Imperial College London

The influence of maternal metabolic phenotype on breast milk composition and on the maternal and infant urinary metabolome

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Declaration of Originality

I, Nicholas Andreas, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated. Portions of this thesis have been published in peer reviewed journals, this has been indicated where this is the case.

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Abstract

The main objective of my research is to describe the influence of maternal phenotype on human breast milk composition. My primary objective is to establish whether maternal Body Mass Index (BMI) influences breast milk metabolomic and endocrine profiles, macronutrient content, and maternal and infant urinary metabolomes.

I first focused on milk sample preparation techniques prior to metabonomic analysis. Due to milk containing complex structures, such as milk lipid globules and casein micelles, it is difficult to analyse because of interference with the analytical techniques employed. I therefore optimised sample preparation methods prior to analysis. I also conducted a systematic review of all published literature relevant to the hormonal content of human milk.

I investigated human milk metabolomic profiles using ^{1}H NMR spectroscopy and liquid chromatography-mass spectrometry. I examined appetite regulating hormones using immunoassay techniques, and macronutrient content using mid infrared spectroscopy. I also investigated the metabolomic profile of mothers' and infants urine using ^{1}H NMR spectroscopy.

My principal conclusions from this work are that breast milk metabolomic profile and macronutrient content are highly conserved across mothers, and not influenced by maternal BMI. The systematic literature review indicated that milk leptin concentration is positively correlated with maternal BMI, whilst evidence pertaining to other hormones was either inconclusive or lacking. Of particular note in the metabonomic analyses were the diverse profiles observed of Human Milk Oligosaccharides secreted into mothers' milk. These are genetically determined, and have developmental and protective effects for infants.

With regard to urinary metabolomic profiles, an association was observed between maternal BMI and maternal metabolome, but not infant urinary metabolome. Metabolites discriminating between mothers' with different BMI were present across a range of chemical shifts, from the aromatic region associated with metabolites resulting from co-metabolism with the gut microbiota, to the aliphatic region.

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List of abbreviations

2'-FL: 2'-Fucosyllactose
3'-FL: 3'-Fucosyllactose
3'-SL: 3'-sialyllactose
6'-SL: 6'-sialyllactose
AA: Arachidonic acid
ANOVA: Analysis of variance
AT: Adipose tissue
BF: Breast fed
BMI: Body mass index
CE-MS: Capillary electrophoresis-mass spectrometry
CI: Confidence interval
DHA: Docosahexaenoic acid
ELISA: Enzyme-linked immunosorbent assay
FF: Formula fed
FID: Free induction decay
GC-MS: Gas chromatography-mass spectrometry
HMO: Human milk oligosaccharides
HMW: High molecular weight
JRES: J-resolved spectrometry
LC-MS: Liquid chromatography-mass spectrometry
LDFT: Lactodifucotetraose
Le: Lewis
LNDFHI: Lacto-N-difucohexaose I
LNFPII: Lacto-N-fucopentaose II
LNFPIII: Lacto-N-fucopentaose III
LNFP V: Lacto-N-fucopentaose V
LPL: Lipoprotein lipase
LS: Longitudinal study
MeSH: Medical Subject Heading
mM: Millimolar
MS: Mass Spectrometry
NMR: Nuclear magnetic resonance
NOESY: Nuclear Overhauser effect Spectroscopy
OPLS-DA: Orthogonal partial least squares discriminant analysis
PCA: Principal component analysis
PLS: Partial least squares projections to latent structures
PLS-DA: Partial least square-discriminant analysis
ppm: Parts Per Million
PROBIT: Promotion of breastfeeding intervention trial
RD: Relaxation delay
RF: Radiofrequency
SD: Standard deviation
Se: Secretor
SFT: Skin fold thickness
STOCSY: Statistical Correlation Spectroscopy
STORM: Subset optimization by reference matching
TAT: Total adipose tissue
tm: Mixing time
TMAO: Trimethylamine *N* Oxide
TMS: Tetramethylsilane
TSP: Trimethylsilyl propionic acid
UPLC-MS: Ultra performance liquid chromatography-mass spectrometry
U.V: Unit Variance
WAT: White adipose tissue
WHO: World Health Organization

Chapter 1
Background

1.1 Introduction

How infants are best fed is an evocative question with people's beliefs, environment, and socioeconomic status, in addition to many other factors, all influencing infant feeding. Infants have always been fed substances other than their own mother's breast milk due to these cultural, health and practical reasons. Artificial formula manufacturers have incrementally improved their formulations to more closely reflect that of human breast milk. For example, many manufacturers now include long chain polyunsaturated fatty acids such as Docosahexaenoic acid (DHA) and arachidonic acid (AA), citing evidence of improved intelligence scores for infants receiving increased intakes of these compounds (Birch et al., 2000).

