolelactate OR 5-Methoxyindoleacetate OR Cinnamoylglycine	**0.033**	0.176
209.09203 (+)	Kynurenine OR Formyl-5-hydroxykynurenamine	**0.024**	0.214
227.0791 (+)	Tryptophan OR N-Alpha-acetyllysine OR N6-Acetyl-L-lysine (>3)	**0.048**	0.241
232.15433 (+)	Isobutyryl-L-carnitine OR O-Butanoylcarnitine	**0.043**	0.241
235.13046 (+)	12-Oxo-9(Z)-dodecenoic acid OR, Traumatin	**0.048**	0.241
265.17741 (+)	3-Oxotetradecanoic acid	**0.044**	0.156
267.1567 (+)	1,11-Undecanedicarboxylic acid	**0.012**	0.186
308.09868 (-)	N-Acetylneuraminate	**0.021**	0.154

(>3); indicates more than three possible putative annotations.


Glycerol
Fold change
PA
Mild
Moderate
Severe
N-Methylnicotinamide
Tryptophan
Acetone
2,3-Butanediol
D-Erythrose 4-phosphate

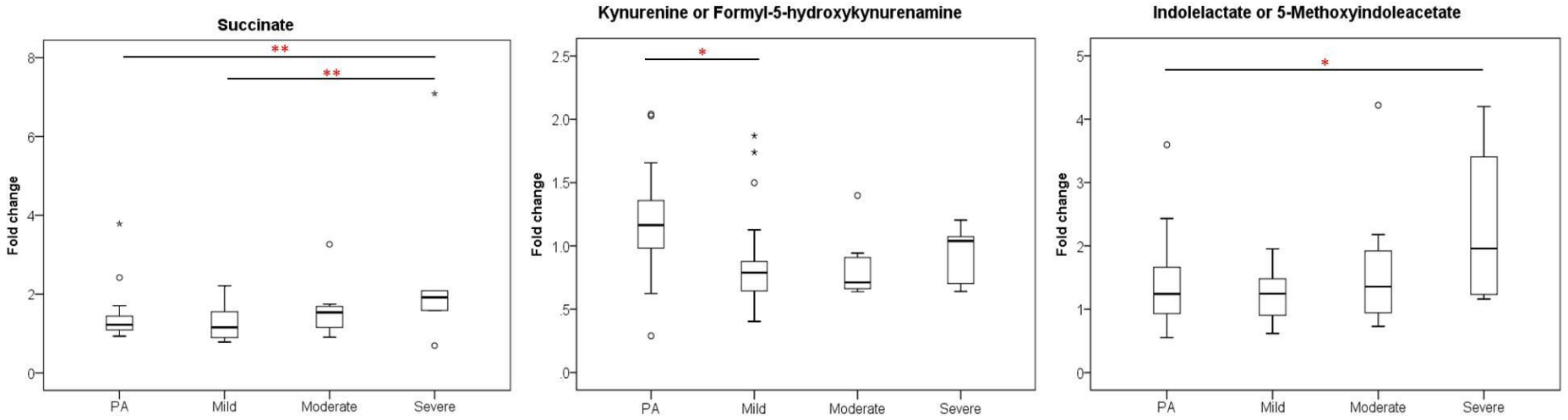


Figure 3.3 Metabolite fold changes stratified by disease severity. Fold changes compare 1:1 matched cases to healthy controls in each disease group; perinatal asphyxia, mild HIE, moderate HIE and severe HIE.The black line and red * denotes statistical significance of ≤0.05, ** ≤0.01 or * ≤0.001 between two groups.**

3.4.3 Multivariate analysis

Multivariate analysis was performed for three comparison groups; (i) HIE *vs.* matched controls, (ii) PA *vs.* matched controls, and (iii) HIE *vs.* non-HIE (perinatal asphyxia and two control groups), using all metabolic features detected in nESI(+) and nESI(-) respectively. Figure 3.4presents the PCA plots constructed using the first two principle components for each comparison group.

A metabolite model which could discriminate infants with HIE from all other infants at birth, would be the most clinically useful. Therefore a PLS-DA models were constructed for HIE *vs.*non-HIE groups using a single latent variable and metabolic features detected in nESI(+) and nESI(-) respectively. The nESI(+)model had an $R^2 = 0.18$, $Q^2 = 0.09$, classification error = 0.35 and an AUC of 0.70, with a corresponding sensitivity (62%) and specificity (69%) for predicting HIE (any grade). Then ESI(-)model performed similarly, having an $R^2 = 0.17$, $Q^2 = 0.1$, classification error = 0.31 and an AUC of 0.74 for predicting HIE(sensitivity (65%) and specificity (71%)).

Permutation testing was performed to test the model performance. The optimal PLS models each comprised of one LV, which was selected as the optimum number of LVs to use based upon the minimisation of classification errors as calculated by cross validating the model rebuilt with increasing numbers of LVs. Permutation testing using all variables produced classification error rates for each class (HIE *vs.* non-HIE) of 0.32 for both nESI(+) and nESI(-), in other words approximately two thirds of samples are classified correctly using these model. The significance of the power of the models to predict class was tested using 50 random permutations and found to be $p < 0.001$ each.

To improve on this model by eliminating noise and further discriminate the mass spectral features responsible for differences in class (HIE *vs.* non-HIE), a forward selection of variables was performed. For then ESI(+) model, 384 variables were selected to build an 'optimal model' producing a minor improvement in the classification error rate 0.30 ($p< 0.001$). While analysis of large numbers of variables may result in some false positives, certain metabolites highlighted by this forward selection method for example, glycerol and succinate have previously been associated with HIE (7) while others such as uridine, kynurenine, homovanillate and phenylalanine confirm the results from the univariate analysis, supporting their use in the model. For the nESI(-) model, the forward selection analysis constructed a model using 10 mass

features which had a classification error rate of 0.23 (p< 0.001), uridine being the only putatively annotated feature.

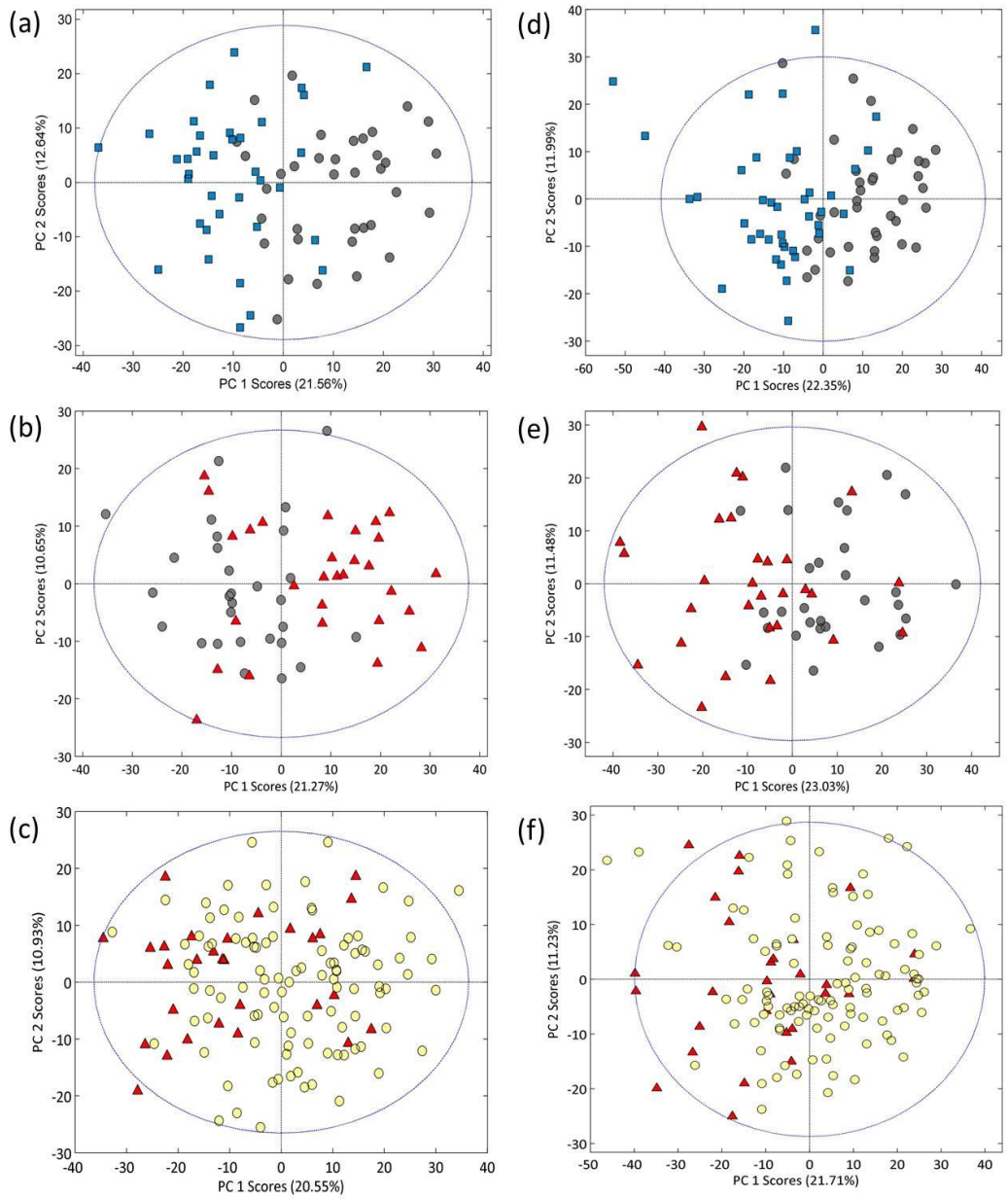


Figure 3.4 PCA plots for the three comparison groups; (a)(d) PA *vs.* matched controls, (b)(e) HIE *vs.* matched controls and (c)(f) HIE *vs.* non-HIE (perinatal asphyxia and two control groups). The metabolic profile of infant groups are represented by the following; blue squares = PA, grey circles = controls, red triangles = HIE and yellow circles = non-HIE. Plots (a)(b)(c) were built using all polar nESI(+) features and plots (d)(e)(f) using non-polar nESI(-) features.

3.4.4 Pathway analysis

The precise mass values obtained by DI-FT-ICR-MS were compared and assigned to empirical formula(e) and known compounds present in human metabolome pathways compiled in the KEGG database. To probe potential biochemical pathway alterations in perinatal asphyxia and HIE, the significantly altered compounds found in the case *vs.* matched control comparisons from both positive and negatively charged ionic datasets were combined. Certain metabolites were detected using both ionisation modes, therefore an over-representation of the pathway may occur. However, this enabled the comparison of metabolites and ion adducts from nESI(+) and (-) to assess whether fold changes were in agreement, providing additional confidence in our findings.

Figure 3.5 displays the foremost perturbed metabolic pathways in perinatal asphyxia and HIE from the cord blood metabolome. The percentage perturbation of a biochemical pathway was determined by calculating the ratio of the number of empirical formulae significantly altered in that pathway, by the total number of empirical formulae detected from that pathway. This calculation was performed for the comparisons ΔPA and ΔHIE separately, then for empirical formulae altered in both groups, to distinguish compounds related solely to the pathophysiology of either perinatal asphyxia or HIE.

Only two pathways, pyrimidine metabolism and tryptophan metabolism exhibited an increasing perturbation as the injury progressed from perinatal asphyxia to HIE. The pyrimidine pathway displayed perturbations of 75% in HIE, with three unique empirical formulae implicating glutamine (m/z 181.03847, p=0.044) hydroxypropanoate (m/z 113.02091, p=0.015) and dihydrothymine (m/z 151.04777 p=0.045). Uridine and its derivatives were altered in both groups, passing false discovery correction (Table 3.2), while urea was significantly decreased in HIE. Eightempirical formulae were detected from the tryptophan metabolism pathway which was 50% perturbed in HIE. Putatively annotated kynurenine and tryptophan were unique to HIE (Figure 3.6), while melatonin (m/z 269.08776, p=0.001) displayed the largest fold change (95%CI) of 1.9 (1.4, 2.8).

The overlapping biosynthesis of the phenylalanine, tyrosine and tryptophan pathway, as well as the individual metabolic pathways of these amino acids was disturbed, Figure 3.5. This disruption tended to be more wide spread in perinatal asphyxia, with tryptophan metabolism

being the exception. Our analysis detected 10 empirical formulae related to the biosynthesis of phenylalanine, tyrosine and tryptophan, implicating seven unique metabolites. Tryptophan was the only metabolite to be decreased in HIE, the others including phenylalanine and tyrosineincreased in both HIE and perinatal asphyxia. Increases were also detected for erythrose-phosphate, deoxy-ketofructose phosphate and (hydroxyphenyl)pyruvate whose alterations were attributable to perinatal asphyxia alone.

Pathways previously reported as being altered in perinatal asphyxia including the tricarboxylic acid (TCA) cycle and oxidative phosphorylation were detected. The TCA cycle was the only pathway 100% perturbed for perinatal asphyxia and HIE with alterations in three metabolites succinate, citrate and oxaloacetate. The closely linked reactions of oxidative phosphorylation are also 100% altered in perinatal asphyxia, caused by changes in inorganic phosphate (Pi) and adenosine di-phosphate (ADP) critical cellular energy molecules.

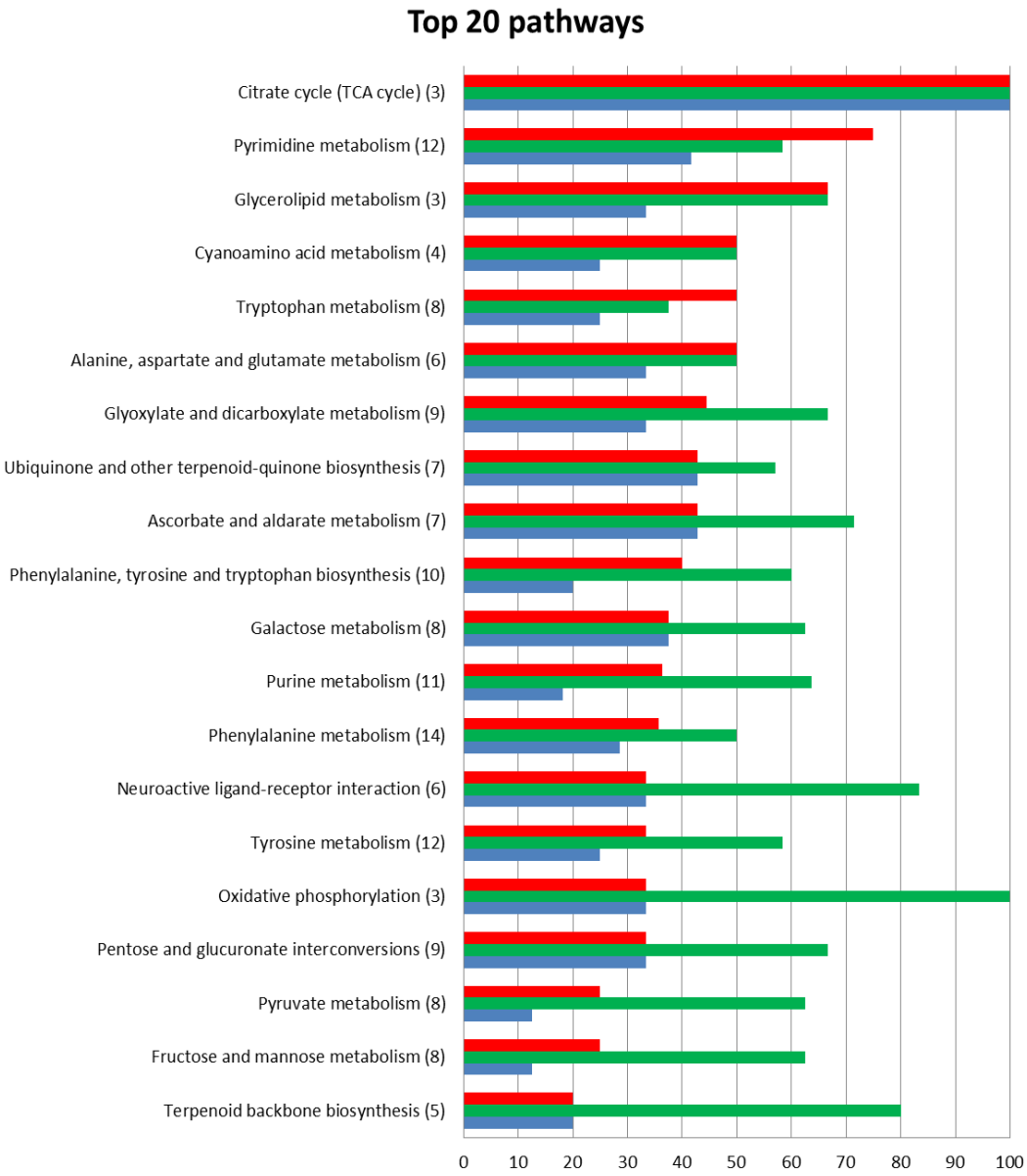


Figure 3.5 Pathway analysis. Altered metabolomic pathways and networks of UCB serum as a result of HIE (ΔHIE in red), PA (ΔPA in green) and those altered in both comparisons (ΔHIE and ΔPA in blue). The y-axis displays the pathway name alongside the total number of empirical formulae from that pathway detected in the analysis. The x-axis displays the % perturbation for each pathway. For example, the TCA cycle was 100% perturbed meaning the three empirical formulae were detected were all altered as a result of PA and HIE.

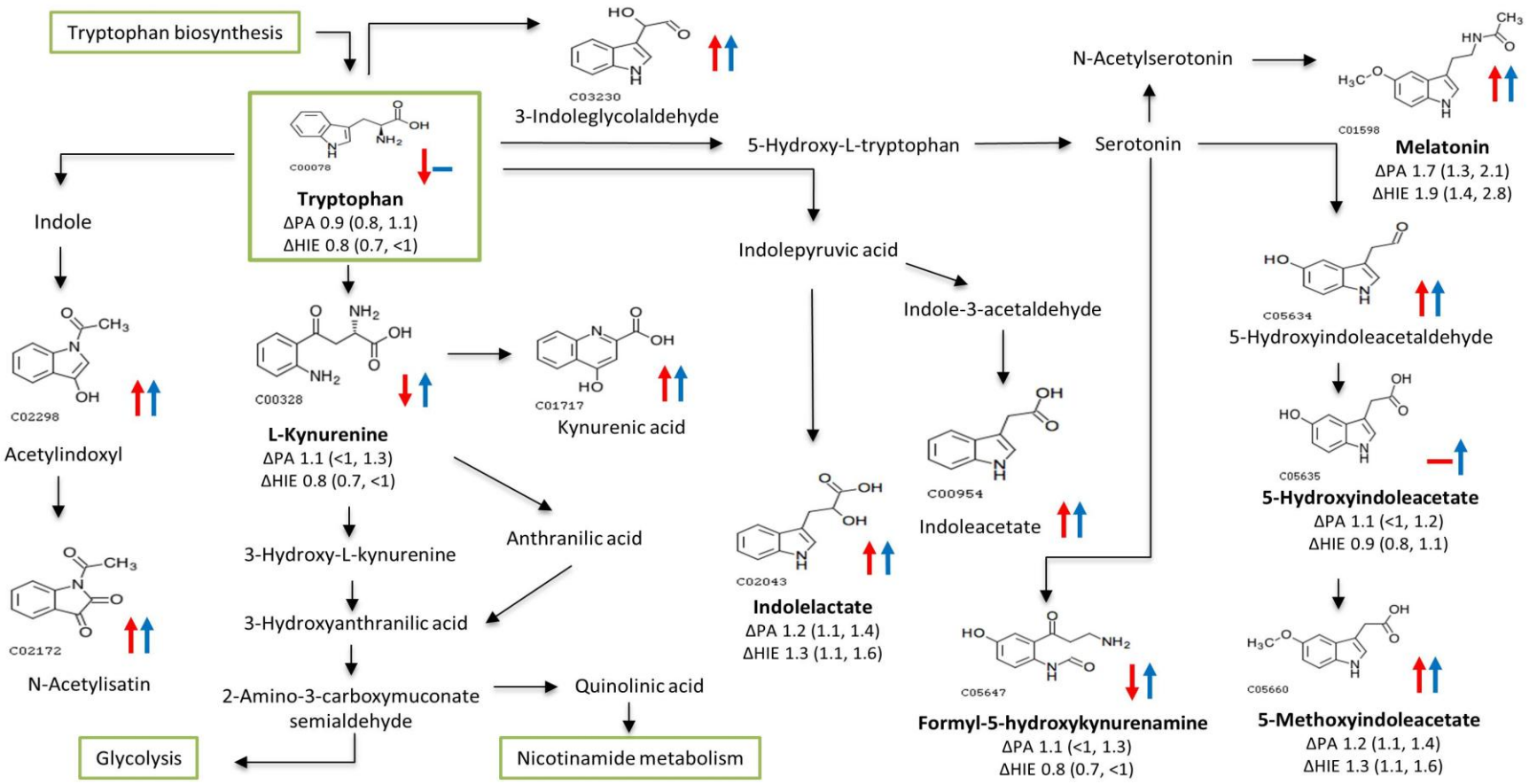


Figure 3.6 Alterations in Tryptophan metabolism. The structure is shown for the compounds identified by FT-ICR-MS. Arrows indicate an increases (↑) or decreases (↓) in metabolite fold change; ΔHIE (red) and ΔPA (blue), while metabolite IDs in bold indicate the significantly altered compounds with fold change (95%CI) included.

3.5 Discussion

This is the first study to characterise the human metabolome of perinatal asphyxia and neonatal HIE using an untargeted metabolomic approach. Our aim was to identify novel metabolic pathway alterations to enhance understanding of the complex aetiology of perinatal asphyxia and HIE. This would enable us to identify important metabolic markers with the potential to accurately diagnose HIE at birth, thus meriting further investigation.

A key strength extends to our ability to verify our findings through the implementation of a cross validation system. Upon biobanking this cohort, several micro-aliquots of serum were frozen from each infant allowing samples to be analysed on multiple metabolomic platforms. The current study represents the third time samples from this neonatal cohort have undergone metabolomic analysis, first a targeted and then a semi-targeted approach was taken (7, 8). No single analytical platform has the sensitivity to detect all metabolites therefore this three pronged approach ensures maximum coverage of the metabolome is achieved and increases confidence in our findings. For example, using ^{1}H-NMR we have previously identified alterations in acetone, lactate, glycerol, creatine and succinate all of which were also found to be significantly altered using DI FT-ICR MS (7). Using a combined targeted DI and liquid chromatography (LC) tandem mass spectrometry (MS/MS) approach, we also found changes in several amino acids that are confirmed herein (8). The varying metabolomic detection methods used have ranged in quantitative ability, DI FT-ICR MS being relatively quantitative, therefore we report fluctuating degrees of metabolite changes but importantly the direction of change has remained consistent.

The heterogeneity of HIE suggests several pathway mechanisms will play a role in its pathophysiology. This study evaluated the metabolic components identified using DI FT-ICR MS that were significantly different between perinatal asphyxia and HIE versus their respective matched control groups. We reveal alterations in a wide range of metabolite classes highlighting several major metabolic pathways associated with the conditions.

Tryptophan metabolism

This study presents extensive coverage of the tryptophan metabolism pathway in perinatal asphyxia and HIE. In infants with HIE, we found total pathway disturbances in 50% of detected

metabolites. Tryptophan is an essential amino acid necessary to maintain cellular health and homeostasis. It functions as a biochemical precursor for two important metabolic pathways; (i) serotonin synthesis and (ii) kynurenine pathway which produces nicotinamide adenine dinucleotide (NAD^+). We have shown alterations in metabolites central to and stemming from, both of these pathways as displayed in Figure 3.6.

We report a significant decrease in tryptophan levels associated with neonatal HIE. Interestingly when grades of HIE were examined, we found tryptophan levels were at their lowest in the infants with mild and moderate HIE. Three of the five severe infants for which tryptophan was detected, had a fold change increase in the metabolites. In the adult population, tryptophan depletion has been investigated in a wide variety of psychological, behavioural, and physical processes as well as psychiatric disorders (226). In particular, tryptophan decreases have been demonstrated to affect a number of cognitive processes, for example, learning and memory skills in healthy individuals (227). Several indole metabolites, metabolised from tryptophan to produce biologically active metabolites, were detected in the current study. Indolelactate was significantly increased up to 1.5 fold in HIE and interestingly, is generally found at significantly higher levels in umbilical foetal plasma (228).

The serotonergic network has been proposed as a candidate network which is disrupted after neonatal hypoxia-ischaemia (229, 230). This network is one of the first transmitters systems to peak in the developing brain. Serotonin functions as a neurotransmitter and is synthesised via 5-hydroxy-L-tryptophan by two enzymatic reactions including the hydroxylase and decarboxylase of tryptophan. Several metabolites downstream of serotonin were detected including significant alterations in hydroxyindoleacetate, methoxyindoleacetate and formyl-hydroxykynurenamine. Serotonin can also be converted to the neurohormone melatonin via n-acetylserotonin. Melatonin was the most significantly raised metabolite detected, with levels elevating 1.7 fold in the perinatal asphyxia population compared to matched controls, and further still in HIE to 1.9 fold (p = 0.001). Melatonin is a naturally occurring neurohormone synthesised from tryptophan in the pineal gland (231). Involved in physiological processes it is considered a natural neuroprotectant and free radical scavenger, with the ability to promote the production of other antioxidant enzymes (232).

The neuroprotective effects of administered melatonin have been demonstrated in animal models of hypoxic brain injury, by decreasing microglial activation and astroglial reactions which prevents white matter myelination defects, as well as reducing the number of apoptotic cells (233, 234). Melatonin's protective effects against induced oxidative mitochondrial damage, oxidative lipid and DNA damage has also been demonstrated (235, 236). Robertson et al. recently showed that melatonin augmented therapeutic hypothermia, as evident by a reduction in lactate/creatine and lactate/N-acetyl aspartate ratios in deep grey matter and an increase in the whole brain nucleotide/exchangeable phosphate pool (237). Maternal melatonin treatment prior to the hypoxic-ischaemic insult reduced inflammation and apoptosis markers (238), and delayed seizure onset (239).*In vitro*, melatonin decreased the expression of CD11b in neutrophils, a marker of inflammation in neonatal encephalopathy (240).

Interestingly oral dose melatonin is currently being trialled as a therapeutic agent; in term infants with moderate and severe HIE in St Louis USA, with neurodevelopmental outcome at 18 months being the primary efficacy measure (NCT01904786) (241). Reports on how endogenous melatonin responds to a hypoxic ischaemic insult are scarce however, elevated levels have been documented in critically ill children with the authors hypothesising its rise may counteract the oxidative stress response (242).

This study detected two metabolites belonging to the kynurenine pathway. Kynurenine metabolites, enzymes and intermediates are neuroactive and responsible for the synthesis of coenzyme NAD^+, which plays an important role in cellular respiration, energy production and DNA repair (243). Tryptophan becomes deoxygenased to produce formyl-kynurenine which is rapidly metabolised to kynurenine. We report a significant reduction in kynurenine in HIE and raised levels of its neighbouring metabolite kynurenic acid in HIE and PA though not reaching statistical significance. Protective effects of kynurenic acid have been proposed, it is thought to reduce excitotoxin induced neuronal death by antagonising excitatory glutamate receptors (244). Conversely, a significant increase was previously reported in kynurenine/hydroxykynurenine in a piglet model of hypoxia(5). Kynurenine itself may progress down another branch of metabolism to produce hydroxykynurenine and hydroxyanthranilic acid which may cause either apoptotic or necrotic neuronal death in cultures (244). Further downstream lies quinolinic acid, the focus of much study due to its interactions with N-methyl-

D-aspartate receptors causing convulsion and excitotoxicity, therefore implicated in the pathogenesis of several human neurological diseases (245).

Pyrimidine metabolism

We report alterations in 75% of detected pyrimidine pathway metabolites. Pyrimidines are heterocyclic organic compounds whose derivatives cytosine, thymine and uracil are the constituents of nucleotides, in other words the building blocks RNA and DNA production. Uridine is the principle pyrimidine in humans and a precursor of brain membrane phospholipids in the form of UTP (246). We have found a marked increase in uridine and pseudouridine in HIE. Beneficial effects of uridine have been proposed, hinged on its ability to enhance levels of cytidine-5-diphphosphocholine (CDP-choline) (247), a metabolite found to be increased in the retinal tissue metabolome after hypoxia-derived brain damage (178). Uridine administered post hypoxic-ischaemic insults reduced infarct volume and expression of caspase-3 a marker of apoptosis (248). Other protective functions include its potential to increase the synthesis of membrane lipid components which may ameliorate neuronal functions or its ability to maintain brain metabolism during ischaemia, as the catabolism of pyrimidines produces citric acid cycle intermediates(249).Uridine's neuroprotective action may also be related to the stimulation of P2Y receptors by the ligands UTP and UDP, previously shown to reducing apoptosis (250).

Pyrimidine synthesis is either recycled via a salvage pathway from normal catabolism or *de novo* through enzymes in the orotate pathway. *De novo* synthesis is the major source of UMP, UDP and UTP in the highly active tissue of the developing brain, though its use gradually declines throughout lifespan making the salvage pathway more important in adulthood (251). Van Os et al. measured spinal fluid purine and pyrimidine metabolites of hypoxic near term lambs. In agreement with our findings, they found the concentration of the pyrimidine metabolites uridine, pseudouridine and uracil were significantly higher in these lambs than controls, and corresponded with decreased electrocortical brain activity (252). Though potentially promoting protective mechanism, the accumulation of these pyrimidines during hypoxemia may also be attributed to the absence of high energy phosphate compounds (ATP) needed to synthesise derivatives such as UTP and UDP (253).

Early studies measuring pyrimidine and purine metabolites (e.g. ATP, GTP, UTP, and CTP) in rodents discovered a marked decrease in metabolite levels compared with controls.

Pyrimidines UTP and CTP displayed the greatest depletion, UTP having the slowest, most incomplete restoration to normal levels and it was the total uridine nucleotide pool had the largest decline of 53% (254). As mentioned the decline in these compounds may be due to depletion of the ATP needed for their formation. Their delayed restoration could indicate decreased synthesis or perhaps increased consumption. Similarly, in neonatal HIE we have found a significant decrease in UTP and dUTP. Finally, sugar conjugates of pyrimidines can modulate cellular and tissue pathophysiology, through interactions with G protein coupled receptors. We detected raised levels of UDP sugar conjugates UDP-D-xylose or UDP-L-arabinose (>2 putative annotations) in perinatal asphyxia and higher again in HIE and significant alterations in n-acetylneuraminate, a nucleotide sugar metabolism compound, in severe HIE compared to other grades.

Amino acids

Amino acids were another particular class of metabolites found disrupted in this cohort, and their alterations in both animal models of injury and infants have been previously reported (176, 255, 256). Pathway analysis revealed metabolites involved in the biosynthesis and metabolism of phenylalanine, tryptophan and tyrosine were disturbed particularly in both perinatal asphyxia and HIE. These interlinking amino acids are precursors to acetyl-CoA, the driving metabolite in the TCA cycle of energy production, whose derailment in perinatal asphyxia has been well documented (4). Phenylalanine, an essential amino acid, synthesises tyrosine using the catalytic enzyme phenylalanine hydroxylase, and both are intrinsically linked to tryptophan. Tyrosine easily passes the blood-brain barrier and in dopaminergic cells of the brain, acts as the precursor for the neurotransmitters dopamine, norepinephrine and epinephrine. D-Erythrose-4-phosphate, an intermediate of phenylalanine, tyrosine and tryptophan biosynthesis and also an important intermediate in the pentose phosphate pathway, was found to be significantly altered in this cohort, particularly in mild and moderate HIE, therefore may have potential as a biomarker of injury in these infants. Consistent with this study, increased levels of cord blood phenylalanine and tyrosine have previously been detected in both arms of this cohort (7, 8). It has also been documented that hypothermia can suppress tyrosine levels (177).

Several other amino acids were also putatively identified as altered in the current analysis including; taurine, isoleucine or leucine, arginine, glutamate and glutamine. We have confirmed alterations in taurine levels in perinatal asphyxia (8). The branched-chain amino acids (BCAA) leucine and isoleucine have previously displayed alterations in infants with perinatal asphyxia and HIE (7, 8). BCAA act as alternate energy sources for the brain and muscles (174, 175). Their increase has previously been documented in asphyxiated neonates (176), while ratios of alanine and glycine to BCAA, in combination with the energy metabolites, correlate with the duration of hypoxia (5), perhaps illustrating their mobilisation under conditions of reduced ATP production. Another important function of BCAA is their contribution of nitrogen to the glutamate/glutamine cycle. Astrocytes are thought to take up excess glutamate converting it to glutamine, to prevent excitotoxicity. Glutamine is shuttled back to the presynaptic neurons for recycling into glutamate, and the nitrogen taken is returned to astrocytes via branched chain

amino acids (257). We report elevations in cord blood glutamate and minor elevations in glutamine for both perinatal asphyxia and HIE.

Energy and oxidative phosphorylation

The hypoxic-ischaemic injury quickly diminishes the supply of high energy phosphate metabolites such as ATP, while inadequacies in oxygen availability inhibit normal function of the TCA cycle and electron transport chain. The current study identified alterations in TCA cycle intermediates; succinate, citrate and oxaloacetate in both perinatal asphyxia and HIE alike. Several metabolomic studies have reported on the accumulation and delayed recovery of these TCA cycle metabolites post insult as well as; fumarate, malate and α-keto-glutarate (5-7, 169, 170). They showed increased succinate and citrate with hypoxia, as did we with 1.3 and 1.5 fold increases in the perinatal asphyxia population which raised to 1.6 in HIE. Solberg et al. hypothesised that due to reduced ATP availability these metabolites cannot enter the respiratory chain for energy production and may be possible cofactors in lactate formation (5).

Reducing equivalents (NADH, $FADH_2$) from the TCA cycle are converted to energy via oxidative phosphorylation (OXPHOS), thus providing a biological explanation for our detected alterations in this pathway. OXPHOS operates via a series of redox reactions, creating a proton (H^+) gradient which becomes phosphorylated by ADP and free inorganic phosphate (Pi) to release ATP into the mitochondrial matrix. Both ADP and Pi metabolites were significantly increased in perinatal asphyxia, perhaps in a futile attempt to produce ATP before glucose stores diminished entirely. A minor fold change increase was seen in the HIE population at birth. Studies using magnetic resonance (MR) spectroscopy have documented altered brain energy stores whereby OXPHOS is inhibited, in the form of the increased P_i, and decreased nucleotide triphosphates (ATP and UTP) have been demonstrated during hypoxia (53, 54, 258).

The present study had some limitations. Accurate metabolite identification is the primary challenge facing the metabolomics community, particularly for studies such as this one undertaking non-targeted techniques whereby a large number of features are detected (259). Putative annotation is often necessary due to the extensive cost and technical challenges of identifying large numbers of unknown metabolites. To increase confidence in our identification we applied a 'transformation mapping' approach to associate the empirical formula detected to known metabolic pathways (225). This revealed several linked metabolites in a single

pathway providing a higher level of confidence in the assigned metabolic annotation for each of the individual mass spectral features and enabled us to achieve the primary goal of the study. To translate these findings to the clinic, confirmation of identification and quantification using metabolite standards is needed. Future work will focus on confirming the identity of metabolic features reported with multiple putative identifications including a comparison to authentic chemical standards by LC-MS/MS.

Herein, hundreds of metabolites were statistically tested simultaneously increasing the likelihood of false positive findings. To compensate for multiple comparisons a Benjamini-Hochberg false discovery rate of 5% was applied (148). Greater confidence is placed in metabolites which passed false discovery correction and their potential as biomarkers. However the nature of this study is exploratory, therefore it would be overly conservative to eliminate metabolites previously reported in the literature, and especially those inter-related through biochemical pathways because they did not pass stringent false discovery corrections. For this reason pathway analysis was based on metabolites deemed significant (p<0.05) prior to the correction being applied, however for completeness both the p-value and q-value have been reported, see Table 3. Future work to confirm injury biomarkers will focus on validating the findings in an independent cohort of infants, the optimal method to rule out false discoveries (144).

Multivariate analysis of this neonatal dataset could not effectively separate infant groups. It seemed the inherent variation of our biological data, and relatively small sample size inhibited the predictive value of these methods. A metabolic fingerprint may not be useful for diagnosing HIE, instead an algorithm based on only a few key metabolites or a metabolic pathway could be more powerful, with the added advantage of being easier to translate to the cot-side.

A clear understanding of perinatal asphyxia and HIE at the biochemical level is an important challenge in the field of paediatrics and child health. Metabolomics now provides researchers with the tools to gain further insight into the disease mechanisms, pathophysiology and onset, through examination of metabolic pathway perturbations at the time of birth. This will enable the discovery of potential biochemical markers as well as new diagnostic and therapeutic strategies for neonatal disorders.

This is the first study to employ a highly sensitive and mass accurate, untargeted metabolomic technique to examine the cord blood metabolome of infants with perinatal asphyxia and HIE. We have identified novel biochemical pathway changes, for example that of tryptophan and pyrimidine metabolism, which have not previously been elucidated in HIE. Due to the novelty of these findings the mechanisms and interactions of some pathway metabolites in relation to HIE is unclear, however some have been detected previously in animal studies. Furthermore, by using a third separate metabolomic approach on this rigorously biobanked and phenotyped cohort we have validated many of our previous findings, and can now with confidence progress these metabolites to the next stage of biomarker validation in a larger and alternate cohort. Importantly, this study has discovered perturbed metabolic pathways that may be specific to HIE. Therefore, measuring the interrelated metabolites of these HIE pathways may provide the robust biomarker panel needed for the early diagnosis of HIE.

Chapter 4

4 Lipid metabolite alterations in perinatal asphyxia and neonatal hypoxic ischaemic encephalopathy

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4.1 Abstract

Background

Evidence suggests early alterations in lipid metabolites arise in infants with perinatal asphyxia (PA) and its sequelae hypoxic ischaemic encephalopathy (HIE). Further investigation into lipid alterations in this population is warranted. This study aims to examine lipid alterations in infants with perinatal asphyxia and HIE, by performing the first untargeted mass spectrometry metabolomic analysis of non-polar cord blood serum metabolites.

Methodology

As described in Chapter 3, 30 cases of HIE defined by EEG at 24 hours and 41 perinatal asphyxia with no encephalopathy were included alongside 71 matched controls. The cord blood lipid profile was analysed using direct injection FT-ICR mass spectrometry in positive and negative ion mode. For each reproducibly detected metabolic feature, mean fold differences were calculated between HIE vs. controls (ΔHIE) and PA vs. controls (ΔPA). Putative lipid classes and annotations were assigned to metabolic features.

Results

Significant metabolite alterations were detected from six putatively identified lipid classes including fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids and prenol lipids. Fatty acyl compounds were predominantly altered (approx. 1.3 mean fold change) in the ΔHIE comparison, alongside some prenol and glycerolipids, while glycerophospholipids were significantly altered across severity grades of HIE.

Conclusion

Early lipid alterations may provide key insights in to the pathogenesis of HIE, and have the potential to present novel avenues for biomarker identification and neuroprotective possibilities. Future work will focus on the validation of these findings in an independent neonatal cohort.

4.2 Introduction

Neonatal hypoxic ischaemic encephalopathy (HIE) can cause long-term neurological disability and has an incidence of approximately 2 per 1000 term live births or as high as 26 per 1000 in the developing world(1, 9). Perinatal asphyxia (PA) describes the compromise of placental gas exchange to the foetus during labour, which can then be compounded by the occurrence of ischaemia. HIE arises from these two pathogenic mechanisms hypoxia and ischaemia which deprive the perinatal brain of oxygen. Current understanding of HIE suggests the immediate injury response is metabolic. The diminished oxygen and glucose supply associated with hypoxia ischaemia leads to a derangement of aerobic metabolism(4). The resulting energy deficit sparks a chain of pathogenic processes which ultimately leads to permanent neuronal cell death (18). Given that metabolic pathways are highly interconnected in large networks it is likely that a global disturbance of metabolism will quickly occur. However the full extent of metabolic change which occurs in response to HIE remains undefined.

Metabolomics enables the comprehensive study of all low molecular weight biochemicals or 'metabolites' present in a biological system (114). The recent application of metabolomics in the field of perinatal asphyxia and HIE has great potential to uncover novel metabolic perturbations. Emerging work in both animal models and human neonates has described novel metabolic alterations in response to hypoxia ischaemia and has allowed previously unsuspected metabolites to be implicated (255).

Lipid metabolites are a particular subset of naturally occurring metabolites found perturbed in these early studies. Solberg et al. (5, 178) found significant alterations in sterol and fatty acyl lipids were associated with prolonged hypoxia in a piglet model, and in a separate study, three retinal lipid metabolites closely linked to glycerophospholipid metabolism could discriminate between hypoxia and controls. Furthermore, elevations in the unsaturated fatty acyl arachidonic acid, an important constituent of the phospholipid bilayer, were reported in a macaque model of perinatal asphyxia (6). Finally,our group has performed the first targeted metabolomic analysis of umbilical cord blood from infants with perinatal asphyxia and HIE, and reported alterations in two lipid classes, glycerophospholipids and fatty acyls (8). In this accurately phenotyped cohort, acylcarnitines were significantly increased in both perinatal asphyxia and HIE versus their matched controls while phosphatidylcholines were significantly

altered in the perinatal asphyxia population alone. The important biological functions of lipids includes energy storage, signalling and cell membrane components (260). Broadly speaking, lipids are non-polar hydrophobic or amphipathic metabolites, often soluble in organic solvents (261). Recently an updated classification system has defined eight lipid categories (261): fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids and polyketides.

Further investigation into lipid alterations in this population is warranted. Identifying metabolic disturbances could shed light on previously unknown pathogenic mechanisms, having the potential to present novel avenues for biomarker identification and neuroprotective possibilities. This study aims to examine lipid alterations in infants with perinatal asphyxia and HIE, by performing the first untargeted mass spectrometry metabolomic analysis of non-polar umbilical cord blood metabolites.

4.3 Methodology

Details for study population, sample collection and storage are described in Chapter3, section 3.3, with more detailed information provided inAppendix E, section E.1.

4.3.1 Metabolomic analysis of UCB serum with DI FT-ICR MS

Sample preparation

A modified Bligh and Dyer biphasic extraction method was used to extract lipid metabolites from UCB serum (142, 219). Samples were defrosted in methanol on ice to minimise metabolic activity. Ice cold methanol, chloroform and water were added to the serum to achieve a ratio of 2:2:1.8. Each sample was vortex mixed and separated on ice (10 min) and centrifuged at 1800 *g* for 15 min at 4 °C. Lipids are hydrophobic by definition and so become partitioned into the lower chloroform phase. Aliquots of the lower lipid (non-polar) phase were dried under nitrogen and stored at -80 °C until MS analysis.

For maximum coverage of the human metabolome, the lipid fractions from each sample was analysed in both positive and negative ion mode mass spectrometry, full details of which are described below. Samples were re-suspended in 2:1, MeOH:H_2O w. 5 mM ammonium acetate for analysis.A 'solvent blank' consisting of the re-suspension solution was analysed as per a real sample, at the beginning and end of each analysis to detect any mass spectral features not of biological origin and to investigate and correct for any carryover of lipid species.

DI FT-ICR MS analysis and signal processing

The order of sample analysis was randomised however the technical replicates for a given sample were measured consecutively. All metabolomic analyses used a hybrid linear ion trap/Fourier transform ion cyclotron resonance 7-T FTICR mass spectrometer (LTQ FT Ultra, Thermo Fisher Scientific, Bremen, Germany) with a chip-based direct infusion nanoelectrospray ionisation (nESI) assembly (Triversa, Advion Biosciences, Ithaca, NY). Full details of nanoelectrospray and mass spectrometry conditions are provided in AppendixG.3. To increase metabolome coverage and maintain mass accuracy, spectra were collected using the 'SIM

stitching' method (220), whereby spectra are acquired as transients, across seven selected ion monitoring (SIM) mass ranges across a total SIM mass range of between 100-2000 Da for non-polar compounds.

Duplicate replicate analyses of each sample were taken and filtered into a single peak list via a three step filtering process to enhance spectral reliability (221). For statistical analysis, the final intensity matrices were normalised using the probable quotient normalisation (PQN) method (224) then logarithm (log_{10}) transformed.

A more detailed description is provided inAppendix G.

Quality assurance

As detailed in Chapter3 section 3.3.2, pooled quality control (QC) samples, representative of the study population, were injected at the beginning of each batch to equilibrate the instrument, and then injected every fifth sample throughout the batch to assess and correct the precision and reproducibility of individual mass spectral features (222, 223).

To reduce inter and intra batch variation the Quality Control-Robust Spline Correction (QC-RSC) batch correction algorithm (135) was applied, in conjunction with three spectral cleaning algorithms, to remove unreliable, inconsistent or unreproducible features.

4.3.2 Statistical analysis

All statistical analyses were performed using MatLab (version R2013, MathWorks, Natick, MA, USA).

Univariate analysis of metabolomic data

The relative standard deviation (RSD) of each reproducibly detected mass feature was calculated for the QC samples (RSD^{QC}). Mass features were included for further analysis only if the RSD^{QC}< 20%, a measure of analysis precision (165). Normality assumptions for each class were tested using the Lilliefor's test. Comparisons between both HIE and matched controls (ΔHIE) and perinatal asphyxia (no encephalopathy) and matched controls (ΔPA) are reported. Alterations in each mass feature were calculated using a paired Student's t-test for all matched case-control pairs with a Benjamini-Hochberg false discovery correction for multiple testing based on an assumed false discovery rate of 10% (148). This yielded an adjusted p value,

termed q value, for each variable. Any pair of samples with one or more missing values for that mass feature was excluded. For each mass feature, mean fold change differences were calculated using the $\log_{10}$ transformed data for ΔHIE and ΔPA and reported with 95% confidence intervals. In order to compare the univariate results from the two main arms of the study (ΔHIE and ΔPA), biplots of mean fold differences for those mass features significant in either comparison were constructed. Using the PQN data, the differences in effect size between the arms of the study (ΔHIE vs ΔPA) were tested using a 2 sample t-test and associated p-values reported. To determine if metabolites differed across the grade of injury, a one way analysis of variance (ANOVA) with Tukey's post hoc analysis was performed on the mean fold differences of the matched case-control pairs. Analysis was focussed on those peaks for which putative annotations could be determined.

Multivariate analysis of metabolomic data

Prior to multivariate analysis, missing values were imputed using k-nearest neighbour and the data was autoscaled (145). Multivariate analysis was conducted using PLS toolbox v. 7.9 (Eigenvector Research Inc, Manson, WA, USA) within Matlab. Unsupervised principal component analysis (PCA) was used as an unbiased method to visualise overall metabolic differences between groups. All of the reproducible mass features for a given comparison were included. Supervised multivariate predictive models using Partial Least Squares Discriminant Analysis (PLS-DA) were constructed using PLS toolbox, with internal cross validation. The optimal number of latent variables (LV) in each model was selected using a stratified ten-fold cross validation (venetian blinds) approach anddetermined by the minimum classification error. The associated squared correlation coefficient (R^2) and averaged correlation coefficient (Q^2) statistics were calculated as a measure of "goodness-of-fit" and "goodness-of-prediction" respectively. Model utility was determined using the area under receiver operating characteristic curve (AUROC), sensitivity and specificity.

4.3.3 Putative metabolite annotation

Putative annotation was performed by applying the Metabolite Identification Package (MI-Pack) workflow described previously (225). A single peak search was conducted using the HMDB (117), KEGG (homo sapiens metabolite library) (154) and LIPIDMAPS (153) databases to putatively annotate metabolites.

It is important to remember that several metabolites can be detected with the same accurate mass therefore a single metabolic feature may have multiple annotations. Likewise, multiple spectral features with different accurate masses can define a single metabolite, as different adducts can be formed from the same metabolite. Herein, metabolic features were identified by putative annotation based on accurate mass, equivalent to 'level 2' identification as defined by The Metabolomics Standards Initiative (155).
A more detailed description is provided in Appendix G.5.

4.4 Results

Details of the study population are provided in Chapter3, Section 3.4.1.

4.4.1 Univariate analysis

The DI FT-ICR MS analysis of umbilical cord blood serum yielded a total number of 591 and 760 non-polar mass spectral features in nano-electrospray ionisation (nESI) positive (+) and negative (-) mode respectively. Following the removal of unreliable, inconsistent or unreproducible metabolic features during spectral cleaning, 511 nESI(+) and 696 nESI(-) were subject to analysis(135)(Figure 4.1).

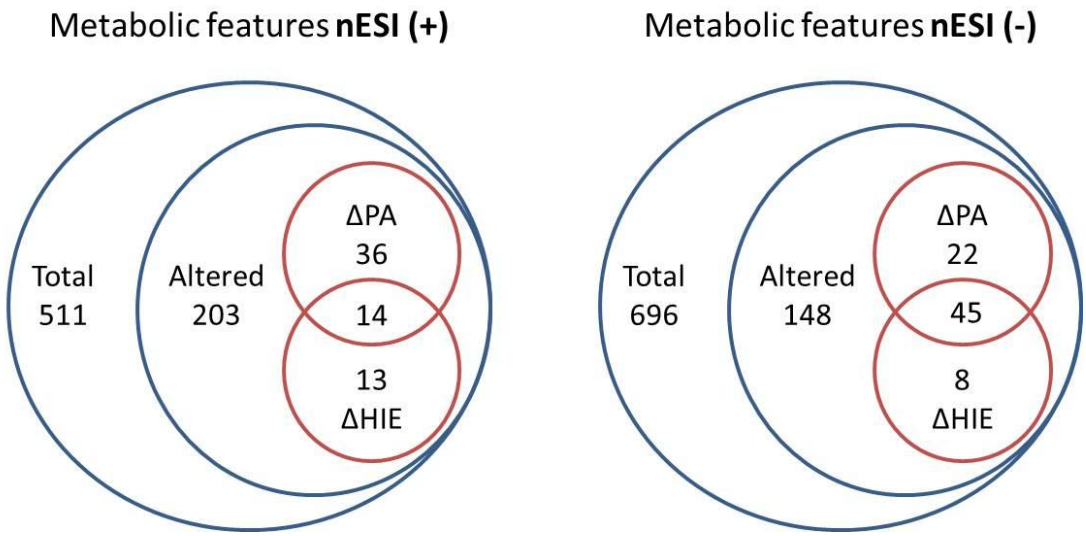

Figure 4.1Summary of the total number of metabolic features detected and significantly altered in nESI(+) and nESI(-) respectively. Red Venn diagrams show the distribution of putatively annotated metabolic features, of those significantly altered for each comparison groups; perinatal asphyxia *vs.* matched controls (ΔPA) and HIE *vs.* matched controls (ΔHIE).

To select the most important spectral features associated with perinatal asphyxia and HIE a fold change comparison, of two separate groups, perinatal asphyxia *vs.* matched controls (ΔPA) and HIE *vs.* matched controls (ΔHIE) was performed. In total 40% nESI(+) metabolic features were significantly altered ($p<0.05$): 82 specific to ΔPA and 46 specific to ΔHIE and 75 were altered in both groups. For nESI(-), 21% were significantly altered providing a breakdown of: 62 specific to

ΔPA, 71 specific to ΔHIE, with 15 features altered in both groups.Figure 4.2depicts a biplot of the mean fold differences in mass feature intensity, for those deemed significantly different in either ΔHIE, ΔPA or both comparisons with respect to the matched control samples. Fold change increases seem to progress from the ΔHIE group to the ΔPA group, with the largest fold change increases seen in the features which changed in both groups.

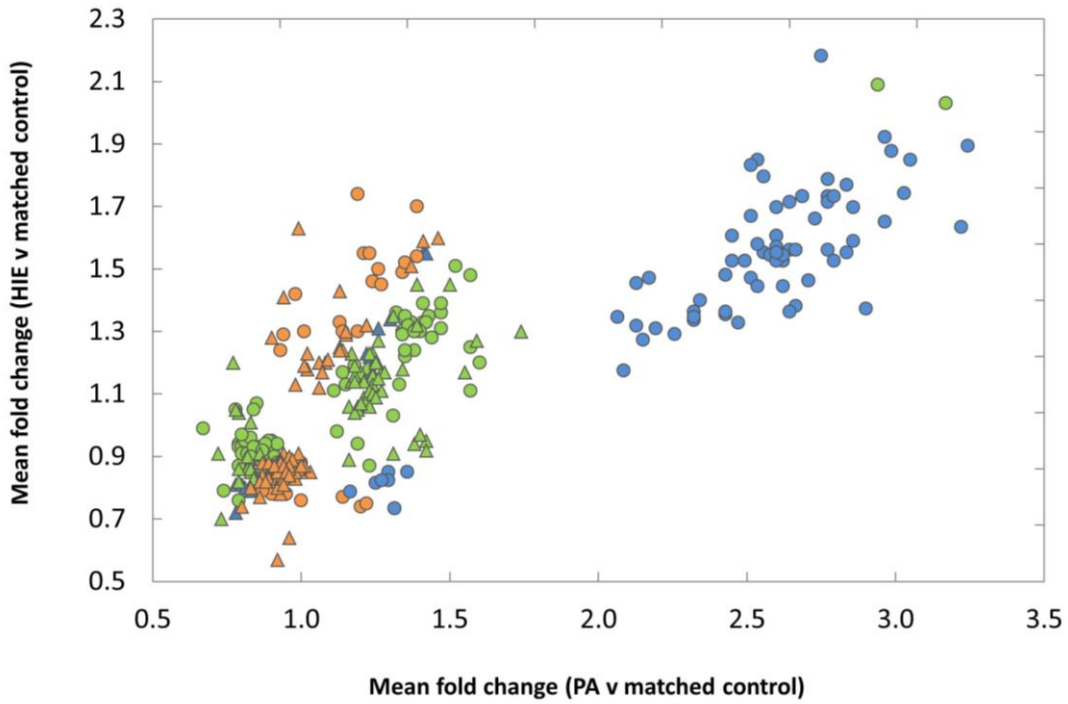

Figure 4.2 Biplot of the mean fold change with respect to the control of the significantly altered metabolic features between the matched case-control pairs; ΔPA *vs*. ΔHIE comparisons. Dots indicate features detected in nESI(+) and triangles nESI(-) modes and the colours orange, green and blue represent the features significantly altered ΔHIE only, ΔPA only and both comparison groups (ΔPA and ΔHIE) respectively.

To derive biological relevance each metabolic feature was compared to known endogenous human metabolites of a similar mass (<2ppm error), to ascribe a putative annotation. A putative metabolite name was assigned to 35% nESI(+) and 43% nESI(-) of detected masses. Of the 203 nESI(+) and 148 nESI(-) statistically different metabolic features 63 (31%) and 75 (50%) were putatively identified from their respective ion modes (Figure 4.1). See Appendix H, Table H.2, which lists the identification parameters of these putatively identified features. It is important to note that metabolite identifications discussed should be viewed as preliminary identifications until fully validated using chemical standards. Where no putative annotations could be ascribed, an empirical formulae was calculated, see Appendix I, Table I.2 (225). Assessment of the QC measurements for each putatively annotated metabolic feature confirmed good technical reproducibility for features with a median RSD^{QC} of 8% for nESI(+) and 11% nESI(-) (223).

Figure 4.3plots the mean fold change of the significantly altered features, putatively identified from either arm of the study. Metabolite alterations from six lipid classes are evident; fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids and prenol lipids. Fatty acyl compounds are predominantly altered (~1.3 mean fold change) in the ΔHIE comparison, alongside some prenol and glycerolipids. The glycerophospholipids and sphingolipids display minimal fold differences in either comparison groups. Sterol lipids display two clusters, one with small decreases in both groups, the other increasing around 1.3 fold in ΔHIE. Similarly glycerolipids display a cluster of marginally decreasing fold changes in ΔHIE however several features display and a second cluster with higher fold changes of over 1.5 fold for the ΔPA comparison.

Out of 21 putatively identified fatty acyl metabolites, 18 were significantly elevated, see Table 4.1. Two fatty acyls significantly altered in ΔHIE had 2 fold increases including the putatively annotated fatty amide compounds; N-palmitoyl serine and N-stearoyl serine ($q<0.056$), while arachidonoyl serotonin, L-palmitoylcarnitine, linoleyl palmitateand octadecenoic acid (oleic acid) all had fold changes of ≥1.5 in ΔHIE. Notably, changes in polyunsaturated fatty acids (PUFA) account for the majority of fatty acid alterations.