Breast milk has been shown to confer many advantages to the infant, with a systematic review and meta-analysis, including 43 studies, reporting breast feeding to be associated with a reduction in the risk of acute otitis media, severe lower respiratory tract infections, atopic dermatitis, asthma, obesity, type 1 and 2 diabetes, childhood leukaemia, sudden infant death syndrome and necrotising enterocolitis (Ip et al., 2007).

The World Health Organisation (WHO) and many other international institutions recommend that infants are exclusively breastfed for the first six months of their lives (Kramer and Kakuma, 2004). This recommendation is based on evidence demonstrating breast fed infants have a significantly reduced risk of gastrointestinal infections (Kramer and Kakuma, 2004).

Despite decades of research into improving infant formula, it is not possible to capture the vast complexity of human breast milk within a standardised formula. The composition of human breast milk is tailored specifically towards infant requirements, changing in response to factors such as time since last feed and length of lactation, matching the infant's needs according to its age and other factors, such as sex (Fujita et al., 2012). This means the composition of breast milk is widely believed to be specifically tailored by each mother to reflect the needs of her infant.



Figure 1: Advertisement (left) for infant formula, on the right a poster promoting breast-feeding in the UK. The global baby food market size in 2010 was estimated to be worth 36.7 billion U.S. dollars (Statista, 2014). http://www.independent.co.uk/life-style/health-and-families/health-news/the-posters-that-celebrate-cool-multitasking-breasts-816224.html. http://www.wikiart.org/en/alphonse-mucha/nestl%C3%A9-s-food-for-infants-1897

Even with the known beneficial outcomes associated with breastfeeding, it has been proposed that human breast milk may not always be the most appropriate source of nutrition for some infants. In one such study, researchers demonstrated a long term negative metabolic effect of breast feeding among infants of diabetic mothers. In this study, infant Body Mass Index (BMI) at two years of age was found to be positively correlated with the quantity of 'diabetic' breast milk the babies received during the first week of life (odds ratio 2.47, 95% CI 1.25–4.87), (Plagemann et al., 2002).

Similarly, an analysis of outcomes in children of mothers with either gestational diabetes or type 1 diabetes found a positive association between the volume of diabetic breast milk ingested in the first week post-partum and increased relative body weight of children (Rodekamp et al., 2005).The authors adjusted for type of maternal diabetes and maternal blood glucose, however, it is difficult to isolate the effects of altered breast milk composition from the other effects of diabetes, for example alterations in the gut microbiota.

The same authors also investigated the impact of breast feeding on psychomotor and neuropsychological development in children of diabetic mothers. Here, the authors did not find any

impact of receiving breast milk from diabetic mothers on the infant psychomotor parameters, although they did observe an association between the volumes of breast milk received from diabetic mothers and delayed onset of speaking. However, after the model was adjusted for various factors, including birth weight and socioeconomic status, the hazard ratio was reduced to non-significance, suggesting that this association was explained by other confounding factors (Rodekamp et al., 2006).

Another example of when breast milk is not considered optimal or sufficient for infant requirements is for premature or low birth weight infants. In the case of these infants, breast milk may not match the nutritional needs, and may require fortification in order to increase the energy, protein, vitamin, sodium, calcium and phosphorus content, (Edmond and Bahl, 2006).

Since the last century various studies have examined the effects of maternal characteristics on breast milk composition. Important factors influencing breast milk composition–such as the gradual increase in fat concentrations throughout a feed have well defined effects and have robust evidence supporting them. Other potential influences, including mode of delivery and maternal BMI, have less high quality evidence to support the claims made on their effect on breast milk composition.

What is clear is that there is a lack of knowledge regarding how certain maternal characteristics influence breast milk composition, and how these alterations in composition may subsequently impact infant and later health outcomes.