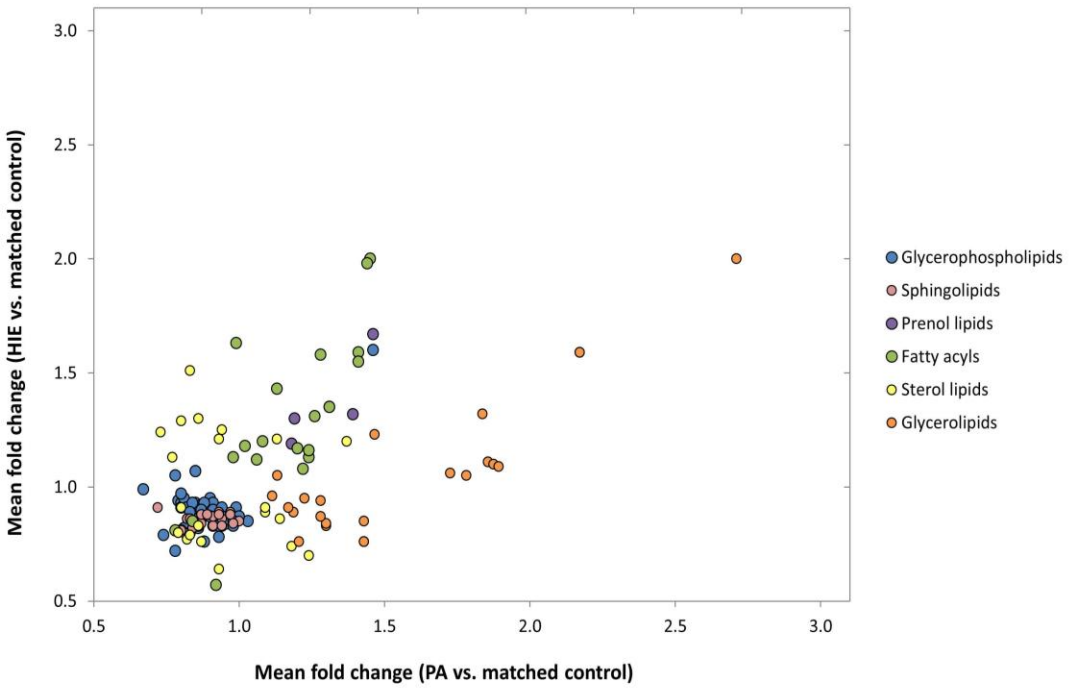

Figure 4.3 Biplot of the mean fold change for the putatively annotated features in the ΔPA and ΔHIE comparisons. Lipid categories are indicated by colour.

Twenty-three putatively annotated glycerolipids from two metabolite classes, diradylglycerols and triradylglycerols were found to be altered, see Appendix H, Table H.2 for annotations. Interestingly the two metabolic features which altered in both ΔPA and ΔHIE comparison groups displayed the largest fold increases. For example m/z 551.50371 increased from 1.7 fold (1.2, 2.3 95%CI) in ΔPA to 2 fold (1.4, 2.9 95%CI) in ΔHIE, while m/z 879.74216 increased from 1.4 fold (1, 1.9 95%CI) in ΔPA to 1.6 fold (1.1, 2.4 95%CI) in ΔHIE. As evident from the biplot (Figure 4.3), fold changes for glycerophospholipids cluster between 0.7–1.1 fold for both ΔPA and ΔHIE comparisons. Glycerophosphocholines (PC) were the class most predominantly detected, along with glycerophosphoethanolamines (PE), glycerophophoserines (PS) and glycerophophoglycerols (PG). One PE metabolite (m/z 498.29743) deviated from this cluster, having a fold change increase of 1.6 (1, 2.5 95%CI) in ΔHIE (p=0.041).

Several sterol lipids including steroids and vitamin D3 derivatives were putatively identified. Notably a reduction in sterol features significantly altered in ΔPA was evident. However small increases were predominantly seen in those significantly altered in ΔHIE. For example,

putatively annotated hydroxycholesterol sulfate was significantly altered (p=0.026) in ΔHIE with a fold change of 1.5 (1.1, 2.2 95%CI). Four prenol lipids were detected, two isoprenoids and two hopanoids. All displayed raised fold changes in both comparison groups with the fold change increasing as the injury progressed from perinatal asphyxia to HIE. Finally, 21 sphingolipids were putatively identified. Interestingly they were principally altered in the HIE population, with all showing fold change decreases. For example, sphingosyl-phosphocholine displayed small yet significant decreases in both groups, (p=0.003) ΔPA and (p=0.011) ΔHIE.

For each putatively annotated feature, mean fold change differences across the grades of HIE compared to their matched controls (ΔMild n=17, ΔModerate n=8 and ΔSevere n=5) and ΔPA (n=41) were analysed using a one-way ANOVA ($p<0.05$). Nine putatively annotated features were statistically significant across grades including three sterol lipids, one triradylglycerol (m/z 757.55715) and five glycerophospholipids putatively annotated as either PCs or PEs. Boxplots for the triradylglycerol and glycerophospholipids all detected in nESI(+) are shown inFigure 4.4. Tukey post hoc analysis revealed a significant difference between ΔPA *vs.* Δmoderate and Δmild *vs.* Δmoderate for features m/z 757.55715 and m/z 730.53876, and between ΔPA *vs.* Δsevere and Δmild *vs.* Δsevere for features m/z 546.35584 and m/z 850.66926. Though the fold change differences in these lipids were less pronounced than other metabolite classes a mean fold increase is apparent for the infants with both moderate and severe HIE compared to perinatal asphyxia and mild HIE. These results must be interpreted with caution due to small sample numbers, and the disparity between sample numbers in each group.

Examination of the effect size ΔPA vs. ΔHIE revealed the glycerophospholipid m/z 780. 55289 was also statistically significant (p=0.02) between infant populations. In agreement, the C^{13} isotope of this feature m/z 781.55635 is also significantly different (p=0.03), along with a fatty acyl (m/z 372.31094) and glycerolipid (m/z 753.56307 nESI(-) metabolite. Significant features without a putative annotation were ascribed an empirical formula(e) and are listed in Appendix I, Table I.2.

Table 4.1 Putatively identified significantly altered compounds from the fatty acid category of lipids.

m/z	Putative ID	Class	Perinatal asphyxia *vs.* matched controls (ΔPA)		HIE *vs.* matched controls (ΔHIE)		ΔPA to ΔHIE
			p-value	mean fold (95%CI)	*p*-value	mean fold (95%CI)	
223.17032	HETE OR EET OR Hydroxyarachidonic acid OR Beyerol OR Trihydroxy-decipiadiene (>5)	ES	**0.002**	1.3 (1.1, 1.5)	**0.003**	1.3 (1.1, 1.6)	↔
251.20166	Hexadecadienoic acid OR Hydnocarpic acid OR Palmitolinoleic acid	FA&C	**0.005**	1.2 (1.1, 1.4)	0.067	1.2 (>1, 1.4)	↔
277.21731	Linolenic acid OR lamenallenic acid OR Catalpic acid OR Columbinic acid OR Crepenynate (>5)	FA&C	**0.030**	1.2 (>1, 1.5)	0.340	1.2 (0.9, 1.6)	↔
315.20965	Linoleic acid OR laballenic acid OR 16:2(2E,4E)(4Me,6MeS) OR Octadecadienoic acid OR Malvalic acid (>5)	FA&C	0.394	1.1 (0.9, 1.5)	**0.045**	1.4 (>1, 2)	↑
319.22247	Oleic acid OR Octadecenoic acid OR Octadecylenic acid OR Petroselaidic acid OR Vaccenic acid (>5)	FA&C	0.963	1 (0.7, 1.3)	**0.013**	1.6 (1.1, 2.4)	↑
319.22783	11-deoxy-16,16-dimethyl-PGE2 OR 16,16-dimethyl-PGA1	ES	0.685	0.9 (0.6, 1.4)	**0.036**	0.6 (0.3, <1)	↓
325.23844	Ceriporic acid A	FA&C	**0.024**	1.2 (>1, 1.5)	0.554	1.1 (0.8, 1.4)	↓
344.27963	N-palmitoyl serine	FA	**0.019**	1.5 (>1.1, 2)	**0.002**	2 (1.3, 3)	↑
363.25168	Eicosanedioic acid OR PGF1a alcohol	FA&C	0.437	1.1 (0.9, 1.3)	**0.037**	1.2 (>1, 1.4)	↑
372.31094	N-stearoyl serine	FA	**0.004**	1.4 (1.1, 1.8)	**<0.001**	2 (1.5, 2.6)	↑

381.30108	21:2(5Z,14Z) OR 21:2(5Z,16Z)	FA&C	0.185	1.1 (1, 1.2)	**0.045**	1.1 (>1, 1.3)	↔
385.23605	15R-PGA2 methyl ester OR acetate OR Digoxigenin OR gitoxigenin	ES	**0.007**	0.8 (0.7, 0.9)	**0.010**	0.8 (0.7, 0.9)	↔
389.23348	14:2(6,9) OR Tetradecadienoic acid OR Alepric acid OR cucujolide III	FA&C	**0.044**	1.2 (>1, 1.5)	0.429	1.1 (0.8, 1.6)	↓
395.31671	13,16-Docosadienoic acid OR 22:2(3Z,16Z) OR 22:2(7Z,13Z) OR 22:2(7Z,15Z)	FA&C	0.797	1 (0.9, 1.1)	**0.042**	1.1 (>1, 1.3)	↑
400.34225	L-Palmitoylcarnitine	FE	**0.017**	1.3 (>1, 1.6)	**0.001**	1.6 (1.2, 2)	↑
421.33245	24:3(15Z,18Z,21Z)	FA&C	0.771	1 (0.9, 1.2)	**0.044**	1.2 (>1, 1.4)	↑
476.27823	HETE-DA, HETE-VA	FA	**0.031**	0.8 (0.7, <1)	0.059	0.9 (0.7, 1)	↑
497.2941	Arachidonoyl Serotonin	FA	0.087	1.4 (0.9, 2.1)	**0.045**	1.6 (>1, 2.5)	↑
559.47286	34:4(19Z,22Z,25Z,28Z) OR Linolenyl palmitatoleate OR Palmitoleyl linolenate	FA&C	**0.048**	1.3 (>1, 1.7)	0.101	1.3 (0.9, 1.9)	↔
561.48858	Linolenyl palmitate OR Linoleyl palmitatoleate OR Palmitoleyl linoleate OR Palmityl linolenate	FE	**0.031**	1.4 (>1, 1.9)	0.210	1.3 (0.8, 2)	↓
563.50427	Linoleyl palmitate OR Oleyl palmitoleate OR Palmitoleyl oleate OR Palmityl linoleate	FE	**0.015**	1.4 (1.1, 1.9)	**0.030**	1.5 (>1, 2.3)	↑

(>5) indicates greater than 5 putative annotations. ES; Eicosanoids, FE; Fatty esters, FA; Fatty amides, FA&C; Fatty acids and conjugates.
Arrows indicate decrease (↓), increase (↑) or no change (↔) between mean fold changes from ΔPA to ΔHIE.
>1 or <1 indicates a confidence interval of greater or less than 1, whose decimal points have been rounded.

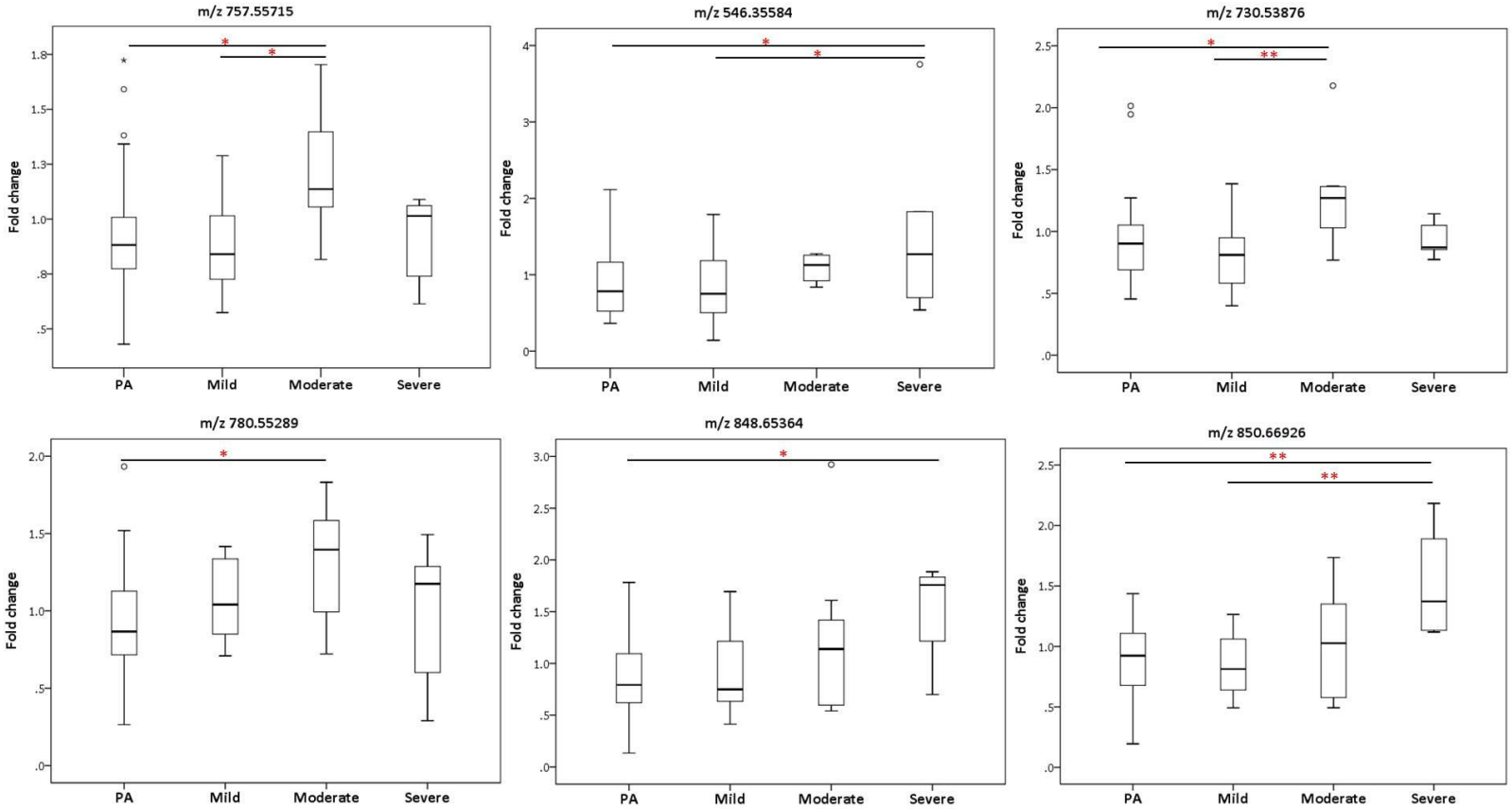

Figure 4.4 Metabolic feature fold-changes stratified by disease severity; perinatal asphyxia (PA) mild HIE, moderate HIE and severe HIE, each compared to their respective matched controls. Mass feature m/z 757.55715 is a triradylglycerol, all others mass features are glycerophosphocholines.The black line and red * denotes statistical significance of ≤0.05, ** ≤0.01 or * ≤0.001 between two groups.**

4.4.2 Multivariate analysis

Figure 4.5presents PCA plots for three comparison groups; (i) PA *vs.* matched controls, (ii) HIE *vs.* matched controls, and (iii) HIE *vs.* non-HIE (perinatal asphyxia and two control groups). The plots were constructed using all mass spectral features detected in nESI(+) and nESI(-), and all samples bar two extreme outliers from the nESI(+) control group. The latter comparison group, HIE *vs.* non-HIE, is the most clinically relevant to investigate whether a lipid profile could discriminate infants with HIE at birth. These plots explain 50.9% (4 PCs) nESI(-) and 70% (9 PCs) nESI(+) of the total variance in metabolic data between samples (Figure 4.5 (c) and (f)). A PLS-DA model for this comparison group was then built using two and one latent variable in nESI(+) and nESI(-) respectively. Using nESI(+) features the model had an R^2 = 0.28, Q^2 = 0.10, classification error = 0.39 and an AUROC of 0.71. The corresponding sensitivity and specificity for predicting HIE (any grade) was 46% and 75% respectively. Using nESI(-) features the model had an R^2 = 0.13, Q^2 = 0.07, classification error = 0.37 and an AUROC of 0.69. The corresponding sensitivity and specificity for predicting HIE (any grade) was 60% and 65% respectively.

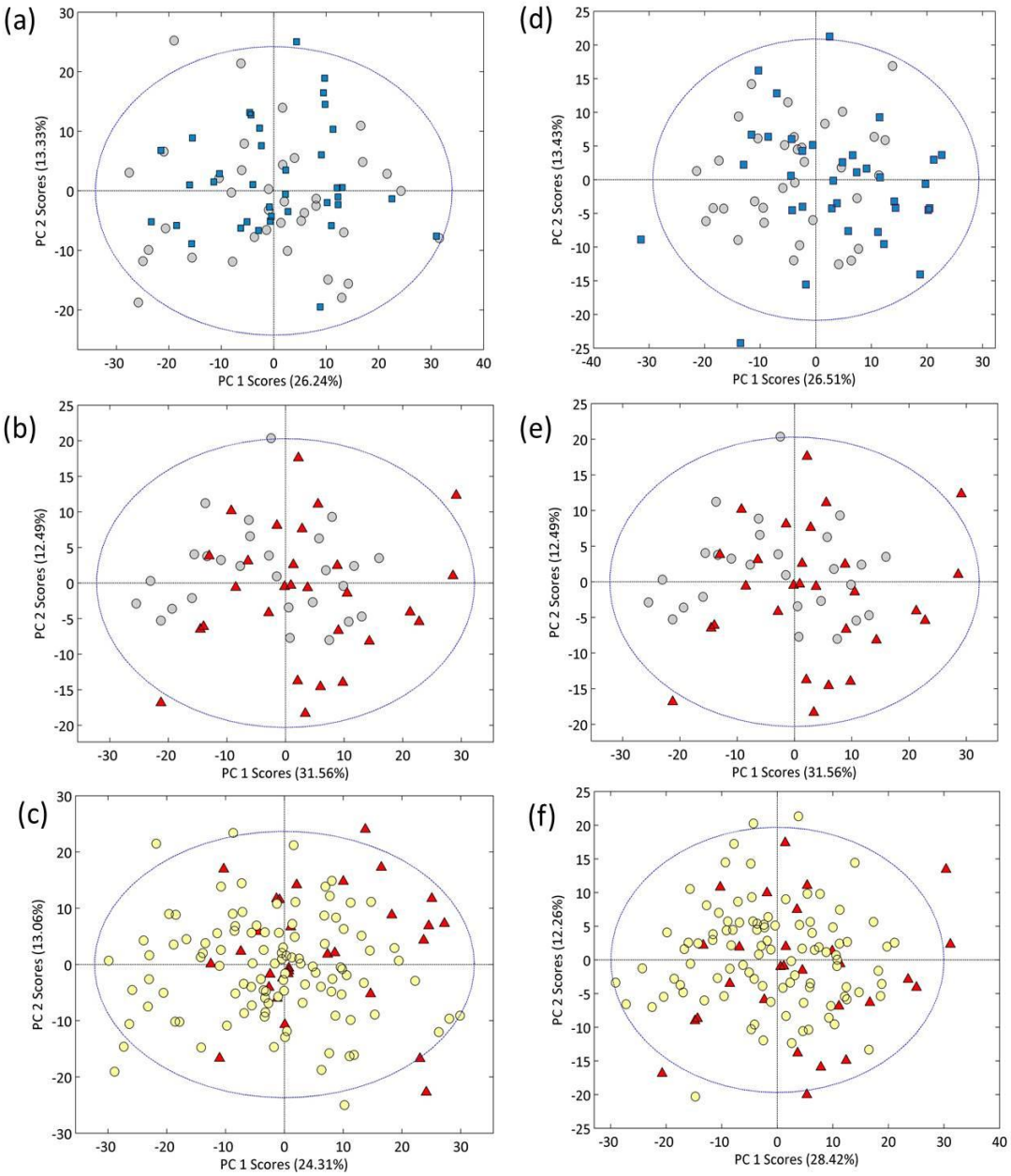

Figure 4.5 PCA plots for the three comparison groups; (a) (d) PA *vs.* matched controls,(b) (e) HIE *vs.* matched controls and (c) (f) HIE *vs.* non-HIE (perinatal asphyxia and two control groups). The metabolic profile of infant groups are represented by the following; blue squares = PA, grey circles = controls, red triangles = HIE and yellow circles = non-HIE. Plots (a)(b)(c) were built using all non-polar nESI(-) features and plots (d)(e)(f) using nonpolar nESI(+) features.

4.5 Discussion

In this population of infants with perinatal asphyxia and HIE, which was confirmed both clinically and by EEG analysis, we have performed the first untargeted mass spectrometry metabolomic analysis of non-polar umbilical cord blood metabolites. We have characterised lipid alterations in these infants, putatively identifying significant alterations in six lipid categories; fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids and prenol lipids. This study illustrates significant elevation of fatty acyl compounds in the HIE population along with derangements in glycerolipids and sterol lipids in both infant groups. Interestingly, although minimal fold changes were evident in glycerophospholipids, metabolic features belonging to this class could significantly differentiate between grades of HIE.

Under normal aerobic conditions pyruvate is the major product of glycolysis. It enters the mitochondrion where it is converted to acetyl-CoA via the enzyme complex pyruvate dehydrogenase. This vital complex is regulated by the ATP/ADP ratio, whereby an increase in the ratio inhibits complex activity. Primarily acetyl CoA enters the tricarboxylic acid (TCA) cycle for energy production. However its use extends to fatty acid and cholesterol biosynthesis, and the synthesis of acetylcholine, a component of glycerophospholipid metabolism(4). Under anaerobic conditions like that of hypoxia-ischaemia, the ATP/ADP ratio will decrease promoting complex mediated production of acetyl-CoA, and may lead to changes in fatty acid, cholesterol and glycerophospholipid metabolism.

The newborn brain is supported almost exclusively by oxidative metabolism and its neuronal membranes are rich in long chain polyunsaturated fatty acids (PUFA), an important nutrient during the late foetal and neonatal period, involved in neuronal cell growth and development (262). Hypoxia-ischaemia is associated with a rapid rise in lactic acid and reactive oxygen metabolites (ROM), whose overproduction disturbs the normal redox state of cells, causing toxicity. ROM initiate the production of peroxides which damage important cellular components especially lipids (lipid peroxidation), disrupting cell signalling, increasing membrane permeability and potentially causing cell death (57).

ROM arise during early reperfusion, consistent with the presences of an oxidative attack on membrane lipids (263). Important sources of ROM include the oxidation of hypoxanthine to xanthine by xanthine oxidase, and onto uric acid (264) or prostaglandin synthesis derived from

the breakdown of free fatty acids. Our metabolomic analysis measured lipids from umbilical cord blood collected at birth, potentially too early to capture metabolic alterations of reperfusion oxidative stress. However, analysis of polar metabolites from this cohort indicated an increase in hypoxanthine (1.2 fold) and significant increase in uric acid (1.3 fold) in HIE compared to matched controls (see Chapter 3). The activation of these purine pathway metabolites, may indicate ROM generation processes which we are measuring in cord blood.

The neonatal brain is particularly vulnerable to this oxidative stress due to high levels of endogenous lipid cell membrane components, approximately 35% of which are composed of omega 3 and omega 6 PUFA (265) and the limited or underdeveloped antioxidant activity to counteract the increased ROM generation (266). Highly abundant in neuronal cell membranes, docosahexanoic acid (DHA) and arachidonic acid are the primary PUFA present in fetal plasma. Released from the phospholipid cell membrane, they act both directly or as precursors to regulate membrane functions including, cell growth, inter and intra cellular communication and protein function (267).

We have detected several putatively annotated long chain PUFA, which increased in response to HIE. One such metabolite is linolenic acid (m/z 277.21731), which in the α-linolenic acid form is a precursor to DHA. Accumulating evidence suggests that omega-3 PUFA, in particular DHA, may attenuate brain injury. Animal studies have shown that enriching the maternal diet with long chain PUFA, for example flaxseed (a rich source of α-linolenic acid), eicosapentaenoic acid (EPA) or DHA prior to induced hypoxic-ischaemia reduces brain damage and improves neurological outcomes in the off-spring (268, 269). Pre-hypoxia treatment of rat pups with intraperitoneal (i.p.) injections of DHA improved functional outcomes (270). Moreover, administration of omega-3 fatty acid rich triglyceride emulsion, specifically containing DHA, both before and after cerebral hypoxia-ischaemia reduced infarct volume in neonatal mice (271).

Deficient levels of DHA in the brain are associated with impaired visual function, cognitive and behavioural abnormalities and reduced activity of membrane proteins and receptors. Reports have shown omega–3 fatty acid depletion alters monoaminergic neurotransmitter metabolism, for example the serotoninergic and dopaminergic systems (272). Notably, we have also identified alterations in serotoninergic metabolites in infants with HIE (see Chapter 3). We

hypothesise, that perhaps DHA is depleted during hypoxia-ischaemia, so to compensate, other PUFA particularly α-linolenic acid become upregulated.

We detected a two fold increase in putatively identified linoleic acid, a PUFA found in lipid cell membranes. Linoleic acid is a precursor in the biosynthesis of arachidonic acid, which in turn is metabolised in the brain, among other sources, to produce hydroxyeicosatetraenoic acids (HETEs), epoxyeicosatrienoic acids (EETs)prostaglandin, prostacyclin, thromboxane, and leukotrienes (273). We found significant differences in the HETE and/or EET (m/z 319.22783 and 476.27823, Table 4.1) for both study populations. These metabolites play an important role as paracrine factors and secondary messengers in the control of cardiac and vascular functions, as well as modulating inflammatory responses (273). Alterations in arachidonic acid metabolites in the CSF of newborn infants with HIE have previously been reported (274) while previous metabolomic investigations reported a marked elevation of arachidonic acid in a macaque model of perinatal asphyxia, suggesting a disturbance of the cell membrane(6).Examining the trajectory of DHA, arachidonic acid and linoleic acid in the days after a hypoxic ischaemic insults, showed that the natural accumulation of these metabolites associated with brain development in the neonatal rat was impaired (275). The clinical development of *in vivo* techniques for directly assessing PUFA are underway recognising the importance of monitoring PUFA as a marker of oxidative stress and subsequent brain damage in neonatal HIE (276). Finally arachidonoyl serotonin is another putatively annotated fatty acid metabolite highly increased (around 1.6 fold) in both populations compared to controls, although only statistically significant in HIE. As an inhibitor of fatty acid amide hydrolase, it has previously demonstrated both analgesic and anti-convulsant activity in rodent models (277, 278) and therefore may potentially be elevated in HIE as a protective response.

The present analysis also detected alterations in several tentatively identified sterol lipids. In particular several vitamin D3 derivatives were significantly altered, three of which displayed significant elevations in infants with HIE. Evidence suggests vitamin D has neuroprotective and antioxidant properties, having the ability to attenuate oxidative injuries and reduce ischaemia derived damage (279-281). A recent study of vitamin D status measured in venous samples from infants with HIE reported significant depletions in vitamin D levels in the first day of life but with raised levels of the antioxidant enzymes (282). We report significant decreases (0.8

fold) and significant increases (1.2-1.3 fold) for three metabolites of the vitamin D3 derivatives class, measured at birth in neonates with HIE.

Steroids and steroid conjugates were another group of sterols displaying noteworthy alterations. Cholesterol sulfate was significantly decreased in both the perinatal asphyxia and HIE comparison groups. This sterol lipid has important functions as regulatory molecule and cell membrane component (283). Cholesterol is an immediate precursor for steroids and bile acids, and its sulfonication is an important modification. Through adrenal mitochondria side-chain cleavage (SCC) enzyme systems, cholesterol sulfate serves as a substrate for the synthesis of adrenal steroids such as pregnenolone sulfate and dehydroepiandrosterone (DHEA) sulfate (283). Previous analysis of polar metabolites detected significant depletions in both of these endogenous steroid hormones in perinatal asphyxia and HIE (see Chapter 3). Here we detected slightly elevated levels of pregnenolone sulfate in HIE, however this was not statistically significant from controls. Mitochondria isolated from human foetal adrenal glands could effectively convert large volumes of cholesterol sulfate to pregnenolone sulfate, however those isolated from adult adrenals did not display the same effects (284). The mixed hydrophobic/hydrophilic properties of cholesterol sulfate mean it's suited to interactions with cell membrane constituents where it has a stabilizing role. Notably, cholesterol sulfate has also been shown to modulate cellular lipid biosynthesis (285) and arachidonic acid metabolism (286).

The present study had some limitations. Putative metabolite annotation was used to ascribe identities. Accurate metabolite identification is both expensive and technically demanding, particularly for studies using untargeted techniques where a large number of features are detected (259). Identification confidence relies on the analytical platform, robustness of methods and completeness of databases used (156). Numerous studies have demonstrated the ability for DI FT-ICR MS, especially when coupled to nanoelectrospray ionisation, to capture large amounts of metabolic data with high accuracy (220, 287, 288). We used stringent protocols to ensure robustness of methods including the standardisation of sample collection and storage, spectral cleaning and batch correction.

To assess the likelihood of false discoveries a Benjamini-Hochberg false discovery rate of 10% was applied to compensate for the multiple comparisons being tested (148). Only two

putatively annotated features passed this correction procedure: exogenous lidocaine administered via an epidural during labour and N-stearoyl serine ($q<0.056$). The aim of this exploratory study was to identify potential metabolite alterations. Therefore we did not rule out metabolites particularly those with pertinent links to previous literature, based on the false discovery evaluation. However to progress these findings we believe the optimal method is to validate the findings in an independent cohort of infants (144).

Unsupervised multivariate analysis was unable to separate the neonatal populations under study, possibly due to the inherent variation in this human biological data and relatively small sample numbers. Supervised analysis did not perform much better at separating HIE from all other outcome states. Though each dataset (positive and negative) could diagnose HIE with an AUC of 0.7, sensitivity of the model was poor and the classification error indicated that typically 40% of infants would be misclassified. A metabolite fingerprint may not be useful for diagnosing HIE, instead an algorithm based on only a few key metabolites or a metabolic pathway could be more powerful.

Strengths of the study include our accurately phenotyped cohort of neonates with perinatal asphyxia and HIE. The injury was defined clinically and then confirmed using continuous multichannel EEG, the gold standard measurement (97). To reduce biological variation unrelated to the injury, each case was matched with a healthy control, as far as was possible for several variables including; gender, gestational age, birthweight, date of birth and method of delivery. The highly unpredictable nature of HIE and low disease prevalence (0.2-0.3%) make recruiting a sufficient and appropriate study population difficult. This cohort (n=41 perinatal asphyxia and n=30 HIE) is the largest infant cohort of its kind used in metabolomic studies (255). All infants had umbilical cord biobanked within 3 hours of birth using rigorous and identical procedures. We believe metabolomic analysis of umbilical cord blood offers the potential to provide a representative snapshot of the metabolic derangements associated with hypoxia-ischaemia, prior to postnatal intervention. However no single specimen can reflect the total metabolomic disturbance of an organism. Cord blood sampling has the unique advantage of being non-invasive, making it a suitable as an early diagnostic and prognostic specimen.

The current study is limited by the lack of an independent validation cohort to assess biological validity. However, a key strength is the system of cross validation implemented by our group to

discover and confirm technical validity of metabolite alterations in these infants by using several analytical techniques separately. Using a combined DI and LC MS/MS targeted metabolomic approach we previously measured a defined list of acylcarnitines, glycerophospholipids and sphingolipids (8). We reported significantly increased cord blood acylcarnitine levels in both perinatal asphyxia and HIE, however the increase in these fatty acid compounds was more pronounced in the HIE population. We also reported significant alterations in six phosphatidylcholines in infants with perinatal asphyxia. The present study supports these finding, also showing significant alterations in these lipid classes. For example the fatty acid L-palmitoylcarnitine was previously identified as increasing by up to 80% in HIE, herein we found a 1.6 fold increase in the same population, which instils additional confidence in our current findings.

Identifying interconnected metabolic disturbances could shed light on previously unknown pathogenic mechanisms, having the potential to present novel avenues for biomarker identification and neuroprotective possibilities. To translate these findings to the clinic, confirmation of identification and quantification using metabolite standards is needed. Future work will focus on validating the findings in an independent cohort of infants.

Chapter 5

5 Cord metabolite model as a predictor of outcome in perinatal asphyxia and hypoxic ischaemic encephalopathy

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5.1 Abstract

Background

An early objective biomarker to predict the severity of hypoxic-ischaemic encephalopathy (HIE) remains elusive. Metabolomic analysis of umbilical cord blood (UCB) from infants with perinatal asphyxia and HIE has revealed two promising metabolite models for this purpose, comprised of five and four metabolites respectively. This work aims to evaluate the ability of these metabolite biomarker models to predict three-year neurodevelopmental outcome in infants with perinatal asphyxia and HIE.

Methodology

From September 2009 to June 2011 infants at risk of perinatal asphyxia were recruited in a single maternity hospital. UCB was drawn and biobanked at delivery. Neonates were monitored for development of encephalopathy both clinically and electrographically. Infants were assessed at 36-42 months using the Bayley Scales of Infant and Toddler Development Ed. III (BSID-III).

Results

Thirty six (model A) and thirty one (model B) infants had both UCB metabolomic analysis and outcome available. Both metabolite model scores significantly correlated with outcome R=0.429 p=0.009 (model A) and R=0.549 p=0.001 (model B) respectively. Model B was particularly robust to predict both severe outcome (AUROC of 0.915, p=0.004) and intact survival (AUROC of 0.800, p=0.006). Neither metabolite model score correlated with performance in the individual BSID-III subscales (cognitive, language or motor).

Conclusion

Both metabolite models outperformed other standard biochemical markers at birth for prediction of outcome at three years. While further validation is required, these metabolite models could be used to provide important prognostic information to assist management planning in infants with perinatal asphyxia and HIE.

5.2 Introduction

Hypoxic-ischaemic injury in the perinatal period remains an important cause of neonatal encephalopathy and of subsequent death and disability in children worldwide (1). The ability to predict severity in a timely manner is becoming increasingly important. Currently available assessment tools in hypoxic ischaemic encephalopathy (HIE) suffer from fundamental flaws that limit their usefulness (37). Foetal heart monitoring, Apgar scores, perinatal pH and lactate, lack sufficient sensitivity and specificity for the prediction of outcome (66, 75, 78, 289-291). The value of clinical and electrographic assessment is dependent on the availability of resources and expertise and remains difficult to interpret due to the evolving nature of the underlying hypoxic-ischaemic injury(97, 292-294). An early, robust, user-independent test that could aid prognostication is urgently needed.

Metabolomic analysis of umbilical cord blood (UCB) is becoming an increasingly popular investigative field in maternal-neonatal medicine(166). Metabolomics can provide a phenotypic biochemical fingerprint by measuring complex interactions and concentration changes of multiple low molecular weight metabolites(122), while UCB provides a potent source of early metabolic information. In HIE, metabolomics has the unique potential to detect biochemical pathway alterations in response to the hypoxic-ischaemic environment(255). These changes may provide useful biomarkers to predict severity or quantify the hypoxic-ischaemic insult.

Using metabolomics, we have previously described alterations in the UCB metabolite profile of infants with perinatal asphyxia and HIE (7, 8). Linear regression analysis revealed two separate metabolite models; the first based on alterations of decenoyl-L-carnitine, 3,5-tetradecadiencarnitine, PC ae C38:0, phenylalanine and proline (model A) detected using a targeted direct infusion (DI-) and liquid chromatography (LC-) tandem mass spectrometry (MS/MS) approach, the second based on alterations of succinate, glycerol, 3-hydroxybutyrate and O-phosphocholine (model B) detected using proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. These models were promising for prediction of HIE, the first predicting HIE from all other outcomes (perinatal asphyxia and controls) with an area under the curve (AUC) of 0.92, second predicting severe HIE with an AUC of 0.95. The long-term significance of altered metabolite fingerprints in UCB is unknown. Therefore, the aim of this study is to correlate these metabolite models with three-year neurodevelopmental outcome in this cohort, applying

a simplified version of the previous models for greater clinical utility. We also compare the performance of these models against other standard biochemical, physiological and clinical markers available in the early neonatal period.

5.3 Methodology

5.3.1 Patient selection

Recruitment took place from September 2009 to June 2011 in a single maternity hospital with 9000 deliveries per annum. Infants of ≥ 36 weeks gestation were recruited if they met any of the following risk factors for perinatal asphyxia: umbilical cord pH <7.1, 5 minute Apgar score ≤ 6 or the need for intubation or cardiopulmonary resuscitation at delivery. Eligibility required the infant to be in-born to allow the collection of UCB,which was biobanked within three hours of birth for metabolomic analysis. Written informed parental consent was obtained for inclusion. The Clinical Research Ethics committee of the Cork Teaching Hospitals provided ethical approval.

All consented cases had demographic and clinical details collected prospectively including Apgar scores at 1 and 5 minutes, degree of resuscitation and pH, lactate and base deficit measurements from arterial and venous cord sampling, and first postnatal sampling. Degree of resuscitation was classed as (i) none required, (ii) intermittent positive pressure ventilation >1min, (iii) intubation, (iv) CPR or (v) adrenaline given.

Cases were divided into (i) infants with clinical or biochemical signs of perinatal asphyxia at birth but recovered quickly with no clinical signs of encephalopathy, or (ii) infants who developed an evolving clinical and electrographic encephalopathy consistent with HIE. HIE severity was determined by a modified Sarnat score assigned at 24 hours of life by a dedicated research fellow (B.H.W).

All cases underwent EEG monitoring. Silver-silver chloride EEG electrodes were applied to the scalp at F3, F4, C3, C4, T3, T4, O1, O2, and Cz (according to the international 10-20 system modified for neonates). The EEG was recorded on a NicOne video-EEG system (Carefusion, Madison, WI). The entire video-EEG was then reviewed by an experienced neonatal

neurophysiologist (G.B.B). One hour epochs of the background EEG were graded at 6 hours and 24 hours after birth where available. An EEG grade was assigned at these time-points, based on a previously described classification system(14, 15), and designated as: (0) normal, sleep cycles present on continuous background; (1) mildly abnormal, e.g. continuous but abnormalities of sleep cycles; (2) moderately abnormal, e.g. discontinuity or presence of seizures and (3/4) severely abnormal, suppressed/isoelectric tracing and/or high seizure burden/status epilepticus.

Therapeutic hypothermia (TH) was commenced at the discretion of the supervising clinician on duty based on compliance with the TOBY criteria (295) within the first 6 hours of life.

5.3.2 Three- year outcome

All eligible cases were invited for neurodevelopmental outcome assessment between 36 to 42 months of age. The Bayley Scales of Infant and Toddler Development Edition III (BSID-III) was administered to the children by a research fellow (C.E.A), trained in administration and scoring and blinded to the clinical background of the infants. Three-year outcome was designated as "normal" if the child scored >85 in the three BSID-III subscales; cognitive, language and motor. A mildly abnormal outcome was determined if 1 or 2 subscales scored ≤85 but >70, and a moderately abnormal outcome was determined if all three subscales scored ≤85 but >70.Due to low numbers, the mild and moderate outcome groups were combined into a "mild/moderate" group. A "severe" outcome included death, dyskinetic or spastic quadriplegic cerebral palsy (CP) or ≤70 in all BSID-III subscales.

5.3.3 Metabolomic analysis

We have previously described the UCB metabolomic profile of infants with perinatal asphyxia and HIE using combined targeted DI- and LC-MS/MS assay (AbsolutIDQ p180 kit, Biocrates Life Sciences AG, Innsbruck, Austria) (model A) and ^{1}H-NMR- spectroscopy (model B). A detailed description of metabolite extraction, spectra acquisition, metabolite identification and quantification is provided in these texts (7, 8).

5.3.4 Statistical analysis

Patient demographic information is presented as mean (standard deviation), median (interquartile range) and n (percentage) as appropriate.

The previously reported five (8) and four (7) metabolite predictive modelswere simplified to provide greater clinical utility, by removing the weightings, using the concentration values and reducing the equations to:

Model A

$$y = \frac{proline \times 3{,}5 - tetradecadiencarnitine \times phenylalanine}{decenoyl - l - carnitine \times PC\ ae\ C38{:}0}$$

Model B

$$y = \frac{succinate \times glycerol}{3 - hydroxybutyrate \times O - phosphocholine}$$

Summary statistics for model scores, y, and other standard biochemical and clinical markers across each outcome group (normal, mild/moderate and severe) are reported. Differences between groups are examined using Kruskal-Wallis, Mann-Whitney U and chi-squared testing as appropriate. Correlation of non-parametric data is performed using Spearman's rho (ρ) correlation coefficient.

The ability of the metabolite combination and other markers to predict neurodevelopmental outcome at three years was assessed using receiver operating characteristic (ROC) curve analysis. Model utility was determined using the area under ROC curve (AUROC), and best cut-off was determined using the coordinates of the curve. Sensitivity and specificity for a given cut-off are reported.

For examining the relationship between BSID-III subscale composite scores and metabolite model scores, metabolite scores were log transformed to normalise data and correlated with

composite scores using Pearson's R. A *p*-value <0.05 was considered significant. All statistical analysis was performed using IBM SPSS statistics 21.0.

5.4 Results

5.4.1 Study population

Model A

DI and LC-MS/MS metabolomic analysis was performed in 71 infants. Five infant samples failed metabolomic analysis and two were retrospectively excluded due to alternative diagnoses; one infant had a spinal cord injury and the other an inborn error of metabolism, which could potentially affect their metabolomic profile at birth and/or outcome. Three-year outcome was available in 36/64 (56%) children; see Table 5.1 for demographic information. Twenty-one of these 36 children were diagnosed with HIE based on a 24 hour Sarnat Score (12 mild, 3 moderate and 6 severe), the remaining 15 were part of the perinatal asphyxia group.

Following neurodevelopment assessment with the BSID-III, 23 children had a normal outcome and 6 children had a mild/moderately abnormal outcome. Five children with a normal outcome underwent TH, (one mild HIE, three moderate HIE and one severe HIE, defined by clinical Sarnat score). Seven children had a severe outcome. Four died during the neonatal period (one infant did not survive to initiation of therapy), and one developed dyskinetic cerebral palsy, microcephaly and epilepsy. This infant did not receive therapeutic hypothermia, as they did not meet eligibility criteria until beyond the six-hour treatment window. All five of these infants had severe HIE in the neonatal period. Two further infants were diagnosed with severe autism, one of whom had perinatal asphyxia at birth, the other mild HIE.

Model B

For ^{1}H-NMR spectroscopy, twelve infant samples from the cohort were excluded due to insufficient sample volume therefore metabolomic analysis was performed in 59 infants. As above, two infants were retrospectively excluded due to alternative diagnoses. Outcome was available on 31/57 (54%) children; see Table 5.1for demographic information. Nineteen of these children were diagnosed with HIE based on a 24 hour Sarnat Score (10 mild, 3 moderate and 6 severe), the remaining 12 were part of the perinatal asphyxia group.

Determined using BSID-III, 20 children had a normal outcome and 6 children had a mild/moderately abnormal outcome. Four children with a normal outcome underwent TH,

(three with a clinical grade of moderate HIE and one with severe HIE). All five children with a severe outcome were severely encephalopathic, four died during the neonatal period, and one developed dyskinetic cerebral palsy, microcephaly and epilepsy. There were no late deaths in the cohort and no child had a severely abnormal BSID-III result.

Table 5.1Demographic details of the cohort.

	Model A				Model B			
	Normal (n=23)	**Mild/Moderate** (n=6)	**Severe** (n=7)	***p*-value**	**Normal** (n=20)	**Mild/Moderate** (n=6)	**Severe** (n=5)	***p*-value**
Gestational Age (wks)	40.6 (39.5-41.2)	40.3(38.1-40.8)	41.0(40.0-41.4)	0.458†	41.1 (40.2-41.2)	40.3 (38.1-40.8)	41.1 (40.2-41.4)	0.209†
Gender (Male/Female)	12/11	5/1	6/1	0.150††	11/9	5/1	4/1	0.326††
Birth Weight (g)	3630 (3170-4130)	2955 (2842-3900)	3430 (3220-4230)	0.336†	3860 (3390-4137)	2955 (2843-3900)	3430 (3285-4345)	0.172†
Sarnat Score								
PA/Mild/Mod/Severe	14/5/3/1	0/6/0/0	1/1/0/5		12/4/3/1	0/6/0/0	0/0/0/5	
Therapeutic hypothermia	5	0	3		4	0	3	

†; Kruskal Wallis,††; chi-squared, PA; perinatal asphyxia

5.4.2 Prediction of outcome

Model A

The median (IQR) metabolite model score correlated significantly with outcome (ρ 0.429 *p*=0.009) and significantly increased (*p*=0.032) as outcome deteriorated from normal 2240.6 (1909.6-3066.5) to mild/moderately 3252.1 (2808.3-4596.5) abnormal to severely abnormal 4067.5 (2393.8-5448.6) see Figure 5.1. Inspection of pair-wise comparisons revealed the median metabolite model score for normal outcome was significantly different compared to the mild/moderate outcome group (*p*=0.043, *q*=0.128) and severe outcome group (*p*=0.039, *q*=0.117), though not when adjusted for false discovery. However the model score could not differentiate between a mild/moderate and severe outcome (*p*=0.945, *q*=1.000).

This model may provide utility in predicting an abnormal outcome, therefore mild/moderate and severe outcome were grouped together (abnormal outcome group n=13) and compared to normal outcome (n=23). The metabolite model was statistically altered between these two groups *p*=0.008. The model could predict an abnormal outcome with an AUROC of 0.77 (0.61, 0.93 95%CI) *p*=0.009, see Table 5.2. Examination of the ROC curve revealed a score cut-off of y ≥ 2661.2 predicted an abnormal outcome with a sensitivity of 85% and a specificity of 65%.

The UCB metabolite model score had a stronger correlation with outcome group (ρ=0.429, *p*=0.009) than any of the standard biochemical markers assessed, see Table 5.2. No correlation existed between outcome group and pH, lactate or base deficit (BD), either from cord sampling or first postnatal sample. Of the standard clinical markers, Apgar scores at 1 and 5 minutes, and degree of resuscitation correlated with outcome group, and each significantly predicted an abnormal outcome.

We next examined the ability to differentiate BSID-III outcome in surviving children for whom BSID-III was performed. When examining overall BSID-III outcome category i.e. normal (n=23) versus mild/moderate (n=6) groups, the median model score was significantly different between groups (2240.6 (1909.6-3066.5) *vs.* 3252.1 (2808.3-4596.5), *p*=0.036). However when agreement between individual children's log transformed metabolite model scores and the composite scores of each BSID-III subscale were assessed, no significant correlation between

the model scores, and the individual cognitive (R=0.055, *p*=0.777), language (R=-0.208, *p*=0.308) or motor (R 0.138, *p*=0.511) composite scores was found (Table 5.3 and Figure 5.2).

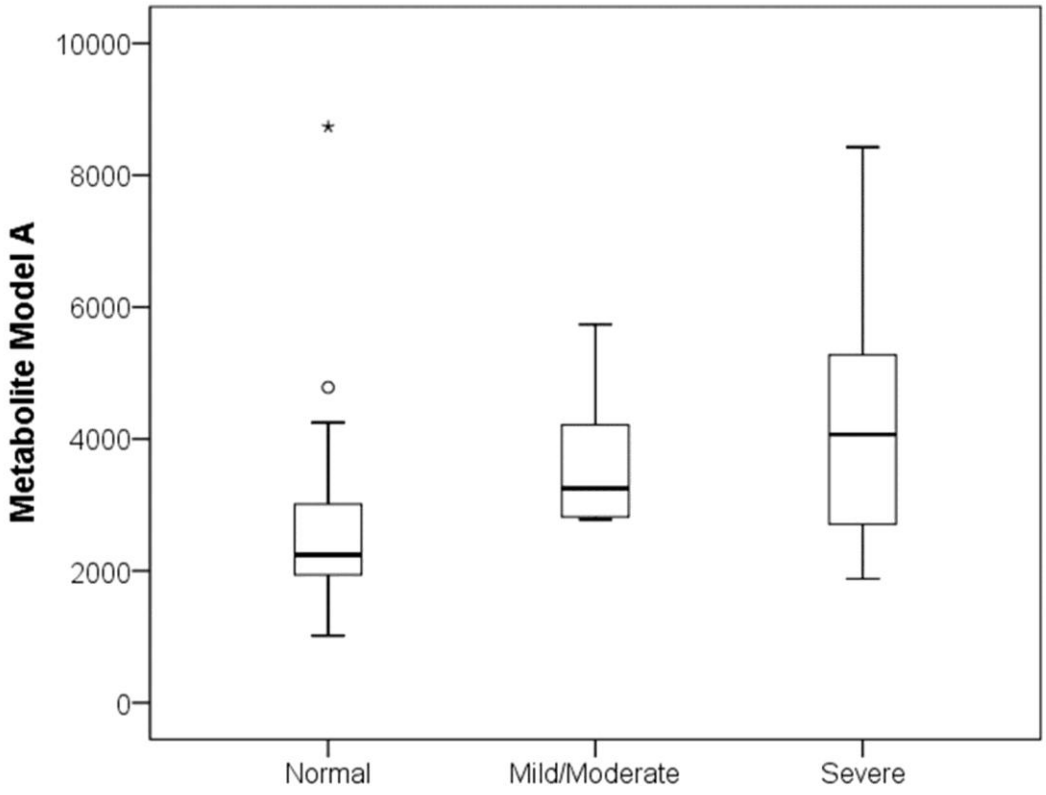

Figure 5.1 Boxplot of metabolite model A scores across outcome groups.

Table 5.2Model A: Median (IQR) values for a selection of clinical and biochemical makers available at delivery across outcome groups.

	Normal	Mild/Moderate	Severe		Correlation		Abnormal Outcome[#]	
	(n=23)	(n=6)	(n= 7)	*p*-value[†]	ρ[††]	*p*-value	AUROC (95%CI)	*p*-value
Metabolite Model A	2240.6 (1909.6-3066.5)	3252.1 (2808.3-4596.5)	4067.5 (2393.8-5448.6)	0.032	0.429	0.009	0.77 (0.61, 0.93)	0.009
Biochemical Markers								
pH	7.13 (7.04-7.25)	7.04 (6.94-7.14)	7.12 (6.67-7.18)	0.426	-0.224	0.261	0.65 (0.43, 0.87)	0.209
Lactate	9.0 (6.0-12.6)	10.7 (7.1-12.0)	11.0 (10.5-20.5)	0.337	0.261	0.207	0.63 (0.40. 0.85)	0.292
Base deficit	9.8 (4.4-14.1)	9.1 (6.4-12.4)	19.0 (10.1-22.5)	0.135	0.281	0.164	0.64 (0.42, 0.86)	0.233
Clinical Markers								
Apgar 1 min	5 (3-6)	3 (2-6)	0 (0-2)	0.018	-0.437	0.008	0.73 (0.55, 0.91)	0.024
Apgar 5 min	8 (5-9)	5 (4-7)	3 (0-7)	0.020	-0.471	0.004	0.77 (0.61, 0.93)	0.008
Resuscitation		-	-	-	0.437	0.009	0.74 (0.56, 0.92)	0.022

Base deficit, pH and lactate were measured on admission to the NICU. † Kruskal-Wallis p-value for the difference across outcome group. ††Spearman's rho (ρ) correlation against outcome groups.[#]AUROC; Area under the receiver operator curve and associated *p*-value, testing the ability of each marker to predict abnormal outcome.

Table 5.3Model A: Median (IQR) values for cognitive, language and motor composite scores across outcome groups.

	Normal Outcome (n=23) Median (IQR)	Mild/Moderate Outcome (n=6) Median (IQR)		Correlation	
			p-value†	Pearson's R	p-value††
Cognitive Composite	95 (95-100)	88 (84-96)	0.013	0.055	0.777
Language Composite	109 (103-112)	88 (79-103)	0.010	-0.208	0.308
Motor Composite	97 (91-103)	85 (79-85)	<0.001	0.138	0.511

† p-value for between group differences calculated with Mann-Whitney U testing.

†† p-value for correlation between composite scores and log transformed metabolite model scores.

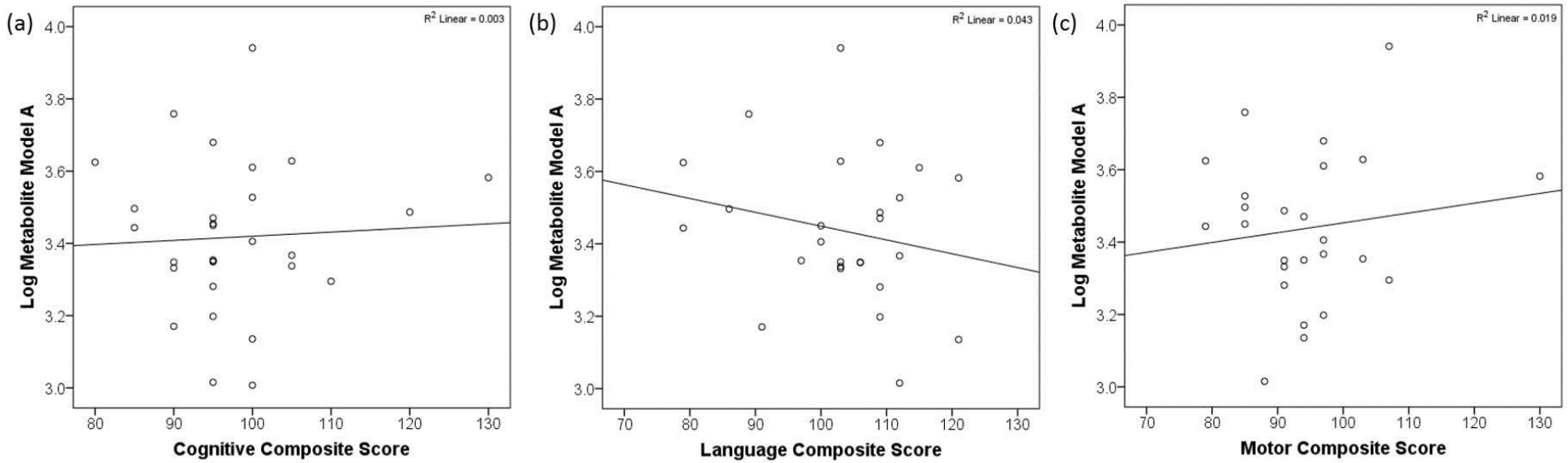

Figure 5.2Correlation between BSID-III composite scores, (a) cognitive, (b) language and (c) motor, and metabolite model Ascores.

Model B

The UCB metabolite model score had a stronger correlation with outcome group (ρ=0.549, *p*=0.001) than any of the standard markers assessed, and accurately predicted both a normal (AUROC of 0.800, *p*=0.006), and a severe outcome (AUROC of 0.915, *p*=0.004), see Table 5.4. Of the standard markers assessed, the Apgar scores at 1 and 5 minutes, and degree of resuscitation correlated with outcome group, and each significantly predicted normal and severe outcome. No correlation existed between outcome group and pH, lactate or base deficit (BD), either from cord sampling or first postnatal sample. Similarly none of these standard biochemical markers could predict a normal outcome. BD drawn from the first post-natal sample did predict a severe outcome.

Unsurprisingly, both the modified Sarnat score and the continuous multichannel EEG's grade of encephalopathy were strong predictors for both severe and normal outcomes. Encouragingly, the UCB metabolite model, drawn hours before either the Sarnat score or the EEG was performed, had comparable predictive abilities to these well-validated tools (Table 5.5).

Based on the coordinates of the ROC curve for the metabolite model, a score cut-off of $y \geq 2.4$ predicts severe outcome with a sensitivity of 80% and a specificity of 100%. This model score cut-off would have detected at birth all of those infants who subsequently died. It would have missed the child who had later developing symptoms and a poor outcome. Meanwhile a model score < 0.13 predicted intact survival with a sensitivity of 65% and specificity of 91%.

Focusing on surviving children for whom BSID-III was performed, i.e. normal (n=20) and mild/moderate (n=6) groups, there was no significant difference between median model scores for each group however a trend towards higher model score is evident in the infants with a mild/moderate outcome (0.15 (0.11-0.32) *vs.* 0.09 (0.05-0.16), [*p*=0.176]). The agreement between individual children's metabolite model scores, and the composite scores for the subscales of the BSID-III was assessed. This showed no significant correlation between the log transformed metabolite model scores, and the cognitive (R -0.205, *p*=0.315), language (R -0.289, *p*=0.180) or motor (R -0.161, *p*=0.463) composite scores (Table 5.6 and Figure 5.3).

Table 5.4 Model B: Median (IQR) values for a selection of clinical and biochemical makers available at delivery across outcome groups.

	Normal	Mild/Moderate	Severe		Correlation		Severe Outcome[#]		Normal Outcome[#]	
	(n=20)	(n=6)	(n= 5)	*p*-value[†]	ρ[††]	*p*-value	AUROC (95%CI)	*p*-value	AUROC (95%CI)	*p*-value
Metabolite Model B	0.09 (0.05-0.16)	0.15 (0.11-0.32)	4.62 (2.18-10.69)	0.007	0.549	0.001	0.92 (0.76, 1)	0.004	0.8 (0.64, 0.97)	0.006
Biochemical Markers										
pH	7.07 (7.02-7.25)	7.04 (6.94-7.14)	6.95 (6.61-7.17)	0.498	-0.250	0.249	0.63 (0.25, 1)	0.417	0.65 (0.41, 0.89)	0.244
Lactate	8.6 (6.2-12.6)	10.7 (7.1-12.0)	14.5 (10.6-21.8)	0.262	0.316	0.162	0.77 (0.53, 1)	0.107	0.65 (0.41, 0.89)	0.256
Base deficit	10.1 (5.7-17.1)	9.1 (6.4-12.4)	19.9 (13.6-23.3)	0.053	0.334	0.128	0.89 (0.73, 1)	0.017	0.63 (0.39, 0.87)	0.317
Clinical Markers										
Apgar 1 min	5 (3-7)	3 (2-6)	0 (0-1)	0.006	-0.512	0.003	0.94 (0.85, 1)	0.002	0.76 (0.58, 0.95)	0.017
Apgar 5 min	8 (5-9)	5 (4-7)	0 (0-5)	0.018	-0.502	0.004	0.86 (0.67, 1)	0.013	0.78 (0.61, 0.95)	0.012
Resuscitation		-	-	-	0.501	0.005	0.88 (0.69-1)	0.008	0.77 (0.58, 0.96)	0.018

Base deficit, pH and lactate were measured on admission to the NICU.† Kruskal-Wallis p-value for the difference across outcome group.
††Spearman's rho (ρ) correlation against outcome groups.[#]AUROC; area under the receiver operator curve and associated *p*-value testing the ability of each marker to predict severe and normal outcome.

Table 5.5 Correlation against outcome groups and predictive ability of metabolite model B, Sarnat score at 24 hours of life and continuous EEG grade at 6 and 24 hours of life for normal or severe outcome.

Clinical Marker	Correlation†		Severe Outcome		Normal Outcome	
	Spearman's ρ	*p*-value	AUROC (95%CI)	*p*-value	AUROC (95%CI)	*p*-value
Metabolite Model	0.549	0.001	0.92 (0.76, 1)	0.004	0.8 (0.64, 0.97)	0.006
Sarnat Score (24h)	0.637	<0.001	0.98 (0.94, 1)	0.001	0.83 (0.68, 0.97)	0.003
EEG (6h)	0.651	<0.001	0.96 (0.9, 1)	0.001	0.84 (0.7, 0.98)	0.002
EEG (24h)	0.745	<0.001	1 (1, 1)	<0.001	0.89 (0.78, 1)	<0.001

† using Spearman's rho (ρ), AUROC; area under the receiver operator curve.

Table 5.6 Model B; Median (IQR) values for cognitive, language and motor composite scores across outcome groups.

	Normal Outcome (n=20)	Mild/Moderate Outcome (n=6)		Correlation	
	Median (IQR)	Median (IQR)	p-value†	Pearson's R	p-value††
Cognitive Composite	95 (95-100)	88 (84-96)	0.023	-0.205	0.315
Language Composite	106 (102-112)	88 (79-103)	0.013	-0.289	0.180
Motor Composite	97 (94-105)	85 (79-85)	<0.001	-0.161	0.463

† p-value for between group differences calculated with Mann-Whitney U testing,

†† p-value for correlation between composite scores and log transformed metabolite model scores

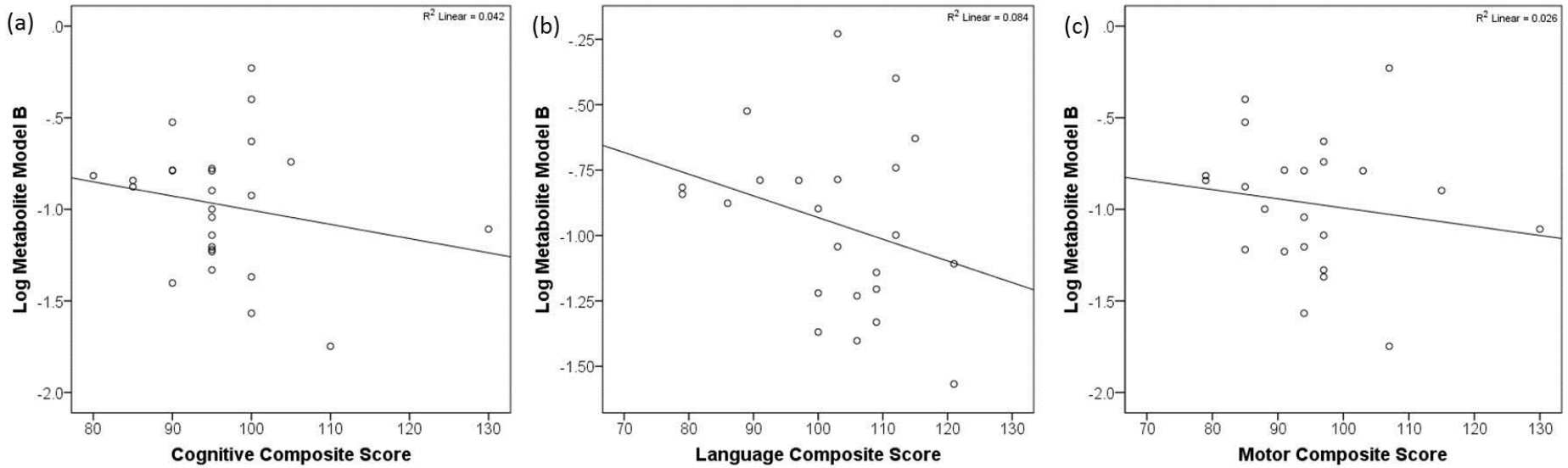

Figure 5.3 Correlation between BSID-III composite scores, (a) cognitive, (b) language and (c) motor, and log metabolite model B scores.

5.5 Discussion

In this cohort we have examined the ability of novel metabolite models to predict 3-year neurodevelopmental outcome in a cohort of infants with perinatal asphyxia and HIE. We compared this to the ability of commonly used biochemical, physiological and clinical markers routinely measured in the perinatal and early neonatal period to predict outcome.

In terms of tools available at delivery, model A scores calculated from endogenous cord blood metabolites measured at birth performed well at predicting an abnormal outcome, as did 1 and 5 minute Apgar and degree of resuscitation. However when the best cut-off score was chosen, ($y \geq 2661.2$) the model suffered from relatively low specificity, misclassifying several normal outcomes.

Model B scores, performed well as did, 1 and 5 minute Apgar scores for the prediction of both severe outcome and intact survival. Severe outcome was however predominated by infants who subsequently died and all infants with a model score above the proposed cut-off of ≥2.4did not survive. Indeed the only child to survive with a severe outcome was not predicted by this marker. This child had delayed evolution of encephalopathic symptoms perhaps related to the timing of injury and failed to meet cooling criteria within the first 6 hours of life. This marker may therefore have greater value in objectively identifying those at higher risk of death and may help to inform difficult decisions regarding redirection of care.

The biochemical markers routinely measured at delivery did not perform well at predicting an abnormal outcome or intact survival with normal neurodevelopment in childhood. The metabolite models outperformed currently available markers in this regard. Indeed for model B, all four children (3 moderate HIE, 1 severe HIE) who received therapeutic hypothermia and had a normal outcome also had a low metabolite model score. Among those who qualify for cooling a low score in this model may identify those who would respond well to treatment.

The metabolite model scores showed superior predictive ability compared with all other biochemical markers available at delivery and yet these markers are in active use as part of clinical decision making and are central to the criteria for initiation of therapeutic hypothermia (295, 296). Even Apgar scores have certain disadvantages. They are generally found to be most useful at extremes but despite this one in five infants with an Apgar score of 0 at 10 minutes of

life will survive to school-age without moderate or severe disability (26). In our cohort even an Apgar score ≥ 6 at 5 minutes could only predict a normal outcome with a sensitivity of 65% and a specificity of between 72-77 %. Apgar scores also suffer from high inter-observer variability (67). As expected, Sarnat score and continuous EEG grading performed at 24 hours of age correlated highly with outcome groups. However while these tools are crucial to longer term prognostication in hypoxic-ischaemic injury, they require significant expertise to utilise to their potential, may not always be available, and provide prognostic information at a later time point compared to cord blood analysis. An objective early user-independent biomarker would be useful for clinicians, particularly in situations necessitating transfer of patients for therapeutic hypothermia.

Metabolite combinations measured at birth could provide an early user-independent test to support clinical decision making. It could also contribute to a management algorithm for perinatal asphyxia alongside clinical markers mentioned above and EEG findings. As technology advances the ability to rapidly measure a combination of metabolites in a point of care device in the labour ward or NICU is a realistic possibility. In addition, this finding could help further elucidate the complex biochemical mechanisms of HIE. The involvement of the metabolites discussed here in the pathophysiology of perinatal asphyxia and subsequent neurological injury has been further explicated in our previous work (7, 8).

To date, this work has been focused on biomarker discovery. Further validation of these metabolite models is needed in alternate prospective cohorts prior to introduction into clinical use. The highly dynamic nature of the metabolome is constantly affected by genetic oversight and environmental influence. Thus metabolite concentrations will change rapidly over time. Studies in animal models of hypoxia-ischaemia will also be helpful in identifying the timed detection of the metabolite alteration. Tracking metabolic changes through post-natal sampling over time will also help establish the pattern of change and speed to recovery of the metabolites. Furthermore, we need to unearth the long-term implications of this early metabolic dysfunction and its potential to lead to ongoing or tertiary brain injury (61, 297).

The novelty of this study relies on the uniquely recruited cohort of term infants, for whom perinatal asphyxia and HIE have been carefully defined both clinically and electrographically, alongside biospecimens that were meticulously biobanked and long-term neurodevelopmental

follow up. A scarce number of biomarker studies have been able to correlate biomarker findings in early life with infant outcome. However, difficulties with study participant retention as well as the low rates of hypoxic-ischaemic injury in the high-income countries where biomarker research is possible present significant ongoing challenges to successful biomarker discovery and validation (1, 298). As a result the number of infants with abnormal outcomes in this cohort is low making careful interpretation of these results necessary. Pre-clinical validation will require a multi-centre approach.

In conclusion, we have demonstrated the potential of metabolite model scoresmeasured at birth to objectively identify infants at high risk of an abnormal outcome or death in the case of a raised score and those with the possibility of a normal outcome if the score is low. Model scores derived from metabolites measured at birthhavethe potential to provide a user-independent test that could guide critical management decisions in the early newborn period.

Chapter 6

6 The effect of haemolysis on the metabolomic profile of cord blood

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6.1 Abstract

Background

Metabolomics is defined as the comprehensive study of all low molecular weight biochemicals, (metabolites) present in an organism. Using a systems biology approach, metabolomics in umbilical cord blood (UCB) may offer insight into many perinatal disease processes by uniquely detecting rapid biochemical pathway alterations. In vitro haemolysis is a common technical problem affecting UCB sampling in the delivery room, and can hamper metabolomic analysis. The extent of metabolomic alteration which occurs in haemolysed samples is unknown.

Methodology

Visual haemolysis was designated by the laboratory technician using a standardised haemolysis index colour chart. The metabolomic profile of haemolysed and non-haemolysed UCB serum samples from 69 healthy term infants was compared using both a ^{1}H-NMR and targeted DI and LC -MS/MS approach.

Results

We identified 43 metabolites that are significantly altered in visually haemolysed UCB samples, acylcarnitines (n=2), glycerophospholipids (n=23), sphingolipids (n=7), sugars (n=3), amino acids (n=4) and TCA cycle intermediates (n=4).

Conclusion

This information will be useful for researchers in the field of neonatal metabolomics to avoid false findings in the presence of haemolysis, to ensure reproducible and credible results.

6.2 Introduction

Interest in the effect of early life status on lifelong health is increasing. As a result, many predictive, diagnostic and prognostic biomarker discovery studies have begun to biobank and use umbilical cord blood (UCB) as their investigative specimen of choice (218). However, haemolysis is a common preanalytical problem that arises in UCB sampling in the delivery room.

Haemolysis (or hemolysis) results when red blood cells are ruptured and their contents are released into the surrounding fluid, following damage to the cell membrane. Haemolysis of UCB can occur in vivo but more commonly in vitro due to a combination of high haematocrit (205) and specimen collection errors. For example, difficult collection or handling, incorrect needle size, unnecessary mixing, under fill of the sample tube and excessive force, can all result in the breakdown of red blood cells which contaminate the surrounding serum or plasma (206).

Metabolomics is the systematic study of temporal interactions between the compliment (metabolome) of low molecular weight biochemicals (metabolites) abundant within living organisms, tissues and cells. In UCB, metabolomics allows the measurement of rapid biochemical alterations and may offer early insight into many perinatal disease processes. Unfortunately haemolysis introduces high preanalytical variability, resulting in potentially unreliable results, and recommendations have been made to avoid using haemolysed samples in metabolomic experiments (197). However, neonatal samples for biomarker discovery are difficult and expensive to collect, and may be collected to examine rare or orphan diseases. Excluding all haemolysed samples is therefore not ideal, and may affect study feasibility.

In the present study, retrospective data from two previous metabolomic investigations (7, 8), were combined to assess whether or not the visual haemolysis of UCB serum affects metabolite concentrations in a healthy control population. Metabolites must demonstrate robustness to haemolysis in order to translate to the clinical setting. Knowledge from this study will facilitate this translation by allowing researchers to avoid false results, and may enable correction for sample haemolysis.

6.3 Methodology

6.3.1 Patient selection and sample collection

Retrospective data from an ongoing birth cohort study, the Cork BASELINE Birth Cohort Study, recruited between June 2010 and July 2011 was analysed (218). Ethical approval was granted by the clinical research ethics committee of the Cork Teaching Hospitals, and written informed consent from all participants was obtained. Healthy control infants were selected based on the following criteria; Apgar scores ≥8 at 1min, ≥9 at 5min, duration of ruptured membrane <24 h, temperature in labour ≤37°C, gestational age ≥36 weeks, cord arterial pH ≥7.2 and birth weight centile ≥10%. This population had no underlying medical issues and normal neonatal exams.

UCB was drawn in all infants using standardised operating procedures (SOPs). In brief, 6 ml was collected in a plain serum tube (BD Vacutainer no. 366431) within 20 min of placental delivery and allowed clot for 30 min at 4 °C, before centrifugation (2400 xg, 10 min, 4 °C). The serum was transferred to a second spin tube and centrifuged (3000 xg, 10 min, 4 °C) before being aliquoted into lithium heparin micro-tubes (VWR no. 89179-704) and stored at -80 °C until analysis. Total time from birth to samples being frozen at -80 °C was always under 3 hours.

6.3.2 Haemolysis

The haemolysis status was designated by laboratory technicians. In order to standardise visual assessment of the separated serum, a haemolysis index colour chart (Mayo Medical Laboratories, T 598) was used. If a sample was dark orange – red in colour (corresponding haemoglobin ≥ 100 mg/dL), it was deemed haemolysed, see Figure 6.1.

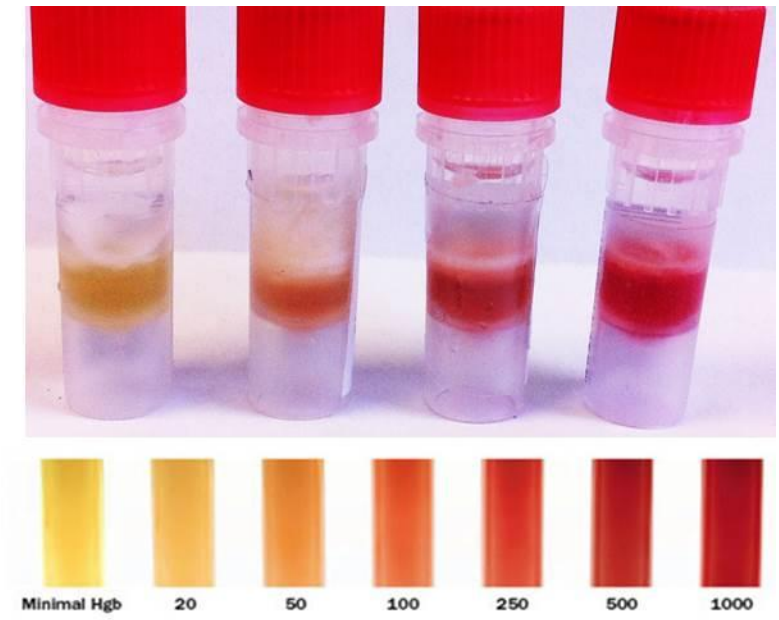

Figure 6.1 Picture of four serum samples being thawed. Left hand side serum is clean (non-haemolysed) and the others range in degree of haemolysis. Below the Mayo Medical Laboratories, haemolysis index colour chart (≥100 mg/dL = haemolysed).

6.3.3 Metabolomic analysis

Using both nuclear magnetic resonance (^{1}H-NMR) spectroscopy and combined targeted direct infusion (DI-) and liquid chromatography (LC-)tandem mass spectrometry (MS/MS) assay (AbsolutIDQ p180 kit, Biocrates Life Sciences AG, Innsbruck, Austria), we have described the metabolomic profile of UCB from healthy term infants (7, 8)and evaluated the pre analytical effects of haemolysis to describe metabolite alterations. Quality control (QC) samples were employed to evaluate the precision and repeatability of the metabolite quantification. A detailed description of the metabolomic methods employed has been previously reported (7, 8).

6.3.4 Statistical Analysis

Statistical analysis was performed using SPSS version 21.0. Metabolite data was normalised using log transformation for parametric tests and the antilog of the mean transformed data was reported; otherwise median and non-parametric tests were used. Statistical comparison

between haemolysis and clean serum groups was tested individually using Mann Whitney U test or using the Student's t-test.

No correction for multiple comparisons was performed, as the aim of this study was not to reduce the probability of false discovery, but to create a non-conservative "watch" list of haemolysis sensitive metabolites that could potentially confound any future biomarker studies.

6.4 Results

UCB serum samples (n = 69) were classified as being with (haemolysed, n = 13) or without (clean, n = 56) haemolysis upon visual inspection post centrifugation. All 69 samples underwent initial mass spectrometry metabolomic analysis using a combined targeted DI and LC -MS/MS approach. For ^{1}H-NMR spectroscopy, ten samples were excluded due to insufficient sample volume, leaving 59 samples for the analysis. See Table 6.1for demographic details of the study cohort.

Using the targeted method 148 metabolites were identified and quantified; 35 metabolites were significantly altered between groups, see Table 2. Separately, 37 were identified and quantified using ^{1}H-NMR spectroscopy and eight unique metabolites were significantly altered between the clean (n = 48) and haemolysed (n = 11) groups,seeTable 6.2.

Evident from the fold change values inTable 6.2, is the magnitude of metabolite concentration change with visual haemolysis. For example, acylcarnitines displayed an average fold change increase of 1.2 and glycerophospholipids an average fold change decrease of 1.3. Metabolites closely associated with the tricarboxylic acid(TCA) cycle, acetate, formate and succinate, and the non-essential amino acid ornithine, all showed large fold increased concentrations of 1.5, 1.8, 1.9 and 1.5 in haemolysed samples respectively.

Table 6.1Clinical and demographic details for the healthy control study populations of two separate metabolomic investigations.

	^{1}H-NMR spectroscopy cohort		Mass spectrometry cohort	
	Haemolysed (n=11)	Clean (n=48)	Haemolysed(n=13)	Clean (n=56)
Gestational Age (weeks)	39.6 (1.2)	40.3 (0.9)	39.7 (1.1)	40.2 (0.9)
Gender (Male/Female)	7/4	32/16	12/12	38/18
Birth Weight (gm)	3430 (595)	3667 (474)	3448 (484)	3627 (480)
Weight Centile (%)	64 (10, 80)	62 (26, 86)	55.4 (32.0)	48.6 (31.0)
Method of Delivery				
Spontaneous vaginal	5 (46%)	12 (25%)	5 (39%)	17 (30%)
Instrumental assisted	4 (36%)	25 (52%)	6 (46%)	26 (46%)
Emergency caesarean section	2 (18%)	10 (21%)	2 (15%)	12 (21%)
Elective caesarean section	-	1 (2%)	-	1 (2%)
Apgar score 1 min	9 (8, 9)	9 (9, 9)	9 (8, 9)	9 (9, 9)
Apgar score 5 min	9 (9, 10)	10 (10, 10)	9 (9,10)	10 (9, 10)
Maternal Ethnicity				
Caucasian	10 (91%)	47 (98%)	12 (92%)	55 (98%)
Indian	1 (9%)	-	1 (8%)	-
Asian	-	1 (2%)	-	1 (2%)
Maternal Age (years)	30.7 (6.7)	29.6 (4.5)	31.0 (6.2)	29.2 (4.7)
Maternal BMI (kg/m^2)	23.7 (4.0)	23.9 (3.5)	24.4 (4.0)	24.2 (4.2)

Values are mean (SD), median (interquartile range), or n (%)

Table 6.2Metabolites significantly different when visually haemolysed and clean UCB serum samples were compared.

Metabolite	Clean serum†	Haemolysed serum†	%RSD[QC]	Fold[#]	up or down	*p*-value
	Mass Spectrometry					
L-Acetylcarnitine	3.8 (3.3,4.2)	4.8 (3.7, 6.1)	3	1.2	↑	0.0205
Hexanoylcarnitine	0.07 (0.07,0.08)	0.08 (0.07,0.1)	14	1.2	↑	0.0287
PC aa C38:0	1.7 (1.5,1.8)	1.3 (1.1,2.0)	6	-1.3	↓	0.0355
PC aa C38:3	40.1 (38.1,43.1)	32.2 (27.3,42.5)	8	-1.2	↓	0.0355
PC aa C38:5	26.4 (24.4,28.2)	20.7 (16.7,25)	8	-1.3	↓	0.0097
PC aa C38:6	75.5 (68.9,82.3)	57.3 (47.2,76.7)	4	-1.3	↓	0.0246
PC aa C40:4	3.2 (2.9,3.6)	2.3 (2.0, 3.1)	4	-1.4	↓	0.0071
PC aa C40:5	6.6 (5.6,7.6)	4.8 (3.6, 5.8)	2	-1.4	↓	0.0060
PC aa C40:6	30.3 (26.7,32.2)	21.8 (15.1,30.1)	5	-1.4	↓	0.0089
PC aa C42:0	0.5 (0.4,0.5)	0.4 (0.2,0.7)	3	-1.2	↓	0.0288
PC ae C32:1	2.4 (2.1,2.6)	1.8 (1.2,2.5)	3	-1.4	↓	0.0209
PC ae C34:3	1.2 (1.1,1.3)	0.9 (0.6,1.4)	3	-1.3	↓	0.0193
PC ae C38:4	8.7 (8.0,9.8)	6.9 (6.1,9.7)	2	-1.3	↓	0.0255
PC ae C38:5	7.7 (7.1,8.2)	6.4 (4.5,9.3)	2	-1.2	↓	0.0478
PC ae C38:6	3.4 (3.2,3.8)	2.7 (1.6,3.8)	3	-1.3	↓	0.0266
PC ae C40:4	1.8 (1.7,1.9)	1.3 (1.2,1.9)	5	-1.3	↓	0.0135
PC ae C40:5	1.6 (1.4,1.7)	1.3 (1.0,1.8)	5	-1.2	↓	0.0222
PC ae C40:6	2.5 (2.3,2.8)	2.0 (1.2,3.1)	3	-1.3	↓	0.0420
PC ae C44:4	0.3 (0.3,0.3)	0.2 (0.2,0.3)	4	-1.4	↓	0.0362
PC ae C44:5	1.1 (1,1.2)	0.8 (0.5,1.1)	2	-1.3	↓	0.0342
PC ae C44:6	0.9 (0.8,0.9)	0.7 (0.4,1.0)	2	-1.3	↓	0.0390
lysoPC a C16:0	59.75 (54.9,62.9)	49.9 (37,63.9)	5	-1.2	↓	0.0355
lysoPC a C16:1	4.5 (3.9,4.9)	3.3 (2.5,4.8)	2	-1.4	↓	0.0156
lysoPC a C18:0	10.3 (9.8,10.7)	8.3 (6.3,9.8)	6	-1.2	↓	0.0129
lysoPC a C18:1	13 (11.6,14.6)	9.1 (6.3,14.1)	5	-1.4	↓	0.0266

SM (OH) C16:1	1.5 (1.4,1.6)	1.4 (1,1.8)	12	-1.1	↓	0.0444
SM (OH) C22:2	3.5 (3,3.7)	2.8 (2,3.6)	4	-1.2	↓	0.0428
SM (OH) C24:1	1 (0.9,1.1)	0.9 (0.6,1.1)	3	-1.1	↓	0.0428
SM C16:1	10.2 (9.5,11.1)	7.8 (7.4,11.1)	4	-1.3	↓	0.0323
SM C22:3	0.4 (0.3,0.4)	0.2 (0.1,0.4)	2	-1.6	↓	0.0121
SM C24:0	16.4 (14.7,17.4)	12.5 (10.3,17.5)	3	-1.3	↓	0.0197
SM C24:1	27.7 (25.7,29.1)	20.9 (12.2,30)	4	-1.3	↓	0.0305
Hexose	4064 (3844,4500)	3451 (3361,4070)	3	-1.2	↓	0.0124
Glycine*	252.5 (236,265)	220 (192,262)	1	-1.1	↓	0.0021
Serine*	128.5 (124.1,133.1)	116.8 (103.7,131.5)	3	-1.1	↓	0.0359
	^{1}H-NMR Spectroscopy					
Acetate	14.7 (12.7, 17.5)	21.3(12.4,46.1)	4	1.5	↑	0.0504
Citrate	35.2 (23.4, 40.4)	44.5 (36.2,52.9)	17	1.3	↑	0.0185
Formate	11.2 (10, 13.3)	20.7 (8.4,42)	14	1.8	↑	0.0293
Glucose*	1666.7 (1575.7, 1763)	1488.7 (1411.8,1569.8)	7	-1.2	↓	0.0037
Glycerol	16.7 (14.6, 19.1)	23.1 (9.8,33.1)	16	1.4	↑	0.0460
Ornithine	17.6 (15.5, 20.2)	26.9 (15.3,36.5)	8	1.5	↑	0.0265
Phenylalanine	26.6(24.9, 30.9)	34.2 (23.6,38.8)	7	1.3	↑	0.0482
Succinate	1.5 (1.2, 1.8)	2.8 (1.2,6.9)	12	1.9	↑	0.0022

QC; quality control, RSD; relative standard deviation, PC; phosphatidylcholines, lysoPC; lysophosphatidylcholines and SM = sphingolipids

[#]Fold change of increased ↑, or decreased ↓ concentration in haemolysed samples.

†Median or *mean µM metabolite concentration with 95% confidence intervals (95%CI)

6.5 Discussion

This study examined the effect of visual haemolysis on UCB serum metabolites using quantitative metabolomic methods DI and LC -MS/MS and ^{1}H-NMR, and found that 43 metabolites were significantly altered when comparing haemolysed and clean serum. These metabolites belonged to several classes; acylcarnitines (n=2), glycerophospholipids (n=23), sphingolipids (n=7), sugars (n=3), amino acids (n=4) and TCA cycle intermediates (n=4), seeTable 6.2. The p-values presented in Table 2 may seem marginal, but it is important to note that this was a retrospective study with only a small number of visually haemolysed samples. However, the fold-change values do indicate potential confounding effects in any true disease biomarker discovery.

Concentration changes for each metabolite were examined as these may arise from the metabolite being either degraded or metabolized by enzymes released from haemolysed erythrocytes (197). Besides haemologlobin, erythrocytes also contain several structural proteins, lipids and carbohydrates which could influence and interfere with the sample (207). Out of a total of 185 quantified metabolites, 56 metabolites were found to have increased concentrations in visually haemolysed samples, 34 and 22 detected by DI and LC -MS/MS and ^{1}H-NMR respectively. Of the significantly altered metabolites, nine displayed concentration increases in haemolysed samples, while the remaining decreased. Increases occurred in both acylcarnitines; L-acetylcarnitine and hexanoylcarnitine, a sugar glycerol, an amino acid phenylalanine, a non-essential amino acid ornithine, and the TCA cycle intermediates, acetate, citrate, formate and succinate. A recent study evaluating the effect of preanalytical variables in blood and plasma found 81 blood metabolites which were significantly altered by haemolysis, with an increase in 50 metabolites and a decrease in 31 (199).

The erythrocytes and monocytes released into the cytoplasm have the ability to convert arginine to ornithine (299), which is in agreement with our results. Though not significantly altered by haemolysis, arginine displayed a concentration decrease in haemolysed serum (78.9 IQR 67.4-97.7 μM) compared to clean (86.8 IQR 74.0-97.7 μM) and ornithine the corresponding increase in concentration. Increasing acetate and decreasing glucose has also been previously shown by Theil et al., examining plasma metabolites from bovine samples (300). Likewise, false elevations in creatinine have been documented in haemolysed samples during clinical testing

(206). We found creatinine concentrations increased from clean (23.0 IQR 17.9-27.2 µM) to visually haemolysed (25.4 IQR 21.5-27.9 µM) serum.

All remaining metabolites, particularly the lipids (phosphatidylcholines, lysophosphatidylcholines and sphingolipids), had decreased concentrations in haemolysed samples, potentially being degraded by erythrocyte enzymes or diluted by chemical interference. A study by Yin et al. using a non-targeted LC metabolomic approach found 69 mass features significantly changed comparing haemolysed and control plasma samples. Two of these mass features were identified as the lysophosphatidylcholines (lyso-PCs) C16:0 and C18:0 which showed increased concentrations in haemolysed samples, while lyso-PC C16:0 showed a strong correlation ($P = 0.0003$; $r = 0.61$) with free haemoglobin (197). Our results indicate that these lipids decrease with haemolysis in UCB.

In the present study, UCB was meticulously biobanked in adherence to strict SOPs (218), to ensure samples were suitable for metabolomic analysis. The prevalence of haemolysis in routine venepuncture samples has been described as 3.3 % (206), however, the prevalence in biobanked UCB is unknown. Visible haemolysis as an indicator of erythrocyte breakdown is only apparent when the serum or plasma has been separated and does not look haemolysed until the concentration of haemoglobin reaches 0.2 g/L (301). A limitation of this study was the subjective visual verification of haemolysis (302). Instead a spectrophotometric technique could be used to quantify haemolysis using the haemolysis index.

Though our results seem biologically plausible, the sample size of our study is relatively small and may be illustrating population differences in metabolites which are highly variable, rather than analytical differences. However our samples were selected from a homogeneous population of normal healthy full term infants in an attempt to leave haemolysis as the sole discernible difference.

The results from our study have indicated potential differences in UCB serum metabolites in visually haemolysed samples. These differences may be the result of chemical interference or concentration changes of analytes, caused by the release of free haemoglobin and other intracellular contents from the erythrocyte. This is the first time UCB metabolite levels have been compared in clean and haemolysed serum, from healthy term infants. Haemolysis remains a major concern for both clinical and research laboratories, by contributing to erroneous results. Excluding haemolysed samples from biomarker discovery is not always

feasible; therefore, future work in the field of metabolomics must concentrate on defining a list of peaks or metabolites that are unreliable to measure in haemolysed samples.

Chapter 7

7 Characterising the effect of haemolysis on the cord blood metabolome by direct infusion mass spectrometry

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7.1 Abstract

Background

Haemolysis (hemolysis) is a common preanalytical error affecting umbilical cord blood (UCB) sampling in the delivery room. Metabolomics in UCB may offer insight into many perinatal disease processes by uniquely detecting rapid biochemical pathway alterations. Unfortunately as with conventional biochemical tests haemolysis may alter endogenous metabolite levels and produce unreliable results. This study aims to examine the feasibility of using haemolysed UCB samples in metabolomic studies and defines a list of mass features that may be affected by haemolysis and therefore unreliable to measure.

Methodology

Two samples of cord blood, one haemolysed (*in vitro*) and one non-haemolysed, were drawn and biobanked at -80 °C within 3 hours of birth for each infant (n=8). Untargeted metabolomic analysis was conducted usingdirect injection FT-ICR mass spectrometry, for polar and non-polar metabolites, in positive and negative ion mode. A quality assurance system was implemented to ensure only robust and reproducible features were analysed.

Results

We report on average 5% of polar and 1.5% of non-polar features were altered between paired haemolysed and clean samples, including putatively annotated carbohydrates, amino acids, sterol and prenol lipids. Unsupervised multivariate analysis concluded that the preanalytical variability introduced by haemolysis was negligible compared with the biological inter-individual variability.

Conclusion

Untargeted metabolomic analysis of UCB serum has revealed that *in vitro* haemolysis alters a low percentage of mass features but that the biological variability between individuals is still greater than the variation between a haemolysed and non-haemolysed sample from the same individual. We conclude that researchers can include haemolysed UCB in metabolomic studies, but must consider whether individual mass features are robust to haemolysis.

7.2 Introduction

Traditionally, analytical data emanating from haemolysed samples has been disregarded, with early investigations into its affects stating “haemolysis must be avoided because of differing concentrations of some metabolites between red cell fluid and serum or plasma” (303). Haemolysis (or hemolysis) is known to alter analyte measurements, producing false elevations and/or dilution effects which ultimately produce spurious results. The haemolysis of blood samples persists today (206), and warrants consideration during clinical investigative research. It is caused by the breakdown of the erythrocyte membrane which releases its intracellular contents into the surrounding fluid, and arises *in vivo* as a result of several clinical conditions but more commonly *in vitro* due to specimen collection and handling errors (214). Besides haemoglobin, erythrocytes contain several structural proteins, lipids and carbohydrates which could influence and interfere with the sample. Several studies have examined the influence of haemolysis on routine biochemical tests and described the extent of chemical alterations that result from it (209-212).

Metabolomics is becoming an increasingly popular analytical tool for the study of perinatal and neonatal conditions, because of its potential to offer early insight into disease pathophysiology (255, 304). Metabolites represent the reactants, products and intermediaries of enzyme-catalysed chemical reactions central to metabolism. They produce a dynamic response to both exogenous and endogenous perturbations, making them ideal markers of disease or injury but also highly susceptible to preanalytical variation (198, 199).

Many prospective cohort studies in the field of maternal-neonatal medicine have begun to biobank and use umbilical cord blood (UCB) as their investigative specimen of choice, because large volumes can be easily and non-invasively collected (166, 218). Unfortunately, UCB frequently becomes haemolysed during collection in the delivery room and there is no opportunity to repeat the sample. Due to the unpredictable nature of birth, the samples can be difficult, labour intensive and expensive to collect. In addition conditions which affect placental function and the condition of the umbilical cord, such as placental insufficiency, intra-uterine growth restriction, and prematurity increase the risk of haemolysis, but are also those of most interest to obstetric and neonatal researchers. Excluding haemolysed samples from further

analysis may not be feasible or justified. However clinically important biomarkers whose measurement may be affected by haemolysis need to be identified at an early stage to allow accurate validation and translation to the clinic.

In the present study, the effect of *in vitro* haemolysis was systematically studied to assess the feasibility of using haemolysed samples in investigative studies and the reliability of potential metabolic biomarkers in haemolysed cord blood. This was achieved by examining the metabolomic fingerprint of cord blood for paired haemolysed versus clean (non-haemolysed) serum samples, collected simultaneously at birth. By directly comparing these paired samples, we characterised the metabolic features, which were significantly altered. To comprehensively study the UCB metabolome, an untargeted direct injection mass spectrometry (DI-MS) approach was employed in both positive and negative ion modes. We hope that this work will be of use to the metabolic biomarker research community, particularly those in the growing field of perinatal metabolomic research.

7.3 Methodology

7.3.1 Sample selection

Ethical approval was granted by the Clinical Research Ethics Committee of the Cork Teaching Hospitals. The study population were full term healthy infants, with no underlying medical issues, normal neonatal examinations and did not require admittance to the intensive care unit. Written informed consent was obtained from all participating parents. All recruited infants had samples of UCB serum collected and biobanked within three hours of birth.
More detailed information on cohort recruitment is provided in Appendix E.2.

Biobanking procedure

UCB serum was drawn within 20 min of placental delivery and placed in two separate additive free serum tubes (6 ml), and left to clot for 30 min at 4°C to quench metabolic activity. Biobanking took place in a sterile environment. Samples were centrifuged at 2400 x g for 10 min at 4°C. Serum was removed, placed in separate clean spin tubes and centrifuged again at 3000 x g for 10 min, at 4°C. The serum was aliquoted into microtubes and stored at -80 °C until metabolomic analysis.

7.3.2 Haemolysis

No intervention was used to deliberately create haemolysis. Haemolysed samples were identified upon visual inspection at the time of biobanking. Each infant included in the study had both clean and haemolysed serum biobanked. For all samples the presence or absence of haemolysis was confirmed using a photometric semi-quantitative automated analyser (Beckman Coulter AU640). Haemolysis is defined as > 50 mg/dL haemoglobin.

7.3.3 Metabolomic analysis of UCB serum with DI FTICR MS

Sample preparation

Eight paired (clean/haemolysed) infant samples were chosen for analysis in which, despite identical collection procedures, one sample was non-haemolysed whilst the other serum sample was significantly haemolysed following serum extraction. For maximum coverage of the infant UCB metabolome, for each sample, polar and non-polar fractions were extracted and analysed in both positive and negative ion modes giving a total of four analyses (polar positive, polar negative, non-polar positive and non-polar negative).

A modified Bligh and Dyer biphasic extraction method was used to extract metabolites from UCB serum (142, 219). Extractions were conducted on ice to minimise metabolic activity. Methanol was added to the frozen serum sample followed by chloroform and water to achieve a ratio of 2:2:1.8. Each sample was vortex mixed and separated on ice (10 min) and centrifuged at 1800 *g* for 15 min at 4 °C. The extraction separated into an upper (polar) phase consisting mainly of methanol/water with hydrophilic metabolites and a lower (non-polar) phase of predominantly chloroform containing hydrophobic metabolites. Proteins and other insoluble molecules formed a solid interphase layer, which separated the two solvent layers. Aliquots of the upper polar layer (MeOH:H_2O) were dried down using a centrifugal evaporator (Thermo Savant, Holbrook, NY) and the lower non-polar layer ($CHCl_3$) was dried under nitrogen, both were stored at -80 °C until MS analysis.

For analysis, polar samples were re-suspended in either 80:20 MeOH:H_2O with 0.25% formic acid solution for positive ion or 80:20 MeOH:H_2O with 20 mM ammonium acetate solution for negative ion, while non-polar samples were re-suspended in 2:1, MeOH:H_2O w. 5 mM ammonium acetate. A “solvent blank”, a sample consisting of the re-suspension solution and analysed as per a normal sample was run at the beginning and end of each analysis batch to detect mass spectral features that were not of biological origin.

DI FT-ICR MS analysis and signal processing

All metabolomic analyses used a hybrid linear ion trap/Fourier transform ion cyclotron resonance 7-T FTICR mass spectrometer (LTQ FT Ultra, Thermo Fisher Scientific, Bremen, Germany) with a chip-based direct infusion nanoelectrospray ionisation (nESI) assembly (Triversa, Advion Biosciences, Ithaca, NY). To increase metabolome coverage and maintain mass accuracy, spectra were collected using the 'SIM stitching' method (220). Duplicate replicate analyses of each sample were taken and filtered into a single peak list via a three step filtering process to enhance spectral reliability (221). To reduce inter and intra batch variation the Quality Control-Robust Spline Correction (QC-RSC) batch correction algorithm was applied(135) in conjunction with three spectral cleaning algorithms. For statistical analysis, the final intensity matrices were normalised using the probable quotient normalisation (PQN) method (224), and logarithm (log_{10}) transformed (Figure 7.1).

Full details of nanoelectrospray and mass spectrometry conditions, as well as signal processing are provided in Appendix G.4 and G.5.

Quality assurance

For each analytical batch quality control (QC) samples were included as a quality assurance measure. Separate polar and non-polar QC samples were prepared by pooling extracted aliquots from each sample, to create a QC sample representative of the infant metabolome being studied. QC samples were injected multiple times at the beginning of each batch for equilibration, as well as every fifth sample and at the end of the batch to assess and correct the precision and reproducibility of individual mass spectral features (222, 223). Following US FDA guidelines, metabolites with a relative standard deviation for QC samples (RSD^{QC}) of > 20% are considered to be below accepted quantification precision. Spectral cleaning algorithms were applied (135) to remove unreliable, inconsistent or unreproducible (> 20% RSD^{QC}) features. A peak was removed if: (i) the difference in median intensity between QC samplesand biological sample intensity was inconsistent between batches, (ii) there was a relatively large difference in median intensity between the QC sample and biological sample (iii) if the technical variation of the peak was considered too high. The extraction and analysis order of each sample was semi-randomised, so that each pair was analysed together but no systematic bias was introduced. However, the technical replicates for a given sample were measured consecutively.

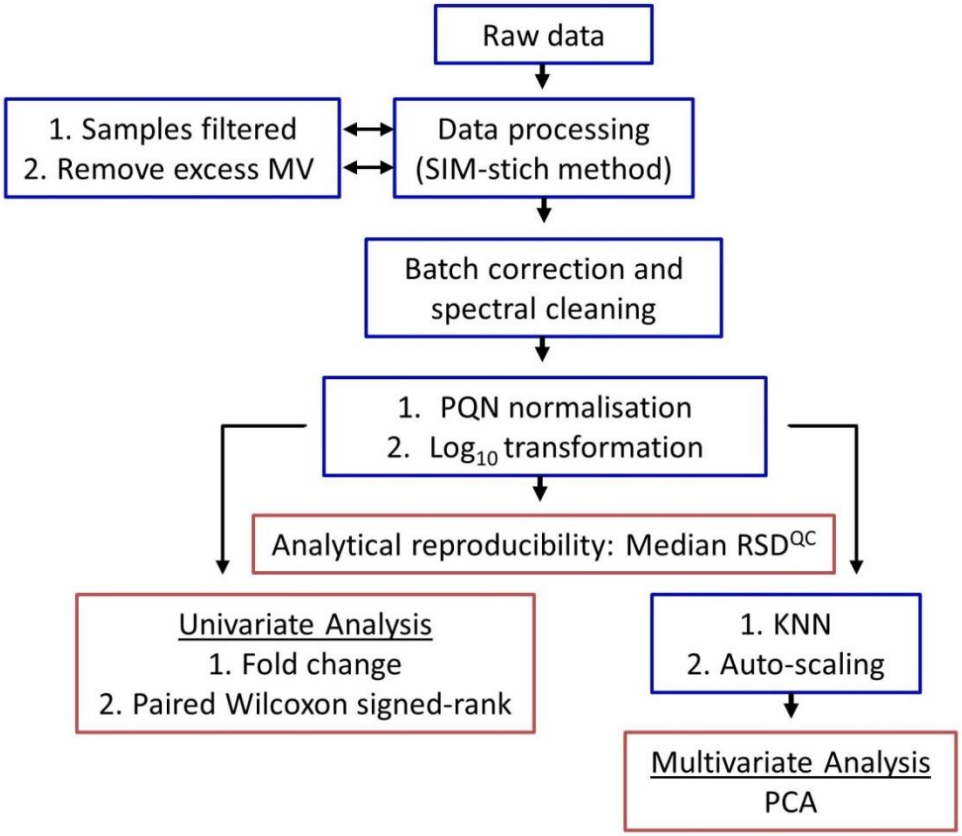

Figure 7.1Computational workflow for the analysis of DI FT-ICR MS data. The resulting four data sets (polar nESI(+), polar nESI(-), non-polar nESI(+) and non-polar nESI(-)) were then analysed to assess data reproducibility and precision, and to characterise the effect of haemolysis, steps highlighted in red.

7.3.4 Putative metabolite annotation

Annotation was performed by applying the Metabolite Identification Package (MI-Pack) workflow as described previously (225). MI-Pack incorporates metabolomic databases for example, the Human Metabolome Database (HMDB), Kyoto Encyclopaedia of Genes and Genomes (KEGG) database and Lipid Metabolites and Pathway Strategy (Lipid MAPS) database to allow the putative annotation of mass features based on accurate mass (117, 154, 261).

A single peak search was conducted using these databases to putatively annotate metabolites. The KEGG database was restricted to the dedicated Homo sapiens (HSA) metabolite library for this process. It is important to remember that metabolite isomers may occur whereby several metabolites can be detected with the same accurate mass therefore a single metabolic feature may have multiple possible annotations. Likewise, multiple spectral features with different accurate masses can define a single metabolite, as different adducts can be formed from the same metabolite, for example, $[M+H]^+$, $[M+Na]^+$, $[M-H]^-$, $[M+Hac-H]^-$. Herein, metabolic features were identified by putative annotation based on accurate mass, equivalent to 'level 2' identification as defined by The Metabolomics Standards Initiative (155). Where no putative annotations could be ascribed a putative empirical formulae was assigned (225).

7.3.5 Data analysis

Statistical analysis was performed using MatLab (version R2013, MathWorks, Natick, MA, USA).

Metabolite fold change differences were analysed using the PQN datasets and calculated as the median mass feature intensity of a haemolysed sample relative to the median intensity of its clean pair. The datasets were $\log_{10}$ transformed and changes in each mass feature were analysed using a Wilcoxon signed-rank test, suitable for paired analysis. A Benjamini Hochberg false discovery rate of 10% was applied (148). Any pair with one or more missing values for that mass feature was excluded.

Unsupervised principal component analysis (PCA) was used as an unbiased method to visualise overall metabolic differences between groups. The cumulative variance, root mean square error of calibration (RMSEC) value and root mean square error of cross-validation (RMSECV) of the models are reported.All of the reproducible mass features for a given comparison were included. Multivariate analysis was conducted using PLS toolbox v. 7.9 (Eigenvector Research Inc, Manson, WA, USA) within Matlab. Prior to PCA, missing values were imputed using k-nearest neighbour (KNN) and the data was autoscaled (145). Traditionally, autoscaling has been regarded as emphasising noise peaks in mass spectrometry. However, as we applied a cleaning step following batch correction using the QC-RSC algorithm (135), only robust and reproducible mass spectral features were retained, enabling the use of autoscaling. Its advantage is that the weighting of each feature in the model is then independent of its variance.

Finally, to assess whether potential biomarkers of perinatal asphyxia and HIE, detected in Chapter 3 and Chapter 4, were in fact potentially altered by haemolysis, the theoretical masses from the compounds relating to both studies were compared.

7.4 Results

For each of the four metabolomic datasets, the number of metabolic features detected and retained after cleaning (223) was calculated (Table 7.1), resulting in 1874 nESI(+), 1503 nESI(-) polar and 552 nESI(+), 1285 nESI(-) non-polar features suitable for analysis. To assess biological plausibility of the findings, we attempted to ascribe putative annotations to each accurate mass feature detected using DI FT-ICR MS, by comparing it to endogenous human metabolites of a similar mass (<1.25ppm error polar and <2ppm error non-polar), listed in the HMDB, KEGG and LIPIDMAPS databases (225). A putative metabolite name was assigned to 13% nESI(+) and 10% nESI(-) of polar and 34% nESI(+) and 41% nESI(-) of non-polar metabolic features.

Table 7.1Summary of metabolic features for each of the four datasets.

	Polar (hydrophilic)		Non polar (hydrophobic)	
Metabolic features	**nESI(+)**	**nESI(-)**	**nESI(+)**	**nESI(-)**
Detected	2341	1864	787	2033
Reproducible[#]	1874	1503	552	1285
Significantly altered (p<0.05)	131 (7%)	40 (3%)	6 (1%)	24 (2%)
Increased	80 (4%)	17 (1%)	3 (0.5%)	19 (1.5%)
Decreased	51 (3%)	23 (2%)	3 (0.5%)	5 (0.5%)

[#]Features post batch correction and spectral cleaning(135)

7.4.1 Multivariate analysis

Assessment of the QC measurements per feature for each dataset confirmed good technical reproducibility with a median RSD^{QC} of 10% for both polar nESI(+) and nESI(-) and 5% for non-polar nESI(+) and 8.5% for nESI(-) ion mode(223). PCA scores plots were used to visualise the reproducibility and variability of the pooled QC samples. Good clustering was observed in both ESI(+) and ESI(-) modes reflecting the systems stability (Figure 7.2).

To examine the effect of haemolysis on the metabolic fingerprint of newborn infants, multivariate PCA plots were constructed using all reproducible features remaining in each dataset. Figure 7.3 displays the PCA plots containing the eight clean-haemolysed sample pairs for each dataset, polar nESI(+), polar nESI(-), non-polar nESI(+) and non-polar nESI(-), modelled on 3 principle components (PCs) (60% variance, RMSEC=0.62, RMSECV=0.86), 6PCs (85% variance, RMSEC=0.38, RMSECV=3.93), 4PCs (71% variance, RMSEC=0.52, RMSECV=3.07) and 5PCs (79% variance, RMSEC=0.45, RMSECV=0.78) respectively. The plots clearly indicate that the biological variation in the data from the sample pairs is greater than the variation introduced by haemolysis. This can be seen by the close proximity of sample pairs, based on their metabolic features compared to the global distribution of samples. The pairing of clean-haemolysed samples allows the identification of biological outliers as opposed to technical outliers, as seen in Figure 7.2 polar ESI(+), where both samples from an individual are shown to be biologically very different from the other infants.

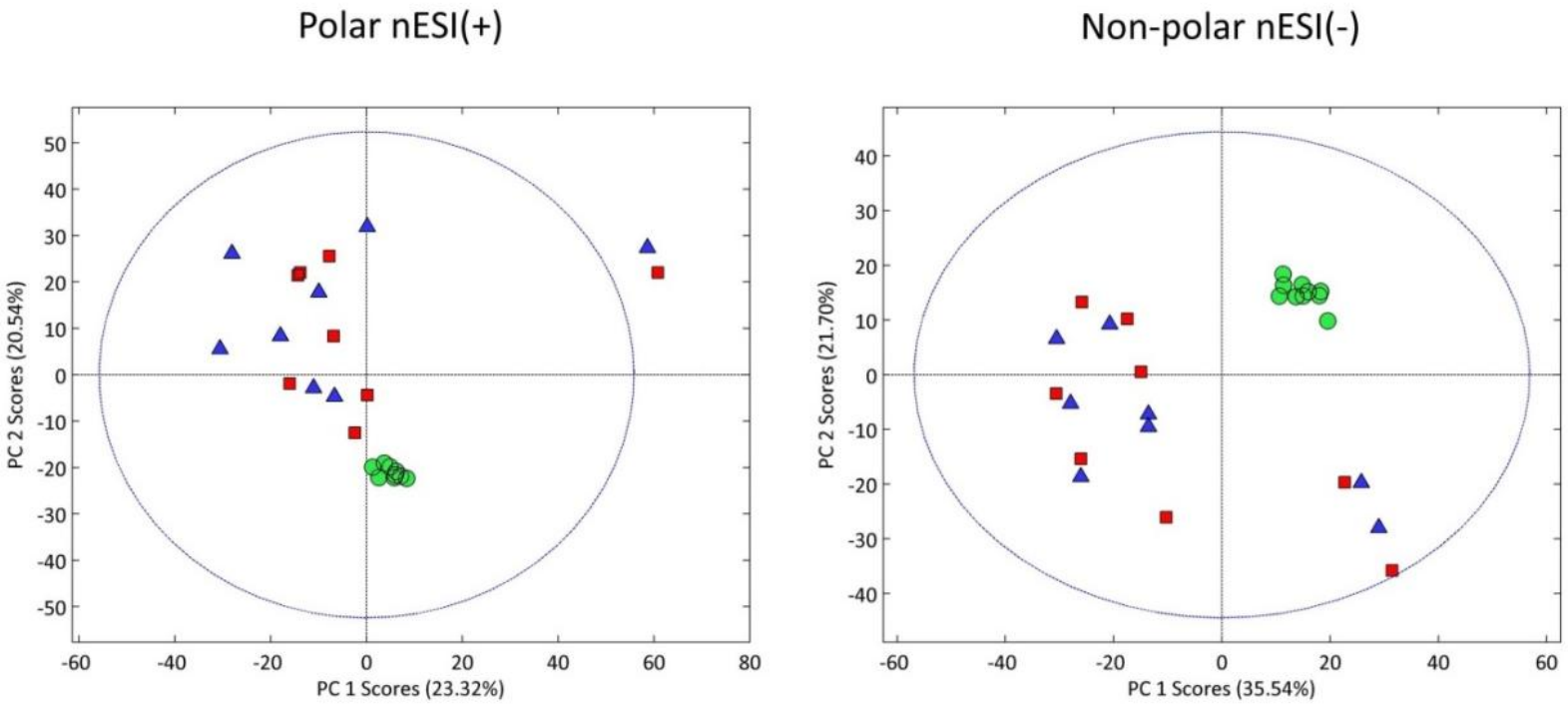

Figure 7.2 PCA plots for two datasets polar nESI(+) and non-polar nESI(-). The cluster of green circles represents the reproducible cluster of pooled QC samples. Blue triangles are the clean serum samples and red squares the haemolysed serum samples.

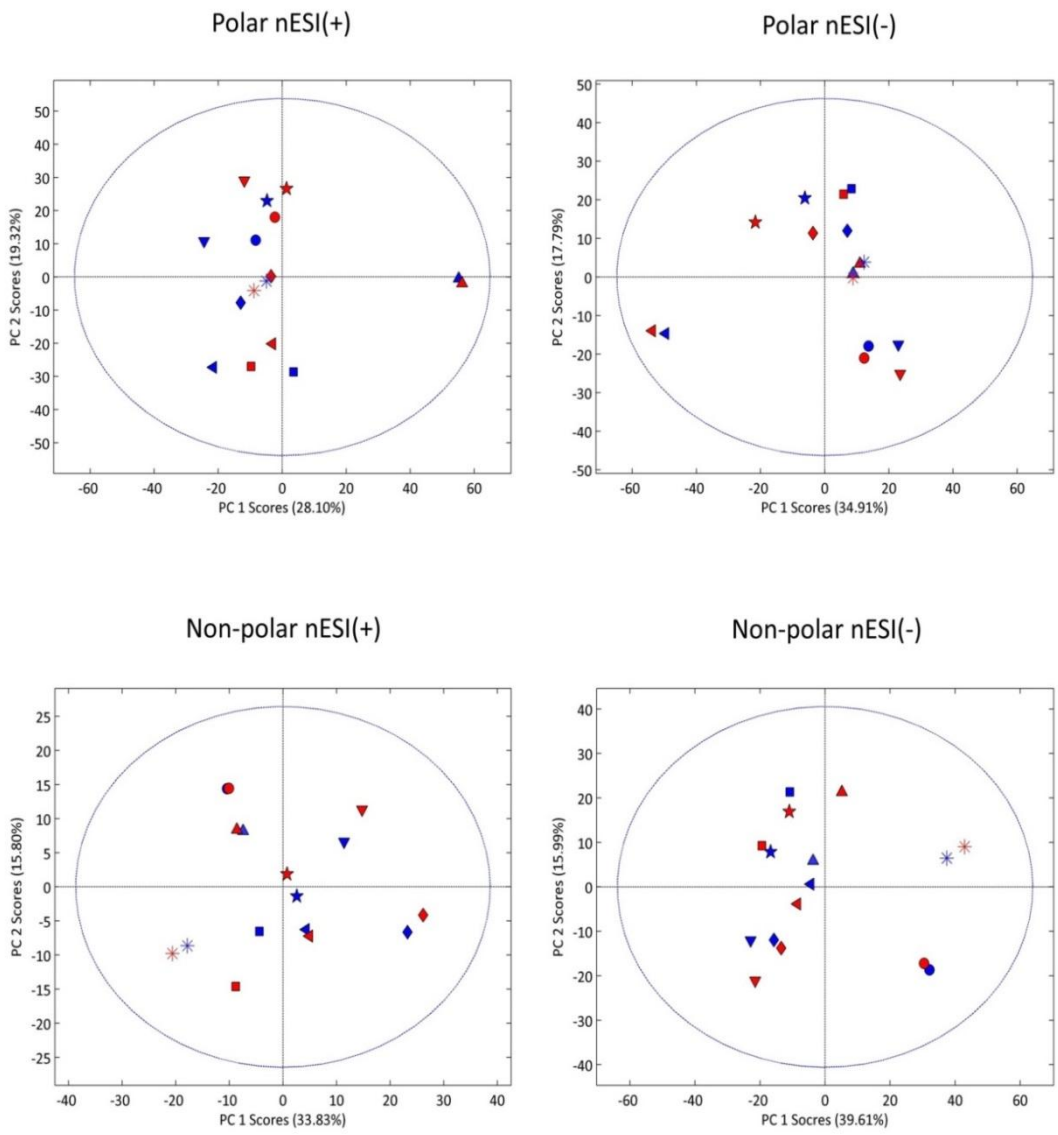

Figure 7.3PCA plots for each of the four metabolomic datasets whereby the greatest variation was seen in the first and second principle components (PC). Each unique shape represents a clean-haemolysed serum pair (n=8), with blue being the clean serum and red the haemolysed serum.