Throughout this thesis the hypothesis that maternal characteristics, specifically maternal BMI, alters breast milk composition, exerting a measurable effect on both the nutritional and hormonal content is investigated.

In this chapter, the current knowledge on breast milk synthesis is reviewed, as well as the different nutritional and non-nutritional components of breast milk. In addition, previous literature investigating the differences between breast milk from overweight and obese mothers in comparison to lean mothers is reviewed. Furthermore, evidence detailing the potential of breast milk to programme infant metabolism, resulting in lifelong effects on offspring physiology and appetite regulation is reviewed.

Previous researchers' observations on factors known to influence breast milk composition are assessed, and this information is used to inform the design of this study, to reduce the effects of confounding factors as far as possible. Also evaluated are the advantages and disadvantages of methodologies previously used to measure breast milk composition, and the metabonomic techniques used throughout this thesis are subsequently described. Finally, areas of uncertainty present in this field are summarized, and investigated in the subsequent chapters.

1.2 Breast milk synthesis

The mammary epithelium employs five separate processes operating in parallel during the synthesis of breast milk, transforming precursors derived from either the blood or interstitial fluid into breast milk components. These five processes are; *exocytosis*, *lipid synthesis* and secretion, *transmembrane secretion*, *transcytosis* and the *paracellular pathway,* outlined in Figure 2.

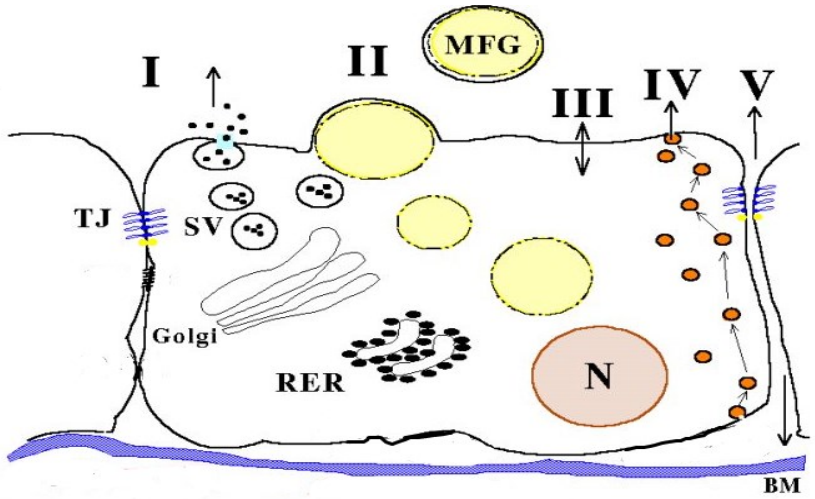


Figure 2: Diagram of an alveolar cell from a lactating mammary gland. BM, basement membrane; MFG, milk fat globule; N, nucleus; TJ, tight junction; SV, secretory vesicle; RER, rough endoplasmic reticulum. Adapted from (Neville, 1998).

Exocytosis contributes towards the majority of components secreted into the aqueous fraction of breast milk (Pathway I, Figure 2), including citrate, nucleotides, calcium, phosphate and glucose, which are packaged into secretory vesicles. Secretory vesicles then fuse with the apical plasma membrane, releasing their contents into the milk (Neville, 1998).

Lipid synthesis and secretion involves fatty acids and glycerol being combining into triacylglycerides in the smooth endoplasmic reticulum of mammary alveolar cells. The triacylglycerols then merge into droplets which travel to the apex of the alveolar cell (Pathway II, Figure 2). The lipid becomes encased in the apical plasma membrane, and eventually buds off as a milk fat globule into the milk. The milk fat globule membrane provides the infant with dietary phospholipids and cholesterol as well as preventing the fat globules from coalescing together, maintaining lipid as an emulsification (Neville, 1998).

Transport across the apical membrane appears to be restricted to a few small molecules such as sodium and potassium. It is unclear what the function of this pathway is (Pathway 3, Figure 2) (Neville, 1998).

Transcytosis allows whole proteins to cross the mammary epithelium from the interstitial fluid. (Pathway IV, Figure 2). At the basal surface of the mammary alveolar cells, proteins bind to their respective receptors; this protein-receptor complex is then taken up via endocytosis and transferred to the apical membrane where the extracellular portion of the receptor is cleaved and secreted, together with the protein. Proteins including immunoglobulins, hormones and growth factors are thought to be secreted into milk via this mechanism (Neville, 1998).