7.4.2 Univariate analysis

To maximise the use of paired samples and to reduce the effect of unwanted biological variability, paired statistical analysis was undertaken using a Wilcoxon signed-rank test. Statistically significant ($p<0.05$) alterations were detected in 131 (7%) and 40 (3%) for polar nESI(+) and nESI(-) metabolic features respectively as well as, 6 (1%) and 24 (2%) for non-polar nESI(+) and nESI(-) features. Fifteen significant features with an RSD^{QC} of >20% were excluded due to their potential unreliability. Over half of the significant feature alterations from the polar nESI(+) and non-polar nESI(-) increased, while more decreased for the polar nESI(-) datasets (Figure 7.1). Of the 184 polar and 32 non-polar features altered by haemolysis, 26 and 12 were assigned putatively annotated respectively, as listed in Table 7.2 with the identification parameters inAppendix Table H.3. All other significantly altered compounds were assigned empirical formulae; see AppendixTable I.3 and Table I.4.

For the polar metabolites, 24 putative identifications implicated 25 unique empirical formulae for which elevations in the fold change were predominantly associated with haemolysis. Though statistically significant, for the majority of compounds the fold change increases were small, between 1.1-1.2 fold. Differences were detected for carbohydrate and energy metabolism compounds for example, dehydroascorbic acid (m/z 212.97956) an intermediate in the isomerization of citrate to isocitrate in the tricarboxylic acid (TCA) increased 1.4 fold (1.2, 1.8 95%CI), while glucose (m/z 203.05265, p=0.039), and deoxy-galactose (m/z 185.043, p=0.016) had small yet significant elevations. Three features putatively ascribed amino acid identifications were also found to be increased, including glutamine (m/z 169.05842, p=0.039), glutamate-5-semialdehyde (m/z 154.04749, p=0.039) and tyrosine (m/z 216.04322, p=0.016). The two metabolites which decreased as a results of haemolysis were docosatetraenoylethanolamine (m/z 416.27509) and N-(mercaptoheptanoyl)threonine-O-phosphate (m/z 402.09919) whose levels declined 0.8 fold (0.6, 0.9 95%CI) and 0.8 fold (0.7, 0.9 95%CI) respectively. Boxplots of the peak intensities for a range of putatively annotated metabolites are provided inFigure 7.4 and indicate clear differences between the clean *vs.* haemolysed samples.

Analysis of the non-polar datasets, which include most lipid metabolites, revealed 12 significantly altered metabolic features implicating 14 unique empirical formula. Interestingly,

for lipid metabolites the fold change elevations associated with haemolysis were greater than that of the altered polar compounds. Boxplots of the feature intensity for each serum group are displayed in Figure 7.5. Larger fold change increases were seen for fat-soluble vitamin metabolites. For example, didesmethyl tocotrienol (m/z 367.26432) a putatively annotated vitamin E related compound increased 4.8 fold (2.3, 12.6 95%CI) in haemolysed serum compared to clean (p=0.008). Vitamin D derivatives (m/z 429.37301, m/z 445.36803 and m/z 537.37957) were detected as being significantly raised by 2.8 fold (1.4, 4.2 95%CI), 1.4 fold (1.1, 3.6 95%CI) and 1.4 fold (1.2, 1.7 95%CI) respectively. Finally m/z 445.36881 which is putatively identified as either vitamin D or E related compounds or a sterol lipid such as cholesterol, was significantly (p=0.016) increased2.3 fold (1.3, 3.2 95%CI) by haemolysis. Slight decreases associated with haemolysis were evident for three lipid compounds, two being glycerolipids (m/z 745.59421 and m/z 761.66639) and one unsaturated fatty acid (m/z 365.27).

A Benjamini-Hochberg correction for multiple comparisons was performed(148), however no metabolic features passed false discovery.

Table 7.2Features identified as being statistically altered between clean-haemolysed sample pairs. Updated

m/z	Putative identification	Fold change (95%CI)	*p*-value	RSD^{QC}	
Polar features					
121.06241	Cyclohexanone OR 3-Hexenal	1.2 (0.9,1.5)	0.039	13	↑
125.05733	2-Methylbutyrate OR 3-Methylbutanoic acid OR Pentanoate	1.2 (1,1.4)	0.039	14	↑
127.01643	Aromatic aldehyde	1.2 (1,1.4)	0.039	17	↑$^{###}$
142.0475	L-Allothreonine OR L-Homoserine OR L-Threonine	1.1 (1.1,1.2)	0.016	4	↑
154.04749	3-Hydroxy-L-proline OR 5-Amino-2-oxopentanoic acid OR L-Glutamate 5-semialdehyde (>3)	1.1 (1,1.3)	0.039	6	↑
169.05842	Glutamine OR 3-Ureidoisobutyrate OR Alanylglycine	1.1 (1,1.2)	0.039	3	↑$^{##}$
184.0581	L-2-Aminoadipate OR O-Acetyl-L-homoserine OR 3-Amino-2,3-dideoxy-scyllo-inososе	1.2 (1,1.4)	0.039	8	↑
185.04300	6-Deoxy-L-galactose OR Rhamnose OR D-Fucose (>3)	1.1 (1,1.2)	0.016	14	↑$^{#}$
190.08392	Methoxytyramine OR Phenylephrine OR Synephrine	1.1 (1,1.4)	0.039	11	↑
203.02918	1,2-Dihydronaphthalene-1,2-diol OR Isosafrole	1.2 (1.2,1.3)	0.008	10	↑
203.05265	Glucose OR Fructose OR myo-Inositol (>3)	1.1 (1,1.2)	0.039	3	↑$^{#}$
203.05391	Theophylline OR 1,7-Dimethylxanthine OR Theobromine	1.1 (1,1.2)	0.031	3	↑
205.04482	Phenylbutyric acid OR Benzenebutanoic acid OR Eugenol	1.2 (1.1,1.2)	0.016	4	↑
207.02406	Phenyllactate OR 3-Phenoxypropionic acid OR 4-Methoxyphenylacetic acid (>3)	1.1 (1,1.2)	0.008	7	↑
212.97956	Dehydroascorbic acid OR Aconitate	1.4 (1.2,1.8)	0.016	9	↑
214.06868	2-Amino-3,7-dideoxy-D-threo-hept-6-ulosonic acid	1.1 (1,1.3)	0.023	4	↑

216.04322	(R)-Salsolinol OR 2(N)-Methyl-norsalsolinol OR Tyrosine OR 3-Amino-3-(4-hydroxyphenyl)propanoate OR 4-Hydroxy-4-(3-pyridyl)-butanoic acid (>3)	1.2 (1.1,1.4)	0.016	13	↑###
244.07929	N-Acetyl-D-glucosamine OR N-Acetyl-D-mannosamine OR N-Acetyl-β-D-galactosamine (>3)	1.1 (1,1.2)	0.039	4	↑
402.09919	N-(7-Mercaptoheptanoyl)threonine 3-O-phosphate	0.8 (0.7,0.9)	0.008	11	↓
416.27509	7,10,13,16-Docosatetraenoylethanolamine	0.8 (0.6,0.9)	0.023	4	↓###
441.15868	2-Methoxyestradiol-17-β-3-sulfate	1.1 (1,1.1)	0.008	8	↑###
443.17437	3β,16α-Dihydroxyandrostenone sulfate	1.1 (1,1.2)	0.039	9	↑###
487.19446	15-hydroxynorandrostene-3,17-dione glucuronide OR 16-Glucuronide-estriol OR 16-α,17-β-estriol 17-β-D-glucuronide (>3)	1.1 (1,1.3)	0.047	6	↑
519.29341	Pregnanediol-3-glucuronide OR 3-α,20-α-dihydroxy-5-β-pregnane 3-glucuronide	1.2 (1,1.3)	0.016	7	↑###
Non polar features					
365.27000	5,8,11-Eicosatrienoic acid OR Icosatrienoic acid OR Oncobic acid (>3)	0.9 (0.9,1)	0.023	10	↓
367.26432	Didesmethyl tocotrienol	4.9 (2.3,12.6)	0.008	7	↑#
389.23347	Acetate OR 15R-PGA2 methyl ester OR digoxigenin OR 11-Deoxycorticosterone OR hydroxyprogesterone OR 1α-hydroxy-20-oxo-22,23,24,25,26,27-hexanorvitamin D3 / 1α-hydroxy-20-oxo-22,23,24,25,26,27-hexanorcholecalciferol (>3)	1.1 (1,1.2)	0.023	13	↑#
429.37301	1-Hydroxyvitamin D5 OR (24R,24R)-Fucosterol epoxide OR (6R)-6,19-ethano-25-hydroxy-6,19-dihydrovitamin D3 / (6R)-6,19-ethano-25-hydroxy-6,19-dihydrocholecalciferol (>3)	2.8 (1.4,4.2)	0.016	2	↑
445.36803	11α-ethyl-1α,25-dihydroxyvitamin D3/11α-ethyl-1α,25-dihydroxycholecalciferol OR 1α,25-dihydroxy-24a,24b-dihomo-20-epivitamin D3/1α,25-dihydroxy-24a,24b-dihomo-20-	1.4 (1.1,3.6)	0.016	8	↑

	epicholecalciferol OR 1α,25-dihydroxy-24a,24b-dihomovitamin D3/1α,25-dihydroxy-24a,24b-dihomocholecalciferol (>3)				
445.36881	Cholesterol OR Lathosterol OR toxisterol3 R1/6,19-dihydrovitamin D3/6,19-dihydrocholecalciferol OR 13-hydroxy-α-tocopherol OR 24-hydroperoxy-24-vinylcholesterol OR Nebrosteroid M (>3)	2.3 (1.3,3.2)	0.016	19	↑
524.27814	LysoPE(0:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)) OR LysoPE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0) (>3)	1.1 (1,1.2)	0.023	9	↑###
537.37957	1α,25-dihydroxy-2α-(3-hydroxypropoxy)-19-norvitamin D3 / 1α,25-dihydroxy-2α-(3-hydroxypropoxy)-19-norcholecalciferol OR 1α,25-dihydroxy-2β-(3-hydroxypropoxy)-19-norvitamin D3 / 1α,25-dihydroxy-2β-(3-hydroxypropoxy)-19-norcholecalciferol (>3)	1.4 (1.2,1.7)	0.008	6	↑
715.57588	SM(d18:1/17:0) OR SM(d19:1/16:0)	1.1 (1,1.2)	0.016	11	↑###
733.59866	DG(18:1(9Z)/22:2(13Z,16Z)/0:0) OR DG(18:2(9Z,12Z)/22:1(13Z)/0:0) OR DG(18:3(6Z,9Z,12Z)/22:0/0:0)(>3)	1.2 (1.1,1.3)	0.008	9	↑
745.59421	DG(18:1(11Z)/24:1(15Z)/0:0) OR DG(20:0/22:2(13Z,16Z)/0:0) OR DG(24:1(15Z)/18:1(9Z)/0:0) (>3)	0.9 (0.9,1)	0.039	4	↓
761.66639	TG(12:0/13:0/20:1(11Z))iso6 OR TG(12:0/14:0/19:1(9Z))iso6 OR TG(12:0/14:1(9Z)/19:0)iso6 (>3)	0.9 (0.9,1)	0.023	6	↓

(>3); indicates more than three possible putative annotations, OR; indicates more than one possible empirical formulae.↑; increasing, ↓; decreasing with haemolysis. Potential biomarkers of #perinatal asphyxia, ##HIE or both ###perinatal asphyxia and HIE are indicated.

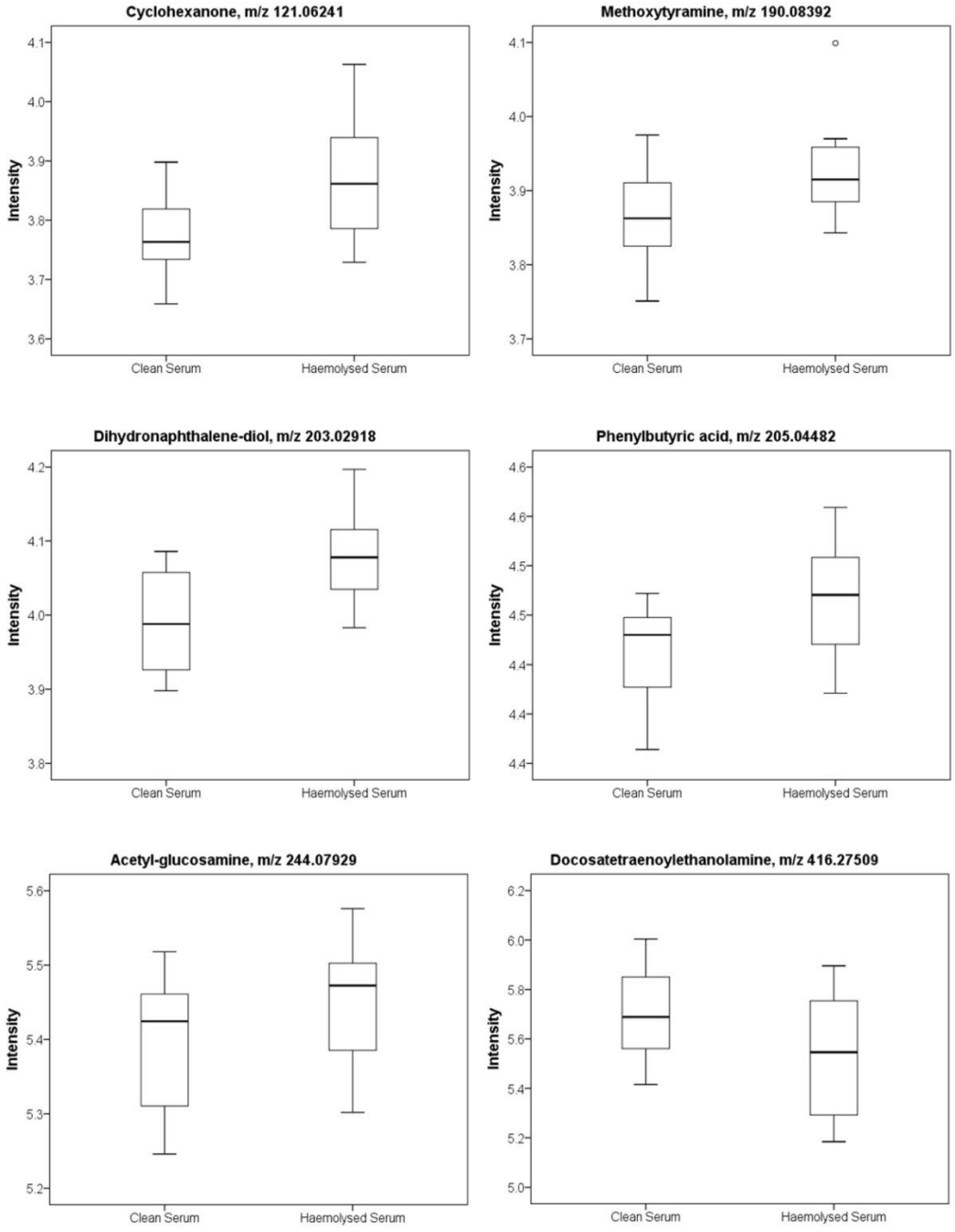

Figure 7.4 Boxplots for putatively annotated polar metabolic features significantly altered between UCB serum types. Feature intensity (log_{10}) is plotted for clean (n=8) and haemolysed (n=8) groups.

7.4.3 Comparison to potential biomarkers

Of the 142 study UCB samples used in Chapter 3 and Chapter 4 to detect potential biomarkers of perinatal asphyxia and HIE, 25% were haemolysed. Of the 30 HIE samples included 8 were haemolysed (4 of 5 Severe HIE, 1 of 8 Moderate HIE and 3 of 17 Mild HIE), while 9 of the 41 perinatal asphyxia were haemolysed. Comparison between compounds altered by haemolysis and potential biomarkers of perinatal asphyxia and/or HIE (reported in Chapter 3 and Chapter 4) revealed 9 polar and 4 non-polar compounds significantly altered in both studies (Table 7.2).

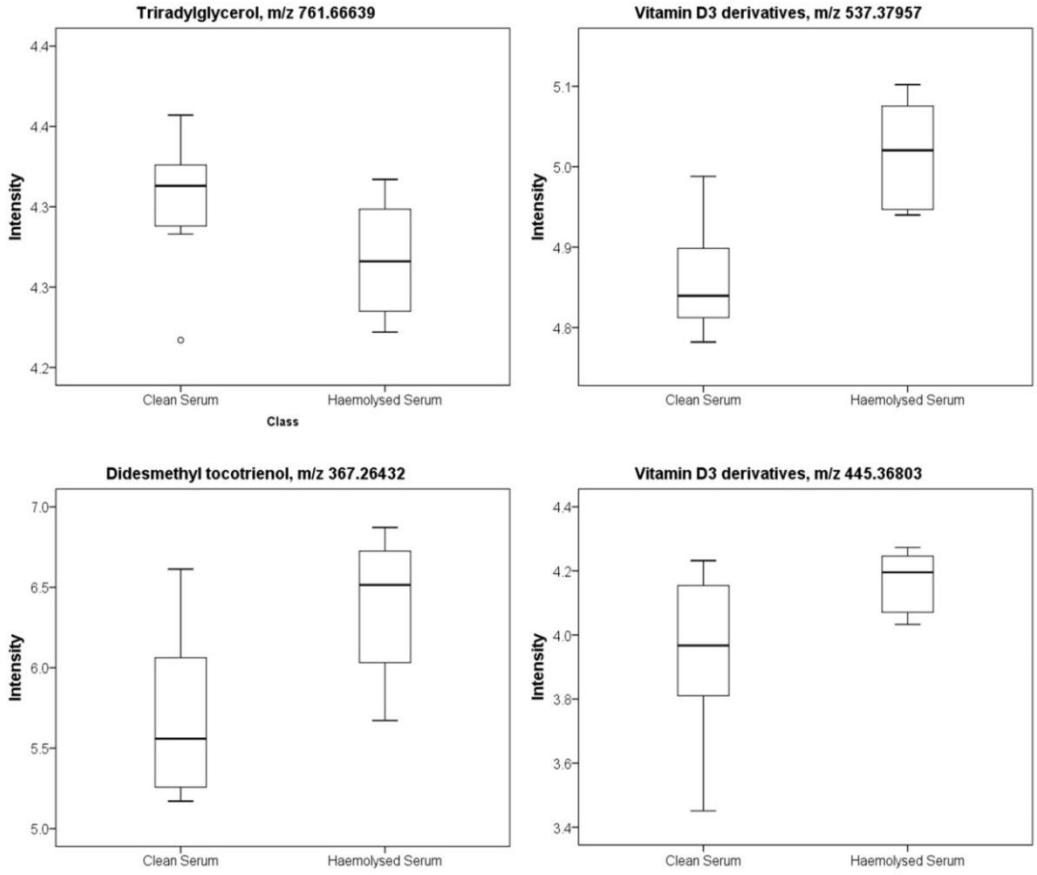

Figure 7.5 Boxplots for putatively annotated non-polar metabolic features significantly altered between UCB serum types. Feature intensity (log_{10}) is plotted for clean (n=8) and haemolysed (n=8) groups.

7.5 Discussion

This is the first untargeted metabolomic study to characterise the extent of metabolic changes caused by haemolysis in umbilical cord blood. DI FT-ICR MS, a highly sensitive and mass accurate analytical platform enabled the comprehensive study of the cord blood metabolome including both polar and non-polar (lipid) metabolites. Despite a number of individual metabolic features being significantly altered by haemolysis, metabolomic profiling revealed the preanalytical variability was negligible compared with inter-individual biological variability. A key strength of this study is that the infant samples are paired allowing the effects of haemolysis to be studied independent to the inherent biological variability of a neonatal population.

Umbilical cord blood is a specimen well suited to biomarker discovery and investigational analysis in the maternal-neonatal population, with its use in metabolomic investigations becoming wide spread (8, 163, 164, 167, 305, 306). Large volumes (~20 ml) can be non-invasively extracted from the placenta by clamping the cord at each end, enabling whole blood, serum and plasma to be biobanked. Unfortunately, umbilical cord blood frequently becomes haemolysed when large volumes are collected in the delivery room. Our research centre has been biobanking cord blood for over a decade. We rely on clinical staff to extract the cord blood sample, due to the unpredictable nature of birth. An in-house analysis of a large population biobank revealed 21% of individuals with samples biobanked had haemolysed serum as a result of *in vitro* collection errors [2014 unpublished data], a much higher rate of haemolysis compared to the 3% reported occurrence in clinical samples routinely collected (214). In vitro collection errors include excess force or pressure, incorrect needle size, unnecessary mixing and under fill of the sample tube which cause the breakdown of red blood cells (207). Anecdotally, higher rates of *in vitro* haemolysis occur in cases of neonatal injury, for example perinatal asphyxia, preterm birth and intra-uterine growth restriction. These samples are of great interest to researchers. However, the cord or placenta is more susceptible to damage, prolapse or compression, or indeed sampling may be delayed or rushed due to the unfolding medical emergency.

Multivariate metabolomic analysis revealed the biological variance between infant samples remained greater than the technical variance introduced by haemolysis. Importantly, clusters of haemolysed or indeed non-haemolysed (clean) samples are not evident in the PCA plots indicating the samples could not be differentiated by haemolysis. This suggests the unique metabolomic fingerprint of each infant remained intact, despite the sample being haemolysed. This has important implications for future neonatal studies examining metabolome changes in relation to neonatal disease, injury or indeed epidemiological exposures. It indicates that a haemolysed sample can still provide a representative snapshot of an individual infant's metabolism at a point in time and therefore can be included in untargeted metabolomic studies.

However, upon inspection of individual mass features, alterations did occur between cord blood pairs. We report on average 5% of polar and 1% of non-polar features changed as a result of haemolysis, much lower than anticipated. Using untargeted approaches, Yin et al. reported a total of 18% of ion mass signals were significantly changed by haemolysis (197) while Kamlage et al. found that 18% were altered by grade 1 haemolysis, which increased to 30% as haemolysis increased in severity to grade 2 (199). This study also reported that metabolites were predominately decreased in grade 1 haemolysis but increased in grade 2 (199). In general, concentration increases are observed for metabolites present at higher concentrations in erythrocytes than serum itself, while decreases are due to dilution effects or metabolic uptake by enzymes released during erythrocyte breakdown (208). Depending on the ratio of metabolite concentration between erythrocyte and serum, even slight haemolysis can produce significant derangement. Herein the significantly altered polar features were both increased and decreased by haemolysis in equal measure, whereas a slightly higher proportion of non-polar features decreased.

To assess the biological relevance of our findings, putative annotations were ascribed to features, and potential differences were detected for metabolites from several metabolite classes. Metabolites of carbohydrate and energy metabolism were significantly elevated between clean-haemolysis pairs including glucose and deoxy-galactose. Using targeted and unpaired metabolomic data, we have previously reported that hexose is potentially altered in haemolysed cord blood (307). Herein we detected six glucose adducts, 3 in each respective ion

mode, however only one glucose adduct was statistically significant, indicating a potential false discovery. Two studies examining the effect of haemolysis on human and bovine plasma metabolites found no difference in glucose (199, 300). Conversely historical studies into the effects of haemolysis which used conventional biochemical tests report decreased glucose measurements in its presence (209-211). Previous studies have not found differences in lactate (199, 300), though we found a significant difference the RSD^{QC} was 22% above the acceptable variability limit which decreases our confidence in the reproducibility of this result.

Dehydroascorbic acid, an intermediate in the isomerization of citrate to isocitrate in the tricarboxylic acid (TCA) cycle, increased 1.4 fold, in agreement with our previous finding of a 1.3 fold significant increase in citrate (307). Putatively identified amino acids compounds, glutamine, glutamate-5-semialdehyde and tyrosine, all increased with haemolysis. Glutamate was previously shown to be influenced by other preanalytical errors for example processing time (197). Finally the analysis of polar metabolites detected a significant rise in theophylline, a methylxanthine drug structurally and pharmacologically similar to caffeine, for which positive and negative alterations are known to occur in the presence of haemolysis (308).

We report large fold change alterations in putatively annotated prenol and sterol lipid metabolite classes for example, the sub-classes 'vitamin E' and 'vitamin D and derivatives'. To our knowledge alterations to these lipid sub-classes as a result of haemolysis have not previously been reported. Significant alteration were also found in two glycerolipids and one unsaturated fatty acid, compound classes in which Kamlage et al. reported significant alterations associated with grade 2 haemolysis (199). Elevations in cholesterol, a sterol lipid putatively identified and significantly altered herein, are known to occur in haemolysed samples using routine clinical tests (209-211). However both cholesterol sulfate and hydroxycholesterol sulfate were also identified and displayed no fold change alterations between samples, despite cholesterol sulfate's ability to regulate the activity of serine proteases involved in blood clotting and fibrinolysis (283), processes exacerbated by red blood cell lysis (309). Fibrin itself was putatively annotated and varied largely in fold change 1.7 (1.2, 1.8 95%CI) however was not statistically significant p= 0.094. Other analytes reportedly altered by haemolysis include uric acid, urea and bilirubin (210). We identified four urate adducts and two urea adducts, which all displayed slight fold change increases associated with haemolysis

however none were statistically significant. Similarly the non-polar analysis putatively identified bilirubin yet we found no change in bilirubin between clean-haemolysed pairs despite reports suggesting its decrease (212, 308).

It is worth noting that 60% of features had reasonably narrow fold change confidence intervals (≤0.3), providing a high level of certainty that the true effect of haemolysis lies within the interval range. However around 16% of features had wide confidence intervals (≥0.6) indicating haemolysis may produce a high level of variance in these metabolites. Though beyond the scope of this study, it may be possible to develop a haemolysed adjusted normal range, for example a range whereby you could measure a biomarker in a haemolysed sample and still determine if it's in the normal limits for haemolysis.

Some of our findings conflict with previous studies evaluating haemolysis, which have measured metabolites either in isolation or as one of the scarce number of metabolomic studies. This is not surprising as the degree of interference caused by haemolysis depends on the methodology and individual instrumentation in use. Measuring the effects of haemolysis is method dependant (209), matrix affects may impact on the ionisation of analytes impacting on the precision and accuracy of the bioanalytical method (310), leading to a dramatic variation in results. Studies will undoubtedly vary with regards to other preanalytical variables such as storage and processing times, and temperatures, which also affect metabolite stability (198). Furthermore different specimen types, plasma and serum, will have a unique reaction to haemolysis, due to the different metabolomic profiles to begin with (311). Changes may also relate to the length of time the serum or plasma remains exposed to the contaminant erythrocyte enzymes, before enzymatic activity is quenched.

Our samples are stored and processed at 4°C in an endeavour to quench enzymatic activity as soon as the sample is drawn before being stored at -80°C. Methanol is added before thawing for metabolite extraction to ensure enzymatic activity remains suppressed. We implement a strict biobanking standard operating procedure (SOP) to minimise other preanalytical errors (204). For example, the maximum time to freezer is defined as less than three hours of birth, and blood processing times and temperatures are maintained. Storing samples in micro-aliquot volumes that are sufficient for one metabolomic experiment, eliminated the need for freeze thaw cycles (198).

Finally in the previous studies (Chapter 3 and 4) 25% of the UCB samples used to detect biomarkers of perinatal asphyxia and HIE were haemolysed. These samples were collected in an identical manner to the samples in this study. We have identified nine polar and four non-polar compounds which are altered in perinatal asphyxia and HIE, but also altered by haemolysis. Polar compounds include two hexose sugars,glucose and deoxy-galactose found altered in perinatal asphyxia, two sulfate steroid compounds, methoxyestradiol-sulfate and dihydroxyandrostenone sulfate reportedly different in both perinatal asphyxia and HIE compared to matched controls, and two amino acids, tyrosine which is altered in both groups and glutamine in HIE alone. Haemolysis would cause marginal increase of 1.1-1.2 fold in all of these compounds bar one, below the level of fold increase reportedly casued by HIE. Herein we found docosatetraenoylethanolamine decreased 0.8 fold because of haemolysis, however alteration of 1.6 fold were detected in perinatal asphyxia and HIE. Non polar compounds include a sphingolipid and glycerophospholipid, both increased marginally with haemolysis but decreased marginally 0.9 fold in perinatal asphyxia and HIE. The mass feature m/z 389.23347 was matched with two possible empirical formulae, putatively annotated as the steroid hydroxyprogesterone or acetate. This feature and resulting compounds was also found to be statistically altered in perinatal asphyxia. It is unclear if haemolysis alters these compounds to a degree which renders their alteration in perinatal asphyxia and HIE invalid. Nevertheless, because of the potential effect of haemolysis on these metabolites their usefullness as biomarkers would require additional validation.

A limitation to this study is the small number of paired samples analysed compared to the large number of features analysed. A Benjamini Hochberg false discovery correction was applied, however no features passed this false discovery rate of <0.1 which is based on a ranking system (148). The study may be underpowered to assess false discovery however this does not impact on the aim of the study to create a list of features potentially altered by haemolysis therefore less conservative estimates are acceptable.

Putative metabolite annotation was used to ascribe identities, rather than assigning accurate metabolite identification which is both expensive and technically demanding, particularly for untargeted metabolomic investigations which detect large numbers of features (259). Identifications were not required to assess how haemolysis affected the metabolic fingerprint

of cord blood, while putative metabolite annotation was sufficient to determine biological plausibility of the results. Numerous studies have demonstrated the ability for DI FT-ICR MS, especially when coupled to nanoelectrospray ionisation, to capture large amounts of metabolic data with high accuracy (220, 287, 288), and we have employed stringent analytical measures to ensure high quality data (135). Publishing a list of accurate mass values, whether ascribed putative annotations or empirical formulae, will hopefully prompt researchers in the field to ensure their findings are robust to haemolysis and will act as tool for the comparison of metabolites.

Successful biomarker discovery is hinged upon producing high quality data in the investigative stage which enables reliable interpretation, validation and translation to the clinic (201).In an ideal world, clinical trials would include a haemolysed subset to assess the effects and reliability of measuring biomarker concentrations in haemolysed samples from healthy and test individuals. However this would add to the number of clinical samples required and to the cost of any trial. Similarly excluding haemolysed UCB samples altogether is not feasible due to the time, cost and effort invested in their collection, and may render the biomarkers impractical. Non targeted broad scale analysis of haemolysed versus clean samples may act as a proxy indicator for future studies to predict the risk that a likely biomarker candidate may require an adjusted normal range or cannot be used when assessed in the context of a haemolysed sample.

Chapter 8

8 Concluding Summary

8.1 Summary of main findings

This research was conducted with the overarching aim of making novel contributions to the field of metabolomic investigations in perinatal asphyxia and HIE, to discover candidate metabolomic markers which have thepotential to identify infants at risk ofneonatal HIE. This thesis has performed the first untargeted metabolomic mass spectrometry analysis of any cohort of infants with HIE. As my interest lay in the translation of metabolomic markers to clinical use, I also examined the ability of a metabolite modelto predict long-term neurodevelopmental outcome, and the robustness of metabolite measurements to withstand the preanalytical variable haemolysis.

This thesis had three main aims; the first aim was to identify novel metabolomic markers of perinatal asphyxia and HIE through untargeted metabolomic analysis. This was achieved by using a direct injection mass spectrometry (DI FT-ICR MS) metabolomic workflow and cord blood samples from a previously recruited cohort of infants with perinatal asphyxia and HIE (Appendix E.1). This highly sensitive untargeted method was performed for both polar (hydrophilic) and non-polar (lipid) compounds enabling the broad characterisation of the cord blood metabolome and its response to hypoxia-ischaemia compared to healthy matched controls. To our knowledge this is the most extensive depiction of the cord blood metabolome in a cohort of infants with perinatal asphyxia. It enabled previously unsuspected individual metabolites, metabolic pathways and lipid classes, which were altered in response to hypoxia-ischaemia to be elucidated. In particular tryptophan and pyrimidine pathway metabolites displayed significant alterations associated with HIE alongside lipid classes including fatty acyl, prenol and glycerolipids. Individual metabolites from the tryptophan and pyrimidine pathway, and some glycerophospholipids showed potential to differentiate between injury severities and infants with or without an encephalopathy.

The second aim of this thesis was to perform the technical study validation of previously identified cord blood metabolomic markers, and to evaluate their potential to predict long-term outcome. Technical validation was carried out by repeating the metabolomic analysis using samples from the same cohort of infants previously analysed by our group using targeted (8) and semi-targeted (7) approaches, but this time employing an alternate untargeted DI FT-

ICR MS workflow. This highly sensitive method allowed changes in previously identified metabolites to be detected and confirmed, including changes in amino acid compounds, energy metabolites for example succinate, glycerol and acetone, and fatty acid compounds. To our knowledge this is the first metabolomic biomarker study in HIE which has performed a technical study validation. It not only strengthens our previous work but also provides confidence in the untargeted workflow to detect novel alterations and progress these findings toward to the final step of validation in an independent neonatal cohort.

Our group has previously reported two separate metabolite models predictive of HIE at birth; the first composed of decenoyl-L-carnitine, 3,5-tetradecadiencarnitine, PC ae C38:0, phenylalanine and proline (model A)(8),the second composed of succinate, glycerol, 3-hydroxybutyrate and O-phosphocholine (model B) (7). This thesis examined the ability of early metabolite models to correlate with three year neurodevelopmental outcome in infants with HIE. Both model scores significantly correlated with outcome, model A showing potential to predict an abnormal outcome and model B being particularly robust to predict both a severe outcome and intact survival at three years. Most importantly the models outperformed other biochemical markers used routinely in clinic. Though further validation is required this highlights the potentially powerful clinical utility that early metabolite measurements in cord blood possess.

Finally the third aim was to examine the robustness of metabolite markers to haemolysis. To do this I first conducted a preliminary study using retrospective metabolomic data from a healthy control population, and reported alterations in lipid metabolites including acylcarnitines, glycerophospholipids and sphingolipids, as well as sugars, amino acids and TCA cycle intermediates. Due to two limitations, the first being that haemolysis was confirmed visually and the second that differences may be due to inherent biological variability as haemolysed/non-haemolysed samples were taken from different infants, this preliminary study was not sufficient to draw concrete conclusions but did indicate that further investigation was warranted. Therefore I conducted the first paired analysis of cord blood serum samples using an untargeted metabolomic method similar to that used in the investigation of biomarkers of HIE, so that results from the two studies could be compared. Importantly this study revealed that the biological variability between individuals is still greater than the variation between a

haemolysed and non-haemolysed sample from the same individual. This means that the use of a haemolysed sample in investigative metabolomics is possible, if haemolysis is documented and controlled for in the interpretation of results. Several individual metabolic features were affected by haemolysis including somefound altered in perinatal asphyxia and HIE (see Chapter 3 and 4), such as steroid compounds and the amino acids glutamine and tyrosine. Haemolysed samples were used in the investigation for biomarkers of HIE therefore results from these features should be approached with caution. Their susceptibility to interference by haemolysis may hamper validation and more importantly it may not be possible to robustly measure them at the cot-side.

8.1.1 Strengths

The primary strength of this study is the unique cohort of infants with perinatal asphyxia and HIE, which has enabled a thorough and methodological approach to biomarker discovery. This prospective cohort, namely The Biomarkers in Hypoxic Ischaemic Encephalopathy Study (The BIHIVE Study, Appendix E.1) was identified and recruited by my supervisors Dr D Murray and Prof G Boylan, along with clinical research fellow Dr Brian Walsh in Cork University Maternity Hospital, Ireland between 2009 and 2011. Several aspects of this cohort have strengthened the work in this thesis:

- Demographic, biochemical, physiological and clinical data (>80 data points) was collected for each infant enrolled in The BIHIVE Study.
- HIE was graded using a modified Sarnat score at 24hours, and the encephalopathy was confirmed using continuous multichannel EEG by an experienced electroencephalographer (Prof G Boylan).
- Each infant had UCB drawn, processed and stored within three hours of birth. All samples were meticulously biobanked using strict standard operating procedures.
- Infants were enrolled in follow-up and underwent standardised three year neurodevelopmental outcome assessment.
- A healthy control population was recruited simultaneously as part of the Cork BASELINE Birth Cohort Study (218), providing matched controls.

Upon examining the literature, previous biomarker studies in this population not only metabolomic studies, are very heterogeneous; having different sample types, inclusion criteria and analysis methods, making comparison between studies and validation difficult. The thorough phenotyping of our cohort has enabled us to accurately grade perinatal asphyxia and HIE. Controls were similarly phenotyped at birth, which enabled the one to one matching of cases and controls for several variables including gender, gestational age and birthweight in an attempt to reduce biological variation unrelated to the injury itself. Biobanking high quality cord blood samples within three hours of birth, in an identical manner for both cases and controls, ensured that the samples were a representative snapshot of the metabolome at the time of collection (122). Post-natal samples are difficult to collect at a consistent time-point, and are inherently confounded by the rapidly changing metabolism. Importantly, samples were biobanked in microaliquots to allow multiple metabolomic experiments to be carried out for each infant enabling technical study validation. Finally infant follow up to three years of age is a rarity in biomarker studies therefore its availability in this cohort has strengthened the clinical utility of the proposed metabolite models and will enable truly translational research.

The untargeted metabolomic analysis was strengthened by the use of DI FT-ICR MS, a highly sensitive and mass accurate analytical platform. This platform enabled the high-throughput analysis of >200 samples of very small volume <20µl. This is the first time a platform with such sensitivity has been coupled with a population of this type, enabling the extensive characterisation of the metabolomic response to perinatal asphyxia and HIE. Most importantly it allowed us to achieve our aims in identifying novel unsuspected markers, which less sensitive methods such as NMR would be unable to detect, and also in technically validating previous markers. DI FT-ICR MS was coupled to an untargeted workflow, which was designed to produce only robust and reproducible data.

8.1.2 Limitations

Certain limitations are recognised. The study cohort has a relatively small number of infants with HIE (n=30) and therefore may be underpowered, particularly when injury severity was

examined (17 mild, 8 moderate, and 5 severe). Howeverthis is the largest cohort of infants used to date, when comparing this cohort to all metabolomic studies in the field of perinatal asphyxia and HIE. Neonatal biomarker studies are limited by the difficulty in recruiting a sufficient and appropriate study population, primarily because disease prevalence of HIE is low (0.3%) in high income countries, and the injury is highly unpredictable. Recruitment requires a regimented and multidisciplinary approach to identify participants and collect samples.

Biological samples possess inherently large variation, for example serum is a complex biofluid containing thousands of possible metabolites(312). Though cases and controls were matched one to one for several variables to reduce biological variation, many confounding factors, which may alter the metabolomic fingerprint are outside the scope of this study including maternal diet, genetics and endogenous microbiotaetc. The heterogeneous nature of the HI injury may also introduce variation between infants depending on the timing and nature of the insult suffered. These factors may have affected our ability to discriminate between infant outcome groups (HIE and perinatal asphyxia) using the full metabolomic fingerprints. A metabolic fingerprint may not be useful for diagnosing HIE, instead a model based on a few key metabolites or a metabolic pathway could be more powerful, with the added advantage of being easier to translate to the cot-side.

Finally due to limitations with regards cost and time, metabolites were ascribed putative annotations and not definitive metabolite identifications. This is the primary challenge facing the metabolomics community, particularly for those undertaking untargeted metabolomic techniques whereby a large number of features are detected (259). Metabolite identification is particularly important to allow meaningful biological interpretation of the data. In this thesis we applied bioinformatics techniques such as 'transformation mapping' and metabolic pathways searching to increase our confidence in identifications(225). Our use of a highly mass accurate platform and robust data processing methods further increased our identification confidence. Despite putative annotations being used we were able to achieve the primary aims of the thesis, however future work will use targeted metabolomicswith pure compound standards to confirm identification and quantifymetabolite changes.

8.2 Impact of thesis

This thesis contributes significantly to research in the area of metabolomic investigations in perinatal asphyxia and neonatal HIE. It has elucidated novel biochemical markers and pathway alterations associated with HIE, and performed the first step in validating previously proposed metabolomic markers. Importantly, the novel insight into the pathophysiological mechanisms of HIE provided by this thesis could direct future research by posing potential therapy targets. The association of metabolite markers with neurodevelopmental outcome, and ultimately comparison to standard early biochemical markers illustrates the potential clinical use of metabolites at the cot-side.

The work has also highlighted a critical and previously overlooked preanalytical variable, haemolysis, as an important consideration in biomarker discovery. A recent editorial in Clinical Biochemistry cited the publication of our preliminary investigation into the effect of haemolysis on the metabolomic profile of umbilical cord blood (307) (see Chapter 6), stating 'This is important information, that is seldom reported in studies derived from biobanks which may have profound implications in biomarker discovery, especially in the metabolomic field' (313).

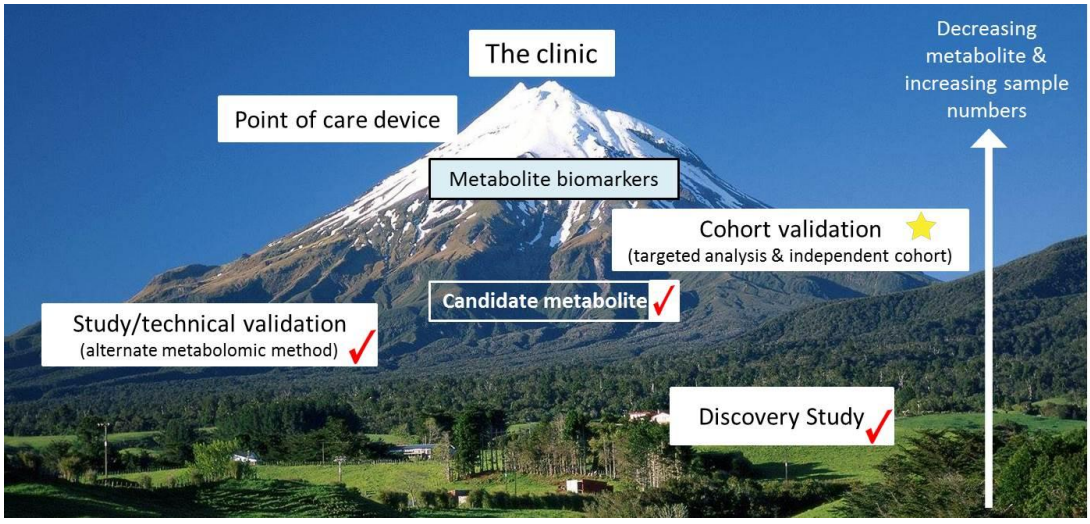

Figure 8.1 The journey of a metabolite biomarker discovery study. This thesis enabled the completion of metabolite discovery and technical validation, from which a list of candidate metabolites has been complied.

8.3 Future work

This thesis has enabled the discovery and technical validation of early cord blood metabolite markers. To translate these findings to the clinic and ultimately the cot-side, validation of markers in an independent cohort of infants is needed. I, along with several team members have recruited a prospective validation cohort, namely The Validation of Biomarkers in Hypoxic Ischaemic Encephalopathy Study (The BIHIVE2 Study, Appendix E.3). Recruitment took place in two large single maternity hospitals, Cork University Maternity Hospital, Ireland and Karolinska Maternity Hospital, Sweden between March 2013 and July 2015. Informed parental consent was a requirement in all cases and ethical approval for this study was granted by our local ethics board.All infants had UCB drawn, processed and biobanked within three hours of birth and neonatal HIE was carefully graded using early continuous multichannel EEG. Over 28 months,we have recruited in total 275 cases (175 Cork and 118 Karolinska) and 225 controls (139 Cork and 86 Karolinska), and biobanked their cord blood. Of the 275 cases, 34 developed HIE with the breakdown of injury severity being, 21 mild, 12 moderate and 1 severe. This cohort has undergone detailed physiological and demographic phenotyping, and all infants are enrolled in a programme of neurodevelopmental follow up.

This final stage of metabolomic biomarker validation will use a targeted approach, measuring a shortlist of pre-defined markers. Our shortlist will likely consist of metabolites from the tryptophan and pyrimidine pathways, several amino acids, acylcarnitines, energy metabolites and glycerophospholipids, including those compounds described in our previously reported models, seeTable 8.1. This list will also depend on the availability of commercial pure compound standards. During my thesis I have established collaborations with Dr Jennifer Kirwan and Prof Warwick Dunn, metabolomic experts from the University of Birmingham, UKwho have a strong track record in the field of human metabolomic analysis. I have also strengthened our ties with Prof David Broadhurst, an experienced metabolomic biostatistician from the University of Alberta, Edmonton. Working with our collaborators, it is envisaged the targeted validation will take place in the University of Birmingham, using a tailored metabolomic approach, for example, extensive sample preparation and chromatographic separation to isolate the candidate markers prior to detection by a highly sensitive analytical platform such as MS/MS and ultimately quantification using pure compounds as a reference.

Then we will need to complete bio-statistical modelling to identify the ideal combination of metabolites to provide optimum test sensitivity and specificity.

Table 8.1 Potential shortlist of metabolites suitable for cohort validation.

Metabolite	Class	DI- & LC-MS/MS	^{1}H-NMR	DI FT-ICR MS	
1. Tetradecadiencarnitine	Acylcarnitines	✓			
2. Butyrylcarnitine	Acylcarnitines	✓		✓	
3. Decenoylcarnitine	Acylcarnitines	✓			
4. L-Acetylcarnitine	Acylcarnitines	✓		✓	
5. L-Palmitoylcarnitine	Acylcarnitines	✓		✓	
6. Phenylalanine	Amino acid	✓	✓	✓	
7. Alanine	Amino acid	✓	✓		
8. Isoleucine	Amino acid	✓	✓	✓	
9. Leucine	Amino acid	✓	✓	✓	
10. Methionine	Amino acid	✓	✓		
11. Proline	Amino acid	✓			
12. Tryptophan	Amino acid			✓	
13. Tyrosine	Amino acid	✓		✓	
14. Valine	Amino acid	✓	✓		
15. Creatine	Amino acid related		✓	✓	×
16. Erythrose 4-phosphate	Amino acid related			✓	
17. Taurine	Biogenic Amines	✓		✓	
18. Glycerol	Carbohydrate		✓		×
19. O-phosphocholine	Choline		✓		
20. Indolelactate	Tryptophan Met.			✓	
21. Kynurenine	Tryptophan Met.			✓	
22. Melatonin	Tryptophan Met.			✓	
23. Succinate	Energy Met.		✓	✓	
24. PC ae C38:0	Glycerophospholipid	✓			
25. 3-hydroxybutyrate	Ketone		✓		
26. Acetone	Ketone		✓	✓	×
27. Oleic acid	MUFA			✓	
28. Linolenic acid	PUFA			✓	
29. Linoleic acid	PUFA			✓	
30. Uridine	Pyrimidine Met.			✓	
31. Dihydrothymine	Pyrimidine Met.			✓	
32. Hydroxypropanoate	Pyrimidine Met.			✓	
33. Glutamine	Pyrimidine Met.			✓	

PUFA; poly unsaturated fatty acid, MUFA mono unsaturated fatty acid, Met; metabolism.

✓ ; Alterations detected using the corresponding analytical platform.

× ; Potentially altered by haemolysis.

In addition to being accurate, the practicalities of a biomarker are important, to enable its seamless translation to the clinic. Ultimately the ideal biomarker panel will be measured quickly and cheaply, without user expertise being necessary. Though mass spectrometry is an extremely useful tool for biomarker discovery, the complex methods and statistical analysis, which require time and in depth knowledge would hamper its use clinically. Not to mention, each maternity hospital would require this expensive analytical instrument and technicians on site to complete the analysis in sufficient time for therapeutic hypothermia to be initiated.

A more feasible approach is to create a point of care diagnostic tool, which could measure a panel of metabolomic markers immediately at the cot-side using a sample of cord blood. Here, small molecules have the advantage because they are well understood and many have been measured in the clinic for decades. For example hand held point of care devices which measure bilirubin, glucose, lactate, cholesterol and ketones from small sample volumes are routinely in use, therefore this type of device would require minimal staff training.

To enhance the diagnostic accuracy of a metabolite panel, developments in bioinformatics and machine learning technology could also allow metabolite measurements to be combined with available clinical and physiological markers including foetal heart rate assessment, gender, gestational age, Apgar score and degree of lactic acidosis as well as EEG and heart rate variability. This would enable the development of a clinical support algorithm, incorporated into the point of care device to diagnose infants suitable for intervention and ‘at risk’ of HIE, with an optimal sensitivity and specificity > 90%.

This thesis has completed an important step in the advancement of a rapid, low cost, robust, non-user dependent method for quantifying the severity of HIE and guiding therapy. The next steps include the validation of promising metabolite markers in an independent cohort and ultimately the development of a point of care device.

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Appendix A. List of Abbreviations

ADP	Adenosine diphosphate
ANOVA	Analysis of variance
Approx.	Approximately
ATP	Adenosine triphosphate
AUC	Area under the curve
BASELINE	Babies after SCOPE: Evaluating Longitudinal Impact using Neurological and Nutritional Endpoints
BCAA	Branched chain amino acids
BIHIVE	Biomarkers in hypoxic ischaemic encephalopathy
CDP	Cytidine diphphosphocholine
CI	Confidence interval
CNS	Central nervous system
CPR	Cardiopulmonary resuscitation
CREC	Clinical research ethics committee
CTP	Cytidine triphosphate
CUMH	Cork university maternity hospital
DI FT-ICR MS	Direct injection Fourier-transform ion cyclotron resonance mass spectrometry
DI MS	Direct injection Mass spectrometry
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EEG	Electroencephalogram
ESI	Electrospray Ionisation
FADH	Flavin adenine dinucleotide
FT-ICR	Fourier transform – ion cyclotron resonance
GABA	γ-aminobutyric acid
GTP	Guanosine triphosphate
HI	Hypoxic ischaemic
HIE	Hypoxic ischaemic encephalopathy
HMDB	Human metabolome database
H-NMR	Proton nuclear magnetic resonance
HO	Hydroxyl radical
HPLC	High performance liquid chromatography
IL	Interleukin
IQR	Interquartile range
KEGG	Kyoto Encyclopaedia of Genes and Genomes
Log	Logarithm
LPS	Lipopolysaccharide
LV	Latent variable
M/Z	Mass to charge ratio
MR	Magnetic resonance
MRI	Magnetic resonance imaging
MS	Mass spectrometry
NADH	Nicotinamide adenine dinucleotide

nESI	Nano electrospray ionisation
NICU	Neonatal intensive care unit
NMDA	N-methyl-d-aspartic acid
NO	Nitric oxide
NOS	Nitric oxide synthase
OXPHOS	Oxidative phosphorylation
PA	Perinatal asphyxia
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
PLSDA	Partial least squares discriminate analysis
PQN	Probable quotient normalisation
QA	Quality assurance
QC	Quality control
RMSEC	Root mean squared error of calibration
RMSECV	Root mean square error of cross-validation
RNA	Ribonucleic acid
ROC	Receiver operating curve
ROM	Reactive oxygen metabolites
RPM	Revolutions per minute
RSD	Relative standard deviation
Sec	Seconds
SHO	Senior house officer
SIM	Selected ion monitoring
SOP	Standard operating procedure
TCA	Tricarboxylic acid
TIC	Total ion current
UCB	Umbilical cord blood
UDP	Uridine diphosphate
UK	United Kingdom
UMP	Uridine monophosphate
USA	United States of America
UTP	Uridine triphosphate

Chemical formulae

Ca	Calcium
K	Potassium
Na	Sodium
$\cdot O_2^-$	Superoxide anion radical
$CHCl_3$	Chloroform
H_2O_2	Hydrogen peroxide
MeOH	Methanol

Units of measurement

°C	Degrees Celsius
Da	Dalton
g	Gravitational constant
Kv	Kilovolts

min	Minutes
ml	Millilitre
mM	Millimolar
PPM	Parts per million
PSI	Pound/square inch
µl	Microliter

Appendix B. List of Figures

Appendix Figures

Appendix C. List of Tables

Appendix Tables

Appendix D. Dissemination of Work

D.1. Peer-reviewed publications

Year	Title
Published manuscripts	
2015	**Denihan NM**, Walsh BH, Reinke SN, Sykes BD, Mandal R, Wishart DS, Broadhurst DI, Boylan GB, Murray DM. The effect of haemolysis on the metabolomic profile of umbilical cord blood. Clinical Biochemistry 2015 48: 534-537
2015	**Denihan NM,** Boylan GB, Murray DM. Metabolomic Profiling in Perinatal Asphyxia: A Promising New Field. BioMed research international 2015, 9.
2014	**Denihan NM.** Who is the cool kid? Biomarkers to help treat newborn brain injury. The Boolean, June 2014, 13-17.
2013	**Denihan NM**, Looney A, Boylan GB, Walsh BH, Murray DM. Normative levels of Interleukin 16 in umbilical cord blood. Clinical Biochemistry 2013 46: 1857-1859.
Accepted manuscripts	
2015	Ahearne CE, **Denihan NM**, Walsh BH, Reinke SN, Sykes BD, Kenny LC, Broadhurst DI, Boylan GB, Murray DM. Cord metabolite model as a predictor of outcome in perinatal asphyxia and hypoxic ischaemic encephalopathy.Neonatology
Manuscripts in preparation	
2015	**Denihan NM,** Kirwan JA, Walsh BH, Hallberg B, Dunn WB, Broadhurst DI, Boylan GB, Murray DM. Untargeted metabolomic analysis and pathway discovery in perinatal asphyxia and hypoxic ischaemic encephalopathy
2015	**Denihan NM,** Kirwan JA, Walsh BH, Hallberg B, Dunn WB, Broadhurst DI, Boylan GB, Murray DM. Lipid metabolite alterations in perinatal asphyxia and hypoxic ischaemic encephalopathy
2015	**Denihan NM,** Kirwan JA, Dunn WB, Broadhurst DI, Boylan GB, Murray DM. Characterising the effect of haemolysis on the cord blood metabolome by direct infusion mass spectrometry

D.2. Conference presentations

Year	Title
Oral presentations	
2015	**Denihan NM**, Kirwan JA, Walsh BH, Dunn WB, Broadhurst DI, Boylan GB, Murray DM. Untargeted metabolomic analysis and pathway discovery in perinatal asphyxia and hypoxic ischaemic encephalopathy. 9th International Conference on Brain Monitoring

	and Neuroprotection in the Newborn, 3rd October 2015, Cork Ireland.
2014	**Denihan NM**. Who is the 'cool' kid? Finding a biomarker to help treat HIE. University College Cork Doctoral Showcase Finalist, 11th June 2013, Cork, Ireland.
2013	**Denihan NM**. The Validation of Early Biomarkers in Hypoxic Ischemic Encephalopathy. Molecular Medicine Ireland Annual Meeting 14th March 2013, Dublin, Ireland.

Poster presentations

2015	**Denihan NM**, Kirwan JA, Broadhurst DI, Boylan GB, Murray DM. Characterising the effect of haemolysis on cord blood metabolites using direct infusion mass spectrometry. Irish Centre for Fetal and Neonatal Translational Research, Research day, College of Medicine and Health UCC, 16th June 2015, Cork, Ireland.
2015	Ahearne CE, **Denihan NM**, Walsh BH, Reinke SN, Sykes BD, Kenny LC, Broadhurst DI, Boylan GB, Murray DM. ^{1}H-NMR Measurement of cord metabolite model predicts severe encephalopathy and outcome in neonatal hypoxic-ischaemic encephalopathy. The Pediatric Academic Societies Annual Meeting 25-28th April 2015, San Diego, USA.
2015	**Denihan NM**, Kirwan JA, Broadhurst DI, Boylan GB, Murray DM. Characterising the effect of haemolysis on cord blood metabolites using direct infusion mass spectrometry. Molecular Medicine Ireland Annual Meeting 24th March 2015, Dublin, Ireland.
2014	**Denihan NM**, Walsh BH, Looney AM Boylan GB, Murray DM. Matrix metalloproteinase-9 is not raised at birth in neonatal hypoxic ischaemic encephalopathy. The 5th Congress of the European Academy of Paediatric Societies 17-20th October 2014, Barcelona, Spain.
2014	Ahearne CE, **Denihan NM**, Walsh BH, Reinke SN, Sykes BD, Kenny LC, Broadhurst DI, Boylan GB, Murray DM. ^{1}H-NMR measurement of cord glycerol succinate and ketones predicts severe encephalopathy and outcome in neonatal hypoxic-ischaemic encephalopathy. The 5th Congress of the European Academy of Paediatric Societies 17-20th October 2014, Barcelona, Spain.
2014	Looney AM, Walsh BH, **Denihan NM**, Boylan GB, Murray DM. Activin-A: A biomarker of severe encephalopathy. The 5th Congress of the European Academy of Paediatric Societies 17-20th October 2014, Barcelona, Spain.
2014	**Denihan NM**, Walsh BH, Reinke SN, Sykes BD, Mandal R, Wishart DS, Broadhurst DI, Boylan GB, Murray DM. the effect of haemolysis on umbilical cord blood metabolites. The 10th Annual International Conference of the Metabolomics Society 23-26th June 2014, Tsuruoka, Japan.
2014	**Denihan NM**, Boylan GB, Murray DM. The effect of haemolysis on metabolomic markers of hypoxic ischaemic encephalopathy.

	Molecular Medicine Ireland Annual Meeting 12th March 2014, Dublin, Ireland.
2014	**Denihan NM**, Looney AM, Boylan GB, Walsh BH, Murray DM. Normative levels of Interleukin 16 in umbilical cord blood. The 8th International Conference on Brain Monitoring and Neuroprotection in the Newborn 16-18th January 2014, Florida, USA.
2013	**Denihan NM**, Looney AM, Walsh BH, Boylan GB, Murray DM. Normative levels of Interleukin 16 in umbilical cord blood. Neuroscience Ireland, 12-13th September 2013, Cork,Ireland.
2013	**Denihan NM**, Looney AM, Walsh BH, Boylan GB, Murray DM. Normative levels of Interleukin 16 in umbilical cord blood. Research Day, College of Medicine and Health UCC, June 2013, Cork, Ireland.
2012	**Denihan NM**, Looney AM, Walsh BH, Boylan GB, Murray DM. Normative levels of Interleukin 16 in umbilical cord blood. The 4th Congress of the European Academy of Paediatric Societies 5-9th October 2012, Istanbul, Turkey.

D.3. Other achievements

International placements

Month/Year	Duration	Location
April 2014	4 weeks	Biosciences Metabolomic Facility, University of Birmingham, United Kingdom
August 2013	2 weeks	The Metabolomic Innovation Centre, University of Alberta, Canada

Student mentoring

Year	Student	Project title
2014-2015	Eadaoin O'Donovan	The evaluation of factors which potentially contribute to the haemolysis of umbilical cord blood
2013-2014	Dr Aimi Rashid	Metabolomic profile of umbilical cord blood following normal deliveries

Modules and other training

Year	Title
2014	Case studies in drug discovery and development
2014	Case studies in research ethics
2014	Skills and professional development
2014	Principles of medical device design
2013	Introduction to SPSS
2013	Regulation of medicines and medical devices

2013	Epidemiology
2013	Intellectual property in the research context
2013	Project management in the research context
2012	Integrated pharmacology
2012	Molecular mechanisms of disease pathogenesis
2012	Molecular medicine of human disease
2012	Introduction to biomarker discovery
2012	Cardiovascular biology
2012	Biostatistics
2012	Drug development and clinical trial design
2012	Stem cells and gene therapy
2012	Gastrointestinal health
2012	Communication, team work and research ethics
2012	Fundamental biological imaging

Prizes and awards

Month/Year	Award
March 2014	Pfizer Ireland innovation through teamwork award, audience selected prize winner.
June 2014	College of Medicine and Health travel bursary of €1000.
June 2013	UCC Doctoral showcase, finalist of the three minute thesis category.

Certificates

Year	Award
2014	Process continuous improvement (LEAN) – White belt training
2012&2014	Good clinical practise
2012	Laboratory animal science and training

Other contributions

Year	Title
2014-2016	College of Medicine and Health UCC, graduate student committee member.
2015	Registration desk duty – INFANT Research day hosted by the College of Medicine and Health, UCC.
2015	Peer reviewer for Volume 5 of The Boolean Journal, UCC.
2014-2015	Author/editor - College of Medicine and Health UCC, graduate student newsletter.

Appendix E. Cohort identification

E.1. Historical prospective cohort - The BIHIVE Study

Study and setting

My supervisors Dr D Murray and Prof G Boylan, along with Dr B Walsh assembled a previous prospective cohortof infants with perinatal asphyxia and neonatal HIE, namely The Biomarkers in Hypoxic Ischaemic Encephalopathy Study (The BIHIVE Study). This study was based in Cork University Maternity Hospital and recruited between September 2009 and June 2011. Infants had clinical assessment, early multichannel EEG recorded, along with umbilical cord blood (UCB) and postnatal samples biobanked. This cohort has provided the potential for investigative metabolomic biomarker discovery studies, allowing two infant groups to be analysed (i) perinatal asphyxia group (no encephalopathy) and (ii) HIE group.

Ethics and recruitment

Ethical permission for this historical prospective cohort study was obtained from the Clinical Research Ethics Committee of the Cork Teaching Hospitals in 2009. Infants were recruited by Dr Walsh in a large single site maternity hospital with 9000 deliveries per annum. Eligibility required all infants to be in-born to allow UCB to be collected and biobanked within three hours of birth. Infants were recruited if they were born at ≥ 36 weeks gestational age and met one or more of the following criteria;

- Apgar score ≤ 6 at 5 minutes
- Cord arterial pH < 7.1
- Requiring intubation or cardiopulmonary resuscitation at birth

Infants were excluded if they were born less than 36 weeks gestational age, or if they were found to have an alternate, potentially confounding pathology (e.g. neonatal sepsis, cyanotic congenital cardiac lesions, neonatal stroke, CNS malformations or inherited neurological disorders). All infants with dysmorphic features or a confirmed chromosomal condition were excluded. UCB was collected from the placental cord within 20 minutes of birth, and samples were processed and stored within three hours of delivery (see Appendix F).Following

resuscitation parents were approached and the study aims and procedures explained. Written informed consent was obtained. At this point if parents did not wish to take part in the study, the samples were discarded and no clinical details retained.

Clinical data collection

All demographic and clinical data from the neonatal period was collected by Dr Walsh prospectively, who compiled an excel file of over 85 data points for each infant. In addition, continuous multichannel video-EEG monitoring was commenced shortly after delivery for all cases whose parents consented. Monitoring continued until the background normalized or electrographic seizures had ceased for 24 hours. Using the NicoletOne video-EEG system (Carefusion, Madison, WI), silver-silver chloride electrodes were applied to the scalp using the international 10/20 system of electrode placement, modified for neonates (F3, F4, C3, C4, T3, T4, O2, O1, and Cz). The application and maintenance of the EEG was performed by Dr Walsh and two additional research fellows, Dr N Lynch and Dr E Low.

Cases of perinatal asphyxia were defined asinfants with clinical or biochemical signs of asphyxia at birth (meeting the recruitment criteria), but who recovered quickly with no clinical signs of encephalopathy. Cases of HIE were defined as infants who developed an evolving clinical and electrographic encephalopathy consistent with HIE.Clinical grade of HIE was assigned using a modified Sarnat score at 24 hours. The EEG was visually analysed by an experienced neonatal neurophysiologist (Prof G Boylan) and a grade of encephalopathy was assigned at 24 hours. EEG grading of HIE in this cohort has been previously described (217) using a validated and published modified grading system (14, 15), outline in Table 2.2. Clinical examination was performed using the Amiel-Tison standardised newborn assessment on days 1, 2, 3 of lifeand at discharge(314).

A Bayley Scales of Infant and Toddler Development Edition III (BSID-III) (111) structured developmental assessment was performed between 36-42 months. This included the Bayley's Social-Emotional and Adaptive Behaviour Questionnaire (315) which was performed by the BIHIVE2 study research fellow Dr C Ahearne, as well as, the Child Behaviour Checklist (316) and Ages and Stages QuestionnaireThird Edition 3 (ASQ-3)(317).

E.2. Healthy prospective cohort - The BABYCORD Study

Study and setting

A prospective cohort study methodology was chosen. My aim was to recruit 50 healthy term infants and biobank their UCB for future metabolomic analysis to assess the effect of preanalytical variables. Recruitment was conducted over a three week period between January and February 2013 in the single maternity unit, Cork University Maternity Hospital.

Ethics

In my ethical submission I included a study specific proposal, consent form and patient information leaflet, staff information leaflet and protocol. Ethical approval to recruit a cohort of healthy term infants was granted in December 2012, from the Clinical Research Ethics Committee of the Cork Teaching Hospitals. The ethically approved consent form and parent information leaflet are provided in Appendix E.2.1, and the staff information leaflet in Appendix E.2.2. In addition all amendments to the protocol were submitted through the CREC before proceeding. The list and details of ethical amendments, and the reason for the amendments is given in Appendix E.2.3.

Recruitment

Consent was obtained antenatally while the mother was in the early stages of labour by eithermyself, consultant obstetrician (Dr M O'Riordan) or obstetric SHO (Dr A Rashid). Parents were given time to read the information leaflet and consent form and a detailed verbal explanation was also given. The consent form was reviewed by the parents in their own time, and any questions they had were answered by myself, the consultant obstetrician or obstetric SHO. All study participation was entirely voluntary and full informed consent was taken in line with Good Clinical Practice (GCP) guidelines.

Once consented healthy term infants of ≥37 week's gestational age were recruited to the study following normal uncomplicated deliveries if met the following criteria;

- Apgar scores ≥8 at 1 minute and ≥9 at 5 minutes
- Cord arterial pH ≥7.2

- Duration of ruptured membrane <24 h

This population had no clinical signs of asphyxia, no underlying medical issues, had normal neonatal exams and did not require admittance to the neonatal intensive care unit. Both maternal diabetes and foetal distress were the two main exclusion criteria.

For all recruited participants, a sample of UCB (two serum bottles, one tempus tube) was drawn by either the midwife or obstetric SHO after birth. The time the sample was taken was recorded and I was notified immediately. I collected the UCB sample from the labour ward and assigned a study number before Iprocessed and biobanked it, see Appendix F.1.

Clinical data collection

I collected both maternal and infant clinical data on each patient recruited by examining the patient charts and completing my own data collection sheet, see Appendix E.2.4.

E.2.1 Information and consent form

Profile of umbilical cord blood following normal deliveries

Information and Consent form

Introduction

The doctors and researchers in the Cork University Maternity Hospital are studying babies soon after birth to try to find ways of identifying which babies have had difficult or stressful pregnancies and labours. As part of this research we first need to study healthy babies. We would like you to read this form carefully and then ask the researchers any questions you may have.

Do I have to take part in this study?

It is entirely your choice. If you agree we will ask you to sign a consent form, and you will be given a copy of this to keep. You are free to change your mind and withdraw your baby from the study at any time, without giving a reason. The care that you or your baby receives now or in the future will not be affected in any way by your decision.

What will happen to my baby if I agree to take part?

Normally after you give birth the umbilical cord is disposed of, along with any blood it contains. If you agree to take part in this study a sample of blood will be taken from the baby's umbilical cord and stored (this does not hurt as there is no sensation in the cord). This blood will be analysed later to measure its levels of chemicals and proteins.

We are collecting these samples because we hope to find chemicals and proteins which are normally present in a healthy baby's blood, after delivery. It is important to know what chemicals are present in the blood of a healthy baby, because it may be able to help us identify babies who may have had injury during pregnancy or birth.

After your baby is born, we will be able to gather information about your baby's birth and your labour, from your maternity notes and the baby's notes. These will be used to look at the effect of the length of your labour, or the fluid that you were given during labour on the levels of chemicals in your baby's blood.

By looking at the chemicals and proteins present in healthy baby blood, we will be able to compare it to the blood profile of a baby who has had a stressful delivery. This will help us to identify babies who may need treatment in the first few hours after birth.

	Y/N
• I have read the information leaflet about this research and have been given a copy to keep. The information has been fully explained to me and I have been able to answer questions. I understand why the research is being done and any risks involved.	☐
• I agree to donate a sample of my baby's umbilical cord blood for this research project. I understand that giving a sample for this research is voluntary and that I am free to withdraw my approval at any time without my medical treatment being affected.	☐
• I give permission for research personnel to look at my baby's medical records to obtain information about my baby's progress after birth, and the records of my labour and delivery. I have been assured that this information will be kept confidential.	☐
• I agree to donate a sample that can be used now or in the future for commercial collaborative research to develop screening tests for brain injury in children. I understand that I will not benefit financially if this research leads to a development of a new treatment or medical test.	☐
• I give permission for my baby's sample and information collected about my baby to be stored for possible future research related to this study *without my further consent being required* and subject to approval by a research ethics committee.	☐

Role	**Print Name**	**Signature**	**Date**
Parent / Legal guardian (1)			
Parent / Legal guardian (2)			
Investigator			
Witness			

Principle investigator: Dr Deirdre Murray

E.2.2 Staff information leaflet

BABYCORD study

Background

The BABYCORD study is a study conducted by three postgraduate researchers here in CUMH, Ms Niamh Denihan, Ms Ann Marie Looney and Dr Aimi Rashid, under the supervision of Dr Deirdre Murray and Dr Mairead O'Riordan

The BABYCORD study aims to characterise the chemical profile of umbilical cord blood in healthy term infants who have had a normal and uneventful birth. This research will contribute to a larger project which aims to find ways of identifying babies who have experienced difficulties or stress during antenatal or intranatal period.

What we are doing

First, we will seek consent from the mother to partake in our study. If consent is obtained, we will then take a sample of the umbilical cord blood after the baby is born. We will also gather necessary information about the mother's pregnancy and labour, and the baby's birth, from the maternal and baby's charts.

Why we are doing it

We are collecting these samples in order to characterise the metabolomic profile of a healthy baby's cord blood, that is to identify the chemicals and proteins which are normally present in a healthy baby's blood after birth. This profile will then be compared to that of a baby who has had a stressful birth. By doing this research, we hope to identify specific markers in the cord blood that could help clinicians recognise babies at risk of developing neurological complications, as soon as possible after birth, so that the treatment can be initiated immediately.

We will also assess whether factors such as; length of labour, maternal fasting or fluids given, have any effect on the chemical profile of a baby's umbilical cord blood.

Any Questions?

Please contact Niamh on 087-9488598 or email n.denihan@ucc.ie

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E.2.3 Amendments to ethical approval

List of ethical amendments made in chronological order (The BABYCORD Study)

1. Co-investigators, study population and number of subjects
2. Co-investigator
3. Analysis outside of University College Cork

1. The additional co-investigators were added to the study to assist in the identification of the study population , recrutiment and consent.
    - Dr Mairead O'Riordan, Consultant Obstetrician & Gynaecologist/Senior Lecturer
    - Dr Aimi Rahayu Abdul Rashid, Obstetric SHO

    The study population was originally specified as normal term vaginal deliveries. This amendment was made to include pre-labour elective caesarean section deliveries. This was to allow us to compare the metabolomic profile of umbilical cord in deliveries with and without labour, ultimately to describe the effect of labour on metabolites.

    The number of subjects was raised from 20 to 80 infants, to increase power.

2. The additional co-investigator was added to the study.
    - Rachel Ronan

3. Permission for samples collected in Cork to be analysed in the University of Birmingham. Approved on the 14 February 2015.

E.2.4 Data collection sheet

Niamh Denihan
Neonatal Brain Research Group
Dept. of Paediatrics

BABYCORD study

Data Sheet

Maternal Hospital Number	
Study Number	
Time sample was taken (hh:mm)	

Baby	
Gender	
Date of Birth (DD/MM/YYYY)	
Time of delivery (hh:mm)	
Gestational Age (Wks + days)	
Birth Weight (gm)	
Birth Weight Centile (%)	
Apgar Score 1 min	
Apgar Score 5 min	

Mother	
Date of Birth	
Mode of Delivery	
Onset of Labour	
Maximum temperature in labour (°C)	
IV fluids (yes/no)	
Duration of Labour (hh:mm)	
Duration of ruptured membrane (hh:mm)	
Fasting state	

Exclusion Criteria

- Maternal Diabetes
- Fetal Distress

E.3. Prospective validation cohort - The BIHIVE2 Study

Study and setting

The prospective BIHIVE2 validation cohort was recruited in two large maternity hospitals based on separate sites; Cork University Maternity Hospital and Karolinska University Maternity Hospital, both with annual birth rates of approximately 9,000 deliveries per annum. The incidence of perinatal asphyxia is reported to be up to 20 per 1000 deliveries, with approximately 2 per 1000 progressing to HIE. Therefore, over a 24 month period (March 2013 – March 2015) with full recruitment at both sites we aimed to recruit approximately 300 cases of perinatal asphyxia, 60 of which would develop HIE, 20-30 cases of moderate-severe HIE and 30 cases of mild HIE per annum. Meanwhile, we simultaneously aimed to recruited 300 healthy control infants. All infants had UCB drawn, processed and biobanked within three hours of birth and encephalopathy was carefully graded using early continuous multichannel EEG. Detailed phenotyping is central to the BIHIVE study allowing us to track infant development and identify confounding factors that may arise in our cohort.

Ethics and recruitment

Ethical approval for this study was granted by Clinical Research Ethics Committee (CREC) of the Cork Teaching Hospitals before proceeding (2012). In addition all amendments to the protocol were submitted to and approved by the CREC. Informed parental consent was a requirement in all cases and controls. Consent forms and parent information leaflet for cases and controls are provided in Appendices E.3.1, E.3.2 and E.3.3, E.3.4 respectively.

A prospective longitudinal cohort study was chosen as the most appropriate design to collect adequate numbers of infants with HIE for biomarker research. The inclusion criteria encompassed broad signs of birth asphyxia in infants (161), which inevitably meant that infants with perinatal asphyxia but without an encephalopathy were recruited. This recruitment method aimed for greater sensitivity in detecting all infants who would develop HIE, albeit with lower specificity. Approximately 20% of infants with HIE could be missed using a more rigid criteria such as the current criteria for therapeutic hypothermia (43), as some infants progress to display more severe clinical signs later in the postnatal period.

Due to the interdisciplinary nature of the study and the acute clinical setting, we relied on the co-operation of the medical and nursing staff of the neonatal unit, and the obstetric and midwifery staff of the labour ward. We depend on these staff to identify study candidates, draw a sample of UCB and notify the research team immediately after delivery. Prior to initiation I educated members of clinical staff on the labour ward about the study and visited at regular intervals to re-inform them of the study aims and recruitment criteria. I placed laminated posters with the recruitment criteria and on-call contact details in the labour ward and in theatre, with smaller versions on each resuscitaire and blood gas machine. I also hung posters with pictures of the BIHIVE researchers in the labour ward so that staff would recognise us when we collected the cord blood samples.

Infants were recruited if they were born at ≥ 36 weeks gestational age, with one or more of the following clinical markers of asphyxia:

- Apgar score ≤ 6 at 5 minutes
- Cord arterial pH< 7.1
- Requiring intubation or cardiopulmonary resuscitation at birth

Infants were excluded if they were born less than 36 weeks gestational age, or if they were found to have an alternate, potentially confounding pathology (e.g. neonatal sepsis, cyanotic congenital cardiac lesions, neonatal stroke, CNS malformations or inherited neurological disorders). All infants with dysmorphic features or a confirmed chromosomal condition were excluded.

Once the obstetric and/or midwifery staff had identified study candidates, they drew an UCB sample from the placenta and filled four tubes; two serum, one plasma and one whole blood. Samples were placed in a 4°C dedicated research fridge and I, or another member of the BIHIVE2 team was contacted immediately. In all cases we ensured that less than 3 hours had elapsed from the time of delivery to the sample being processed and stored at -80 °C, see AppendixE.3.1 and Table E.1.

For all perinatal asphyxia and HIE infants, informed parental consent was sought after delivery once clinically appropriate due to the inability to identify cases prior to birth in the acute setting. All healthy control infants were consented prior to birth on both the antenatal and

labour ward while the mother was in early stages of labour. The parent information leaflet was provided and a detailed verbal explanation was given. Parents were given time to review the consent form and information, then any questions they had were answered. When possible consent was obtained with both parents present, as in keeping with best practice (318). At all times it was explained to the parents that refusal of entry into this study would in no way impact upon the care of their child, nor was there any pressure upon them to enter into the study. If the parents refused entry, the UCB samples were immediately discarded.

Clinical data collection

Using a secure online database we systematically collected over 350 data points on each infant related to clinical, demographic, physiological and lifestyle factors. For example, early outcome data collected includes; admission to the neonatal intensive care unit (NICU), length of ventilation, neurological examination, clinical and EEG grade of encephalopathy, occurrence of seizures and neurological status on discharge. Each infant is given a unique identifier in the database and each member of the study team had individual passwords so that data was securely stored and logged.

A bedside EEG was performed as soon as possible during the first day of life. EEG was recorded for as long as feasible, only when appropriate, both clinically and taking the parents' wishes into consideration. The EEG was recorded using the Viasys NicOne video-EEG system (Viasys International, Madison, WI), silver-silver chloride electrodes were applied to the scalp using the international 10/20 system of electrode placement, modified for neonates (F3, F4, C3, C4, T3, T4, O2, O1, and Cz). This system records the aEEG and multichannel-EEG simultaneously, making comparisons with either method possible. Application and maintenance of the EEG was performed by either neurophysiologists Mr R Goulding and Mr R Lloyd, or clinical research fellows Dr C Ahearne and Dr L Kharoshankaya. Anonymised EEGs were uploaded to the database as well as a remote and secure server for later blinded analysis by an experienced clinical neurophysiologist (Prof G Boylan). Clinical grade of HIE was assigned using the modified Sarnat score at 24 hours (88), and confirmed by EEG grade was assigned at 24 hours. EEG grading of HIE in this cohort has been previously described (217) using a validated and published modified grading system (14, 15),outline in Table 2.2. Neurological status was

assessed in all infants (perinatal asphyxia, HIE and controls) on days 1, 2 and 3 of life and prior to discharge using a modified Thompson score (90).

Every child is enrolled in a programme of detailed ongoing neurological assessment and developmental follow up at 18-24 months (Dr C Aherne and Ms E Hennessy) which includes the Bayley Scales of Infant and Toddler Development Edition III (BSID-III) (111), the Bayley Social-Emotional and Adaptive Behaviour Questionnaire, the Child-Behavioural Checklist (316) and assessment using a novel touch screen executive function testing platform. To retain participants for long-term follow up we have adopted a number of strategies including; collecting detailed contact information, sending out study newsletters, updating our website, birthday cards, text messages reminders of appointment details and a questionnaire pack before follow up appointment.

For the current prospective validation cohort I was responsible for the collection, processing and biobanking of both UCB and post-natal samples, as part of a three person rota, 24 hours seven days a week as well as maintenance of the biobank and database. Table E.1 details a summary of recruitment, sample collection and follow up in the BIHIVE2 study, including an outline of my role as on-call laboratory researcher.

Table E.1 Chain of events in the BIHIVE2 study. Highlighted in bold is my role as laboratory (lab) on-call researcher.

Who	What	When
Attending obstetrician or midwife	· Identifies infant meeting inclusion criteria · Clamps umbilical cord and extracts blood sample (serum, whole blood, plasma) · Stores samples in labour ward fridge @ 4°C · Immediately notifies laboratory on call researchers	Within 20 minutes of placental delivery
Lab oncall researcher	· **Collect blood from labour ward in 4 °C cold bag** · **Process and aliquot blood in sterile environment** · **Create patient file and enter sample/anlytical details into database** · **Process and biobank samples @ -80 °C** · **Follow up on infant status (a or b)**	**24/7** **All within 3 hours of birth**
Infant status; two immediate possiblities a) The infant has recovered well after birth and has accompanied the mother to the ward. b) The infant requires further monitoring and has gone to the neonal unit.		
a) **Lab oncall researcher**	· **Notifies the clinical research fellow**	**Monday-Sunday 9am-5pm**
a) Clinical research fellow	· Clinically asess and consent infant to study · Records clinical details · Preform Thompson Score	Prior to discharge
a) Clinical research fellow	· Neurological assessment using the Bayley's III score	18 months of life
b) **Lab oncall researcher**	· **Immediately notifies the EEG oncall specialist**	**24/7**
b) EEG oncall specialist	· Clinically asess and consent infant to study · Records multichannel EEG up to 72 hours	24/7
b) **Lab oncall researcher**	· **Collect postnatal serum sample (1.5 ml) from neonatal unit in 4°C cold bag** · **Process and biobank samples @ -80 °C** · **Enter sample details into database**	**Day 1, 2 and 3 of life**

	Clinical research fellow	· Record clinical details · Perform Thompson Score · MRI for infants with moderate/severe HIE · Neurological assessment using the Bayley's III score	Prior to discharge Day 1, 2 and 3 of life Day 5-10 of life 18-24 months of life

E.3.1 Case consent form

Ospideal Maithreachais na h'Ollscoile Corcaigh
Cork University Maternity Hospital

HRB Health Research Board

UCC Coláiste na hOllscoile Corcaigh, Éire University College Cork, Ireland

BiHIVE 2 STUDY

The Investigation and Validation of Predictive Biomarkers in Hypoxic-Ischaemic Encephalopathy

Information and Consent form

Information and consent leaflet:

Introduction

We would ask you to read the information leaflet given to you again now, and give you the opportunity to ask any questions you may have.

Do I have to take part in this study? It is entirely your choice. If you agree we will ask you to sign a consent form, and you will be given a copy of this to keep. You are free to change your mind and withdraw your baby from the study at any time, without giving a reason. The care that you or your baby receive now or in the future will not be affected in any way by your decision whether or not to take part.

What will happen to my baby if I agree to take part? Because your baby has needed resuscitation at birth, blood has been drawn from the umbilical cord and placenta after the baby was born. This is part of routine care in our hospital and most other modern maternity hospitals. This blood is used to measure acid levels in the blood. We have also drawn extra blood and stored it in 3-4 extra tubes. If you agree to take part in the study this extra blood will be analysed at a later period to measure levels of other chemicals in the blood. This blood is otherwise discarded and if you do not wish to take part in the study we will discard this stored blood immediately. We are collecting these samples because we hope to identify which chemicals and proteins can best predict how babies cope with stressful deliveries and resuscitation. We are looking for markers in the blood which can predict which infants may have suffered significant stress and who may need treatment in the first few hours after birth. Most importantly we want to improve our ability to predict a baby's development over the first few years of life.

We would also like to see if these chemicals change over the first few days of life. Therefore if your baby is having a blood test as part of their routine care, we will take an extra 1.5 milliliters of blood on the first, second and third day of life or if your baby develops seizures (convulsions). This is unlikely, but if it happens seizures can also affect a baby's develop and this is important for us to study. We will only take the sample if your baby requires a blood test or intravenous line for other reasons as part of their medical care.

After your baby is born, we will be able to gather information about your baby's birth, and treatment from your maternity notes and the baby's notes.

To accurately measure any effect on your baby's brain we wish to record an EEG (electroencephalograph) and other vital signs such as heart rate, breathing rate and oxygen saturation levels. This is part of routine monitoring in our hospital and carrying out in all babies who have signs of brain irritation or injury. This is usually carried out for up to 24 hours. However as part of the study we may ask you to allow us to monitor your baby's brain waves for longer, so that we can gather extra information.

Lastly as part of the study we will ask to meet you and your baby again between 18 months and 2 years of age to monitor their growth and development. This will involve short questionnaires and a one hour developmental assessment.

Ospideal Maithreachais na h'Ollscoile Corcaigh
Cork University Maternity Hospital

HRB Health Research Board

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BiHIVE INFORMED CONSENT FORM

Principal Investigator: Dr. Deirdre Murray **Phone Number**: +353 21 4901271

EEG ID Number_____ **Patient ID Number for this study**_____

	Y/N
• I have read the information leaflet about this research and have been given a copy to keep. The information has been fully explained to me and I have been able to ask questions. I understand why the research is being done and any risks involved.	☐
• I agree to donate a sample of my baby's umbilical cord blood for this research project. I understand that giving a sample for this research is voluntary and that I am free to withdraw my approval at any time without my medical treatment being affected.	☐
• I agree to donate a sample of DNA from my baby's umbilical cord blood for storage and research related to the project	☐
• I agree to donate a sample of my baby's blood for this research project on day 1, 2 or 3 of life, or if my baby develops seizures. I understand that giving a sample for this research is voluntary and that I am free to withdraw my approval at any time without my medical treatment being affected.	☐
• I agree to allow my baby to take part in the follow up visits required at 18-24 months of age	☐
• I give permission for research personnel to look at my baby's medical records to obtain information about my baby's progress after birth, and the records of my labour and delivery. I have been assured that this information will be kept confidential	☐
• I agree to donate a sample that can be used now or in the future for commercial collaborative research to develop screening tests for brain injury in children. I understand that I will not benefit financially if this research leads to a development of a new treatment or medical test.	☐
• I give permission for my baby's sample and information collected about my baby to be stored for possible future research related to this study *without my further consent being required* and subject to approval by a research ethics committee.	☐

Role	Print Name	Signature	Date
Parent/legal guardian 1			
Parent/legal guardian 2			
Investigator			
Witness			

Insert Hospital Addressogram Label Here	**Local Principal Investigator: Dr Deirdre Murray**

E.3.2 Case patient information leaflet

BiHiVE 2 study

The Investigation and Validation of Predictive Biomarkers in Hypoxic-ischaemic Encephalopathy

Information Leaflet for Parents

What is the BiHiVE study?

Some baby's require resuscitation when they don't cry or move after delivery. This may occur when a baby has had a difficult or stressful time during labour, or before delivery. Sometimes this can even lead to long term injury to the baby. The injury which we worry about the most is newborn brain injury. This type of brain injury is called hypoxic-ischaemic encephalopathy or HIE. As your baby has required resuscitation they may need careful monitoring for the next few days. We have no good way of knowing whether your baby has had a significant injury, and usually can't tell for sure until at least 24 hours after birth. The BiHiVE study hopes to find chemical and protein markers

in the blood which can tell us very soon after birth which babies have suffered significant injury so that we can intervene and improve their outcome. At the moment we have no good reliable early marker which can give us this information straight after birth. We hope that in the future a simple blood test at delivery will be able to give doctors and parents more information within hours of a baby's birth.

Because your baby has needed resuscitation at birth, blood has been drawn from the umbilical cord and placenta after the baby was born. This is the standard care in our hospital and many other modern maternity hospitals. This blood is used to measure acid levels in the blood. We have also drawn extra blood and stored it in 3-4 tubes. If you wish your baby to take part in the study we will store this blood to use for research into newborn brain injury. If you do not wish to take part in the study these samples will be destroyed.

If your baby is admitted to Neonatal Intensive Care Unit, and, if they are having a blood test as part of their routine care, we will ask for an extra 1.5 milliliters of blood on the first, second and third day of life. We will not disturb your baby an extra time for this sample, and if your baby does not require a blood test for other reasons, we will not take this sample. These samples will be frozen and stored for analysis.

Our current best way of looking at those who might be at risk of brain injury is to carry out a recording of their brain waves using an EEG machine. This is routinely done on the neonatal unit. We want to be able to compare what is currently our best method, to the simpler blood test which we hope to develop, and therefore all babies in the study will also have an EEG if they have signs of brain irritation (HIE).

In order to help babies who have difficulties after birth, it is also important for us to study babies who have had normal, uneventful deliveries. These babies act as controls so that we can know that the changes we find in the unwell babies are genuine. If your baby is a control infant, blood is taken from the umbilical cord and stored at birth. We will follow up your baby with an examination the day after birth. This takes about 5 minutes and will not upset your baby in any way. It can be carried out when they are still asleep in many cases. In addition we would like to contact you at 2 years of age to look at the growth and development of your child. The researchers will be very clear with you to reassure you that your baby is a control, or well baby.

What will the study involve?

If you are happy for the research team to follow your baby after they are born, the extra samples of blood taken from your baby's umbilical cord will be frozen, stored and analyzed later when we have collected enough samples. An additional 1.5 milliliters of

blood will be taken if the baby is having other blood samples on day 1, 2 and 3, these will be analyzed once enough samples are collected. We will look at many different chemicals and proteins which may change in babies with significant brain injury. We will compare the levels to those of babies who did not need resuscitation. Your baby's progress after birth will be recorded. If your baby needs any extra medical care the details of this care will be collected from your baby's medical notes. This information will be anonymous, and will be identified only using a study number, not your baby's name or address.

We will also ask you to meet us again at 24 months of age so that we can record the progress and development of your baby. You will be contacted and an appointment will be made for a follow up assessment at a time convenient for you and your family. This will happen in the Cork University Hospital. In these follow up appointments, your baby's development will be assessed and discussed with you. Your baby will not need any further samples to be taken after the initial sample from the placenta for this study.

What are my options?

Being part of the BiHiVE study is completely voluntary. You or your child can withdraw from this study at any stage. Not being part of this study will not affect any part of the medical care of you or your baby.
The BiHiVE study will be managed by the staff of the Department of Paediatrics and Child Health, University College Cork. We will publish our results in international medical journals. Once a year we will organize a meeting to tell families about how the study is progressing.

Where can I get more information?

If you have any questions regarding the BiHiVE study, please contact the research coordinators

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Clinical Investigations Unit,
Cork University Hospital
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E.3.3 Control consent form

BiHIVE INFORMATION LEAFLET AND CONSENT

BiHIVE 2 STUDY

The Investigation and Validation of Predictive Biomarkers in

Hypoxic-Ischaemic Encephalopathy

Consent Form Control Group (Well babies)

Information and consent leaflet:

Introduction

We would ask you to read the information leaflet given to you again now, and give you the opportunity to ask any questions you may have.

Do I have to take part in this study? It is entirely your choice. If you agree we will ask you to sign a consent form, and you will be given a copy of this to keep. You are free to change your mind and withdraw your baby from the study at any time, without giving a reason. The care that you or your baby receive now or in the future will not be affected in any way by your decision whether or not to take part.

What will happen to my baby if I agree to take part? In order to study differences in normal healthy births and those that needed resuscitation at birth we need to study a healthy control group. Your baby had an uneventful normal delivery and this is why we are asking your permission to include your baby as a **control** (well baby) in this study. We are asking if you would allow us to draw blood from the umbilical cord and placenta after your baby is born. This blood is used to measure acid levels in the blood. We store it and it will be analysed at a later period to measure levels of other chemicals in the blood. We are collecting these samples because we hope to identify which chemicals and proteins can best predict how babies cope with stressful deliveries and resuscitation. We will be looking at normal levels in the control group and comparing them to the study group of resuscitated babies. We are looking for markers in the blood which can predict which infants may have suffered significant stress and who may need treatment in the first few hours after birth. Most importantly we want to improve our ability to predict a baby's development over the first few years of life.

After your baby is born, we will be able to gather information about your baby's birth, and treatment from your maternity notes and the baby's notes.

Lastly as part of the study we will ask to meet you and your baby again between 18 months and 2 years of age to monitor their growth and development. This will involve short questionnaires and a one hour developmental assessment.

Ospideal Maithreachais na h'Ollscoile Corcaigh
Cork University Maternity Hospital

HRB Health Research Board

ucc

BiHIVE INFORMED CONSENT FORM

Principal Investigator: Dr. Deirdre Murray **Phone Number**: +353 21 4901271

EEG ID Number_____ **Patient ID Number for this study**_____

	Y/N
• I have read the information leaflet about this research and have been given a copy to keep. The information has been fully explained to me and I have been able to ask questions. I understand why the research is being done and any risks involved.	☐
• I agree to donate a sample of my baby's umbilical cord blood for this research project. I understand that giving a sample for this research is voluntary and that I am free to withdraw my approval at any time without my medical treatment being affected.	☐
• I agree to donate a sample of DNA from my baby's umbilical cord blood for storage and research related to the project.	☐
• I agree to allow my baby to take part in the follow up visits required at 18-24 months of age	☐
• I give permission for research personnel to look at my baby's medical records to obtain information about my baby's progress after birth, and the records of my labour and delivery. I have been assured that this information will be kept confidential	☐
• I agree to donate a sample that can be used now or in the future for commercial collaborative research to develop screening tests for brain injury in children. I understand that I will not benefit financially if this research leads to a development of a new treatment or medical test.	☐
• I give permission for my baby's sample and information collected about my baby to be stored for possible future research related to this study *without my further consent being required* and subject to approval by a research ethics committee.	☐

Role	Print Name	Signature	Date
Parent/legal guardian 1			
Parent/legal guardian 2			
Investigator			
Witness			

Insert Hospital Addressogram Label Here	**Local Principal Investigator: Dr Deirdre Murray**

E.3.4 Control patient information leaflet

BiHiVE 2 study
The Investigation and Validation of Predictive Biomarkers in Hypoxic-ischaemic Encephalopathy

Information Leaflet for Parents Control Group (well babies)

What is the BiHiVE study?

The BiHiVE study is a research study currently taking place in your hospital looking at infants who have needed resuscitation after birth. This happens in about 2% of deliveries. Some of these infants may be at risk of long term problems and need intervention in the first few days of life. At the moment we have no good reliable early marker which can give us this information straight after birth. Through this study we hope to find chemical and protein markers in the umbilical cord blood which can tell us very soon after birth which babies have suffered significant injury so that we can intervene and improve their outcome. We hope that in the future a simple blood test at delivery will be able to give doctors and parents more information within hours of a baby's birth.

In order to help babies who have difficulties after birth, it is also important for us to study babies who have had normal, uneventful deliveries. These babies act as **controls (well babies)** so that we compare them to infants who are unwell. We are asking for your baby to be a control infant for the study. If your baby is a control infant, blood is taken from the umbilical cord and stored at birth. We will follow up your baby with an examination the day after birth. This takes about 5 minutes and will not upset your baby in any way. It can be carried out when they are still asleep in many cases. In addition we would like to contact you at 2 years of age to look at the growth and development of your child. The researchers will be very clear with you to reassure you that your baby is a control (well baby).

What will the study involve?

If you are happy for the research team to follow your baby after they are born, the extra samples of blood taken from your baby's umbilical cord will be frozen, stored and analyzed later when we have collected enough samples. We will look at many different chemicals and proteins and we will compare their levels to those of babies who needed resuscitation. Your baby's progress after birth will be recorded. If your baby needs any extra medical care the details of this care will be collected from your baby's medical notes. This information will be anonymous, and will be identified only using a study number, not your baby's name or address.
We will also ask you to meet us again at 24 months of age so that we can record the progress and development of your baby. You will be contacted and an appointment will be made for a follow up assessment at a time convenient for you and your family. This

will happen in the Cork University Hospital. In these follow up appointments, your baby's development will be assessed and discussed with you.

What are my options?

Being part of the BiHiVE study is completely voluntary. You or your child can withdraw from this study at any stage. Not being part of this study will not affect any part of the medical care of you or your baby.
The BiHiVE study will be managed by the staff of the Department of Paediatrics and Child Health, University College Cork. We will publish our results in international medical journals. Once a year we will organize a meeting to tell families about how the study is progressing.

Where can I get more information?

If you have any questions regarding the BiHiVE study, please contact the research coordinator.

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Appendix F. Sample biobanking

F.1. Umbilical cord blood (UCB)

Though UCB is not routinely stored, large volumes can be non-invasively extracted from the placenta, allowing whole blood, serum and plasma to be collected, see Table F.1. This research used a mixed arterial/venous cord sample to simplify collection and user friendly testing in the acute setting.

UCB was drawn from the placenta within 20 minutes of birth and immediately stored at 4°C before processing, to quench enzymatic activity and ensure subsequent biological observations are accurate. Cool pack bags at 4°C were used to transport samples between the labour ward/neonatal unit and the laboratory. All samples are collected, processed and stored within three hours of birth using strict standard operating procedures (SOPs) generated for UCB specimens. Time of birth and time in -80°C freezer were noted in the online BIHIVE2 database and laboratory book, if three hours had elapsed before samples were biobanked in full, samples were discarded. The online database presented in Figure F.1 was used to track each sample with details such as; barcode, freezer location (row, column, box, shelf and freezer), collection centre, usage status, time and date at -80 °C, and preanalytical details were recorded including the presence of haemolysis.

Consumables**:** Serum tube (BD Vacutainer no. 366431)

Micro-tube (VWR no. 89179-704, SCT-050-SS-B-S)

TEMPUS blood RNA tube (Applied Biosystems no. 4342792)

EDTA tube (BD Vacutainer no. 367863)

Filter pipette tips (PIP7876)

Second spin tubes (VWR no, 60818-500)

Plastic Pasteur pipettes (Fisher Scientific no.FB55348)

Table F.1Required blood collection tubes. Filling order is from left to right.

Sample	Plain serum	Whole blood	Plain serum	EDTA plasma
Cap Top	Red	Blue	Red	Purple
Volume (ml)	6	3	6	6

Serum biobanking SOP

UCB was collected in a plain serum tube, ≤ 20 min of placental delivery and allowed to sit at 4°C for at least 30 min post collection for the clot to form.

- Keep all tubes on ice until aliquots are complete.
- Check clot is present prior to centrifugation.
- Centrifuge the 6 ml red capped tube at 2400 x g for 10 min at 4°C.
- Using asterile Pasteur pipette, transfer theserum into a sterile labelled second spin tube and centrifuge at 3000 x g for 10 min at 4°C.
- Collect the red capped barcoded micro-tubes from the -20°C freezer.
- In a sterile fume hood, aliquot250 µlof serum into micro-tubes on ice, using filtered pipette tips.
- Fit cap, scan barcode into the BIHIVE database and store samples at -80°C until analysis.

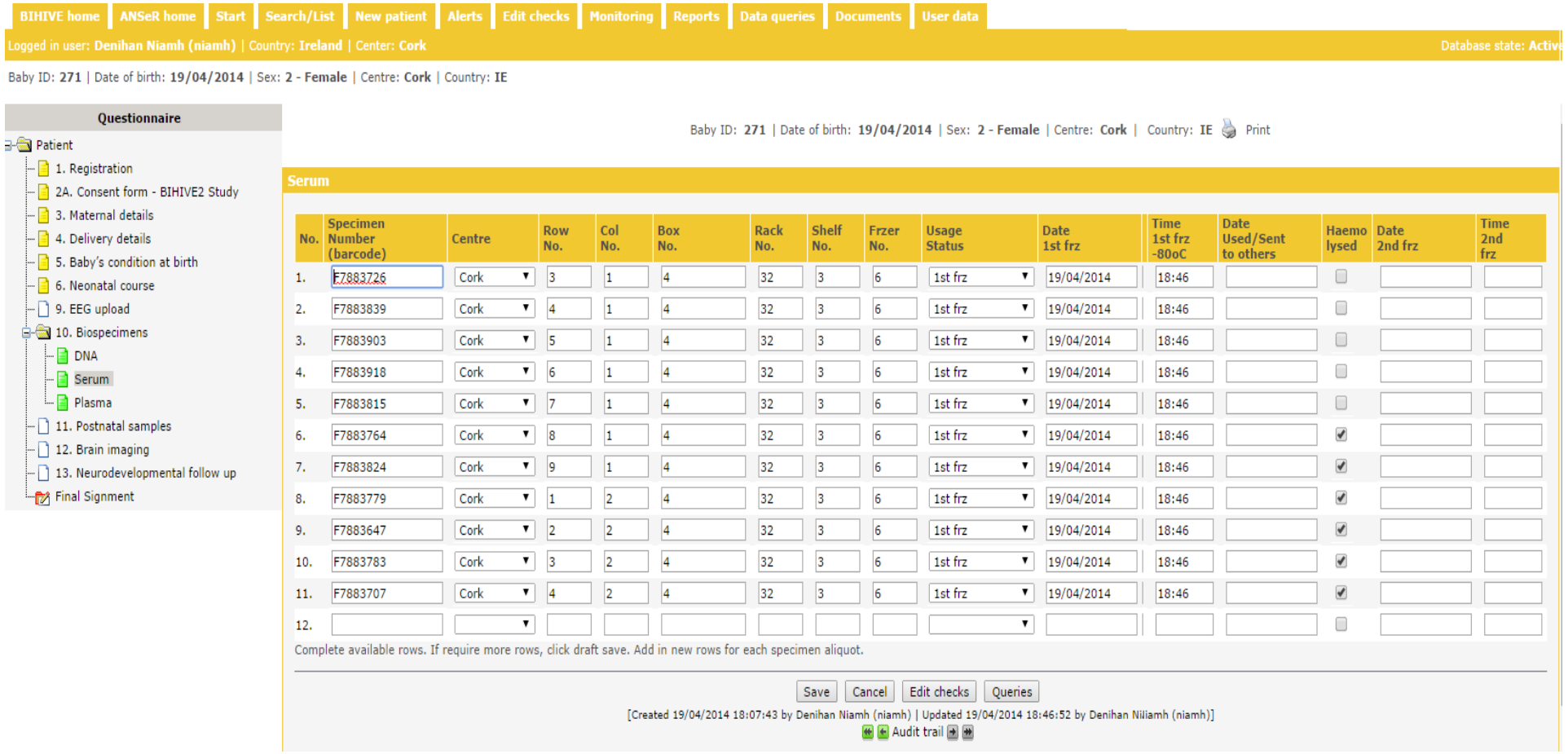

Figure F.1Test screenshot of the biospecimen section of the BIHIVE2 database.

Plasma and buffy coatSOP

EDTA plasma tubes will be utilized in two ways:

i. EDTA plasma
ii. Buffy coat for later DNA extraction

EDTA plasma

- Keep all tubes on ice until aliquots and buffy coat are completed.
- Centrifuge the 6 ml purple capped tubeat 2400 x g for 10 min at 4°C.
- Using a sterile Pasteur, pipette the plasma into a sterile labelled second spin tube and centrifuge at 3000 x g for 10 min at 4°C.
- Collect the lavender capped barcoded micro-tubesfrom the -20°C freezer.
- In the sterile fume hood, aliquot 250 µlaliquots of plasma into the micro-tubes using filtered pipette tips
- Fit caps, scan the barcodes into the BIHIVE database and store at -80 °C.

EDTA buffy coat

- Following removal of the plasma, use a sterile Pasteur pipette to carefully remove the white cells (buffy coat) just above the red blood cells (interface between the plasma and red blood cells).
- Transfer the buffy coat into a sterile, white topped barcoded micro-tube and centrifuge at 2400 x g for 10 min at 4 °C.
- Using new sterile pipette tips remove residual plasma from the buffy coat and discard.
- Fit caps, scan the barcodes into the BIHIVE database and store at -80 °C.

Whole blood SOP

- Draw 3 ml of whole blood directly into the Tempus blood RNA tube.

Note: Filling the tube to the black mark on the tube label indicates the collection of approximately 3 ml of blood.

- To stabilise the sample, shake the tube vigorously immediately after filling to ensure that the applied biosystems reagent makes uniform contact with the sample.
- Place a barcode sticker on the Tempus tube, scan into the BIHIVE database and store at -80°C.

IMPORTANT: Failure to mix the stabilizing reagent with the blood leads to inadequate stabilization of the gene expression profile and the formation of micro-clots that can potentially clog the purification filter, hindering later analysis.

F.2. Postnatal samples

If parents gave consent, post-natal research samples were drawn at the same time as clinical samples. An attempt was made to draw these at 24 hours, 48 hours and 72 hours post birth. If the infant did not require a clinical blood sample, no post-natal research sample was drawn.

The circulating blood volume of a neonate is approximately 80 ml per kg body weight; therefore, the average 3.5 kg infant will have only 280 ml of blood. Ethical guidelines limit sampling for research purposes to 1% of circulating blood volume per 24 hours giving an upper limit of 3 ml for postnatal sampling, or approximately 1-1.3 ml of serum or plasma. Regular 2 ml serum collection tubes from neonatal unit were used to collect this sample. Once the post-natal samples were drawn, they were processed using a standardised SOP similar to the UCB samples.

Postnatal Serum

UCB was collected in a serum tube and allowed to sit at 4 °C for ≥ 30 min post collection for the clot to form.

- Keep all tubes on ice until aliquots are complete.
- Check the clot is present prior to centrifugation.
- Centrifuge the 6 ml red capped tube at 2400 x g for 10 min at 4°C.
- Using asterile Pasteur pipette, transfer theserum into a sterile labelled second spin tube and centrifuge at 3000 x g for 10 min at 4°C.
- Collectthe red capped barcoded micro-tubes from the -20°C freezer.
- In a sterile fume hood, aliquot 250 µl of serum into micro-tubes on ice, using filtered pipette tips.
- Fit cap, scan barcode into the BIHIVE database and store samples at -80°C until analysis.

F.3. Sample storage and transport

Storage

All samples are stored at -80 °C in dedicated biobank freezers housed in a well ventilated room containing generator backup power. Freezers are monitored 24/7 using internal and external temperature probes, to ensure the safe preservation of samples. In the BIHIVE study, an automatic emergency call is made to either myself or a member of the laboratory on-call team if a freezers temperature rises above -75°C . We immediately respond, day or night, to rectify any problem and ensure the temperature never rises above -70°C for more than three hours.

Aliquotes are stored in small made for purpose boxes, with the position of each aliquot recored in the online BIHIVE database and laboratory book. The aliquots row-column position in the box is noted, along with the box, rack, shelf and freezer identification numbers.

Transport

Samples required transport to the University of Birmingham prior to the metabolomic analysis. To ensure that samples maintained the cold chain through-out, they were transported on dry-ice which was topped up at regular intervals and monitored constantly using an internal temperature probe. Samples were transported by Marken (www.Marken.com), an international courier company who specialise in the transport of biological samples who could ensure their safe and appropriate transport.

Appendix G. Metabolomic analysis

I conducted the metabolite extraction, sample preparation and mass spectrometry analysis in the Biomolecular Analysis Facility, School of Biosciences, University of Birmingham, UK under the supervision of Dr J Kirwan.

G.1. Metabolite extraction

A modified Bligh-Dyer biphasic extraction method was used to obtain polar and non-polar fractions from UCB serum, as described previously (142, 219). Extractions were conducted on ice to minimise metabolic activity. Methanol was added to the frozen serum followed by chloroform and water to achieve a ratio of 2:2:1.8. All solvents were HPLC grade, pre-chilled and kept on ice 4 °C to reduce evaporation. Chloroform was transferred via a Hamilton glass syringe with metal plunger. To avoid cross contamination the syringe was washed three times in three separate wash solutions (MeOH:H_2O Rinse, $CHCL_3$ Rinse and $CHCL_3$) between every sample.

Equipment

1.8 ml and 7 ml glass vials with aluminium lined caps (Fisher TUL 520 006 J)

2 ml micro test tubes (Eppendorf, Germany)

Glass Pasteur pipettes

Hamilton syringe (Fisher Scientific UK 500 µl)

Nitrogen sample concentrator (Techne, Bibby Scientific, UK)

Solvents

100 % Methanol (MeOH) (Fisher Scientific, UK)

100 % Chloroform ($CHCl_3$) (Pesticide Grade)

100 % Water (H_2O) (J.T. Baker)

Protocol

- Assuming 90% water content of serum(312), 450 µl MeOH was added directly to the 200 µl frozen serum samples while on dry ice, after which, they defrosted on ice for 20 min and were vortex mixed for 15 sec.
- Separately, 1350 µl MeOH, 1800 µl CHCl3, and 1440 µl H_2O was placed in 7 ml glass vials on ice.
- The defrosted serum:MeOH sample was transferred into the pre-filled solvent vials using a glass Pasteur pipette.
- Vials were vortex mixed for 15 sec and left on ice for 10 min to separate.
- Vials were centrifuged at 1800 x g for 10 min, at 4 °C and left at room temperature for 5 min for the phases to fully separate.

The extracted samples were now biphasic, with proteins and other insoluble molecules forming a solid interphase layer which separated the two solvent layers, the upper (polar) and lower (non-polar) layers, see Figure G.1.

- Using a pipette, 4 x 400 µl aliquots (1 positive, 1 negative and 2 extra) of the upper polar layer (MeOH:H_2O) was transferred into micro-tubes. 80 µl aliquots from each sample were pooled in a separate micro tube to use later as a quality control (QC) sample.
- Polar samples were dried using a vacuum centrifuge until dry (approx. 10 hours) at 35°C.
- The lower non-polar layer ($CHCl_3$) was removed using a Pasteur pipette, placed in 1.8 ml glass vials and centrifuged at 1800 x g at 4 °C for 10 min to remove any particulate matter from the protein interface.
- Using a glass syringe 4 x 200 µl aliquots (1 positive, 1 negative and 2 extra) of the non-polar layer were transferred into micro-tubes, washing the syringe three times between samples. A 120 µl aliquot from each sample was placed into a separate vial as a QC sample.Care was taken not to remove any of the interface region, 1-2mm either side of the protein debris layer.
- Non-polar samples were dried under nitrogen to reduce oxidation.
- All dried samples were stored at -80 °C until mass spectrometry (MS) analysis.

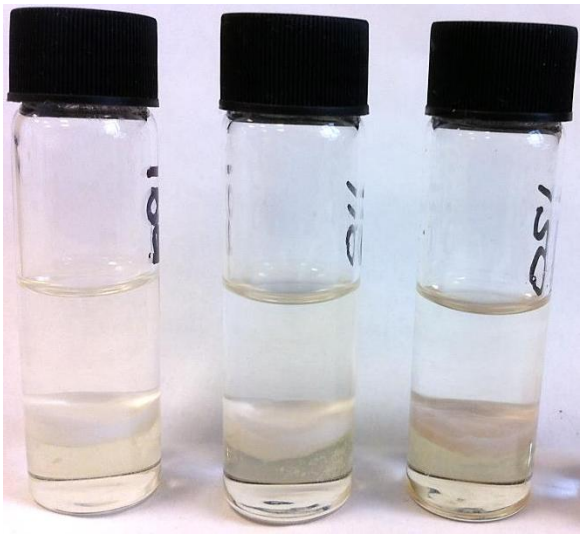

Figure G.1 Picture of three biphasic samples mid-extraction process. Three distinct sections are visible, the upper polar layer (hydrophilic metabolites) the middle interface consisting of protein and other insoluble debris and the lower non-polar layer (hydrophobic lipid metabolites).

G.2. Sample preparation for mass spectrometry

Polar samples

- Samples were re-suspended on ice in 400 µl of 4 °C MeOH: H_2O, 80:20 w. 0.25% formic acid (positive ion analysis)or 4 °C MeOH: H_2O, 80:20 w. 20 mM ammonium acetate (negative ion analysis).
- Samples were sonicated for 5 min, vortex mixed for 30 sec and centrifuged at 1800 x g for 10 min at 4°C to remove solid contaminants.
- Without disturbing the sample, 20 µl of the supernatant was transferred by manual pipette into individual wells on a 384-well polymerase chain reaction (PCR) plate (ABgene, Thermo Fisher Scientific, USA), and covered immediately with strips of self-adhesive foil.
- When all samples were loaded, the self-adhesive foil strips were replaced by a single heat sealed foil sheet (ALPS 50V, Thermo Fisher Scientific, USA).

Non-polar samples

- Samples were re-suspended on ice in 50 µl of 4 °C MeOH: H_2O, 2:1 w. 5 mM ammonium acetate, for both ion modes.
- Samples were vortex mixed for 30 sec and centrifuged at 1800 x g for 10 min at 4°C to remove solid contaminants.
- The 384-well PCR plate was cooled on ice for 10 min before 10 µl of the supernatant was transferred by manual pipette with carbon pipette tips (Advion, USA) and covered immediately with strips of self-adhesive foil.
- When all samples were loaded, the self-adhesive foil strips were replaced by a single heat sealed foil sheet (ALPS 50V, Thermo Fisher Scientific, USA).

G.3. FT-ICR mass spectrometry – acquisition of spectra

Metabolomic analysis was performed using a hybrid 7-Tesla FT-ICR mass spectrometer (LTQ FT Ultra, Thermo Fisher Scientific, Bremen, Germany) via chip-based direct infusion nanoelectrospray ionisation (nESI) source (Triversa, Advion Biosciences, Ithaca NY) in both positive and negative ion mode. Nanoelectrospray conditions were controlled using ChipSoft software (version 8.1.0) and mass spectrometry conditions are controlled using Xcalibur software (version 2.0, Thermo Fisher Scientific). Conditions for both are listed in Table G.1.

To increase metabolome coverage and maintain mass accuracy, spectra were collected using the 'SIM stitching' methodology (220, 221, 319). Spectra are acquired as transients, across seven selected ion monitoring (SIM) mass ranges or 'windows' to cover a total mass range of 70 – 590 Da for polar compounds (Figure G.2) and 100-2000 Da for non-polar compounds (Figure G.3). The individual SIM mass ranges overlap with each other by30 Da and are stitched together during data processing. Data from each sample was collected as both a Thermo.raw mass spectral file and as multiple individual transient files.

Table G.1 Nanoelectrospray and mass spectrometry conditions.

Parameters	Polar settings		Non-polar setting	
	(+)	(-)	(+)	(-)
Nanoelectrospray conditions				
Flow rate (nl/ml)	200	200	200	200
Gas pressure (PSI)	0.3	0.3	0.4	0.4
Spray voltage (kV)	+1.7	-1.7	+1.2	-1.2
Mass spectrometry conditions				
No. of windows	7	7	7	7
Window width (Da)	100	100	200-480	200-480
Range (m/z)	70 – 590	70 – 590	100-2000	100-2000
Automatic gain control	1×10^6	1×10^6	1×10^6	1×10^6
Mass resolution	100,000	100,000	100,000	100,000
Acquisition time (s)	2.25	2.25	2.5	2.5
Sample volume (µl)	8	8	6	6

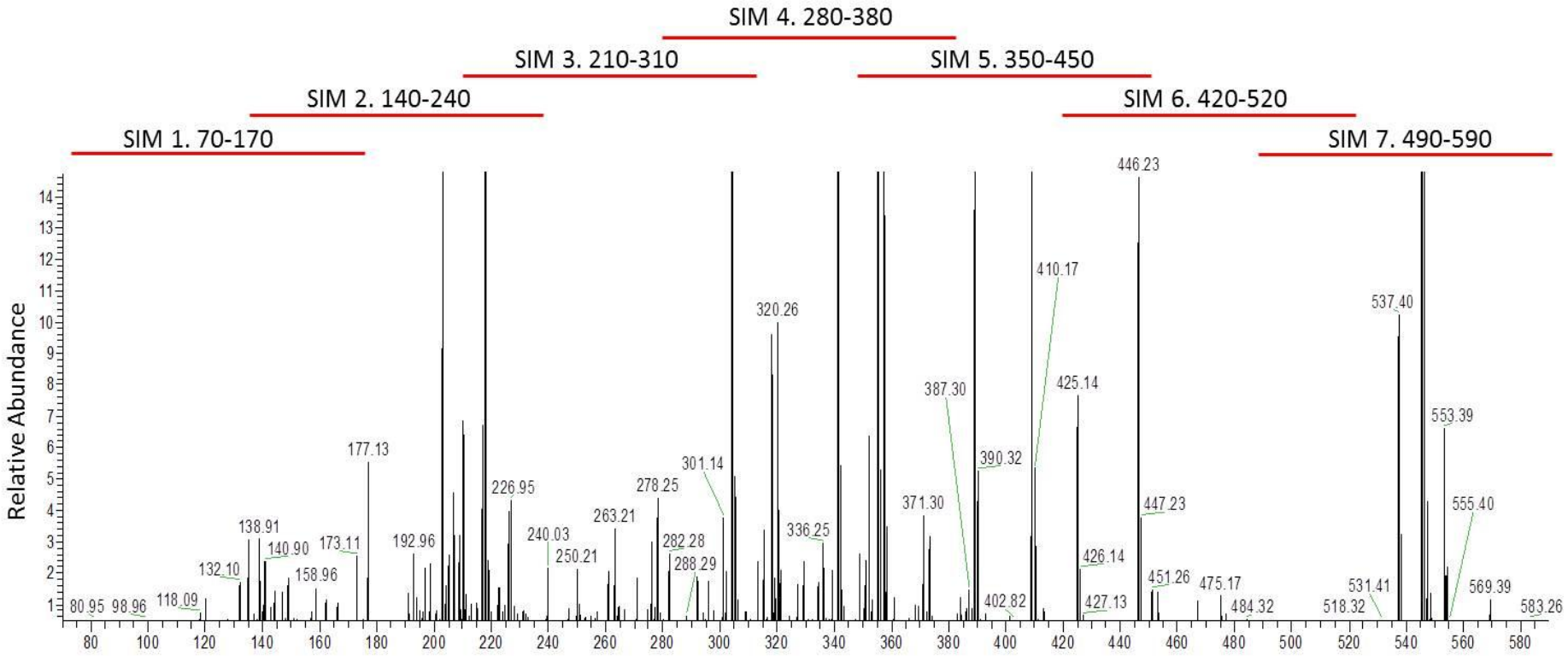

Figure G.2 SIM-windows used during polar DI FT-ICR MS analysis. A typical pre-processing spectrum from umbilical cord blood metabolite extract is shown with SIM-windows and their corresponding range above in red.

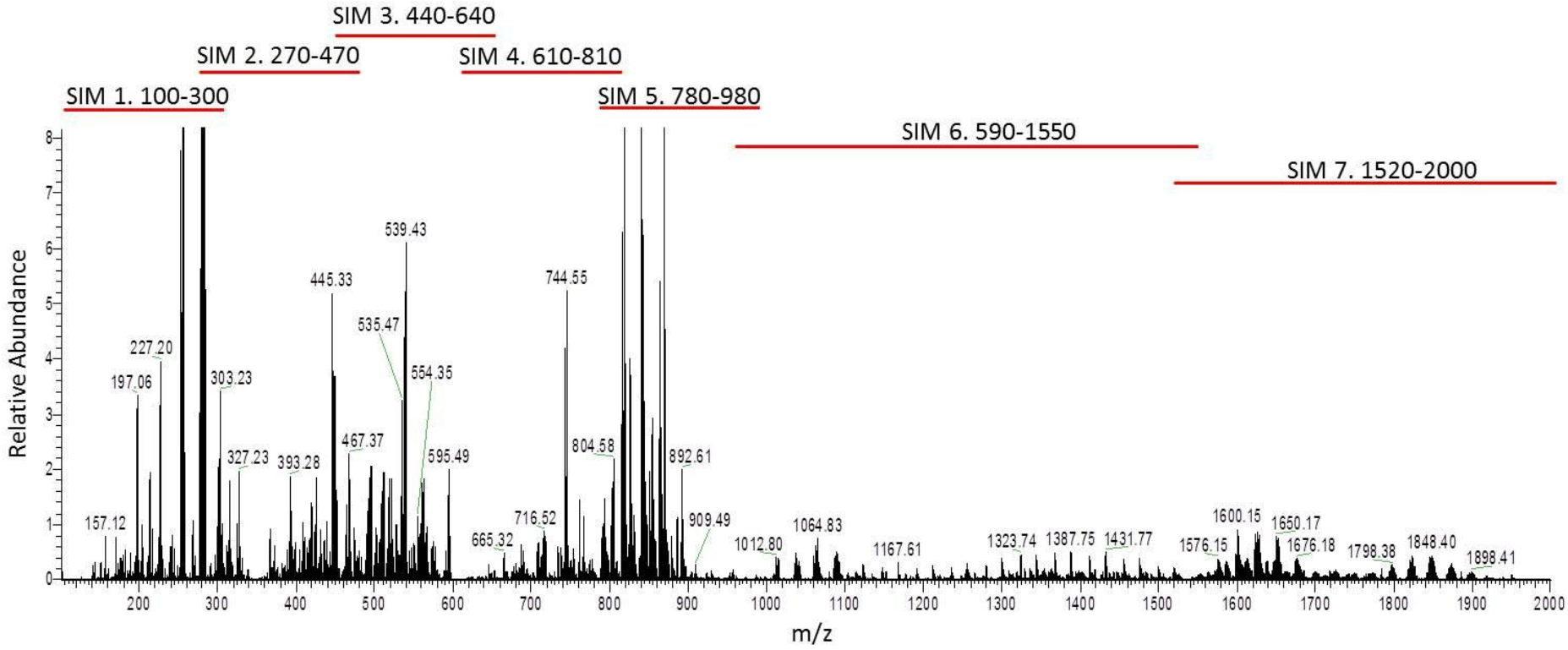

Figure G.3 SIM-windows used during non-polar DI FT-ICR MS analysis. A typical pre-processing spectrum from umbilical cord blood metabolite extract is shown with SIM-windows and their corresponding range above in red.

G.4. Processing mass spectral data

Each.raw mass spectral files were assessed manually to ensure only spectra of sufficient quality were processed. Samples were removed based on eletrospray stability and the subsequent total ion current (TIC) profile. A failed electrospray current was perceived by a recording of zero in one or more scans in the TIC profile. For each patient sample a minimum of two replicates (two spectra per sample) were required to pass TIC inspection, otherwise the sample was re-analysed on a subsequent plate.

The SIM-stitch work flow was implemented using a custom written Matlab code (The MathWorks R2009a, Natick, MA) (221). The transients containing the mass spectral data were averaged and converted from a time to frequency domain. To yield a single spectrum for each replicate containing only real peaks, peak-picking was performed above a selected signal-to-noise ratio threshold (SNR = 10) with regularly observed 'high noise regions' removed [74.04-74.08, 90.5-90.53, 101.75-101.9, 105.3-105.4, 116.35-116.4]. External mass calibration was then used to convert the frequency domain to *m/z* values. Calibrants of known mass value were determined at the FT-ICR mass spectrometer's weekly calibration and are contained in the instruments .RAW files. Next, for each individual replicate the seven SIM windows were 'stitched' together into a single file via the alignment of peaks in overlapping (30 Da) windows. The overlap allowed the very outer regions of each SIM window to be removed. This file contains the frequency, resolution, intensity and SNR for each peak.

A three step filtering process was used to enhance spectral reliability (221). First, a 'replicate filter' combined the replicate spectra for each sample to retain peaks which were present in a fraction of the spectra within an error range (<2ppm) but remove random noise and yield a single robust peak list per sample. Secondly, a 'blank filter' removed peaks from the biological samples that were also present in the blank samples, namely a threshold of 10-fold was set such that peaks in the biological samples which were less than 10 fold greater than intensity in the blank sample were removed. Finally 'sample filtering' removed peaks not present in 80 % of the biological samples per single class.

The resultant data file is displayed as an intensity matrix with rows and columns, corresponding to samples (quality control or biological) and *m/z* values of spectral features. Missing intensity

values may occurs in the matrix where no mass feature of sufficient intensity and reproducibility was detected at the specified m/z for a particular sample. To further enhance data quality samples with a high percentage of missing values were identified and removed, the sample filtering process was repeated to yield a refined matrix.

Following US FDA guidelines, metabolites with a relative standard deviation for QC samples (RSD^{QC}) of > 20% are considered to be below accepted quantification precision. To reduce inter and intra batch variation the Quality Control-Robust Spline Correction (QC-RSC) batch correction algorithm was applied in conjunction with three spectral cleaning algorithms (135) to remove unreliable, inconsistent or unreproducible (>20% RSD^{QC}) features. A peak was removed if: (i) the difference in median intensity between QC samplesand biological sample intensity was inconsistent between batches, (ii) there was a relatively large difference in median intensity between the QC sample and biological sample (iii) if the technical variation of the peak was considered too high.

The intensity matrix was processed further to allow statistical analyses. First, probable quotient normalisation (PQN) normalised the sample TICs to each other. This step is necessary due to differences in measured TIC arising from inherent sample differences that affect measurement by a mass spectrometer, for example minor differences in sample concentration, by using intensities of consistently arising peaks (224). For each peak present in 100% of samples, quotients of intensity over mean intensity across all spectra were calculated. The median of all quotients within a SIM-window was defined as the most probable quotient and used to normalise all peaks within that window. The intensity matrix can be dominated by the more intense peaks which have intrinsically higher variation. To normalise and reduce technical variation the dataset underwent logarithm (log_{10}) transformation. Finally to allow for multivariate statistical analysis, any remaining missing intensity values were imputed using k-nearest neighbour and the data was autoscaled (145).

G.5. Putative metabolite annotation and pathway analysis

A list of metabolic features containing their associated mean intensities was input into MI-Pack software (version 2 beta), and annotation was performed by applying the Metabolite

Identification Package (MI-Pack) workflow described previously (225). MI-Pack incorporates metabolomic databases for example, the Human Metabolome Database (HMDB) (117), Kyoto Encyclopaedia of Genes and Genomes (KEGG) database (154) and Lipid Metabolites and Pathway Strategy (Lipid MAPS) database (153) to allow the putative annotation of features based on accurate mass.

A single peak search was conducted using these databases, KEGG being restricted to the dedicated Homo sapiens (HSA) metabolite library, to putatively annotate metabolites. For polar features 'Transformation mapping' was also performed using the HSA subsection of the KEGG database to increase confidence in metabolite annotations. This method associates the empirical formula difference of a detected pair of peaks to a known difference between substrate-product pairs derived from a known metabolic pathway (225). A chain of linked metabolites in a single pathway would be deemed to have a higher level of confidence in the identification of each of the individual metabolites than alternative annotations which placed each metabolite in a different pathway. Where no putative annotations could be ascribed, the putative empirical formulae could be calculated based on the following conditions(225);

- Thirteen atoms (C, ^{13}C, H, N, O, P, S, ^{34}S, NaCl, Na(37Cl), Ca, Fe, Cu),
- Four positive ion adducts ($[M+H]^+$, $[M+Na]^+$,$[M+K]^+$, $[M+(41K)]^+$)**or** six negative ion adducts ($[M-H]^-$, $[M+Na-2H]^-$,$[M+K-2H]^-$, $[M+(37Cl)]^-$, $[M+Cl]^-$, $[M+Hac-H]^-$)
- Three isotopes C, S, Cl
- Mass error of <1.25 ppm for polar and <2ppm for non-polar features

It is important to remember that metabolite isomers may occur whereby several metabolites can be detected with the same accurate mass therefore a single metabolic feature may have multiple 'identifications'. Likewise, multiple spectral features with different accurate masses can define a single metabolite, as different adducts can be formed from the same metabolite, for example ($[M+H]^+$, $[M+Na]^+$ or $[M-H]^-$, $[M+Hac-H]^-$). Herein, metabolic features were identified by putative annotation based on accurate mass, equivalent to 'level 2' identification as defined by The Metabolomics Standards Initiative (155).

Appendix H. Putatively annotated metabolic features

Table H.1 The identification parameters of putatively identified polar metabolic features, detected in both positive and negative ionisation mode, which were significantly altered (p<0.05) between perinatal asphyxia *vs*. matched control and/or HIE *vs*. matched control. (See Chapter 3)

m/z	Empirical Formula	Ion form	Theoretical mass (Da)	Mass error (ppm)	%RSD^{QC}	Putative identifications
83.02159	CH_4N_2O	$[M+Na]^+$	60.032363	0.07	17	N-nitrosomethanamine OR Urea
94.9904	C_3H_6O	$[M+K-2H]^-$	58.041865	-0.75	9	Acetone OR Propanal
94.99064	$C_2H_4O_2$	$[M+Cl]^-$	60.02113	1.14	12	Acetate OR Glycolaldehyde
113.02091	$C_3H_6O_3$	$[M+Na]^+$	90.031695	-0.06	17	Lactate OR 3-Hydroxypropanoate OR Glyceraldehyde (>3)
115.03656	$C_3H_8O_3$	$[M+Na]^+$	92.047345	-0.06	13	Glycerol
125.00092	$C_4H_8O_2$	$[M+K-2H]^-$	88.05243	-0.93	9	2-Methylpropanoate OR Acetoin OR Butanoic acid
	OR	OR	OR	OR		OR
	$C_2H_7O_4P$	$[M-H]^-$	126.008198	-0.01		Ethylphosphate
127.01656	C_7H_6O	$[M+Na-2H]^-$	106.041865	0.21	9	Aromatic aldehyde
	OR	OR	OR	OR		OR
	$C_4H_{10}O_2$	$[M+K-2H]^-$	90.06808	-1		2,3-Butanediol
132.07674	$C_4H_9N_3O_2$	$[M+H]^+$	131.069477	-0.1	12	3-Guanidinopropanoate OR Creatine
132.94622	H_3O_4P	$[M+Cl]^-$	97.976898	-0.6	7	Orthophosphate
137.07092	$C_7H_8N_2O$	$[M+H]^+$	136.063663	-0.14	9	N-Methylnicotinamide
141.01582	$C_4H_6O_4$	$[M+Na]^+$	118.02661	-0.08	10	Methylmalonate OR Succinate OR Threonolactone (>3)
148.00386	$C_2H_7NO_3S$	$[M+Na]^+$	125.014666	-0.19	7	Taurine
148.06041	$C_5H_9NO_4$	$[M+H]^+$	147.053159	-0.17	11	Glutamate OR L-4-Hydroxyglutamate semialdehyde OR O-Acetyl-L-serine (>3)

151.04777	$C_5H_8N_2O_2$	$[M+Na]^+$	128.058578	-0.19	8	5,6-Dihydrothymine OR γ-Amino-γ-cyanobutanoate
154.08382	$C_6H_{13}NO_2$	$[M+Na]^+$	131.094629	-0.2	12	6-Aminohexanoate OR L-Isoleucine OR L-Leucine (>3)
159.02771	$C_5H_4N_4O$	$[M+Na]^+$	136.038511	-0.14	11	Hypoxanthine
164.07161 OR 204.04209	$C_9H_{11}NO_2$	$[M-H]^-$ OR $[M+K]^+$	165.078979	-0.56 OR -0.24	8 OR 9	4-Hydroxy-1-(3-pyridinyl)-1-butanone OR L-Phenylalanine OR 3-Pyridinebutanoic acid (>3)
167.02099	$C_5H_4N_4O_3$	$[M-H]^-$	168.028341	-0.45	9	Urate
167.03144	$C_6H_8O_4$	$[M+Na]^+$	144.04226	-0.25	13	3-Hexenedioic acid OR 3-Methylglutaconic acid OR Methylitaconate (>3)
170.09237	$C_7H_{11}N_3O_2$	$[M+H]^+$	169.085127	-0.2	10	1-Methylhistidine OR N(pi)-Methyl-L-histidine
171.02741	$C_5H_{10}O_5$	$[M+Na-2H]^-$	150.052825	-0.49	5	2-Deoxyribonic acid OR D-Ribose OR D-Xylose (>3)
171.06275	$C_6H_{12}O_4$	$[M+Na]^+$	148.07356	-0.18	27	(R)-2,3-Dihydroxy-3-methylpentanoate OR (R)-Mevalonate OR (R)-Pantoate
181.03847	$C_5H_{10}N_2O_3$	$[M+Cl]^-$	146.069143	-0.41	12	3-Ureidoisobutyrate OR Glutamine OR Alanylglycine (>3)
181.0471	$C_7H_{10}O_4$	$[M+Na]^+$	158.05791	-0.17	18	Isopropylmaleate OR Succinylacetone
188.17571	$C_9H_{21}N_3O$	$[M+H]^+$	187.168462	-0.15	9	N1-Acetylspermidine OR N8-Acetylspermidine
191.01966	$C_6H_8O_7$ OR $C_4H_4O_5$	$[M-H]^-$ OR $[M+Hac-H]^-$	192.027005 OR 132.005875	-0.36 OR -0.36	9 OR	Citrate OR Isocitrate OR 5-Dehydro-4-deoxy-D-glucarate (>3) OR 2-HydroxyethylenedicarboxylateOR Oxaloacetate OR trans-2,3-Epoxysuccinate (>3)
193.0471	$C_8H_{10}O_4$	$[M+Na]^+$	170.05791	-0.16	15	1,2-Dihydroxy-6-methylcyclohexa-3,5-dienecarboxylate, 1,6-Dihydroxy-5-methylcyclohexa-2,4-dienecarboxylate, 2-Hydroxy-6-oxo-octa-2,4-dienoate (>3)
196.05907	$C_7H_{13}NO_4$	$[M+Na-2H]^-$	175.084459	-0.29	7	N-Carboxyethyl-g-aminobutyric acid
197.09369	$C_9H_{18}O_2$	$[M+K]^+$	158.13068	-0.76	14	Nonanoic acid OR Oenanthic ether
199.02234	$C_6H_{10}O_6$	$[M+Na-2H]^-$	178.04774	-0.34	16	3-Keto-beta-D-galactose OR Galactonolactone OR L-Gulono-1,4-lactone (>3)
199.97885	$C_5H_9NOS_2$ OR	$[M+(37Cl)]^-$ OR	163.012558 OR163.030	-0.8 OR	7	3-Methylsulfinylpropyl isothiocyanate OR

	$C_5H_9NO_3S$	$[M+K-2H]^-$	316	-0.36		Acetylcysteine
200.06825	$C_7H_{15}NO_3$	$[M+K]^+$	161.105194	-0.52		L-Carnitine
	OR	OR	OR	OR	13	OR
	$C_{10}H_{11}NO_2$	$[M+Na]^+$	177.078979	0.25		1,2-Dehydrosalsolinol OR 5-Hydroxytryptophol
201.01594	$C_6H_{10}O_5$	$[M+K]^+$	162.052825	-0.22		(2R,3S)-2,3-Dimethylmalate OR 2(R)-Hydroxyadipic acid OR Glucosan (>3)
	OR	OR	OR	OR	16	OR
	$C_4H_9O_7P$	$[M+H]^+$	200.008593	0.35		D-Erythrose 4-phosphate (>3)
203.03158	$C_9H_8O_4$	$[M+Na]^+$	180.04226	0.49	10	3-(4-Hydroxyphenyl)pyruvate OR Caffeate OR 3-Hydroxyphenylpyruvic acid (>3)
	OR	OR	OR	OR		OR
	$C_6H_{12}O_5$	$[M+K]^+$	164.068475	-0.27		1,5-Anhydro-D-glucitol OR L-Rhamnose OR 2-Deoxy-D-galactose (>3)
204.06656	$C_{11}H_{11}NO_3$	$[M-H]^-$	205.073894	-0.28	8	5-Methoxyindoleacetate OR Cinnamoylglycine OR Indolelactate
204.99449	$C_6H_6N_4S$	$[M+K]^+$	166.031318	0.06	8	6-Methylmercaptopurine
205.01749	$C_{11}H_8N_2$	$[M+K-2H]^-$	168.068748	0.66	6	Beta-Carboline
205.0471	$C_9H_{10}O_4$	$[M+Na]^+$	182.05791	-0.15	7	3,4-Dihydroxyphenylpropanoate OR 3-(2,3-Dihydroxyphenyl)propanoate OR 3-(4-Hydroxyphenyl)lactate (>3)
205.06822		$[M+Na]^+$		-0.2	6	
OR	$C_6H_{14}O_6$	OR	182.07904	OR	OR	D-Sorbitol OR Galactitol OR Mannitol (>3)
241.09284		$[M+Hac-H]^-$		-0.22	7	
209.06662	$C_7H_{14}O_7$	$[M-H]^-$	210.073955	-0.28	12	Sedoheptulose
	OR	OR	OR	OR		OR
	$C_{11}H_{12}N_2$	$[M+(37Cl)]^-$	172.100048	0.58		1,2,3,4-tetrahydro-beta-carboline
209.09203	$C_{10}H_{12}N_2O_3$	$[M+H]^+$	208.084793	-0.19	10	Formyl-5-hydroxykynurenamine OR L-Kynurenine
212.00226	$C_8H_7NO_4S$	$[M-H]^-$	213.009581	-0.21	6	Indoxyl sulfate
213.07469	$C_6H_{14}N_4O_2$	$[M+K]^+$	174.111676	-0.68	12	D-Arginine OR L-Arginine
214.04745	$C_7H_{13}NO_4$	$[M+K]^+$	175.084459	-0.79	6	N-Carboxyethyl-g-aminobutyric acid
	OR	OR	OR	OR		OR
	$C_{10}H_9NO_3$	$[M+Na]^+$	191.058244	-0.07		5-Hydroxyindoleacetate OR 5-Phenyl-1,3-oxazinane-2,4-dione
215.01757	$C_4H_8N_4O_4$	$[M+K]^+$	176.054556	-0.68	10	Allantoate

217.06239	$C_{11}H_{14}O_2$	$[M+K]^+$	178.09938	-0.69	13	5-Phenylvaleric acid
	$C_9H_8O_5$	$[M+Na]^+$	196.037175	0.47		3-(3,4-Dihydroxyphenyl)pyruvate
219.0265	OR	OR	OR	OR	6	OR
	$C_6H_{12}O_6$	$[M+K]^+$	180.06339	-0.23		D-Glucose OR 3-Deoxyarabinohexonic acid OR myo-Inositol (>3)
219.04168	$C_{10}H_{12}O_3$	$[M+K]^+$	180.078645	-0.57	12	3-Methoxybenzenepropanoic acid OR Coniferyl alcohol
220.03701	$C_9H_{11}NO_3$	$[M+K]^+$	181.073894	-0.2	7	4-Hydroxy-4-(3-pyridyl)-butanoic acid OR L-Threo-3-Phenylserine OR L-Tyrosine (>3)
225.03456	$C_9H_{12}O_4$	$[M+(41K)]^+$	184.07356	-1.23	9	3-Methoxy-4-hydroxyphenylethyleneglycol OR cis-3-(Carboxy-ethyl)-3,5-cyclo-hexadiene-1,2-diol
225.06151	$C_5H_{10}O_6$	$[M+Hac-H]^-$	166.04774	-0.37	13	D-Xylonate OR L-Arabinonate OR D-Ribonate
226.10498	$C_9H_{17}NO_4$	$[M+Na]^+$	203.115759	0	8	L-Acetylcarnitine
227.07910	$C_8H_{16}N_2O_3$	$[M+K]^+$	188.116093	-0.67	6	Glycyl-leucine OR N-Alpha-acetyllysine OR N6-Acetyl-L-lysine
	OR	OR	OR	OR		OR
	$C_{11}H_{12}N_2O_2$	$[M+Na]^+$	204.089878	0		D-Tryptophan
232.05918	$C_{10}H_{13}NO_4$	$[M+Na-2H]^-$	211.084459	0.23	9	3-Methoxytyrosine OR Methyldopa anhydrous
232.15433	$C_{11}H_{21}NO_4$	$[M+H]^+$	231.147059	-0.02	7	Isobutyryl-L-carnitine OR O-Butanoylcarnitine
233.07853	$C_{11}H_{14}O_4$	$[M+Na]^+$	210.08921	0.42	30	Sinapyl alcohol
233.11482	$C_{12}H_{18}O_3$	$[M+Na]^+$	210.125595	0.02	14	(+)-7-Isojasmonic acid OR (-)-Jasmonic acid
235.13046	$C_{12}H_{20}O_3$	$[M+Na]^+$	212.141245	-0.03	15	12-Oxo-9(Z)-dodecenoic acid OR, Traumatin
237.14611	$C_{12}H_{22}O_3$	[M+Na]+	214.156895	-0.03	17	3-Oxododecanoic acid
241.01200	$C_7H_{10}O_7$	[M+Cl]-	206.042655	-0.23	10	(R)-2-Hydroxybutane-1,2,4-tricarboxylate OR 2-Methylcitrate OR Homoisocitrate(>3)
	OR	OR	OR	OR		OR
	$C_6H_{11}O_8P$	$M-H]^-$	242.019158	0.49		6-Deoxy-5-ketofructose 1-phosphate OR D-myo-Inositol 1,2-cyclic phosphate
267.05878OR		$[M+Na]^+$		0.08	7	
243.06225	$C_9H_{12}N_2O_6$	OR	244.069538	OR	OR	Pseudouridine OR Uridine
247.13046	$C_{13}H_{20}O_3$	$[M-H]^-$	224.141245	-0.05	10	(+)-7-Isomethyljasmonate OR Methyl jasmonate

255.09918	$C_{11}H_{20}O_4$	$[M+K]^+$	216.13616	-0.55	9	Undecanedioic acid
255.13569	$C_{12}H_{24}O_3$	$[M+K]^+$	216.172545	-0.06	35	(R)-3-Hydroxydodecanoic acid OR 12-Hydroxydodecanoic acid OR 3-Hydroxydodecanoic acid
262.12852	$C_{11}H_{19}NO_6$	$[M+H]^+$	261.121239	0.02	14	Methylglutarylcarnitine
262.97611	$C_6H_{11}O_6PS$	$[M+Na-2H]^-$	242.0014	0.16	8	2,3-Diketo-5-methylthiopentyl-1-phosphate OR 2-Hydroxy-3-keto-5-methylthiopentenyl-1-phosphate
263.00829		$[M+H]^+$		1.02	5	
OR	$C_5H_{12}O_8P_2$	OR	262.000746	OR	OR	1-Hydroxy-2-methyl-2-butenyl 4-diphosphate
298.94947		$[M+K-2H]^-$		0.39	8	
265.17741	$C_{14}H_{26}O_3$	$[M+Na]^+$	242.188195	-0.02	11	3-Oxotetradecanoic acid
265.21381	$C_{15}H_{30}O_2$	$[M+Na]^+$	242.22458	0.03	12	Pentadecanoic acid
267.1567	$C_{13}H_{24}O_4$	$[M+Na]^+$	244.16746	0.07	11	1,11-Undecanedicarboxylic acid
269.08776	$C_{13}H_{16}N_2O_2$	$[M+(37Cl)]^-$	232.121178	0.48	17	Melatonin
276.14418	$C_{12}H_{21}NO_6$	$[M+H]^+$	275.136889	0.05	23	Glutarylcarnitine
280.09207	$C_8H_{20}NO_6P$	$[M+Na]^+$	257.102827	0.08	9	Glycerophosphocholine
293.24513	$C_{17}H_{34}O_2$	$[M+Na]^+$	270.25588	0.1	11	Heptadecanoic acid
307.26083	$C_{18}H_{36}O_2$	$[M+Na]^+$	284.27153	0.26	21	Octadecanoic acid
308.09868	$C_{11}H_{19}NO_9$	$[M-H]^-$	309.105984	-0.09	13	N-Acetylneuraminate
325.17765	$C_{19}H_{26}O_3$	$[M+Na]^+$	302.188195	0.72	18	11beta-Hydroxyandrost-4-ene-3,17-dione OR 19-Oxotestosterone OR 2-Methoxyestradiol-17beta(>3)
	OR	OR	OR	OR		OR
	$C_{16}H_{30}O_4$	$[M+K]^+$	286.21441	0.25		Hexadecanedioic acid
329.04344	$C_{14}H_{14}O_7$	$[M+Cl]^-$	294.073955	0.25	9	Cyclic de-hypoxanthine futalosine
330.9958	$C_6H_{14}O_{10}P_2$	$[M+Na]^+$	308.006226	1.07	6	(R)-5-Diphosphomevalonate OR (R)-Mevalonic acid-5-pyrophosphate
331.04028	$C_{15}H_{14}O_6$	$[M+(41K)]^+$	290.07904	-0.11	6	(+)-Catechin OR (-)-Epicatechin OR cis-3,4-Leucopelargonidin
367.15846	$C_{19}H_{28}O_5S$	$[M-H]^-$	368.165747	-0.03	4	Dehydroepiandrosterone sulfate OR Epitestosterone sulfate OR Testosterone sulfate
373.02921	$C_9H_{19}O_{11}P$	$[M+K]^+$	334.066503	-1.21	7	sn-glycero-3-Phospho-1-inositol

383.15335	$C_{19}H_{28}O_6S$	$[M-H]^-$	384.160662	-0.09	5	3b,16a-Dihydroxyandrostenone sulfate		
389.03021	$C_{12}H_{16}O_{12}$	$[M+(37Cl)]^-$	352.06418	-1.08	5	4-(4-Deoxy-alpha-D-gluc-4-enuronosyl)-D-galacturonate		
395.18974	$C_{21}H_{32}O_5S$	$[M-H]^-$	396.197047	-0.08	8	Pregnenolone sulfate		
416.27487	$C_{24}H_{41}NO_2$	$[M+(41K)]^+$	375.313729	-0.33	10	7,10,13,16-Docosatetraenoylethanolamine		
425.08048	$C_{13}H_{22}N_4O_8S_2$	$[M-H]^-$	426.08791	-0.36	10	Cysteineglutathione disulfide OR S-Glutathionyl-L-cysteine		
426.02184	$C_{12}H_{16}N_5O_8P$	$[M+K-2H]^-$	389.073653	-0.98	14	ADP OR dGDP OR Adenosine 3',5-bisphosphate		
	OR	OR	OR	OR		OR		
	$C_{10}H_{15}N_5O_{10}P_2$	$[M-H]^-$	427.029421	-0.71		Acetyl adenylate		
441.15886	$C_{19}H_{26}O_6S$	$[M+Hac-H]^-$	382.145012	-0.01	9	2-Methoxyestradiol-17beta 3-sulfate		
471.03289	$C_{10}H_{14}N_4O_{10}P_2$	$[M+Hac-H]^-$	412.018522	1.09	6	dIDP		
502.94334	$C_9H_{15}N_2O_{14}P_3$	$[M+Cl]^-$	467.973622	0.63	6	dUTP		
519.29317	$C_{27}H_{44}O_8$	$[M+Na]^+$	496.30362	0.63	6	3-alpha,20-alpha-dihydroxy-5-beta-pregnane Pregnanediol-3-glucuronide	3-glucuronide	OR
524.92947	$C_9H_{15}N_2O_{15}P_3$	$[M+(41K)]^+$	483.968537	-0.65	17	UTP		
531.96968	$C_{10}H_{18}N_3O_{14}P_3$	$[M+Cl]^-$	497.000171	0.2	7	2-Deoxy-5-hydroxymethylcytidine-5-triphosphate		
534.29592	$C_{24}H_{50}NO_7P$	$[M+K]^+$	495.332492	0.5	8	LysoPC(16:0)		
557.25259	$C_{33}H_{34}N_4O_3$	$[M+Na]^+$	534.263091	0.5	7	Pyropheophorbide a		

(>3); indicates more than three possible putative annotations, OR; indicates more than one possible empirical formulae or multiple adducts.

Table H.2The identification parameters of putatively identified non-polar metabolic features, detected in both positive and negative ionisation mode, which were significantly altered (p<0.05) between perinatal asphyxia *vs*. matched control and/or HIE *vs*. matched control. (See Chapter 4)

m/z	Formula	Ion form	Theoretical mass (Da)	Mass error (ppm)	%RSD^{QC}	Putative identifications
179.05739	$C_7H_8N_4O_2$	$[M-H]^-$	180.064726	-0.33	14	1,7-Dimethylxanthine, Theobromine, Theophylline
223.17032	$C_{14}H_{24}O_2$	$[M-H]^-$	224.17763	-0.15	9	14:2(6,9), 5,8-Tetradecadienoic acid, Alepric acid, R-cucujolide III(>3)
235.18054	$C_{14}H_{22}N_2O$	$[M+H]^+$	234.173213	0.22	26	Lidocaine
251.20166	$C_{16}H_{28}O_2$	$[M-H]^-$	252.20893	0.03	10	7,10-hexadecadienoic acid, 7Z,10Z-Hexadecadienoic acid, Palmitolinoleic acid(>3)
261.22135	$C_{18}H_{28}O$	$[M+H]^+$	260.214015	0.22	10	4,5-(methanoxyethano)isolongifol-4-ene, 5-(1-oxopropan-2-yl)isolongifol-5-ene
271.24208	$C_{20}H_{30}$	$[M+H]^+$	270.23475	0.2	10	Axerophthene, Erogorgiaene
277.21731	$C_{18}H_{30}O_2$	$[M-H]^-$	278.22458	0.02	15	(6Z,9Z,12Z)-Octadecatrienoic acid, Crepenynate, octadeca-9Z,11E,15Z-trienoic acid(>3)
315.20965	$C_{18}H_{32}O_2$	$[M+Cl]^-$	280.24023	0.06	19	(R)-laballenic acid, 7-trans,9-cis-octadecadienoic acid, Linoelaidic acid(>3)
319.22247	$C_{18}H_{34}O_2$	$[M+(37Cl)]^-$	282.25588	0.43	18	(11E)-Octadecenoic acid, 17:1(12)(7Me), cis-10-oleic acid(>3)
319.22783	$C_{20}H_{32}O_3$	$[M-H]^-$	320.235145	-0.12	7	18-Hydroxyarachidonic acid, 11(R)-HETE, 14,15-EET(>3)
325.23844	$C_{19}H_{34}O_4$	$[M-H]^-$	326.24571	0.02	18	Ceriporic acid A
344.27963	$C_{19}H_{37}NO_4$	$[M+H]^+$	343.272259	0.27	11	N-palmitoyl serine
359.29559	$C_{24}H_{40}O_2$	$[M-H]^-$	360.30283	0.1	10	5alpha-Cholanoic acid, 5beta-Cholanoic acid, Tetracosatetraenoic acid (24:4n-6)(>3)
363.25168	$C_{20}H_{38}O_4$	$[M+Na-2H]^-$	342.27701	0	13	Eicosanedioic acid, PGF1a alcohol

372.31094	$C_{21}H_{41}NO_4$	$[M+H]^+$	371.303559	0.28	9	N-stearoyl serine
381.30108	$C_{21}H_{38}O_2$	$[M+Hac-H]^-$	322.28718	0.12	8	21:2(5Z,14Z), 21:2(5Z,16Z)
385.23605	$C_{22}H_{36}O_4$	$[M+Na-2H]^-$	364.26136	0.06	23	11-deoxy-16,16-dimethyl-PGE2, 16,16-dimethyl-PGA1
389.23348	$C_{21}H_{30}O_3$	$[M+Hac-H]^-$	330.219495	0.34	10	11-Deoxycorticosterone, 11alpha-hydroxyprogesterone, 17alpha-Hydroxyprogesterone(>3) OR
	OR	OR	OR	OR		
	$C_{23}H_{34}O_5$	$[M-H]^-$	390.240625	0.34		15R-PGA2 methyl ester, 15-acetate, Digoxigenin
395.18983	$C_{21}H_{32}O_5S$	$[M-H]^-$	396.197047	0.15	16	3beta-Hydroxypregn-5-en-20-one sulfate
395.31671	$C_{22}H_{40}O_2$	$[M+Hac-H]^-$	336.30283	0.07	9	13,16-Docosadienoic acid, 22:2(3Z,16Z), 22:2(7Z,13Z)(>3)
400.34225	$C_{23}H_{45}NO_4$	$[M+H]^+$	399.334859	0.29	8	L-Palmitoylcarnitine
407.20756	$C_{20}H_{28}O_5$	$[M+Hac-H]^-$	348.193675	0.08	31	12-oxo-Resolvin E1, 18-oxo-Resolvin E1, Gibberellin A14(>3)
413.3061		$[M-H]^-$		-0.05	11	(22E)-(24R)-1alpha,24-dihydroxy-22,23-didehydrovitamin D3 / (22E)-(24R)-1alpha,24-dihydroxy-22,23-didehydrocholecalciferol, (22E)-(24R)-24,25-dihydroxy-22,23-didehydrovitamin D3 / (22E)-(24R)-24,25-dihydroxy-22,23-didehydrocholecalciferol(>3)
OR	$C_{27}H_{42}O_3$	OR	414.313395	OR	OR	
415.32089		$[M+H]^+$		0.53	11	
421.33245	$C_{24}H_{42}O_2$	$[M+Hac-H]^-$	362.31848	0.28	9	24:3(15Z,18Z,21Z)
433.36787	$C_{28}H_{48}O_3$	$[M+H]^+$	432.360345	0.57	13	22,23-dihydroxycampesterol, 3-Dehydro-6-deoxoteasterone, Cathasterone
435.20253	$C_{21}H_{28}O_6$	$[M+Hac-H]^-$	376.18859	0.2	18	18-Oxocortisol
437.32729	$C_{24}H_{42}O_3$	$[M+Hac-H]^-$	378.313395	0.09	15	C-9,11,21-Trisnor-17-methyl-1alpha,25-dihydroxyvitamin D3
447.34715	$C_{28}H_{46}O_4$	$[M+H]^+$	446.33961	0.59	12	3-Dehydroteasterone, (20S)-1alpha,20,25-trihydroxy-24a-homovitamin D3 / (20S)-1alpha,20,25-trihydroxy-24a-homocholecalciferol, (20S)-1alpha,25-dihydroxy-20-methoxyvitamin D3 / (20S)-1alpha,25-dihydroxy-20-methoxycholecalciferol(>3)
449.36287	$C_{28}H_{48}O_4$	$[M+H]^+$	448.35526	0.74	10	Chlorogenin, Teasterone, Typhasterol
463.34297	$C_{26}H_{44}O_3$	$[M+Hac-H]^-$	404.329045	0.15	14	1,25-dihydroxy-2-nor-1,2-secovitamin D3 / 1,25-dihydroxy-2-nor-1,2-secocholecalciferol, 1alpha,25-dihydroxy-19-norvitamin D3 / 1alpha,25-

						dihydroxy-19-norcholecalciferol, 2-Nor-1,3-seco-1alpha,25-dihydroxyvitamin D3 OR
	OR	OR	OR	OR		
	$C_{28}H_{48}O_5$	$[M-H]^-$	464.350175	0.15		7-oxotyphasterol, Castasterone,Trihydroxycoprostanoic acid(>3)
476.27823	$C_{23}H_{44}NO_7P$	$[M-H]^-$	477.285542	-0.07	9	LysoPE(18:2(9Z,12Z)/0:0)
	OR	OR	OR	OR		OR
	$C_{28}H_{41}NO_4$	$[M+Na-2H]^-$	455.303559	0.01		15-HETE-DA, 15-HETE-VA
478.2939	$C_{23}H_{46}NO_7P$	$[M-H]^-$	479.301192	-0.03	7	LysoPE(18:1(9Z)/0:0), PC(15:1(9Z)/0:0)
480.30949	$C_{23}H_{48}NO_7P$	$[M-H]^-$	481.316842	-0.16	5	LysoPC(15:0), LysoPE(18:0/0:0), PC(14:0/O-1:0) (>3)
481.29926	$C_{27}H_{46}O_5S$	$[M-H]^-$	482.306597	-0.13	20	26-hydroxycholesterol 3-sulfate
494.32444	$C_{24}H_{48}NO_7P$	$[M+H]^+$	493.316842	0.65	10	LysoPC(16:1(9Z)), PC(16:1(9E)/0:0), PE(19:1(9Z)/0:0)
497.2941	$C_{30}H_{42}N_2O_2$	$[M+Cl]^-$	462.324628	0.14	18	Arachidonoyl Serotonin
498.29743	$C_{24}H_{48}NO_6P$	$[M+Na-2H]^-$	477.321927	1.67	16	PE(P-19:1(12Z)/0:0)
500.27813	$C_{25}H_{44}NO_7P$	$[M-H]^-$	501.285542	-0.27	9	LysoPE(20:4(5Z,8Z,11Z,14Z)/0:0)
	OR	OR	OR	OR		OR
	$C_{26}H_{43}NO_6$	$[M+Cl]^-$	465.309039	-0.62		3a,7b,12a-Trihydroxyoxocholanyl-Glycine, Glycocholate
501.28136	$C_{27}H_{46}O_4S$	$[M+Cl]^-$	466.311682	0.55	9	Cholesterol sulfate
502.29373	$C_{23}H_{50}N_2O_5P$	$[M+K-2H]^-$	465.345736	-1.22	9	Sphingosyl-phosphocholine
	OR	OR	OR	OR		OR
	$C_{25}H_{46}NO_7P$	$[M-H]^-$	503.301192	-0.37		LysoPE(20:3(8Z,11Z,14Z)/0:0)
508.34073	$C_{25}H_{52}NO_7P$	$[M-H]^-$	509.348142	-0.27	10	LysoPC(17:0), LysoPE(20:0/0:0), PC(16:0/O-1:0) (>3)
512.29935	$C_{21}H_{44}NO_7P$	$[M+Hac-H]^-$	453.285542	-0.09	13	LysoPE(16:0/0:0), PC(13:0/0:0)
518.32194	$C_{24}H_{50}NO_7P$	$[M+Na]^+$	495.332492	0.44	12	LysoPC(16:0), PC(0:0/16:0), PC(16:0/0:0)rac, PE(19:0/0:0) (>3)
520.34005		$[M+H]^+$		0.54	9	LysoPC(18:2(9Z,12Z)), PC(18:2(2E,4E)/0:0)
OR	$C_{26}H_{50}NO_7P$	OR	519.332492	OR	OR	
542.32182		$[M+Na]^+$		0.2	18	

522.35569	$C_{26}H_{52}NO_7P$	$[M+H]^+$	521.348142	0.52	9	LysoPC(18:1(9Z)), PC(0:0/18:1(6Z)), PC(0:0/18:1(9E)) (>3)
524.2781	$C_{27}H_{44}NO_7P$	$[M-H]^-$	525.285542	-0.32	8	LysoPE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0)
527.29682	$C_{30}H_{50}O_3S$	$[M+K-2H]^-$	490.348067	0.28	20	26,27-diethyl-1alpha,25-dihydroxy-22-thia-20-epivitamin D3 / 26,27-diethyl-1alpha,25-dihydroxy-22-thia-20-epicholecalciferol, 26,27-diethyl-1alpha,25-dihydroxy-22-thiavitamin D3 / 26,27-diethyl-1alpha,25-dihydroxy-22-thiacholecalciferol
538.31485	$C_{25}H_{50}NO_9P$	$[M-H]^-$	539.322322	-0.36	14	PS(19:0/0:0)
546.35584	$C_{28}H_{52}NO_7P$	$[M+H]^+$	545.348142	0.77	10	LysoPC(20:3(8Z,11Z,14Z))
547.35917	$C_{31}H_{54}O_5$	$[M+(41K)]^+$	506.397125	1.4	11	(20R)-24-Hydroxy-19-norgeminivitamin D3, (20S)-24-Hydroxy-19-norgeminivitamin D3
547.43656	$C_{34}H_{60}O_5$	$[M-H]^-$	548.444075	-0.44	14	DG(13:0/18:3(9Z,12Z,15Z)/0:0)iso2, DG(14:1(9Z)/17:2(9Z,12Z)/0:0)iso2
547.47239	$C_{35}H_{62}O_4$	$[M+H]^+$	546.46481	0.55	9	bacteriohopane-32,33,34,35-tetrol
549.48863		$[M-H]^-$		-0.37	10	
OR	$C_{35}H_{66}O_4$	OR	550.49611	OR	OR	1-O-(1Z-Tetradecenyl)-2-(9Z-octadecenoyl)-sn-glycerol
551.50371		$[M+H]^+$		0.59	9	
559.47286	$C_{34}H_{60}O_2$	$[M+Hac-H]^-$	500.45933	-0.58	14	34:4(19Z,22Z,25Z,28Z), Linolenyl palmitatoleate, Palmitoleyl linolenate OR
	OR	OR	OR	OR		2-methylbacteriohopane-32,33,34,35-tetrol, Mayolene-18
	$C_{36}H_{64}O_4$	$[M-H]^-$	560.48046	-0.58		
561.48858	$C_{34}H_{62}O_2$	$[M+Hac-H]^-$	502.47498	-0.45	12	Linolenyl palmitate, Linoleyl palmitatoleate, Palmitoleyl linoleate(>3)
563.50427	$C_{34}H_{64}O_2$	$[M+Hac-H]^-$	504.49063	-0.38	14	Linoleyl palmitate, Oleyl palmitoleate, Palmitoleyl oleate(>3)
568.34011	$C_{30}H_{50}NO_7P$	$[M+H]^+$	567.332492	0.6	9	LysoPC(22:6(4Z,7Z,10Z,13Z,16Z,19Z))
649.50491	$C_{37}H_{66}O_5$	$[M+Hac-H]^-$	590.491025	0.05	7	DG(12:0/22:3(10Z,13Z,16Z)/0:0)iso2, DG(14:0/20:3(8Z,11Z,14Z)/0:0), DG(14:1(9Z)/20:2(11Z,14Z)/0:0) (>3)
661.50495	$C_{38}H_{66}O_5$	$[M+Hac-H]^-$	602.491025	0.11	20	DG(13:0/22:4(7Z,10Z,13Z,16Z)/0:0)iso2, DG(15:0/20:4(5Z,8Z,11Z,14Z)/0:0), DG(15:1(9Z)/20:3(8Z,11Z,14Z)/0:0)iso2(>3)

663.52043	$C_{38}H_{68}O_5$	$[M+Hac-H]^-$	604.506675	-0.15	10	DG(13:0/22:3(10Z,13Z,16Z)/0:0)iso2, DG(15:0/20:3(8Z,11Z,14Z)/0:0), DG(15:1(9Z)/20:2(11Z,14Z)/0:0)iso2(>3)
665.53613	$C_{38}H_{70}O_5$	$[M+Hac-H]^-$	606.522325	-0.07	13	DG(13:0/22:2(13Z,16Z)/0:0)iso2, DG(15:0/20:2(11Z,14Z)/0:0), DG(15:1(9Z)/20:1(11Z)/0:0)iso2(>3)
675.54389	$C_{37}H_{75}N_2O_6P$	$[M+H]^+$	674.536276	0.5	10	SM(d16:1/16:0), SM(d18:1/14:0)
677.53619	$C_{39}H_{70}O_5$	$[M+Hac-H]^-$	618.522325	0.02	12	DG(14:0/22:3(10Z,13Z,16Z)/0:0)iso2, DG(14:1(9Z)/22:2(13Z,16Z)/0:0), DG(16:0/20:3(8Z,11Z,14Z)/0:0) (>3)
685.52906	$C_{38}H_{75}N_2O_6P$	$[M-H]^-$	686.536276	0.09	12	SM(d18:2/15:0)
687.54457	$C_{38}H_{77}N_2O_6P$	$[M-H]^-$	688.551926	-0.12	13	Etn-1-P-Cer(d14:1/18:0), SM(d16:1/17:0), SM(d18:1/15:0)
706.53855	$C_{38}H_{76}NO_8P$	$[M+H]^+$	705.530857	0.59	4	PC(20:0/10:0), PC(9:0/21:0), PE(12:0/21:0) (>3)
707.54195	$C_{41}H_{78}O_6$	$[M+(41K)]^+$	666.57984	1.18	4	TG(12:0/12:0/14:0)iso3, TG(12:0/13:0/13:0)iso3
715.57586	$C_{40}H_{81}N_2O_6P$	$[M-H]^-$	716.583226	-0.13	30	SM(d18:1/17:0), SM(d19:1/16:0)
715.62211	$C_{44}H_{84}O_5$	$[M+Na]^+$	692.631875	1.42	9	DG(19:0/22:1(13Z)/0:0)iso2, DG(19:1(9Z)/22:0/0:0)iso2,
	OR	OR	OR	OR		DG(20:1(11Z)/21:0/0:0)iso2 OR
	$C_{46}H_{82}O_5$	$[M+H]^+$	714.616225	-1.94		DG(21:0/22:4(7Z,10Z,13Z,16Z)/0:0)iso2
716.52346	$C_{39}H_{76}NO_8P$	$[M-H]^-$	717.530857	-0.17	29	PC(19:1(9Z)/12:0), PE(12:0/22:1(11Z)), PE(14:0/20:1(11Z)) (>3)
716.55939	$C_{40}H_{78}NO_7P$	$[M+H]^+$	715.551592	0.73	13	PC(O-14:0/18:2(9Z,12Z)), PE(O-18:0/17:2(9Z,12Z)), PE(P-16:0/19:1(9Z)) (>3)
	OR	OR	OR	OR		OR
	$C_{45}H_{75}NO_4$	$[M+Na]^+$	693.569609	0.78		Cholesteryl nitrolinoleate
719.59811	$C_{46}H_{76}O_2$	$[M+Hac-H]^-$	660.58453	-0.38	8	Ergosteryl oleate
726.66159	$C_{42}H_{85}NO_4$	$[M+Hac-H]^-$	667.647859	-0.17	13	Cer(d18:0/h24:0), Cer(t18:0/24:0)
718.5392		$[M-H]^-$		-0.04	24	
OR	$C_{39}H_{78}NO_8P$	OR	719.546507	OR	OR	PC(21:0/10:0), PC(9:0/22:0), PE(12:0/22:0)
720.55434		$[M+H]^+$		0.77	9	
730.53876	$C_{40}H_{76}NO_8P$	$[M+H]^+$	729.530857	0.86	7	PC(20:2(11Z,14Z)/12:0), PE(13:0/22:2(13Z,16Z)), PE(15:0/20:2(11Z,14Z)) (>3)

730.53914	$C_{40}H_{78}NO_8P$	$[M-H]^-$	731.546507	-0.12	9	PC(12:0/20:1(11Z)), PC(13:0/19:1(9Z)), PE(22:1(11Z)/13:0)(>3)
732.55508	$C_{38}H_{76}NO_6P$	$[M+Hac-H]^-$	673.541027	0.27	11	CerP(d18:1/20:0)
	OR	OR	OR	OR		OR
	$C_{40}H_{80}NO_8P$	$[M-H]^-$	733.562157	0.27		PC(10:0/22:0), PE(22:0/13:0) (>3)
733.5498	$C_{49}H_{76}O_2$	$[M+(37Cl)]^-$	696.58453	-1.61	11	22:6 Cholesteryl ester
742.57485	$C_{42}H_{80}NO_7P$	$[M+H]^+$	741.567242	0.45	5	PC(O-16:0/18:3(6Z,9Z,12Z)), , PC(P-16:0/18:2(9Z,12Z)), PE(P-20:0/17:2(9Z,12Z)) (>3)
744.55417	$C_{41}H_{78}NO_8P$	$[M+H]^+$	743.546507	0.52	5	PC(13:0/20:2(11Z,14Z)), PC(14:1(9Z)/19:1(9Z)), PE(22:2(13Z,16Z)/14:0) (>3)
746.56995	$C_{41}H_{80}NO_8P$	$[M+H]^+$	745.562157	0.69	5	PC(13:0/20:1(11Z)), PC(14:0/19:1(9Z)), PE(22:1(11Z)/14:0) (>3)
747.57334	$C_{44}H_{82}O_6$	$[M+(41K)]^+$	706.61114	1.24	9	TG(12:0/12:0/17:1(9Z))iso3, TG(12:0/13:0/16:1(9Z))iso6, TG(12:0/14:0/15:1(9Z))iso6(>3)
753.56307		$[M+(37Cl)]^-$		1.5	9	TG(12:0/12:0/18:3(6Z,9Z,12Z))iso3, TG(12:0/12:0/18:3(9Z,12Z,15Z))iso3, TG(14:1(9Z)/14:1(9Z)/14:1(9Z))
OR	$C_{45}H_{80}O_6$	OR	716.59549	OR	OR	
757.55715		$[M+(41K)]^+$		0.51	4	
756.55372	$C_{42}H_{78}NO_8P$	$[M+H]^+$	755.546507	-0.08	4	PC(14:0/20:3(8Z,11Z,14Z)), PC(14:1(9Z)/20:2(11Z,14Z)), PE(22:2(13Z,16Z)/15:1(9Z)) (>3)
757.62281	$C_{43}H_{87}N_2O_6P$	$[M-H]^-$	758.630176	-0.12	8	SM(d16:1/22:0), SM(d18:1/20:0)
770.57039	$C_{43}H_{80}NO_8P$	$[M+H]^+$	769.562157	1.24	10	PC(15:0/20:3(8Z,11Z,14Z)), PC(15:1(9Z)/20:2(11Z,14Z)), PE(22:2(13Z,16Z)/16:1(9Z)) (>3)
770.60732	$C_{44}H_{84}NO_7P$	$[M+H]^+$	769.598542	1.95	5	PC(O-16:0/20:3(8Z,11Z,14Z)), PC(O-18:0/18:3(6Z,9Z,12Z)), PC(O-18:0/18:3(9Z,12Z,15Z)) (>3)
772.58547	$C_{43}H_{82}NO_8P$	$[M+H]^+$	771.577807	0.5	4	PC(13:0/22:2(13Z,16Z)), PC(15:0/20:2(11Z,14Z)), PE(22:2(13Z,16Z)/16:0) (>3)
773.58881	$C_{46}H_{84}O_6$	$[M+(41K)]^+$	732.62679	0.96	8	TG(12:0/13:0/18:2(9Z,12Z))iso6, TG(12:0/14:0/17:2(9Z,12Z))iso6, TG(12:0/14:1(9Z)/17:1(9Z))iso6(>3)

774.60122		$[M+H]^+$		0.63	5	
OR	$C_{43}H_{84}NO_8P$	OR	773.593457	OR	OR	PC(13:0/22:1(11Z)), PC(14:1(9Z)/21:0), PE(22:1(11Z)/16:0) (>3)
832.60842		$[M+Hac-H]^-$		1.33	10	
775.60451	$C_{46}H_{86}O_6$	$[M+(41K)]^+$	734.64244	1.02	5	TG(12:0/12:0/19:1(9Z))iso3, TG(12:0/13:0/18:1(9Z))iso6, TG(12:0/14:0/17:1(9Z))iso6(>3)
776.54452	$C_{41}H_{80}NO_{10}P$	$[M-H]^-$	777.551987	-0.25	18	PS(13:0/22:0), PS(14:0/21:0), PS(15:0/20:0) (>3)
780.55289	$C_{42}H_{80}NO_8P$	$[M+Na]^+$	757.562157	1.94	7	PC(12:0/22:2(13Z,16Z)), PE(22:2(13Z,16Z)/15:0), PE-NMe(18:1(9E)/18:1(9E))
	OR	OR	OR	OR		(>3) OR
	$C_{44}H_{78}NO_8P$	$[M+H]^+$	779.546507	-1.14		PC(14:1(9Z)/22:4(7Z,10Z,13Z,16Z)), PC(16:0/20:5(5Z,8Z,11Z,14Z,17Z)), PE(22:4(7Z,10Z,13Z,16Z)/17:1(9Z)) (>3)
781.55635	$C_{47}H_{80}O_6$	$[M+(41K)]^+$	740.59549	-0.53	6	TG(12:0/12:0/20:5(5Z,8Z,11Z,14Z,17Z))iso3, TG(12:0/14:1(9Z)/18:4(6Z,9Z,12Z,15Z))iso6
783.63741		$[M+H]^+$		-0.05	6	
OR	$C_{45}H_{87}N_2O_6P$	OR	782.630176	OR	OR	SM(d18:2/22:1)
841.64374		$[M+Hac-H]^-$		-0.34	9	
784.58616	$C_{44}H_{82}NO_8P$	$[M+H]^+$	783.577807	1.37	4	PC(14:1(9Z)/22:2(13Z,16Z)), PC(16:0/20:3(5Z,8Z,11Z)), PE(22:2(13Z,16Z)/17:1(9Z)) (>3)
785.58902	$C_{47}H_{84}O_6$	$[M+(41K)]^+$	744.62679	1.21	4	TG(12:0/12:0/20:3(8Z,11Z,14Z))iso3, TG(12:0/14:0/18:3(6Z,9Z,12Z))iso6, TG(12:0/14:0/18:3(9Z,12Z,15Z))iso6 (>3)
792.59052	$C_{46}H_{82}NO_7P$	$[M+H]^+$	791.582892	0.44	5	PC(P-18:0/20:5(5Z,8Z,11Z,14Z,17Z)), PC(o-16:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))
793.59386	$C_{43}H_{85}O_{10}P$	$[M+H]^+$	792.588038	-1.83	5	PG(15:0/22:0), PG(16:0/21:0), PG(17:0/20:0) (>3)
794.57036	$C_{45}H_{80}NO_8P$	$[M+H]^+$	793.562157	1.17	7	PC(15:1(9Z)/22:4(7Z,10Z,13Z,16Z)),PC(20:5(5Z,8Z,11Z,14Z,17Z)/17:0), PE(20:1(11Z)/20:4(5Z,8Z,11Z,14Z)) (>3)
798.6023	$C_{45}H_{84}NO_8P$	$[M+H]^+$	797.593457	1.96	6	PC(15:1(9Z)/22:2(13Z,16Z)), PC(17:0/20:3(8Z,11Z,14Z)),

						PE(22:2(13Z,16Z)/18:1(9Z)) (>3)
806.56984	$C_{46}H_{80}NO_8P$	$[M+H]^+$	805.562157	0.5	6	PC(16:0/22:6(4E,7E,10E,13E,16E,19E)), PC(16:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), PE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/19:0) (>3)
807.57326	$C_{49}H_{82}O_6$	$[M+(41K)]^+$	766.61114	1.04	6	TG(12:0/12:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))iso3, TG(12:0/14:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z))iso6, TG(14:1(9Z)/14:1(9Z)/18:4(6Z,9Z,12Z,15Z))iso3
813.62081	$C_{49}H_{88}O_6$	$[M+(41K)]^+$	772.65809	1.77	5	TG(12:0/12:0/22:3(10Z,13Z,16Z))iso3, TG(12:0/14:0/20:3(8Z,11Z,14Z))iso6, TG(12:0/14:1(9Z)/20:2(11Z,14Z))iso6(>3)
818.60602	$C_{48}H_{84}NO_7P$	$[M+H]^+$	817.598542	0.25	8	PC(P-18:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))
820.62241	$C_{48}H_{86}NO_7P$	$[M+H]^+$	819.614192	1.15	7	PC(P-20:0/20:5(5Z,8Z,11Z,14Z,17Z)), PC(o-18:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))
821.62551	$C_{45}H_{89}O_{10}P$	$[M+H]^+$	820.619338	-1.34	8	PG(17:0/22:0), PG(18:0/21:0), PG(19:0/20:0) (>3)
822.63826	$C_{48}H_{88}NO_7P$	$[M+H]^+$	821.629842	1.39	6	PC(O-18:0/22:5(4Z,7Z,10Z,13Z,16Z)), PC(O-18:0/22:5(7Z,10Z,13Z,16Z,19Z)), PC(O-20:0/20:5(5Z,8Z,11Z,14Z,17Z)) (>3)
824.65414	$C_{48}H_{90}NO_7P$	$[M+H]^+$	823.645492	1.66	6	PC(O-18:0/22:4(7Z,10Z,13Z,16Z)), PC(P-20:0/20:3(8Z,11Z,14Z)), PC(o-20:0/20:4(8Z,11Z,14Z,17Z))
829.55525	$C_{44}H_{85}O_9P$	$[M+(41K)]^+$	788.593123	1.03	14	PG(O-16:0/22:2(13Z,16Z)), PG(O-18:0/20:2(11Z,14Z)), PG(O-20:0/18:2(9Z,12Z)) (>3)
829.64395	$C_{44}H_{87}N_2O_6P$	$[M+Hac-H]^-$	770.630176	-0.1	11	SM(d18:2/21:0)
830.64879	$C_{48}H_{91}NO_8$	$[M+Na-2H]^-$	809.674469	-0.42	10	GalCer(d18:1/24:1(15Z)), GlcCer(d18:1/24:1(15Z))
831.65994	$C_{44}H_{89}N_2O_6P$	$[M+Hac-H]^-$	772.645826	0.31	10	SM(d16:1/23:0), SM(d17:1/22:0), SM(d18:1/21:0) (>3)
832.6645	$C_{48}H_{93}NO_8$	$[M+Na-2H]^-$	811.690119	-0.35	9	GalCer(d18:1/24:0), GlcCer(d18:0/24:1(15Z)), Glucosylceramide (d18:1/24:0)
834.60124	$C_{48}H_{84}NO_8P$	$[M+H]^+$	833.593457	0.61	7	PC(18:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)),

						PC(18:0/22:6(9Z,11Z,13Z,15Z,17Z,19)), PE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/21:0) (>3)
834.62192	$C_{45}H_{88}NO_{10}P$	$[M+H]^+$	833.614587	0.07	7	PS(17:0/22:0), PS(18:0/21:0), PS(19:0/20:0) (>3)
	OR	OR	OR	OR		OR
	$C_{47}H_{89}NO_8$	$[M+K]^+$	795.658819	-0.07		GalCer(d18:2/23:0), GlcCer(d18:2/23:0)
835.6045	$C_{51}H_{86}O_6$	$[M+(41K)]^+$	794.64244	0.94	6	TG(12:0/14:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))iso6, TG(12:0/14:1(9Z)/22:5(7Z,10Z,13Z,16Z,19Z))iso6, TG(12:0/16:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z))iso6(>3)
845.67565	$C_{45}H_{91}N_2O_6P$	$[M+Hac-H]^-$	786.661476	0.38	7	SM(d16:1/24:0), SM(d18:1/22:0)
848.62943	$C_{47}H_{92}NO_7P$	$[M+Cl]^-$	813.661142	-1.31	11	PC(P-20:0/19:1(9Z)), PE(O-20:0/22:2(13Z,16Z)), PE(P-20:0/22:1(11Z))
848.65364	$C_{50}H_{90}NO_7P$	$[M+H]^+$	847.645492	1.03	10	PC(O-20:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))
850.66926	$C_{50}H_{92}NO_7P$	$[M+H]^+$	849.661142	0.99	8	PC(P-20:0/22:4(7Z,10Z,13Z,16Z))
858.68022	$C_{50}H_{95}NO_8$	$[M+Na-2H]^-$	837.705769	-0.25	9	GalCer(d18:1/26:1(17Z)), GlcCer(d18:1/26:1(17Z))
859.69186	$C_{46}H_{93}N_2O_6P$	$[M+Hac-H]^-$	800.677126	1.02	8	SM(d16:1/25:0), SM(d17:1/24:0), SM(d18:1/23:0)
860.6957	$C_{50}H_{97}NO_8$	$[M+Na-2H]^-$	839.721419	-0.45	8	GalCer(d18:0/26:1), GalCer(d18:1/26:0), GlcCer(d18:0/26:1(17Z)) (>3)
864.57572	$C_{48}H_{84}NO_{10}P$	$[M-H]^-$	865.583287	-0.34	11	PS(20:1(11Z)/22:4(7Z,10Z,13Z,16Z)), PS(20:3(8Z,11Z,14Z)/22:2(13Z,16Z)), PS(20:4(5Z,8Z,11Z,14Z)/22:1(11Z)) (>3)
873.70799	$C_{47}H_{95}N_2O_6P$	$[M+Hac-H]^-$	814.692776	1.56	9	SM(d18:0/24:1(15Z)), SM(d18:1/24:0)
876.57584	$C_{47}H_{80}NO_8P$	$[M+Hac-H]^-$	817.562157	-0.19	9	PC(17:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), PC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/17:1(9Z)), PE(20:1(11Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)) (>3) OR
	OR	OR	OR	OR		
	$C_{49}H_{84}NO_{10}P$	$[M-H]^-$	877.583287	-0.19		PS(21:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)), PS(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/21:0)
879.74216	$C_{55}H_{100}O_6$	$[M+Na]^+$	856.75199	1.08	13	TG(12:0/18:0/22:3(10Z,13Z,16Z))iso6, TG(12:0/18:1(9Z)/22:2(13Z,16Z))iso6, TG(12:0/18:2(9Z,12Z)/22:1(11Z))iso6 (>3) OR

	OR	OR	OR	OR		TG(12:0/20:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))iso6,
	$C_{57}H_{98}O_6$	$[M+H]^+$	878.73634	-1.66		TG(12:0/20:1(11Z)/22:5(7Z,10Z,13Z,16Z,19Z))iso6,
						TG(12:0/20:2(11Z,14Z)/22:4(7Z,10Z,13Z,16Z))iso6(>3)
885.70648	$C_{48}H_{95}N_2O_6P$	$[M+Hac-H]^-$	826.692776	-0.17	11	SM(d17:1/26:1), SM(d18:2/25:0), SM(d19:1/24:1)
890.59169	$C_{48}H_{82}NO_8P$	$[M+Hac-H]^-$	831.577807	0.03	11	PC(18:1(11Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)),
						PC(18:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)),
	OR	OR	OR	OR		PC(18:2(9Z,12Z)/22:5(4Z,7Z,10Z,13Z,16Z)) (>3) OR
	$C_{50}H_{86}NO_{10}P$	$[M-H]^-$	891.598937	0.03		PS(22:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)),
						PS(22:2(13Z,16Z)/22:4(7Z,10Z,13Z,16Z)),
						PS(22:4(7Z,10Z,13Z,16Z)/22:2(13Z,16Z)) (>3)
892.60723	$C_{50}H_{88}NO_{10}P$	$[M-H]^-$	893.614587	-0.09	12	PS(22:1(11Z)/22:4(7Z,10Z,13Z,16Z)), PS(22:4(7Z,10Z,13Z,16Z)/22:1(11Z))

(>3); indicates more than three possible putative annotations, OR; indicates more than one possible empirical formulae or multiple adducts.

Table H.3The identification parameters of putatively identified polar and non-polar metabolic features, detected in both positive and negative ionisation mode, which were significantly altered (p<0.05) between haemolysed and clean cord blood serum pairs. (See Chapter 7)

m/z	Empirical formula	Ion form	Theoretical mass (Da)	Mass error (ppm)	%RSD^{QC}	Putative annotations
Polar metabolic features						
121.06241	$C_6H_{10}O$	$[M+Na]^+$	98.073165	0.19	13	Cyclohexanone OR 3-Hexenal
125.05733	$C_5H_{10}O_2$	$[M+Na]^+$	102.06808	0.23	14	2-Methylbutyrate OR 3-Methylbutanoic acid OR Pentanoate
127.01643	C_7H_6O	$[M+Na-2H]^-$	106.041865	-0.82	17	Aromatic aldehyde
142.0475	$C_4H_9NO_3$	$[M+Na]^+$	119.058244	0.24	4	L-Allothreonine OR L-Homoserine OR L-Threonine
154.04749	$C_5H_9NO_3$	$[M+Na]^+$	131.058244	0.16	6	3-Hydroxy-L-proline OR 5-Amino-2-oxopentanoic acid OR L-Glutamate 5-semialdehyde (>3)
169.05842	$C_5H_{10}N_2O_3$	$[M+Na]^+$	146.069143	0.33	3	Glutamine OR 3-Ureidoisobutyrate OR Alanylglycine
184.0581	$C_6H_{11}NO_4$	$[M+Na]^+$	161.068809	0.38	8	L-2-Aminoadipate OR O-Acetyl-L-homoserine OR 3-Amino-2,3-dideoxy-scyllo-inosose
185.043	$C_6H_{12}O_5$	$[M+Na-2H]^-$	164.068475	-0.78	14	6-Deoxy-L-galactose OR L-Rhamnose OR β-D-Fucose (>3)
190.08392	$C_9H_{13}NO_2$	$[M+Na]^+$	167.094629	0.37	11	Methoxytyramine OR Phenylephrine OR Synephrine
203.02918	$C_{10}H_{10}O_2$	$[M+(41K)]^+$	162.06808	-0.87	10	1,2-Dihydronaphthalene-1,2-diol OR Isosafrole
203.05265	$C_6H_{12}O_6$	$[M+Na]^+$	180.06339	0.19	3	Allose OR Fructose OR myo-Inositol (>3)
203.05391	$C_7H_8N_4O_2$	$[M+Na]^+$	180.064726	-0.18	3	1,7-Dimethylxanthine OR Theobromine OR Theophylline
205.04482	$C_{10}H_{12}O_2$	$[M+(41K)]^+$	164.08373	-0.91	4	Phenylbutyric acid OR Benzenebutanoic acid OR Eugenol
207.02406	$C_9H_{10}O_3$	$[M+(41K)]^+$	166.062995	-1.02	7	Phenyllactate OR 3-Phenoxypropionic acid OR 4-Methoxyphenylacetic acid (>3)
212.97956	$C_6H_6O_6$	$[M+K]^+$	174.01644	-0.18	9	Dehydroascorbic acid OR Aconitate
214.06868	$C_7H_{13}NO_5$	$[M+Na]^+$	191.079374	0.4	4	2-Amino-3,7-dideoxy-D-threo-hept-6-ulosonic acid

216.04322	$C_{10}H_{13}NO_2$	$[M+K-2H]^-$	179.094629	-0.07	13	(R)-Salsolinol OR 2(N)-Methyl-norsalsolinol
	OR	OR	OR	OR		OR
	$C_9H_{11}NO_3$	$[M+Cl]^-$	181.073894	-0.35		Tyrosine OR 3-Amino-3-(4-hydroxyphenyl)propanoate OR 4-Hydroxy-4-(3-pyridyl)-butanoic acid (>3)
244.07929	$C_8H_{15}NO_6$	$[M+Na]^+$	221.089939	0.53	4	N-Acetyl-D-glucosamine OR N-Acetyl-D-mannosamine OR N-Acetyl-β-D-galactosamine (>3)
402.09919	$C_{11}H_{22}NO_7PS$	$[M+Hac-H]^-$	343.085464	-0.32	11	N-(7-Mercaptoheptanoyl)threonine 3-O-phosphate
416.27509	$C_{24}H_{41}NO_2$	$[M+(41K)]^+$	375.313729	0.2	4	7,10,13,16-Docosatetraenoylethanolamine
441.15868	$C_{19}H_{26}O_6S$	$[M+Hac-H]^-$	382.145012	-0.42	8	2-Methoxyestradiol-17-β-3-sulfate
443.17437	$C_{19}H_{28}O_6S$	$[M+Hac-H]^-$	384.160662	-0.33	9	3β,16α-Dihydroxyandrostenone sulfate
487.19446	$C_{24}H_{32}O_9$	$[M+Na]^+$	464.204635	1.24	6	15-hydroxynorandrostene-3,17-dione glucuronide OR 16-Glucuronide-estriol OR 16-α,17-β-estriol 17-β-D-glucuronide (>3)
519.29341	$C_{27}H_{44}O_8$	$[M+Na]^+$	496.30362	1.09	7	Pregnanediol-3-glucuronide OR 3-α,20-α-dihydroxy-5-β-pregnane 3-glucuronide
Non-polar metabolic features						
365.27000	$C_{20}H_{34}O_2$	$[M+Hac-H]^-$	306.25588	0.73	10	5,8,11-Eicosatrienoic acid OR Icosatrienoic acid OR Oncobic acid (>3)
367.26432	$C_{25}H_{36}O_2$	$[M-H]^-$	368.27153	0.18	7	Didesmethyl tocotrienol
389.23347	$C_{23}H_{34}O_5$	$[M-H]^-$	390.240625	0.31	13	15R-PGA2 methyl ester OR acetate OR Digoxigenin
	OR	OR	OR	OR		OR
	$C_{21}H_{30}O_3$	$[M+Hac-H]^-$	330.219495	0.31		11-Deoxycorticosterone OR hydroxyprogesterone OR 1α-hydroxy-20-oxo-22,23,24,25,26,27-hexanorvitamin D3 / 1α-hydroxy-20-oxo-22,23,24,25,26,27-hexanorcholecalciferol (>3)
429.37301	$C_{29}H_{48}O_2$	$[M+H]^+$	428.36543	0.71	2	1-Hydroxyvitamin D5 OR (24R,24R)-Fucosterol epoxide OR (6R)-6,19-ethano-25-hydroxy-6,19-dihydrovitamin D3 / (6R)-6,19-ethano-25-hydroxy-6,19-dihydrocholecalciferol (>3)
445.36803	$C_{29}H_{48}O_3$	$[M+H]^+$	444.360345	0.92	8	11α-ethyl-1α,25-dihydroxyvitamin D3/11α-ethyl-1α,25-dihydroxycholecalciferol OR 1α,25-dihydroxy-24a,24b-dihomo-20-epivitamin D3/1α,25-dihydroxy-24a,24b-dihomo-20-epicholecalciferol OR

						1α,25-dihydroxy-24a,24b-dihomovitamin D3/1α,25-dihydroxy-24a,24b-dihomocholecalciferol (>3)
445.36881	$C_{27}H_{46}O$	$[M+Hac-H]^-$	386.354865	0.21	19	Cholesterol OR Lathosterol OR toxisterol3 R1/6,19-dihydrovitamin D3/6,19-dihydrocholecalciferol
	OR	OR	OR	OR		OR
	$C_{29}H_{50}O_3$	$[M-H]^-$	446.375995	0.21		13-hydroxy-α-tocopherol OR 24-hydroperoxy-24-vinylcholesterol OR Nebrosteroid M (>3)
524.27814	$C_{27}H_{44}NO_7P$	$[M-H]^-$	525.285542	-0.24	9	LysoPE(0:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z)) OR LysoPE(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/0:0) (>3)
537.37957	$C_{29}H_{50}O_5$	$[M+Hac-H]^-$	478.365825	-0.2	6	1α,25-dihydroxy-2α-(3-hydroxypropoxy)-19-norvitamin D3 / 1α,25-dihydroxy-2α-(3-hydroxypropoxy)-19-norcholecalciferol OR 1α,25-dihydroxy-2β-(3-hydroxypropoxy)-19-norvitamin D3 / 1α,25-dihydroxy-2β-(3-hydroxypropoxy)-19-norcholecalciferol (>3)
715.57588	$C_{40}H_{81}N_2O_6P$	$[M-H]^-$	716.583226	-0.1	11	SM(d18:1/17:0) OR SM(d19:1/16:0)
733.59866	$C_{43}H_{78}O_5$	$[M+Hac-H]^-$	674.584925	-0.16	9	DG(18:1(9Z)/22:2(13Z,16Z)/0:0) OR DG(18:2(9Z,12Z)/22:1(13Z)/0:0) OR DG(18:3(6Z,9Z,12Z)/22:0/0:0) (>3)
745.59421	$C_{45}H_{84}O_5$	$[M+(41K)]^+$	704.631875	1.42	4	DG(18:1(11Z)/24:1(15Z)/0:0) OR DG(20:0/22:2(13Z,16Z)/0:0) OR DG(24:1(15Z)/18:1(9Z)/0:0) (>3)
761.66639	$C_{48}H_{90}O_6$	$[M-H]^-$	762.67374	-0.1	6	TG(12:0/13:0/20:1(11Z))iso6 OR TG(12:0/14:0/19:1(9Z))iso6 OR TG(12:0/14:1(9Z)/19:0)iso6 (>3)

(>3); indicates more than three possible putative annotations, OR; indicates more than one possible empirical formulae or multiple adducts.

Appendix I. Metabolic features assigned empirical formulae

Table I.1Empirical formulae ascribed to the polar metabolic features detected in positive and negative ionisation mode which were significantly altered (p<0.05) when effect size between ΔPA and ΔHIE was examined, and for which a putative annotation could not be ascribed. (See Chapter 3)

m/z	Intensity	Empirical formula	Ion	Theoretical mass (Da)	Mass error (ppm)
		Positive ionisation mode			
359.03024	79111.2	$C_3H_{15}N_{10}OP_3$[NaCl]	$[M+H]^+$ $[M+H]^+$	358.022942	0.060.09
(>5)		$C_8H_7N_{12}OPCa$	$[M+H]^+$ $[M+H]^+$	358.022932	0.110.12
		$C_9H_{18}N_3O_6PCu$	$[M+H]^+$	358.022924	0.16
		$C_{11}H_{20}N_2OS_2Ca$[NaCl]		358.022921	
		$C_{12}H_{21}N_2OPS_2Fe$		358.022907	
380.90488	39007.8	$C_6H_7N_4O_5P_3SCa$	$[M+H]^+$ $[M+H]^+$	379.897598	0.010.06
(>5)		$C_5HN_{10}OPSCa$[Na(37Cl)]	$[M+H]^+$ $[M+H]^+$	379.897579	0.070.08
		$C_5H_6N_9P_3SCu$	$[M+H]^+$	379.897576	0.09
		$C_5H_{12}N_2O_5S_5Ca$		379.897574	
		$C_8H_{14}OS_3Ca$[NaCl][Na(37Cl)]		379.897568	
386.80562	47843.4	$C_7H_2O_8S_2Fe_2$	$[M+H]^+$ $[M+H]^+$	385.798338	0.010.01
(>5)		$C_3H_6N_2O_2P_4S_5$ $C_4H_2N_4OP_2S_2Ca_2$[NaCl]	$[M+H]^+$ $[M+H]^+$	385.79834	0.020.06
		$C_5H_3N_4OP_3S_2CaFe$	$[M+H]^+$	385.798336	0.09
		$C_4H_7NO_2S_2CaCu$[NaCl][Na(37Cl)]		385.798322	
				385.798309	
471.04405	35227.9	$C_7H_{17}N_{14}O_3PCaFe$	$[M+H]^+$ $[M+H]^+$	470.036772	0
(>5)		$C_{13}H_{14}N_{12}SCa$[Na(37Cl)]	$[M+H]^+$ $[M+H]^+$	470.036774	0
		$C_{14}H_{25}N_3O_5SCu$[Na(37Cl)]	$[M+H]^+$	470.036766	0.020.04
		$C_{20}H_{16}N_4O_4P_2S$		470.036754	0.05
		$C_{17}H_{28}N_2S_3Fe$[Na(37Cl)]		470.036749	
263.12538	46559.6	$C_{13}H_{20}O_4$	$[M+Na]^+$ $[M+H]^+$	240.13616	-
		$C_{11}H_{14}N_6O_2$	$[M+H]^+$	262.117824	0.011.06
		$C_5H_{18}N_8O_2Ca$		262.117863	0.91
324.10306	131160.5	$C_7H_{19}N_9P_2S$	$[M+H]^+$ $[M+H]^+$	323.095939	-0.48
(>5)		$C_{13}H_{23}N_3OSFe$	$[M+H]^+$ $[M+H]^+$	323.095796	-
		$C_{12}H_{22}NO_5PS$	$[M+H]^+$	323.095634	0.040.46
		$C_5H_{19}N_9O_4Fe$		323.095613	0.530.71
		$C_{10}H_{22}N_3O_3P$[Na(37Cl)]		323.095553	
338.11872	125161.3	$C_8H_{21}N_9P_2S$	$[M+H]^+$ $[M+H]^+$	337.111589	-0.43
(>5)		$C_{14}H_{25}N_3OSFe$	$[M+H]^+$ $[M+H]^+$	337.111446	-
		$C_{13}H_{24}NO_5PS$	$[M+H]^+$	337.111284	0.010.47
		$C_6H_{21}N_9O_4Fe$		337.111263	0.530.71
		$C_{11}H_{24}N_3O_3P$[Na(37Cl)]		337.111203	
445.16951	11881.2	$C_{17}H_{38}N_2OP_2S_3$	$[M+H]^+$ $[M+H]^+$	444.162155	0.180.19
(>5)		$C_{18}H_{34}N_4Ca_2$[NaCl]	$[M+H]^+$ $[M+H]^+$	444.162151	0.22
		$C_{14}H_{29}N_4O_{10}P$	$[M+H]^+$	444.162134	0.22
		$C_{19}H_{35}N_4PCaFe$		444.162137	0.27

		$C_{24}H_{30}N_2Ca[NaCl]$		444.162112	
447.06506	28140.5	$C_{10}H_{22}O_{17}S$	$[M+H]^+$ $[M+H]^+$	446.057777	0.01
(>5)		$C_{15}H_{28}O_7SCaFe$	$[M+H]^+$ $[M+H]^+$	446.05778	0.01
		$C_{13}H_{29}N_4P_3SCa_2$	$[M+H]^+$	446.057764	0.04
		$C_8H_{10}N_{14}O_7S$		446.057763	0.05
		$C_{14}H_{27}N_5O_2SFeCu$		446.057758	0.06
469.04711	35692.1	$C_{17}H_{27}NO_4S_3Cu$	$[M+H]^+$ $[M+H]^+$	468.039824	0.02
(>5)		$C_8H_{15}N_{10}O_8P[NaCl]$ $C_9H_{26}N_8OP_2S_2Ca_2$	$[M+H]^+$ $[M+H]^+$	468.039821	0.03
		$C_{18}H_{26}N_2OP_2Ca_3$	$[M+H]^+$	468.039809	0.05
		$C_{15}H_{28}N_2O_4Fe_2[Na(37Cl)]$		468.039812	0.05
				468.039805	0.06
497.00216	18699.1	$C_{15}H_{25}N_4O_2PS_2Fe_2$	$[M+H]^+$ $[M+H]^+$	495.994882	0
(>5)		$C_{16}H_6N_{12}O_2Ca[NaCl]$ $C_{10}H_{23}N_9P_4CaCu$	$[M+H]^+$ $[M+H]^+$	495.994882	0
		$C_{10}H_{18}N_{10}OP_2Ca_2[Na(37Cl)]$	$[M+H]^+$	495.994883	0
		$C_{17}H_{17}N_3O_7Cu[NaCl]$		495.994886	0
				495.994874	0.02
541.27509	51117.5	$C_{17}H_{46}N_{10}OP_2SCa$	$[M+H]^+$ $[M+H]^+$	540.267794	0.04
(>5)		$C_{23}H_{42}N_8OP_2S$	$[M+H]^+$ $[M+H]^+$	540.267755	0.11
		$C_{24}H_{48}O_7S[Na(37Cl)]$	$[M+H]^+$	540.26775	0.12
		$C_{28}H_{52}CaFe[NaCl]$		540.267726	0.16
		$C_{23}H_{46}O_{10}[NaCl]$		540.267723	0.17
		Negative ionisation mode			
381.01801	20078.5	$C_{13}H_{25}PSCa_2$	$[M-H]^-$	382.025265	0.06
(>5)		$C_{14}H_{26}P_2SCaFe$	$[M-H]^-$	382.025251	0.09
		$C_9H_{20}O_{10}P_2S$	$[M-H]^-$	382.025248	0.1
		$C_7H_8N_{14}P_2S$	$[M-H]^-$	382.025234	0.14
		$C_{10}H_{21}N_4PS_3$	$[M-H]^-$	382.025223	0.17
463.02103	49254.7	$C_{15}H_{27}N_3P_2SCu$	$[M-H]^-$	464.028317	-0.02
(>5)		$C_{20}H_{19}N_5SCaCu$	$[M-H]^-$	464.028307	0
		$C_{10}H_{26}N_6O_4P_2Fe_2$	$[M-H]^-$	464.028304	0.01
		$C_{11}H_{19}N_{11}S_3Cu$	$[M-H]^-$	464.028304	0.01
		$C_{18}H_{16}N_4O_5S_3$	$[M-H]^-$	464.028287	0.04
545.02366	44075.1	$C_{19}H_{31}N_3O_3Ca_2FeCu$ $C_9H_{23}N_{10}O_8P_3Fe$	$[M-H]^-$	546.030935	0
(>5)		$C_{14}H_{25}N_3O_{13}CaCu$	$[M-H]^-$	546.030936	0
		$C_{15}H_{31}O_9PS_5$	$[M-H]^-$	546.030932	0.01
		$C_7H_{21}N_{15}O_3P_2Cu$	$[M-H]^-$	546.030933	0.01
			$[M-H]^-$	546.030928	0.02
187.00699	126669.4	$C_7H_8O_4S$	$[M-H]^-$ $[M+Cl]^-$	188.014332	-0.35
		$C_4H_8N_4Ca$	$[M+Cl]^-$	152.037487	0.54
		$C_{10}H_4N_2$	$[M+Hac-H]^-$	152.037448	0.75
		$C_5H_4O_2S$		127.993202	-0.35
384.80864	11903.6	$C_4H_4N_6O_3CaFe_3$	$[M-H]^-$	385.815916	0
(>5)		$C_4H_2N_2O_7P_4SCa$	$[M-H]^-$	385.815918	0
		$C_7H_2N_2O_4S_3Fe$	$[M-H]^-$	385.815909	0.02
		$C_7H_7N_3OCa_3FeCu$	$[M-H]^-$	385.815896	0.05
		$C_3HN_7O_2P_4SCu$	$[M-H]^-$	385.815896	0.05
131.09052	8035.4	-	-	-	-
224.06376	29701.9	$C_4H_{11}N_5O_6$	$[M-H]^-$	225.070935	0.45
		$C_8H_{16}N_3PCa$	$[M-H]^-$	225.070776	1.16
		$C_2H_7N_5O_4$	$[M+Hac-H]^-$	165.049805	0.45
295.00472	23895.9	$C_{10}H_{14}N_2O_3SFe$	$[M-H]^-$	296.012127	-0.44
(>5)		$C_7H_{18}N_3PCu$	$[M-H]^-$	296.012057	-0.21
		$C_9H_{13}O_7PS$	$[M-H]^-$	296.011965	0.11
		$C_2H_{10}N_8O_6Fe$	$[M-H]^-$	296.011944	0.18
		$C_5H_{14}N_6Ca_2$	$[M-H]^-$	296.011799	0.67

318.95238	12224.8	$C_7H_{14}N_4Fe_2$	$[M-H]^-$	319.959693	-0.11
(>5)		$C_5HN_{10}O_3PCa$	$[M-H]^-$	319.959664	-0.02
		$C_6H_{12}NO_8PCu$	$[M-H]^-$	319.959656	0
		$C_8H_{14}O_3S_2Ca$	$[M-H]^-$	319.959653	0.01
		$C_9H_{15}O_3PS_2Fe$	$[M-H]^-$	319.959639	0.05
371.04529	29596.3	$C_{10}H_{23}N_4OP_3S_2$	$[M-H]^-$	372.052619	-0.14
(>5)		$C_8H_{15}N_{10}PS$	$[M-H]^-$	372.052573	-0.02
		$C_6H_{17}N_{10}O_2PCa_2$	$[M-H]^-$	372.05254	0.07
		$C_7H_{21}N_6O_2P_3$	$[M-H]^-$	372.052511	0.15
		$C_{12}H_{13}N_8O_2PCa$	$[M-H]^-$	372.052501	0.18
450.11248	10875.8	$C_{16}H_{33}N_5OSFe_2$	$[M-H]^-$	451.119806	-0.11
(>5)		$C_9H_{28}N_9O_4P_3S$	$[M-H]^-$	451.119787	-0.07
		$C_{17}H_{33}NO_4S_3Ca$	$[M-H]^-$	451.119766	-0.02
		$C_7H_{20}N_{15}O_3P$	$[M-H]^-$	451.119741	0.03
		$C_{14}H_{31}N_3O_5SCa$	$[M-H]^-$	451.119658	0.22
476.81948	12749.9	$C_4H_2N_{12}O_2P_2SCa_2Fe$	$[M-H]^-$	477.82676	-0.01
(>5)		$C_{20}H_2N_4FeCu_2$	$[M-H]^-$	477.826756	0
		$C_{10}H_{10}N_2O_3S_6Ca_2$	$[M-H]^-$	477.826757	0
		$C_{15}H_6N_4S_4Fe_2$	$[M-H]^-$	477.826758	0
		$C_{11}H_{15}O_3P_3S_2Cu_2$	$[M-H]^-$	477.826751	0.01
489.0481	7754.1	$C_{18}H_{31}N_2PSCaFe$	$[M-H]^-$	490.055384	-0.02
(>5)		$C_{13}H_{25}N_2O_{10}PS$	$[M-H]^-$	490.055381	-0.01
		$C_{28}H_{17}N_2OP_3$	$[M-H]^-$	490.055377	0
		$C_{19}H_{32}N_2P_2SFe_2$	$[M-H]^-$	490.05537	0.01
		$C_6H_{22}N_{10}O_9Fe$	$[M-H]^-$	490.05536	0.03
543.26307	37401.2	$C_{19}H_{40}N_6O_{12}$	$[M-H]^-$	544.270424	-0.14
(>5)		$C_{30}H_{42}N_4O_2Fe$	$[M-H]^-$	544.270388	-0.08
		$C_{19}H_{47}N_6O_4PS$	$[M-H]^-$	544.270337	0.02
		$C_{23}H_{45}N_4O_6PCa$	$[M-H]^-$	544.270265	0.15
		$C_{22}H_{44}N_9OPCu$	$[M-H]^-$	544.270243	0.19

(>5); indicates more than five possible empirical formulae.

Table I.2 Empirical formulae ascribed to the non-polar metabolic features detected in positive and negative ionisation mode which were significantly altered (p<0.05) when effect size between ΔPA and ΔHIE was examined, and for which a putative annotation could not be ascribed.(See Chapter 4)

m/z	Intensity	Empirical formula	Ion	Theoretical mass (Da)	Mass error (ppm)
		Negative ionisation mode			
393.29724	5714.3	$C_{17}H_{38}N_2O_4$	$[M+Hac-H]^-$	334.283158	0.58
(>5)		$C_{15}H_{38}N_{10}$	$[M+Cl]^-$	358.32809	-0.64
		$C_{23}H_{47}PCa$	$[M-H]^-$	394.304129	0.99
		$C_{19}H_{42}N_2O_6$	$[M-H]^-$	394.304288	0.58
		$C_{14}H_{39}N_{10}OP$	$[M-H]^-$	394.304593	-0.19
409.36877	16988.7	$C_{24}H_{46}O$	$[M+Hac-H]^-$	350.354865	0.13
		$C_{26}H_{50}O_3$	$[M-H]^-$	410.375995	0.13
435.38442	10961.5	$C_{26}H_{48}O$	$[M+Hac-H]^-$	376.370515	0.12
		$C_{25}H_{55}N_2P$	$[M+Na-2H]^-$	414.410286	-1.23
		$C_{28}H_{52}O_3$	$[M-H]^-$	436.391645	0.12
443.35311	11877.6	$C_{27}H_{44}O$	$[M+Hac-H]^-$	384.339215	0.09
		$C_{26}H_{51}N_2P$	$[M+Na-2H]^-$	422.378986	-1.23
		$C_{29}H_{48}O_3$	$[M-H]^-$	444.360345	0.09
		$C_{22}H_{48}N_6OS$	$[M-H]^-$	444.361031	-1.45
454.39828	14828.5	$C_{22}H_{49}N_9O$	$[M-H]^-$	455.406006	-0.99
455.33786	8604.5	$C_{21}H_{45}N_8OP$	$[M-H]^-$	456.345395	-0.57
(>5)		$C_{24}H_{44}O_4$	$[M+Hac-H]^-$	396.32396	0.1
		$C_{26}H_{48}O_6$	$[M-H]^-$	456.34509	0.1
		$C_{18}H_{46}N_{10}S$	$[M+Na-2H]^-$	434.362762	0.94
		$C_{15}H_{46}N_{12}P_2$	$[M-H]^-$	456.344364	1.7
466.39826	5911.6	$C_{25}H_{55}N_3O_3$	$[M+Na-2H]^-$	445.424342	-1.61
		$C_{17}H_{50}N_{13}P$	$[M-H]^-$	467.404975	1.2
		$C_{23}H_{49}N_9O$	$[M-H]^-$	467.406006	-1.01
541.33341	113779.1	$C_{18}H_{45}N_{14}O_2P$	$[M+Na-2H]^-$	520.358754	-0.02
(>5)		$C_{24}H_{51}O_5PS$	$[M+Hac-H]^-$	482.319485	0.13
		$C_{26}H_{55}O_7PS$	$[M-H]^-$	542.340615	0.13
		$C_{31}H_{52}O_4S$	$[M+Na-2H]^-$	520.358632	0.2
		$C_{29}H_{51}N_2O_3P$	$[M+Cl]^-$	506.363731	0.51
556.46601	7558.3	$C_{25}H_{55}N_9O$	$[M+Hac-H]^-$	497.452956	-1.44
(>5)		$C_3HP_5CaFe_3Cu_2$	$[M+K-2H]^-$	519.517265	0.25
		$C_{23}H_{61}N_{13}$	$[M+K-2H]^-$	519.517287	0.21
		$C_{22}H_{59}N_{13}O$	$[M+Cl]^-$	521.496552	0.1
		$C_{27}H_{59}N_9O_3$	$[M-H]^-$	557.474086	-1.44
579.46286	6777.1	$C_{30}H_{61}N_8OP$	$[M-H]^-$	580.470595	-0.79
(>5)		$C_{33}H_{60}O_4$	$[M+Hac-H]^-$	520.44916	-0.27
		$C_{27}H_{62}N_{10}S$	$[M+Na-2H]^-$	558.487962	0.4
		$C_4HP_5Ca_2Fe_2Cu_3$	$[M-H]^-$	580.469843	0.51
		$C_{24}H_{62}N_{12}P_2$	$[M-H]^-$	580.469564	0.99
673.50513	7737.5	$C_5H_4O_9P_6FeCu_3$	$[M+K-2H]^-$	636.556522	0
(>5)		$C_{10}H_3N_3OS_4CaFe_3Cu_2$	$[M+K-2H]^-$	636.556525	0
		$C_6HNO_4P_2S_6Ca_3Fe[NaCl]$	$[M+K-2H]^-$	636.556525	0
		$C_3H_5N_4O_2P_5SCa_2FeCu_3$	$[M+Cl]^-$	638.535729	0
		$C_5H_4N_3OP_7SCa_3Fe_3$	$[M+Na-2H]^-$	652.530459	0
739.51108	79913.9	$C_8H_6NO_{10}PSFe_4Cu_2[Na(37Cl)]$	$[M-H]^-$	740.518328	0.04

(>5)		$N_{10}O_7P_3Ca_3Fe_4$[Na(37Cl)]	$[M-H]^-$	740.518328	0.04
		$C(13C)H_2N_2O_{11}P_5S_6CaCu$[Na(37Cl)]	$[M-H]^-$	740.518328	0.04
		$C_{10}(13C)H_6NP_2S_4(34S)Ca_3Cu_2$[NaCl][Na(37Cl)]	$[M-H]^-$	740.518328	0.04
		$C_{10}(13C)HN_7P_4S(34S)CaFe_4Cu$	$[M-H]^-$	740.518328	0.04
762.50769	261017.8	$C_9(13C)H_4N_2O_3P_7S(34S)Fe_3$[NaCl][Na(37Cl)]	$[M-H]^-$	763.514961	0.01
(>5)		$C_6H_3N_7O_5(34S)Ca_2Fe_4Cu$[NaCl][Na(37Cl)]	$[M-H]^-$	763.514961	0.01
		$C_{13}HN_6OP_2S(34S)CaFe_4Cu$[Na(37Cl)]	$[M-H]^-$	763.514961	0.01
		$C_8(13C)H_4NO_5P_6S_3(34S)Ca_3Cu$[NaCl]	$[M-H]^-$	763.514961	0.01
		$C_2(13C)H_8N_5O_7P_4(34S)CaFe_4Cu$[Na(37Cl)]	$[M-H]^-$	763.514961	0.01
763.51109	122012.4	$H_9N_9O_8PS(34S)Fe_4Cu_3$	$[M-H]^-$	764.518359	0.01
(>5)		$C_7N_5O_8P_2(34S)Ca_2Cu_3$[NaCl][Na(37Cl)]	$[M-H]^-$	764.518359	0.01
		$C_{13}NO_{11}P_2Fe_2Cu_3$[Na(37Cl)]	$[M-H]^-$	764.518359	0.01
		$C_9H_6N_7OP_2S_2Fe_3Cu_3$[Na(37Cl)]	$[M-H]^-$	764.518359	0.01
		$C_5(13C)H_5O_{15}S(34S)Ca_2FeCu_3$[NaCl]	$[M-H]^-$	764.518359	0.01
864.60728	262092.8	$C_3(13C)H_{14}N_6O_{17}PS_3Ca_2FeCu_2$[Na(37Cl)]	$[M-H]^-$	865.614548	0.01
(>5)		$C_{14}(13C)H_{17}N_3O_7P_6(34S)Fe_2Cu_2$[Na(37Cl)]	$[M-H]^-$	865.614548	0.01
		$C_{12}H_8N_5O_{15}P_3S_2Cu_3$[NaCl]	$[M-H]^-$	865.614548	0.01
		$C_5H_{27}NO_{12}P_4S_4(34S)CaCu_3$[NaCl]	$[M-H]^-$	865.614548	0.01
		$C_{12}H_{25}O_8P_6S_5(34S)Cu_3$	$[M-H]^-$	865.614548	0.01
851.56552	11850.3	$CH_{25}N_8O_7P_5SFe_4Cu_3$	$[M-H]^-$	852.572754	0.05
(>5)		$C_{10}(13C)H_6N_{12}P_5S_2Ca_2Cu_3$[NaCl]	$[M-H]^-$	852.572754	0.05
		$C_{18}(13C)H_{10}N_4O_2PS_5(34S)FeCu_3$[NaCl]	$[M-H]^-$	852.572754	0.05
		$C_2(13C)H_{27}N_5O_4P_4S_3Ca_2Fe_2Cu_3$[NaCl]	$[M-H]^-$	852.572754	0.05
		$C_{13}(13C)H_{18}N_4PS_2(34S)Ca_3FeCu_3$[NaCl][Na(37Cl)]	$[M-H]^-$	852.572754	0.05
879.59676	126974.4	$C_{27}H_3N_2O_8P_2(34S)Ca_2Fe_3$[Na(37Cl)]	$[M-H]^-$	880.604028	0.01
(>5)		$C_{12}(13C)H_{16}N_7O_4P_4S(34S)Ca_2Fe_4$[Na(37Cl)]	$[M-H]^-$	880.604028	0.01
		$C_{18}H_3N_{11}OP_5S_5(34S)Ca_2Cu$	$[M-H]^-$	880.604028	0.01
		$C_6H_{13}N_{15}O_2PS_5Ca_2Fe_3Cu$[NaCl]	$[M-H]^-$	880.604028	0.01
		$C_{11}(13C)H_3N_{13}O_6S_6(34S)Fe_2Cu$[NaCl]	$[M-H]^-$	880.604028	0.01
918.54751	19151.4	$C(13C)H_{34}N_6O_5P_6S(34S)CaFe_4Cu_3$	$[M-H]^-$	919.554778	0.01
(>5)		$C_8(13C)H_{25}N_2O_5P_7(34S)Ca_3Cu_3$[NaCl][Na(37Cl)]	$[M-H]^-$	919.554778	0.01
		$C_{19}(13C)H_{16}N_4P_2S_3(34S)Fe_2Cu_3$[NaCl][Na(37Cl)]	$[M-H]^-$	919.554778	0.01
		$C_{19}(13C)H_3N_2O_{13}P_6(34S)Fe_3$[NaCl]	$[M-H]^-$	919.554778	0.01
		$C_{14}H_{25}NO_5P_6S_4CaFe_4Cu$	$[M-H]^-$	919.554778	0.01

Positive ionisation mode

327.22714	8097.9	$C_{21}H_{34}$	$[M+(41K)]^+$	286.26605	-0.57
		$C_{13}H_{32}N_6O$	$[M+K]^+$	288.263759	0.68
		$C_{16}H_{28}N_6$	$[M+Na]^+$	304.237544	1.14
		$C_{11}H_{31}N_6O_3P$	$[M+H]^+$	326.219527	1.03
		$C_{19}H_{35}PS$	$[M+H]^+$	326.21971	0.47
409.23024	4300.5	$C_{19}H_{33}N_6PS$	$[M+H]^+$	408.222504	1.12
(>5)		$C_{14}H_{34}N_6O_4$	$[M+H]^+$	408.222777	0.46
		$C_{15}H_{36}N_6OS$	$[M+H]^+$	408.222804	0.39
		$C_{17}H_{36}N_4O_3S_2$	$[M+H]^+$	408.222885	0.19
		$C_{22}H_{38}OS$	$[M+H]^+$	408.22296	0.01
926.79986	60903.6	$C_2H_{36}N_9O_{15}P_4S_8Ca_3$	$[M+H]^+$	925.792492	0.1
(>5)		$C_{12}H_{41}N_3O_{12}P_7S_4Fe_3$	$[M+H]^+$	925.792492	0.1
		$C_{17}H_{28}N_2O_{18}P_5S_5Cu$	$[M+H]^+$	925.792492	0.1
		$C_{28}H_{20}O_{17}P_4Ca_3Fe$	$[M+H]^+$	925.792492	0.1
		$C_2H_{36}N_9O_{15}P_4S_8Ca_3$	$[M+H]^+$	925.792492	0.1
1290.19044	235573.2	$C_{24}H_2NO_{14}P_3S_7CaFe_4Cu_3$	$[M+H]^+$	1289.183163	0
(>5)		$C_{25}N_4O_7P_5S_8(34S)Ca_2Fe_2Cu_3$	$[M+H]^+$	1289.183163	0
		$C_{23}HN_2O_{15}P_6(34S)Ca_3Fe_4Cu_3$	$[M+H]^+$	1289.183162	0
		$C_{28}N_4O_3P_7S_8Ca_2Fe_2Cu_3$	$[M+H]^+$	1289.183161	0
		$C_{32}HO_6P_7S_3(34S)Ca_3Fe_4Cu_2$	$[M+H]^+$	1289.183159	0

1342.22159	398598.5	$C_{13}H_{13}N_6O_{15}P_6S_6Ca_3Fe_3Cu_3$	$[M+H]^+$	1341.21431	0
(>5)		$C_{31}N_8O_3P_4S_7Ca_3Fe_4Cu_2$	$[M+H]^+$	1341.214312	0
		$C_{25}HN_5O_{11}P_7S_7Ca_3Fe_2Cu_2$	$[M+H]^+$	1341.2143	0.01
		$C_{18}(13C)H_4N_6O_{14}P_6S_8Ca_2Fe_4Cu$	$[M+H]^+$	1341.214292	0.02
		$C_{12}N_{14}O_{14}P_7SCa_3Fe_3Cu_3[NaCl]$	$[M+H]^+$	1341.214288	0.02

(>5); indicates more than five possible empirical formulae.

Table I.3 Empirical formulae ascribed to the polar metabolic features detected in positive and negative ionisation mode which were significantly altered (p<0.05) between haemolysed and clean cord blood serum pairs, for which a putative annotation could not be ascribed.(See Chapter 7)

Mass	Intensity	Empirical formula	Ion	Theoretical mass (Da)	Mass error (ppm)	p-value
		Positive ionisation mode				
119.08964	10679.7	-	-	-	-	0.008
121.97442	16227.9	C_2H_3N	$[M+Na]^+$	98.985172	0.22	0.039
132.10277	3364.7	-	-	-	-	0.039
132.98724	10991.6	C_4H_5OPS	$[M+H]^+$	131.979875	0.67	0.031
135.00289	981662.1	C_4H_7OPS	$[M+H]^+$	133.995525	0.66	0.008
136.00625	34843.7	CH_5N_3O	$[M+H]^+$	134.998935	0.28	0.039
		$C_3H_5NO_3S$	$[M+H]^+$	134.999016	-0.31	
137.00713	7367.9	$C_2H_2N_4O_2$	$[M+Na]^+$	114.017776	0.97	0.039
		C_3H_9P	$[M+H]^+$	135.999861	-0.05	
143.05083	4859.2	-	-	-	-	0.023
149.01854	127171	C_5H_9OPS	$[M+H]^+$	148.011175	0.59	0.008
158.96412	872696.7	CH_5N_2P	$[M+Na]^+$	135.974709	1.19	0.023
		$C_3H_5O_2PS$	$[M+Na]^+$	135.97479	0.68	
		C_4H_4OS	$[M+H]^+$	157.95691	-0.42	
159.96747	19742.8	$CH_4N_3O_2P$	$[M+K]^+$	121.004115	1.22	0.008
		$C_2H_3NO_4S$	$[M+Na]^+$	136.978281	-0.2	
163.1094	21273.2	$C_9H_{16}O$	$[M+Na]^+$	140.120115	0.39	0.016
164.02947	114231.9	$C_5H_{10}NOPS$	$[M+H]^+$	163.022074	0.73	0.008
164.93006	29686.2	$CH_4O_2P_2S$	$[M+Na]^+$	141.940728	0.67	0.008
		$C_5H_2Ca_2$	$[M+Na]^+$	141.940832	0.04	
		C_2H_3OPS	$[M+H]^+$	163.922848	-0.39	
165.01348	87645.9	$C_7H_{10}S$	$[M+K]^+$	126.050322	-0.01	0.023
		$C_3H_9N_2P$	$[M+H]^+$	164.006009	1.18	
		$C_5H_9O_2PS$	$[M+H]^+$	164.00609	0.69	
168.07128	7912.9	-	-	-	-	0.008
179.06211	5988.9	$C_{10}H_{11}OP$	$[M+H]^+$	178.054753	0.45	0.047
		$C_6H_{14}O_2$	$[M+H]^+$	178.055053	-1.23	
188.06935	7595.6	$C_7H_{17}NS$	$[M+(41K)]^+$	147.108171	-0.52	0.023
		$C_5H_{10}N_5OP$	$[M+H]^+$	187.062298	-1.19	
191.04038	544488.1	$C_8H_{10}N_2O$	$[M+(41K)]^+$	150.079313	-1.1	0.008
		$C_6H_{11}N_2OPS$	$[M+H]^+$	190.032973	0.68	
191.07677	219541.1	$C_9H_{14}N_2$	$[M+(41K)]^+$	150.115698	-1.07	0.023
		$C_7H_{15}N_2PS$	$[M+H]^+$	190.069358	0.71	
192.04374	34415.2	$C_3H_9N_5O$	$[M+H]^+$	191.036383	0.42	0.023
		$C_5H_9N_3O_3S$	$[M+H]^+$	191.036464	0	
193.0448	33370.5	$C_9H_{12}O_2$	$[M+(41K)]^+$	152.08373	-1.07	0.008
		$C_9H_{14}S$	$[M+K]^+$	154.081622	0.1	
		$C_5H_{13}N_2P$	$[M+H]^+$	192.037309	1.11	
		$C_7H_{13}O_2PS$	$[M+H]^+$	192.03739	0.69	
194.95861 (>5)	481777.6	$C_5H_6O_2$	$[M+K]^+$	155.995403	0.24	0.039
		$C_5H_8S_2Ca$	$[M+Na]^+$	171.969335	0.27	

		C_4H_7OPS	$[M+H]^+$	193.951198	0.7	
		$C_4H_6N_2Fe$	$[M+H]^+$	193.951333	0	
		$C_4H_5NO_4Cu$	$[M+H]^+$	193.951458	-0.64	
198.10434	13059.4	$C_{10}H_{16}NOP$	$[M+H]^+$	197.096952	0.56	0.023
200.97471	32010.3	$C_3H_7N_2OP$	$[M+Na]^+$	177.985274	1.07	0.008
		$C_5H_7O_3PS$	$[M+Na]^+$	177.985355	0.66	
		$C_4H_6N_2$	$[M+H]^+$	199.967394	0.2	
		$C_6H_6O_2S$	$[M+H]^+$	199.967475	-0.21	
202.1187	31109.8	$C_8H_{15}N_3O_3$	$[M+H]^+$	201.111342	0.4	0.039
202.99036	1839740	$C_5H_2N_6O$	$[M+(41K)]^+$	162.029009	0.37	0.016
(>5)		$C_3H_9N_2OP$	$[M+Na]^+$	180.000924	1.06	
		$C_5H_9O_3PS$	$[M+Na]^+$	180.001005	0.66	
		$C_4H_8N_2$	$[M+H]^+$	201.983044	0.2	
		$C_6H_8O_2S$	$[M+H]^+$	201.983125	-0.2	
203.05101	133101.6	$C_9H_{16}Ca$	$[M+K]^+$	164.087791	0.29	0.039
		$C_3H_4N_{10}$	$[M+Na]^+$	180.06204	-1.24	
		$C_7H_{15}O_2PCa$	$[M+H]^+$	202.043559	0.86	
		$C_3H_{10}N_2O_8$	$[M+H]^+$	202.043718	0.08	
203.99371	79387.9	$C_4H_7NO_5S$	$[M+Na]^+$	181.004496	-0.04	0.016
(>5)		$C_4H_9N_3S_2Ca$	$[M+H]^+$	202.986382	0.25	
		$C_2H_7N_3O_3$	$[M+Na]^+$	181.004415	0.36	
		$C_{10}H_5NS_2$	$[M+H]^+$	202.986343	0.44	
		$C_2H_8N_5OPFe$	$[M+H]^+$	202.98626	0.85	
204.05604	1281409	$C_5H_{11}N_5P_2$	$[M+H]^+$	203.048971	-1.02	0.023
204.05892	37545.5	$C_4H_{11}N_3O_5$	$[M+Na]^+$	181.069872	-0.85	0.016
		$C_{10}H_9N_3S$	$[M+H]^+$	203.051719	-0.37	
		$C_4H_{13}N_5SCa$	$[M+H]^+$	203.051758	-0.56	
204.91861	12374.7	$C_3H_4O_2SCa$	$[M+H]^+$	203.911466	-0.65	0.016
(>5)		$C_2H_2O_5Ca$	$[M+H]^+$	203.911439	-0.51	
		$C_2H_4N_2O_2CaFe$	$[M+Na]^+$	181.929481	-0.45	
		$C_3H_3O_5PFe$	$[M+H]^+$	203.911425	-0.45	
		$C_7HOPSCa$	$[M+H]^+$	203.911166	0.82	
205.06653	30834.4	$C_9H_{18}Ca$	$[M+K]^+$	166.103441	-0.34	0.039
208.96771	324544.8	$C_4H_7N_3OSCu$	$[M+H]^+$	207.960583	-0.71	0.039
(>5)		$C_6H_9PS_3$	$[M+H]^+$	207.960404	0.14	
		C_9H_6OCa	$[M+K]^+$	170.004456	0.45	
		$C_3H_7N_2OPS$	$[M+H]^+$	207.960296	0.66	
		$C_6H_6N_2Ca_2$	$[M+Na]^+$	185.97828	1	
214.05644	20774.2	$C_{10}H_{11}N_3$	$[M+(41K)]^+$	173.095297	-0.62	0.031
		$C_8H_{12}N_3PS$	$[M+H]^+$	213.048957	0.97	
		$C_6H_{15}NO_3S_2$	$[M+H]^+$	213.049338	-0.81	
217.00602	168480	$C_4H_{11}N_2OP$	$[M+Na]^+$	194.016574	1.03	0.008
(>5)		$C_6H_{11}O_3PS$	$[M+Na]^+$	194.016655	0.66	
		$C_5H_{10}N_2$	$[M+H]^+$	215.998694	0.23	
		$C_2H_8N_4O_4S_2$	$[M+H]^+$	215.9987	0.2	
		$C_7H_{10}O_2S$	$[M+H]^+$	215.998775	-0.14	
217.04486	9452.6	$C_{11}H_{12}O_2$	$[M+(41K)]^+$	176.08373	-0.67	0.039
(>5)		$C_3H_{10}N_6O_3$	$[M+K]^+$	178.081439	1.21	
		$C_{11}H_{14}S$	$[M+K]^+$	178.081622	0.36	
		$C_2H_{10}N_8OFe$	$[M+H]^+$	216.037369	0.99	
		$C_9H_{13}O_2PS$	$[M+H]^+$	216.03739	0.89	
217.92613	65528.1	$C_3H_5NOS_2$	$[M+Na]^+$	194.936931	-0.1	0.008
(>5)		$C_2H_3NO_4S$	$[M+Na]^+$	194.936904	0.02	
		$C_4H_6NOPS_2$	$[M+K]^+$	178.962846	0.57	

		$C_6H_3NOSCa_2$	$[M+H]^+$	216.918718	0.62	
		$CH_4N_3O_2P$	$[M+K]^+$	178.962738	1.07	
218.91982	967787.2	C_4H_4OS	$[M+H]^+$	217.912583	-0.18	0.008
(>5)		C_9HNSCu	$[M+H]^+$	217.91257	-0.12	
		$C_3H_4N_2OFe$	$[M+Na]^+$	195.930598	0	
		$C_3H_5O_2PS$	$[M+Na]^+$	195.930463	0.62	
		$C_2H_3O_5P$	$[M+Na]^+$	195.930436	0.74	
218.92121	29557.6	$C_3H_5N_2OPS_2$	$[M+K]^+$	179.958095	-0.2	0.008
(>5)		$C_5H_2N_2OSCa_2$	$[M+H]^+$	217.913967	-0.15	
		$CH_4N_2O_3P_2S_2$	$[M+H]^+$	217.913863	0.32	
		$C_6HN_2PS_2$	$[M+Na]^+$	195.93188	0.5	
		$C_3H_7P_3$	$[M+Na]^+$	195.931737	1.15	
218.92702	23380.1	C_3HN_5OSCu	$[M+H]^+$	217.919781	-0.17	0.016
(>5)		$C_2H_2N_2O_4Ca$	$[M+H]^+$	217.919722	0.1	
		$C_2H_7NO_3P_2Cu$	$[M+H]^+$	217.919719	0.11	
		C_2H_7OP	$[M+Na]^+$	195.937749	0.23	
		C_7H_4NPCu	$[M+Na]^+$	195.937736	0.29	
220.06695	34834.1	$C_9H_{13}N_3O$	$[M+(41K)]^+$	179.105862	-0.86	0.016
		$C_7H_{14}N_3OPS$	$[M+H]^+$	219.059522	0.69	
226.03802	20402.5	$C_4H_{13}N_5OCa$	$[M+K]^+$	187.074601	1.15	0.031
		$C_5H_{11}N_5P_2$	$[M+Na]^+$	203.048971	-0.76	
		$C_4H_{11}N_3O_4$	$[M+H]^+$	225.03063	0.5	
		$C_6H_{11}NO_6S$	$[M+H]^+$	225.030711	0.14	
232.01697	38827.2	$C_4H_{12}N_3OP$	$[M+Na]^+$	209.027473	1.19	0.039
(>5)		$C_6H_{12}NO_3PS$	$[M+Na]^+$	209.027554	0.84	
		$C_5H_{11}N_3$	$[M+H]^+$	231.009593	0.43	
		$C_2H_9N_5O_4S_2$	$[M+H]^+$	231.009599	0.41	
		$C_7H_{11}NO_2S$	$[M+H]^+$	231.009674	0.08	
233.12455	36246.4	$C_8H_{16}N_4O_4$	$[M+H]^+$	232.117156	0.5	0.039
239.1636	18025.3	-	-	-	-	0.039
250.97338	45988.5	$C_6H_{10}O_3Ca$	$[M+Na]^+$	227.984209	-0.2	0.039
(>5)		$C_7H_{11}O_3PFe$	$[M+Na]^+$	227.984195	-0.15	
		$C_2H_8N_4O_4$	$[M+K]^+$	212.010229	-0.03	
		$C_7H_4N_2O_5S$	$[M+Na]^+$	227.984095	0.25	
		$C_8H_{13}PSCa$	$[M+K]^+$	212.010151	0.28	
256.82284	23603.7	$C_4H_2Cu_2$	$[M+Na]^+$	233.833471	0.57	0.047
(>5)		CHN_3P_2FeCu	$[M+Na]^+$	233.833784	-0.64	
		C_2HNS_3Cu	$[M+H]^+$	255.815337	0.88	
		$C_2H_2O_2SFe_2$	$[M+H]^+$	255.815399	0.64	
		C_2HNP_2SCu	$[M+H]^+$	255.815769	-0.8	
259.96505	19704.8	$C_6H_{10}N_2O_2SCu$	$[M+Na]^+$	236.975899	-0.27	0.031
(>5)		$C_7H_{13}NOCaFe$	$[M+K]^+$	221.001917	-0.1	
		$C_5H_4N_5O_2P_3$	$[M+H]^+$	258.957789	-0.06	
		$C_4H_9N_3O_2S_4$	$[M+H]^+$	258.957765	0.03	
		$C_4H_{10}N_4Cu$	$[M+Na]^+$	236.975818	0.04	
262.17787	118549.7	$C_{14}H_{25}NO_2$	$[M+Na]^+$	239.188529	0.46	0.016
269.23374	74043.5	$C_{14}H_{28}N_4O$	$[M+H]^+$	268.226311	0.57	0.047
270.16775	14600.4	$C_{12}H_{25}NO_4$	$[M+Na]^+$	247.178359	0.63	0.039
272.04353	126751.1	$C_7H_7N_9S$	$[M+Na]^+$	249.054513	-0.75	0.023
(>5)		$C_6H_{13}N_5O_2P_2$	$[M+Na]^+$	249.054451	-0.52	
		$C_7H_{13}NO_8S$	$[M+H]^+$	271.036191	0.23	
		$C_4H_7N_{11}P_2$	$[M+H]^+$	271.036115	0.51	
		$C_5H_{13}N_3O_6$	$[M+H]^+$	271.03611	0.53	
280.20389	35682.8	$C_{15}H_{31}NO$	$[M+K]^+$	241.240564	0.59	0.039

		$C_{18}H_{27}N$	$[M+Na]^+$	257.214349	1.14	
		$C_{13}H_{30}NO_3P$	$[M+H]^+$	279.196332	1	
292.2524	14310.1	-	-	-	-	0.039
302.24841	74538	-	-	-	-	0.039
303.24902	468240.7	-	-	-	-	0.039
305.2608	26484.6	$C_{19}H_{38}$	$[M+K]^+$	266.29735	0.95	0.023
305.26468	6104302	-	-	-	-	0.039
307.27133	37832.4	-	-	-	-	0.039
315.95808	41593.6	$C_3H_9N_7O_4Fe_2$	$[M+H]^+$	314.950827	-0.07	0.039
(>5)		$C_6H_{12}N_4O_2Ca_2Cu$	$[M+H]^+$	314.950807	-0.01	
		$C_4H_9N_3O_7S_2Ca$	$[M+H]^+$	314.950787	0.05	
		$C_4H_9N_5O_6Fe$	$[M+K]^+$	276.994897	0.07	
		$C_7H_{11}NO_3Ca$	$[M+H]^+$	314.950781	0.07	
316.93900	90386.4	$C_9H_4N_4O_3P_2$	$[M+K]^+$	277.975867	-0.08	0.023
(>5)		$C_{13}H_7P_3$	$[M+H]^+$	315.931737	-0.04	
		$C_2H_6N_8P_2S$	$[M+Na]^+$	293.949763	0.05	
		$C_8H_{12}N_2O_2CaFe_2$	$[M+H]^+$	315.931693	0.1	
		$C_8H_9NO_5SCu$	$[M+Na]^+$	293.949745	0.11	
334.18867	36952.6	$C_{11}H_{29}N_5O_3S$	$[M+Na]^+$	311.199112	1.01	0.031
(>5)		$C_{16}H_{31}NO$	$[M+Na]^+$	311.199187	0.78	
		$C_{19}H_{25}N_3O$	$[M+Na]^+$	311.199762	-0.94	
		$C_{13}H_{29}N_5OCa$	$[M+Na]^+$	311.199801	-1.05	
		$C_{14}H_{28}N_3O_4P$	$[M+H]^+$	333.181745	-1.05	
334.23089	6591.9	$C_{11}H_{27}N_9O_3$	$[M+H]^+$	333.223686	-0.22	0.016
		$C_{19}H_{31}N_3S$	$[M+H]^+$	333.223869	-0.76	
336.20431	56553.5	$C_{18}H_{34}NP$	$[M+(41K)]^+$	295.242887	0.44	0.039
(>5)		$C_{16}H_{33}NO$	$[M+Na]^+$	313.214837	0.75	
		$C_{19}H_{27}N_3O$	$[M+Na]^+$	313.215412	-0.96	
		$C_{13}H_{31}N_5OCa$	$[M+Na]^+$	313.215451	-1.08	
		$C_{14}H_{30}N_3O_4P$	$[M+H]^+$	335.197395	-1.07	
336.89963	28350.3	$C_3H_{15}N_2P_5S_2$	$[M+K]^+$	297.936482	-0.03	0.016
(>5)		$C_5H_9N_3O_2SCaCu$	$[M+H]^+$	335.892362	-0.02	
		$C_5H_{12}N_2P_4SCa_2$	$[M+H]^+$	335.892354	0	
		$C_6H_{10}N_3O_2PSFeCu$	$[M+H]^+$	335.892348	0.02	
		$C_3H_6N_2O_7S_3$	$[M+H]^+$	335.892342	0.03	
337.23348	143163.2	$C_{19}H_{37}PCa$	$[M+H]^+$	336.225879	0.96	0.031
(>5)		$C_{15}H_{32}N_2O_6$	$[M+H]^+$	336.226038	0.49	
		$C_{10}H_{29}N_{10}OP$	$[M+H]^+$	336.226343	-0.41	
		$C_{17}H_{39}P_3$	$[M+H]^+$	336.226464	-0.77	
339.02770	519139.5	$C_5H_8N_{12}OS_2$	$[M+Na]^+$	316.038547	-0.2	0.023
(>5)		$C_9H_{24}P_4$	$[M+Na]^+$	316.038525	-0.14	
		$C_{20}H_4N_4O$	$[M+Na]^+$	316.038511	-0.1	
		$C_4H_{14}N_8O_3P_2S$	$[M+Na]^+$	316.038485	-0.02	
		$C_{11}H_{18}O_4S_2$	$[M+H]^+$	338.020327	0.28	
362.22000	134989.9	$C_{13}H_{33}N_5O_3S$	$[M+Na]^+$	339.230412	1.01	0.016
(>5)		$C_{18}H_{35}NO$	$[M+Na]^+$	339.230487	0.81	
		$C_{21}H_{29}N_3O$	$[M+Na]^+$	339.231062	-0.78	
		$C_{15}H_{33}N_5OCa$	$[M+Na]^+$	339.231101	-0.89	
		$C_{16}H_{32}N_3O_4P$	$[M+H]^+$	361.213045	-0.89	
365.22048	27184.6	$C_6H_{26}N_{14}O_3$	$[M+Na]^+$	342.231231	0.08	0.039
(>5)		$C_{14}H_{30}N_8S$	$[M+Na]^+$	342.231414	-0.43	
		$C_{17}H_{34}N_4OFe$	$[M+H]^+$	364.212873	0.91	
		$C_{11}H_{30}N_{10}P_2$	$[M+H]^+$	364.213016	0.51	
		$C_{14}H_{35}N_4OP$	$[M+H]^+$	364.213472	-0.73	

365.24952	47830.2	$C_{22}H_{38}Ca$	$[M+Na]^+$	342.259941	0.98	0.008
(>5)		$C_{13}H_{30}N_{10}O$	$[M+Na]^+$	342.260405	-0.29	
		$C_{20}H_{40}P_2$	$[M+Na]^+$	342.260526	-0.62	
		$C_{16}H_{37}N_4OPS$	$[M+H]^+$	364.242571	-0.9	
370.84958	14596.8	$C_4H_6O_{11}SFe_2$	$[M+H]^+$	369.842311	-0.02	0.039
(>5)		$C_3H_{12}OP_4S_2$	$[M+H]^+$	369.842307	-0.01	
		$C_7H_3N_4PCa$	$[M+K]^+$	331.886421	0	
		$C_6H_2N_4O_2P_6$	$[M+Na]^+$	347.860354	0.01	
		$C_2H_7N_4O_4P_3SCaFe$	$[M+H]^+$	369.842295	0.02	
371.14042	51876.2	$C_8H_{22}N_{10}O_2$	$[M+Na]^+$	348.151343	-0.39	0.039
(>5)		$C_{15}H_{34}P_4S$	$[M+H]^+$	370.133174	-0.08	
		$C_8H_{24}N_{10}OP_2S$	$[M+H]^+$	370.133053	0.24	
		$C_{19}H_{30}Fe$	$[M+H]^+$	370.132985	0.43	
		$C_{12}H_{27}N_6P_3$	$[M+Na]^+$	348.151008	0.51	
372.97414	15388.8	$C_8H_{15}NO_9CaCu$	$[M+H]^+$	371.966874	-0.03	0.039
(>5)		$C_{15}H_{13}O_2P_3S$	$[M+Na]^+$	349.984916	0.01	
		$C_{11}H_{18}O_4S_2CaFe$	$[M+H]^+$	371.966857	0.02	
		$C_7H_9N_8OPCa$	$[M+Na]^+$	349.984909	0.03	
		$C_7H_{14}N_6O_4Cu_2$	$[M+H]^+$	371.966852	0.03	
382.85037	31292.5	$C_7H_8N_2OP_2S_2Ca_3$	$[M+H]^+$	381.843106	-0.03	0.008
(>5)		$C_7H_8N_4P_2Ca_2Fe$	$[M+K]^+$	343.887216	-0.01	
		$C_5H_8N_2O_5Cu_2$	$[M+Na]^+$	359.861144	0.01	
		$C_5H_{11}NO_3P_4CaCu$	$[M+Na]^+$	359.861136	0.03	
		$C_5H_6N_2O_4P_2Ca_2$	$[M+Na]^+$	359.861139	0.03	
383.05401	242846.5	$C_{10}H_{16}N_8O_2Ca_2$	$[M+Na]^+$	360.064804	-0.04	0.016
(>5)		$C_{11}H_{15}N_6O_5PCa$	$[M+H]^+$	382.046748	-0.04	
		$C_5H_{17}N_{10}OP$	$[M+H]^+$	382.046739	-0.01	
		$C_{10}H_{14}N_{11}PCu$	$[M+H]^+$	382.046726	0.02	
		$C_{11}H_{20}N_4O_2P_2$	$[M+Na]^+$	360.064775	0.04	
385.34272	19554.2	$C_{22}H_{44}N_2O_3$	$[M+H]^+$	384.335193	0.65	0.047
386.962	8142.2	$C_{12}H_{15}N_4PSFe_2$	$[M+H]^+$	385.95473	-0.02	0.039
(>5)		$C_6H_{10}N_6O_7SFe$	$[M+Na]^+$	363.972783	-0.01	
		$C_4H_{11}N_{10}P_3SCa$	$[M+Na]^+$	363.972767	0.03	
		$C_5H_{10}N_8O_3P_4S$	$[M+H]^+$	385.954711	0.03	
		$C_9H_{16}N_4O_2Ca_3Fe$	$[M+H]^+$	385.954711	0.03	
389.13230	50922.2	$C_{19}H_{29}OP_3$	$[M+Na]^+$	366.143129	-0.13	0.031
(>5)		$C_8H_{16}N_{14}O_3S$	$[M+H]^+$	388.125053	-0.08	
		$C_{15}H_{33}N_3SCu$	$[M+K]^+$	350.169118	0.06	
		$C_6H_{23}N_{12}O_2PCa$	$[M+Na]^+$	366.143047	0.08	
		$C_7H_{22}N_{10}O_5P_2$	$[M+H]^+$	388.124991	0.08	
395.33841	126253.5	$C_{22}H_{42}N_4O_2$	$[M+H]^+$	394.330776	0.9	0.016
		$C_{15}H_{42}N_{10}S$	$[M+H]^+$	394.331462	-0.83	
404.3139	51406.8	$C_{23}H_{43}NO_3$	$[M+Na]^+$	381.324294	0.95	0.031
405.03593	26852.2	$C_{13}H_{27}NS_2Cu$	$[M+Na]^+$	382.046715	-0.02	0.016
(>5)		$C_{13}H_{28}P_4$	$[M+K]^+$	366.072775	-0.01	
		$C_{17}H_{11}N_4O_5P$	$[M+Na]^+$	382.046709	0	
		$C_{18}H_{20}N_2P_2Ca$	$[M+K]^+$	366.072765	0.01	
		$C_9H_{20}N_8P_2S_2$	$[M+K]^+$	366.072762	0.02	
405.09829	145000.8	$C_{10}H_{23}N_8O_3PS$	$[M+K]^+$	366.135147	-0.04	0.023
(>5)		$C_{14}H_{26}N_4P_2S$	$[M+H]^+$	404.091017	-0.01	
		$C_{12}H_{20}N_8O_3Ca_2$	$[M+H]^+$	404.091019	-0.01	
		$C_{12}H_{25}N_6PCa$	$[M+Na]^+$	382.109046	0.06	
		$C_{13}H_{24}N_4O_3P_2$	$[M+H]^+$	404.09099	0.06	
418.32951	66871.7	$C_{24}H_{45}NO_3$	$[M+Na]^+$	395.339944	0.82	0.031

442.76735	74429.6	$C_4H_8N_4SFeCu_2$	$[M+H]^+$	441.760074	0	0.023
(>5)		$C_2H_2N_8OP_2S_3Fe_2$	$[M+Na]^+$	419.778123	0.01	
		$C_6H_6O_4P_2S_3Ca_3$	$[M+Na]^+$	419.778125	0.01	
		$C_{11}H_2N_2OP_2SCaFe_2$	$[M+Na]^+$	419.778126	0.01	
		$C_2H_5N_7OP_2Ca_3FeCu$	$[M+H]^+$	441.760068	0.01	
454.70280	29367.8	$C_6H_2NP_3S_4Cu$	$[M+Na]^+$	431.713573	0.01	0.039
(>5)		$C_3H_3O_7P_3Ca_2Fe_2$	$[M+Na]^+$	431.713575	0.01	
		$C_6HO_2P_3S_8$	$[M+H]^+$	453.69552	0.01	
		$C_6H_3P_7S_2$	$[M+K]^+$	415.739633	0.02	
		$C_4HNO_9Fe_3Cu$	$[M+Na]^+$	431.713569	0.02	
458.69690	44965.8	$C_6HN_2O_4P_3Fe_3$	$[M+K]^+$	419.733758	-0.04	0.039
(>5)		$C_5H_6O_4S_4Fe_3$	$[M+K]^+$	419.733734	0.01	
		$C_{10}H_2O_4Fe_2Cu_2$	$[M+K]^+$	419.733732	0.02	
		$C_4H_4N_2OS_2FeCu_2$	$[M+H]^+$	457.689613	0.02	
		$C_2HN_5O_2P_2SCa_3FeCu$	$[M+H]^+$	457.689607	0.04	
467.12793	5867.8	$C_{14}H_{33}N_6PS_4$	$[M+Na]^+$	444.13872	-0.02	0.039
(>5)		$C_7H_{28}N_{12}O_2S_3$	$[M+H]^+$	466.120657	-0.01	
		$C_{16}H_{28}N_6O_2SCa$	$[M+H]^+$	466.12066	-0.01	
		$C_{11}H_{24}N_8O_9S$	$[M+Na]^+$	444.138699	0.02	
		$C_{17}H_{29}N_6O_2PSFe$	$[M+H]^+$	466.120646	0.02	
468.12457	59470	$C_{11}H_{32}N_5O_5P_3$	$[M+H]^+$	467.117307	-0.03	0.023
(>5)		$C_6H_{23}N_{15}O_4$	$[M+K]^+$	429.161418	-0.02	
		$C_{16}H_{21}N_9O_3$	$[M+Na]^+$	445.135359	-0.02	
		$C_5H_{23}N_{15}O_5SCa$	$[M+Na]^+$	445.135323	0.05	
		$C_{16}H_{29}N_5O_2P_2$	$[M+Na]^+$	445.135324	0.05	
472.69458	16390.4	$C_3HN_2O_4P_3CaFe$	$[M+K]^+$	433.731421	0	0.016
(>5)		$C_6H_3N_3O_3Ca_2Cu_3$	$[M+K]^+$	433.731421	0	
		$C_3H_5N_2O_3PS_2FeCu_2$	$[M+Na]^+$	449.705358	0	
		$C_9H_2S_3Cu_2$	$[M+Na]^+$	449.70536	0	
		$C_3H_6NO_3P_5FeCu$	$[M+K]^+$	433.731418	0.01	
483.09531	42208	$C_{18}H_{23}N_9OCu$	$[M+K]^+$	444.132155	-0.01	0.008
(>5)		$C_{20}H_{30}O_4P_2S_2$	$[M+Na]^+$	460.10608	0.02	
		$C_7H_{24}N_{14}O_2SFe$	$[M+H]^+$	482.088023	0.02	
		$C_{25}H_{20}N_2O_6$	$[M+K]^+$	444.132138	0.03	
		$C_{12}H_{26}N_8O_3SCa$	$[M+Na]^+$	460.106073	0.03	
488.29896	36886.9	$C_{27}H_{47}NOS_2$	$[M+Na]^+$	465.309908	-0.35	0.039
(>5)		$C_{24}H_{45}N_3O_2$	$[M+Na]^+$	465.3098	-0.13	
		$C_{19}H_{43}N_7O_4S$	$[M+Na]^+$	465.309725	0.03	
		$C_{17}H_{43}N_9O_2$	$[M+Na]^+$	465.309644	0.19	
		$C_{24}H_{47}N_3OP_2S$	$[M+H]^+$	487.29151	0.36	
489.08786	21674.7	$C_{11}H_{30}N_8OP_2S_3Ca$	$[M+H]^+$	488.08059	-0.01	0.016
(>5)		$C_{11}H_{30}N_{10}P_2SFe$	$[M+K]^+$	450.1247	0	
		$C_{12}H_{26}N_{10}Ca_3$	$[M+H]^+$	488.080586	0	
		$C_{13}H_{27}N_{10}PCa_2Fe$	$[M+H]^+$	488.080572	0.02	
		$C_9H_{28}N_8O_4P_2S$	$[M+Na]^+$	466.098623	0.03	
519.029	25005.8	$C_{12}H_{23}N_8O_3PS_2$	$[M+K]^+$	480.065842	0	0.016
(>5)		$C_{21}H_{25}N_2OPS_2Ca_2$	$[M+Na]^+$	496.039777	0	
		$C_{13}H_{13}N_{14}P_3$	$[M+H]^+$	518.021723	0	
		$C_{28}H_{21}PS$	$[M+K]^+$	480.065833	0.01	
		$C_{13}H_{25}N_7O_2P_2Cu$	$[M+Na]^+$	496.039771	0.01	
519.09144	42346.1	$C_{10}H_{33}N_8OP_5$	$[M+Na]^+$	496.10222	0	0.047
(>5)		$C_{19}H_{31}N_4O_2PS_2Fe$	$[M+Na]^+$	496.10222	0	
		$C_{15}H_{34}N_2O_7P_2S_2$	$[M+K]^+$	480.128273	0.01	
		$C_{12}H_{26}N_{10}O_4SFe$	$[M+H]^+$	518.084157	0.01	
		$C_{27}H_{28}NPCu$	$[M+H]^+$	518.084159	0.01	

519.29341	109666.3	$C_{18}H_{44}N_8O_4$	$[M+Na]^+$	496.304225	-0.07	0.031
(>5)		$C_{26}H_{42}N_6OS_2$	$[M+H]^+$	518.286153	-0.04	
		$C_{24}H_{49}N_4PSCa$	$[M+Na]^+$	496.304147	0.08	
		$C_{25}H_{48}N_2O_3P_2S$	$[M+H]^+$	518.286091	0.08	
		$C_{17}H_{44}N_{10}O_2Ca$	$[M+H]^+$	518.286084	0.1	
524.68747	45928.6	$C_3H_7N_5P_2S_3CaFeCu$	$[M+K]^+$	485.724312	0	0.031
(>5)		$C_{11}HO_3P_5SCa_2Fe$	$[M+Na]^+$	501.698251	0	
		$C_4H_{10}NP_7SCa_2Cu$	$[M+H]^+$	523.680191	0	
		$C_4H_3N_4O_2PS_5Ca_2Fe$	$[M+H]^+$	523.680191	0	
		$C_7H_6O_5P_2S_2Ca_3Fe_2$	$[M+H]^+$	523.680192	0	
525.08005	44071.9	$C_{20}H_{26}N_4O_3SCa$	$[M+Na]^+$	502.090827	0	0.031
(>5)		$C_{21}H_{25}N_2O_6PS$	$[M+H]^+$	524.072771	0	
		$C_{20}H_{19}N_{10}P_3S$	$[M+H]^+$	524.072776	0	
		$C_9H_{23}N_6O_{16}P$	$[M+Na]^+$	502.090822	0.01	
		$C_{11}H_{26}N_{10}O_3S_3$	$[M+Na]^+$	502.090824	0.01	
531.48665	242430.7	$C_{32}H_{64}N_2O_2$	$[M+Na]^+$	508.496778	1.22	0.016
532.19270	21452.7	$C_{25}H_{42}O_4P_2Cu$	$[M+H]^+$	531.185435	-0.02	0.008
(>5)		$C_{15}H_{44}N_7OP_5$	$[M+K]^+$	493.229548	-0.01	
		$C_{22}H_{40}NO_6PS_2$	$[M+Na]^+$	509.203471	0.01	
		$C_{24}H_{36}N_6Cu$	$[M+H]^+$	531.185416	0.01	
		$C_{15}H_{35}N_7O_8S$	$[M+H]^+$	531.185408	0.03	
532.48991	84705.3	-	-	-	-	0.008
533.27271	158330.4	$C_{25}H_{53}O_2P_3Fe$	$[M+H]^+$	532.265456	-0.04	0.039
(>5)		$C_{20}H_{44}N_8O_2S_2Ca$	$[M+H]^+$	532.265457	-0.04	
		$C_{20}H_{44}N_{10}OFe$	$[M+K]^+$	494.309567	-0.03	
		$C_{18}H_{42}N_8O_5$	$[M+Na]^+$	510.28349	0	
		$C_{26}H_{40}N_6O_2S_2$	$[M+H]^+$	532.265418	0.03	
534.27605	49030	$C_{18}H_{41}N_9O_7$	$[M+K]^+$	495.312896	-0.01	0.031
(>5)		$C_{21}H_{45}N_7OCa$	$[M+Na]^+$	511.286822	0.01	
		$C_{22}H_{44}N_5O_4P$	$[M+H]^+$	533.268766	0.01	
		$C_{21}H_{50}N_6P_2Cu$	$[M+Na]^+$	511.286819	0.02	
		$C_{27}H_{41}N_5O$	$[M+Na]^+$	511.286783	0.09	
535.28833	221207.5	$C_{18}H_{44}N_8O_5$	$[M+Na]^+$	512.29914	-0.06	0.039
(>5)		$C_{26}H_{42}N_6O_2S_2$	$[M+H]^+$	534.281068	-0.03	
		$C_{25}H_{49}N_4P$	$[M+K]^+$	496.325157	0.03	
		$C_{18}H_{46}N_{10}SCa$	$[M+H]^+$	534.281026	0.05	
		$C_{24}H_{49}N_4OPSCa$	$[M+Na]^+$	512.299062	0.09	
536.29158	63569.9	$C_{28}H_{51}NP_2SCa$	$[M+H]^+$	535.284338	-0.06	0.008
(>5)		$C_{16}H_{42}N_9O_9P$	$[M+H]^+$	535.284314	-0.02	
		$C_{21}H_{39}N_9O_6$	$[M+Na]^+$	513.302331	0.05	
		$C_{22}H_{48}N_7OPCa$	$[M+K]^+$	497.328387	0.06	
		$C_{28}H_{44}N_5OP$	$[M+K]^+$	497.328348	0.14	
545.50222	95383.7	$C_2HOP_3S_4FeCu_3$	$[M+(41K)]^+$	504.540726	0.4	0.008
(>5)		$C_3HP_5SCaFeCu_3$	$[M+K]^+$	506.539712	-1.19	
		$C_{33}H_{66}N_2O_2$	$[M+Na]^+$	522.512428	1.05	
		$C_3HP_3S_3Fe_2Cu_3$	$[M+Na]^+$	522.513351	-0.65	
546.50555	32745.3	-	-	-	-	0.008
558.27328	31125.6	$C_{28}H_{50}N_2OS_2Cu$	$[M+H]^+$	557.266056	-0.09	0.023
(>5)		$C_{14}H_{36}N_{15}O_7P$	$[M+H]^+$	557.265978	0.05	
		$C_{26}H_{51}NO_4CaFe$	$[M+Na]^+$	535.284012	0.08	
		$C_{21}H_{45}NO_{14}$	$[M+Na]^+$	535.284009	0.09	
		$C_{25}H_{48}N_4O_2Cu$	$[M+H]^+$	557.265948	0.1	
560.63748	40551.3	$C_5H_6N_2OP_2S_8CaFe$	$[M+K]^+$	521.674318	0	0.039
(>5)		$C_4H_7N_4P_7SFe_3$	$[M+K]^+$	521.67432	0	
		$C_5H_8P_6S_4Ca_2$	$[M+K]^+$	521.674321	0	

		$C_4H_6N_3O_3P_3SCa_2Cu_3$	$[M+Na]^+$	537.648257	0	
		$C_4H_6N_5P_3S_3Fe_3Cu$	$[M+Na]^+$	537.64826	0	
562.83057 (>5)	45880.2	$C_{14}H_2N_{12}Cu_2$	$[M+K]^+$	523.867409	0	0.023
		$C_7H_3N_8O_{13}PSFe$	$[M+K]^+$	523.867409	0	
		$C_9H_{19}N_2O_3P_3S_4Ca$	$[M+K]^+$	523.867409	0	
		$C_9H_6N_{12}S_4Fe$	$[M+K]^+$	523.867411	0	
		$C_7H_{13}N_6O_6PS_3Ca_3$	$[M+K]^+$	523.867411	0	
		Negative ionisation mode				
95.00152	25530.6	-	-	-	-	0.023
96.99856	16598.5	-	-	-	-	0.016
168.02426	10797.5	-	-	-	-	0.047
210.00116	7753	$C_2H_5N_3OS_2$	$[M+Hac-H]^-$	150.987406	-0.47	0.023
		$C_7H_{13}NP_2$	$[M+K-2H]^-$	173.052325	1.09	
		$C_6H_{11}NOP_2$	$[M+Cl]^-$	175.03159	0.8	
		$C_4H_9N_3O_3S_2$	$[M-H]^-$	211.008536	-0.47	
213.58769	47837.9	-	-	-	-	0.039
215.08187	29696.3	C_7H_{14}	$[M+Hac-H]^-$	156.068173	-0.73	0.008
		$C_7H_{12}N_6$	$[M+Cl]^-$	180.112344	0.58	
		$C_8H_{19}O_3P$	$[M+Na-2H]^-$	194.107183	0.09	
		$C_9H_{18}O_2$	$[M-H]^-$	216.089303	-0.73	
236.06512	16463.1	$C_4H_3N_9$	$[M+Hac-H]^-$	177.051141	0.53	0.031
		$C_7H_{14}N_5P$	$[M+(37Cl)]^-$	199.098683	-0.06	
		$C_8H_{13}N_3O_4$	$[M+Na-2H]^-$	215.090607	-0.66	
		$C_6H_7N_9O_2$	$[M-H]^-$	237.072271	0.53	
239.58683	67383.3	-	-	-	-	0.016
260.91016 (>5)	15637.7	$C_7H_3O_4P$	$[M+Na-2H]^-$	239.935521	-0.11	0.039
		$C_2H_8N_2O_2P_2SCa$	$[M+Cl]^-$	225.940767	-0.03	
		$C_8H_4O_2P_2S$	$[M+Cl]^-$	225.940728	0.12	
		$C_2HN_4O_6PS$	$[M+Na-2H]^-$	239.935446	0.17	
		$C_3H_{10}N_2OP_2SCa$	$[M+K-2H]^-$	223.961502	0.2	
300.87702 (>5)	56083.1	$C_3H_9NO_4P_2SCu$	$[M+Na-2H]^-$	279.902356	-0.02	0.039
		$C_2H_4N_6CaFe$	$[M+(37Cl)]^-$	263.91057	-0.01	
		$C_{11}H_2O_2S_3Ca$	$[M-H]^-$	301.884287	0.03	
		$C_3H_5N_6PFe_2$	$[M+(37Cl)]^-$	263.910556	0.04	
		$C_2H_2N_6O_2S_5$	$[M-H]^-$	301.884284	0.04	
308.04202 (>5)	17231.2	$C_{14}H_{15}NO_3S_2$	$[M-H]^-$	309.049338	-0.14	0.031
		$C_7H_{18}N_3O_4PS$	$[M+(37Cl)]^-$	271.075567	0	
		$C_6H_{16}N_3O_7P$	$[M+Cl]^-$	273.07259	0.09	
		$C_4H_{14}N_5O_2PFe$	$[M+Hac-H]^-$	249.028125	0.13	
		$C_6H_{18}N_5O_4PFe$	$[M-H]^-$	309.049255	0.13	
313.1192	21179.7	$C_{15}H_{14}N_2O_2$	$[M+Hac-H]^-$	254.105528	-0.58	0.031
318.95697 (>5)	12170.8	$C_4H_{11}N_4O_3PS$	$[M+Cl]^-$	283.987574	-0.02	0.047
		$C_{16}H_8OS_2Ca$	$[M-H]^-$	319.96425	-0.01	
		$C_7H_8N_6OS_4$	$[M-H]^-$	319.964247	0	
		$C_{16}H_8N_2Fe$	$[M+K-2H]^-$	282.00836	0.01	
		$C_4H_{13}N_6PSFe$	$[M-H]^-$	319.964239	0.02	
328.0777	36607.2	$C_{19}H_{17}NOS$	$[M+Na-2H]^-$	307.103086	-0.17	0.016
340.97869 (>5)	13369.9	$C_{11}H_{16}N_2O_2Ca_2Fe$	$[M-H]^-$	341.985972	-0.02	0.031
		$C_4H_6N_2O_{10}Ca$	$[M+Hac-H]^-$	281.964839	-0.01	
		$C_6H_{10}N_2O_{12}Ca$	$[M-H]^-$	341.985969	-0.01	
		$C_{10}H_{13}N_5CaCu$	$[M+Cl]^-$	306.009285	0.01	
		$C_{14}H_{14}O_2S$	$[M+K-2H]^-$	304.030075	0.02	
344.01870	21325.2	$C_{14}H_{17}N_3S_2Fe$	$[M-H]^-$	345.026003	-0.08	0.039

(>5)		$C_9H_{21}N_3O_2S_2Ca$	$[M+K-2H]^-$	307.070112	-0.05	
		$C_{11}H_{22}NP_5$	$[M+Na-2H]^-$	323.044039	-0.02	
		$C_{15}H_{17}NO_2S_2$	$[M+K-2H]^-$	307.070073	0.06	
		$C_7H_{18}NO_9PS$	$[M+Na-2H]^-$	323.043994	0.11	
359.03107	11267.5	$C_7H_{10}N_6O_2S$	$[M+Hac-H]^-$	300.017219	-0.01	0.031
(>5)		$C_9H_{14}N_6O_4S$	$[M-H]^-$	360.038349	-0.01	
		$C_9H_{16}N_8OSFe$	$[M+Na-2H]^-$	338.056391	0.03	
		$C_5H_{19}N_6O_6PS$	$[M+K-2H]^-$	322.082444	0.05	
		$C_5H_{12}N_6O_4Ca_2$	$[M+Hac-H]^-$	300.017186	0.08	
375.13835	16998.8	$C_{17}H_{31}O_3PCa$	$[M+Na-2H]^-$	354.163674	0.02	0.031
(>5)		$C_{16}H_{24}N_6Ca$	$[M+Cl]^-$	340.168835	0.3	
		$C_4H_{23}N_{15}O_2Cu$	$[M-H]^-$	376.145514	0.3	
		$C_7H_{24}N_{12}S_2$	$[M+Cl]^-$	340.168832	0.31	
		$C_9H_{16}N_8O_5$	$[M+Hac-H]^-$	316.124367	0.34	
376.82742	35000.4	$C_2H_2N_7O_4PCu$	$[M+K-2H]^-$	339.878813	0	0.016
(>5)		$C_7HN_6P_5Fe$	$[M-H]^-$	377.834696	0	
		$C_{11}H_4NO_2PCa_2Cu$	$[M+Na-2H]^-$	355.852748	0.01	
		$C_2H_4N_7O_2PS_2CaCu$	$[M+Na-2H]^-$	355.852745	0.02	
		$C_3H_3N_5O_5P_2S_2Cu$	$[M-H]^-$	377.834689	0.02	
386.76257	16740.9	$C_2H_2P_2S_5Ca_2$	$[M+Hac-H]^-$	327.748718	0	0.008
(>5)		$C_4H_6O_2P_2S_5Ca_2$	$[M-H]^-$	387.769848	0	
		$C_4H_6N_2OP_2S_3CaFe$	$[M+K-2H]^-$	349.813958	0.01	
		$C_6HO_4PS_2Ca_3$	$[M+Cl]^-$	351.793165	0.01	
		$C_5H_2N_4Ca_3Fe$	$[M+K-2H]^-$	349.813954	0.02	
390.75670	16341.7	$C_7H_5O_3P_3Fe_3$	$[M-H]^-$	391.763995	-0.05	0.008
(>5)		$C_3H_2N_5OPS_3CaCu$	$[M+K-2H]^-$	353.808104	-0.03	
		$C_{11}HPCaFe_2$	$[M+Na-2H]^-$	369.782026	0.01	
		$C_2HN_6PS_2Fe_2$	$[M+Na-2H]^-$	369.782023	0.02	
		$C_{12}H_2P_2Fe_3$	$[M+Na-2H]^-$	369.782012	0.05	
398.87473	66915.9	$C_4H_4N_4OP_2S_3$	$[M+Hac-H]^-$	339.860876	0	0.023
(>5)		$C_5H_9N_6PS_3Ca$	$[M+Na-2H]^-$	377.900062	0	
		$C_6H_8N_4O_3P_2S_3$	$[M-H]^-$	399.882006	0	
		$C_8H_3N_4O_5PCa$	$[M+Cl]^-$	363.905323	0.01	
		$C_8H_{15}NOS_5Cu$	$[M+Cl]^-$	363.905323	0.01	
401.1877	17192.2	$C_{15}H_{28}N_4OP_2$	$[M+Hac-H]^-$	342.173837	0.02	0.016
(>5)		$C_{16}H_{33}N_6PCa$	$[M+Na-2H]^-$	380.213023	0.02	
		$C_{17}H_{32}N_4O_3P_2$	$[M-H]^-$	402.194967	0.02	
		$C_{22}H_{29}N_4P$	$[M+Na-2H]^-$	380.212984	0.12	
		$C_8H_{18}N_{14}O_2$	$[M+Hac-H]^-$	342.173716	0.33	
401.97727	16684.3	$C_7H_{11}N_7S_3Fe$	$[M+Hac-H]^-$	342.963421	-0.01	0.008
(>5)		$C_{13}H_9NO_{12}S$	$[M-H]^-$	402.984551	-0.01	
		$C_9H_{15}N_7O_2S_3Fe$	$[M-H]^-$	402.984551	-0.01	
		$C_{13}H_{19}NO_4SCa_2$	$[M+K-2H]^-$	365.028663	0	
		$C_{17}H_{12}N_4SCu$	$[M+Cl]^-$	367.007867	0	
410.74153	28381.6	$C_2H_4N_4P_2S_3Ca_2Fe$	$[M+Cl]^-$	375.772132	-0.01	0.039
(>5)		$C_4H_3O_7P_3S_2Fe$	$[M+K-2H]^-$	373.792925	0	
		$C_2HN_5O_2P_2S_2Cu$	$[M+K-2H]^-$	373.792917	0.02	
		$C_5HN_2O_4PSCa_3Fe$	$[M+Na-2H]^-$	389.766853	0.02	
		$C_2H_3N_5P_2S_4CaCu$	$[M+Na-2H]^-$	389.766849	0.03	
418.0237	11843.2	$C_{11}H_{23}N_3O_2P_2S$	$[M+(37Cl)]^-$	381.057248	0	0.016
(>5)		$C_7H_{22}N_9O_2PSFe$	$[M+K-2H]^-$	381.075093	0	
		$C_9H_{17}N_7O_5Ca_2$	$[M+Cl]^-$	383.0543	0	
		$C_{16}H_{15}N_5O_2SCa$	$[M+(37Cl)]^-$	381.057238	0.02	
		$C_4H_{14}N_{15}P_3S$	$[M+Na-2H]^-$	397.049021	0.02	
447.31151	23549.5	$C_{25}H_{40}O_3$	$[M+Hac-H]^-$	388.297745	-0.2	0.023

(>5)		$C_{19}H_{47}N_6P$	$[M-H]^-$	448.318605	0.41	
		$C_{27}H_{44}O_5$	$[M-H]^-$	448.318875	-0.2	
		$C_{20}H_{47}N_7Cu$	$[M-H]^-$	448.318892	-0.24	
		$C_{22}H_{41}N_8P$	$[M-H]^-$	448.31918	-0.88	
456.76783	14255.4	$C_8H_7N_3OSFe_2Cu$	[M+Cl]-	421.79843	0	0.039
(>5)		$C_4HN_6O_4PS_4Ca_2$	$[M+Na-2H]^-$	435.793162	0	
		$C_8H_7OPSFe_4$	$[M+Hac-H]^-$	397.753973	0.01	
		$C_6H_{12}N_3OPCu_3$	$[M+K-2H]^-$	419.81922	0.01	
		$C_4H_6N_2O_{10}SFe_3$	$[M+Na-2H]^-$	435.793156	0.01	
461.98275	11919.9	$C_4H_{13}N_9O_4SCa_3$	$[M+Hac-H]^-$	402.968896	0	0.023
(>5)		$C_6H_{21}N_7O_4P_6$	$[M+Na-2H]^-$	441.008081	0	
		$C_{21}H_{16}NO_2PS_3$	$[M+Na-2H]^-$	441.008083	0	
		$C_6H_{17}N_9O_6SCa_3$	$[M-H]^-$	462.990026	0	
		$C_{12}H_7N_7O_2S_2$	$[M+Hac-H]^-$	402.96889	0.01	
468.70015	23371.6	$C_4H_5O_5P_3S_4CaFe$	$[M+Na-2H]^-$	447.72548	0	0.023
(>5)		$C_3H_2N_2O_2S_3Fe_4$	$[M+Hac-H]^-$	409.686292	0.01	
		$C_5HN_2O_4PSCa_3Fe$	$[M+Na-2H]^-$	447.725476	0.01	
		$C_9HO_5P_3CaCu_2$	$[M+Na-2H]^-$	447.725478	0.01	
		$C_5H_6N_2O_4S_3Fe_4$	$[M-H]^-$	469.707422	0.01	
470.68774	8967.5	$C_4HNO_6P_2CaFeCu$	$[M+Cl]^-$	435.71834	0	0.016
(>5)		$C_4H_4O_4P_6Ca_2Fe$	$[M+Cl]^-$	435.718332	0.01	
		$C_3H_5N_2OP_5Ca_3Fe$	$[M-H]^-$	471.695011	0.01	
		$C_8H_4P_2S_8Fe$	$[M-H]^-$	471.695014	0.01	
		$C_2H_2N_3O_6PS_3Ca_2Cu$	$[M+K-2H]^-$	433.739122	0.02	
486.68900	13096	$C_9HO_4PS_4Ca_2Fe$	$[M+Na-2H]^-$	465.71433	0	0.016
(>5)		$C_6H_3N_3P_2S_7Cu$	$[M+Na-2H]^-$	465.714326	0.01	
		$C_6H_4N_2P_6S_5$	$[M+K-2H]^-$	449.740386	0.02	
		$C_6H_6O_3P_2SCa_2Fe_2$	$[M+Na-2H]^-$	465.714322	0.02	
		$C_5H_4O_2P_6SCa_2$	$[M+Cl]^-$	451.719585	0.03	
497.21858	34640.9	$C_{13}H_{30}N_{12}O_9$	$[M-H]^-$	498.225873	-0.03	0.023
(>5)		$C_{31}H_{35}N_2PS$	$[M-H]^-$	498.225858	0	
		$C_{24}H_{30}N_8O_2$	$[M+Cl]^-$	462.249172	0.01	
		$C_{22}H_{41}P_3Ca$	$[M+Hac-H]^-$	438.204705	0.04	
		$C_{24}H_{45}O_2P_3Ca$	$[M-H]^-$	498.225835	0.04	
510.7335	19159.5	$C_5H_4N_4P_2S_2Ca_2Cu_2$	$[M+Hac-H]^-$	451.719646	0	0.008
(>5)		$C_{11}H_{12}OP_2S_2Ca_2Fe_2$	$[M+K-2H]^-$	473.784891	0	
		$C_9H_3N_6OPSCaFe_3$	$[M+Cl]^-$	475.764096	0	
		$C_9HN_2O_5P_5S_2Ca$	$[M+Cl]^-$	475.764098	0	
		$C_{17}HNOCa_2FeCu$	$[M+Na-2H]^-$	489.75883	0	

(>5); indicates more than five possible empirical formulae.

Table I.4 Empirical formulae ascribed to the non-polar metabolic features detected in positive and negative ionisation mode which were significantly altered (p<0.05) between haemolysed and clean cord blood serum pairs, for which a putative annotation could not be ascribed.(See Chapter 7)

m/z	Intensity	Empirical formula	Ion	Theoretical mass (Da)	Mass error (ppm)	p-value
		Negative ionisation mode				
229.06623	9988.2	$C_{10}H_{15}N_2P$	$[M+Cl]^-$	194.097286	-2	0.016
		$C_6H_{17}N_4PS$	$[M+Na-2H]^-$	208.091156	1.77	
		$C_{17}H_{10}O$	$[M-H]^-$	230.073165	1.49	
		$C_{11}H_{14}N_2OCa$	$[M-H]^-$	230.073204	1.32	
		$C_9H_{16}N_2OP_2$	$[M-H]^-$	230.073789	-1.23	
253.21977	53795.1	-	-	-	-	0.017
254.22298	12429.3	-	-	-	-	0.016
279.16025	11482.4	$C_{14}H_{20}O_2$	$[M+Hac-H]^-$	220.14633	0.24	0.047
		$C_{13}H_{27}N_2OP$	$[M+Na-2H]^-$	258.186101	-1.86	
		$C_{16}H_{24}O_4$	$[M-H]^-$	280.16746	0.24	
368.26771	338622.3	$C_{15}H_{38}N_7P$	$[M+Na-2H]^-$	347.292631	1.11	0.008
		$C_{21}H_{37}N_3O$	$[M+Na-2H]^-$	347.293662	-1.69	
		$C_{11}H_{35}N_{11}OS$	$[M-H]^-$	369.274676	0.84	
370.26811	31665.5	$C_{13}H_{29}N_9$	$[M+Hac-H]^-$	311.254591	-0.9	0.023
		$C_{10}H_{33}N_{13}$	$[M+Cl]^-$	335.298187	1.41	
		$C_{17}H_{39}N_3O_4$	$[M+Na-2H]^-$	349.294057	-1.66	
		$C_9H_{34}N_{13}OP$	$[M-H]^-$	371.27469	1.88	
		$C_{15}H_{33}N_9O_2$	$[M-H]^-$	371.275721	-0.9	
422.22657 (>5)	30167.6	$C_8H_{33}N_{15}P_2$	$[M+Na-2H]^-$	401.251861	0.1	0.047
		$C_{15}H_{33}N_5OS_2$	$[M+Hac-H]^-$	363.212654	0.15	
		$C_{17}H_{37}N_5O_3S_2$	$[M-H]^-$	423.233784	0.15	
		$C_{21}H_{40}NO_2PS$	$[M+Na-2H]^-$	401.251739	0.38	
		$C_{12}H_{31}N_7O_2$	$[M+Hac-H]^-$	363.212546	0.4	
446.37217	54829.6	$C_{25}H_{51}N_3O_2$	$[M+Na-2H]^-$	425.398127	-1.4	0.016
		$C_{23}H_{45}N_9$	$[M-H]^-$	447.379791	-0.77	
522.46071	30153.6	$C_{29}H_{63}N_3O_3$	$[M+Na-2H]^-$	501.486942	-1.72	0.008
		$C_{21}H_{58}N_{13}P$	$[M-H]^-$	523.467575	0.79	
		$C_{27}H_{57}N_9O$	$[M-H]^-$	523.468606	-1.19	
538.38279 (>5)	32777.4	$C_{27}H_{59}N_3OP_2$	$[M+Cl]^-$	503.413338	0.09	0.008
		$C_{28}H_{61}N_3P_2$	$[M+K-2H]^-$	501.434073	0.21	
		$C_{27}H_{54}N_7PS$	$[M-H]^-$	539.389903	0.3	
		$C_{20}H_{49}N_{13}O_2$	$[M+Cl]^-$	503.413217	0.32	
		$C_{24}H_{56}N_3P_3$	$[M+Hac-H]^-$	479.368711	0.42	
669.45846 (>5)	25629	$C_4H_3N_2OPS_7Fe_3Cu$	$[M+K-2H]^-$	632.509863	-0.01	0.016
		$C_4H_5O_4P_7CaFe_2Cu_3$	$[M-H]^-$	670.465738	0	
		$C_5H_4O_4P_2S_5CaFeCu_3$	$[M+K-2H]^-$	632.509846	0.01	
		$C_7H_5OP_3S_2Fe_3Cu_3$	$[M-H]^-$	670.465729	0.01	

		$C_7HN_2OPS_6CaFe_4Cu$	$[M-H]^-$	670.465721	0.02	
686.53239 (>5)	65380	$C_6H_6N_4OP_2S_8CaFeCu_2$	$[M-H]^-$	687.539664	0	0.008
		$C_5HN_2O_9P_5Ca_2Fe_3$	$[M-H]^-$	687.539664	0	
		$C_5H_7N_6P_7SFe_3Cu_2$	$[M-H]^-$	687.539666	0	
		$C_4H_5O_{10}P_7S_3Fe_3$	$[M-H]^-$	687.539668	0	
		$C_9H_4NO_5PS_5Ca_3Fe_2Cu$	$[M-H]^-$	687.539668	0	
713.56021 (>5)	491391.8	$C_2H_6N_5O_8P_7SFeCu_2[NaCl]$	$[M-H]^-$	714.567486	0	0.0156
		$C_7H_5N_6O_3PS_3Ca_3Cu_3[NaCl]$	$[M-H]^-$	714.567486	0	
		$CH_6N_3O_{14}P_3S_2Fe_4[NaCl]$	$[M-H]^-$	714.567486	0	
		$C_5(13C)H_2N_{10}OP_4S_2Ca_2Fe_4$	$[M-H]^-$	714.567486	0	
		$C_9H_2N_5O_3P_7S_4(34S)Fe_2$	$[M-H]^-$	714.567486	0	
775.71865 (>5)	7729.7	$C_2H_{32}O_{12}P_7S_6Ca_3$	$[M-H]^-$	776.725926	0	0.008
		$C_{21}H_2N_9O_3P_3S_8$	$[M-H]^-$	776.725926	0	
		$C_6H_{15}N_8O_{15}S_3Ca_2Fe_3$	$[M-H]^-$	776.725926	0	
		$C_2H_{21}N_{14}O_5S_5Ca_2Fe_4$	$[M-H]^-$	776.725926	0	
		$C_{25}H_{15}N_2P_5S_3Ca_3Cu$	$[M-H]^-$	776.725926	0	
1277.97179 (>5)	40384.6	$C_{32}H_{68}N_{12}OP_4S_7Ca_3FeCu[NaCl]$	$[M-H]^-$	1278.979066	0	0.031
		$C_{25}H_{60}N_{13}O_{16}P_5S_5CaCu_2$	$[M-H]^-$	1278.979066	0	
		$C_{30}H_{62}N_8O_{18}P_2S_7Fe_2Cu$	$[M-H]^-$	1278.979065	0	
		$C_{28}H_{58}N_{14}O_{11}P_5S_7Ca_2Fe$	$[M-H]^-$	1278.979064	0	
		$C_{34}H_{60}N_{11}O_7P_6S_8CaCu$	$[M-H]^-$	1278.979063	0	
		Positive ionisation mode				
368.33950	1730.2	-	-	-	-	0.039
404.38893	42553.1	$C_{27}H_{49}NO$	$[M+H]^+$	403.3814	0.59	0.023
405.39230	12475.6	$C_{23}H_{50}N_4$	$[M+Na]^+$	382.4035	-1.15	0.039
734.58708 (>5)	11985.6	$C_2H_8N_4O_{12}P_5S_4Fe_2Cu$	$[M+H]^+$	733.5798	0	0.016
		$C_6H_4N_2O_{18}PSFe_4Cu$	$[M+H]^+$	733.5798	0	
		$C_{11}H_8N_2O_3P_6S_7Fe_2$	$[M+H]^+$	733.5798	0.01	
		$C_{15}H_4O_9P_2S_4Fe_4$	$[M+H]^+$	733.5798	0.01	
		$C_{15}H_8O_3P_5S_3Cu_3[NaCl]$	$[M+H]^+$	733.5798	0.01	
800.67254 (>5)	57836.9	$C_{28}H_6O_4P_4S_3Ca_3Fe$	$[M+H]^+$	799.6653	0	0.039
		$C_{10}H_6N_8O_{13}P_3S_2Ca_2FeCu$	$[M+H]^+$	799.6653	0	
		$C_2H_{27}NO_{17}P_2S_3Ca_2Fe_3Cu$	$[M+H]^+$	799.6653	0	
		$C_{17}H_{14}N_2O_5P_5S_8Cu$	$[M+H]^+$	799.6653	0	
		$C_{31}H_{12}OPSCaFe_2Cu_3$	$[M+H]^+$	799.6653	0	

(>5); indicates more than five possible empirical formulae.

Appendix J. Publications arising from thesis

Denihan NM, Boylan GB, Murray DM. *Metabolomic Profiling in Perinatal Asphyxia: A Promising New Field*. BioMed Research International. 2015;2015:9.

Denihan NM, Walsh BH, Reinke SN, Sykes BD, Mandal R, Wishart DS, Broadhurst DI, Boylan GB, Murray DM. *The effect of haemolysis on the metabolomic profile of umbilical cord blood.* Clinical Biochemistry. 2015;48(7–8):534-7.

Denihan NM. *Who is the cool kid? Biomarkers to help treat newborn brain injury*.The Boolean. 2014;4(0):5-9.

Denihan NM, Looney A, Boylan GB, Walsh BH, Murray DM. *Normative levels of Interleukin 16 in umbilical cord blood.* Clinical Biochemistry. 2013;46(18):1857-9.