The paracellular pathway (Pathway V, Figure 2) permits metabolites to be secreted between epithelial cells. This pathway is closed once mature milk begins to be secreted as the tight junctions close, but molecules as large as immunoglobulins are able to be transported in this manner (Neville, 1998).

1.3 Breast milk composition

There is a vast collection of published scientific data documenting the various constituents of human breast milk. The methods employed to measure the presence and concentrations of these compounds are wide and varied.

Generally speaking, the macronutrient composition of mature breast milk, consisting of protein, fat and carbohydrate is approximately 9.0g/L of protein, 39.0 g/L of fat and 71.9g/L of carbohydrate. (Michaelsen et al., 1990). However, as we shall see, this is a gross simplification, as breast milk is an extremely complex and highly variable biofluid, its composition changing due to many different influences.

A database containing a large number of breast milk metabolites, their concentrations, and at which point during lactation they were measured, as documented by previous literature was complied, presented in Appendix 1A. Macromolecules that contribute to the nutritional and immunologic value of breast milk are outlined below.

1.3.1 Protein

Proteins present in milk can be divided into two fractions, casein and whey proteins, according to their solubility. Whey proteins are more soluble, and present in solution, whilst caseins are found as casein micelles, suspended in solution.

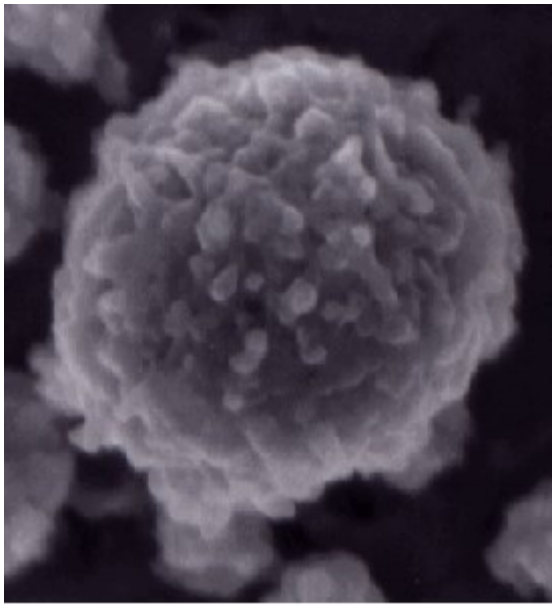


Figure 3: Structure of a casein micelle of bovine origin, image from a scanning electron microscope. Reprinted with permission from Elsevier, International Dairy Journal, Volume 14, Issue 12, Dalgleish et al., 2004.

Proteins present in significant quantities in the whey fraction are α-lactalbumin, lactoferrin, immunoglobulins, serum albumin and lysozyme. a-Lactalbumin is a mammary-specific protein and is the major milk protein found in human milk.

Casein refers to milk proteins possessing a low solubility at pH 4.0-5.0 (Lawrence RA, 2005). Three types of casein are present in human milk α -, β- and k-casein. Stabilising the insoluble α- and β-caseins is k-casein, forming a colloidal suspension, the casein micelle. Calcium phosphate is present in high concentrations in casein micelles, casein itself contains a high proline content and lacks disulfide bonds, causing the micelles to form a tangled web structure (Holt and Horne, 1996). The total protein content of human breast milk consists of 17% casein, the lowest casein concentration of any studied species (Lonnerdal et al., 1987).

A major fraction of breast milk protein content, particularly early on in lactation, is formed by the immunoglobulins found in milk, IgA being the most predominant form, followed by IgG. These provide immunological protection to the infant whist its own immune system matures (Hurley and Theil, 2011).

Lactocytes produce approximately 80-90% of the proteins present in breast milk, these are first synthesised on the rough endoplasmic reticulum and then transported to the Golgi apparatus where they are packaged into secretory vesicles, leaving the cell via exocytosis. The majority of the rest of the proteins secreted into breast milk are taken up via transcytosis (Neville M, 1983).

1.3.2 Non-protein nitrogen

Non-protein nitrogen, incorporating molecules such as urea, creatinine, creatine, uric acid, small peptides and free amino acids are known to vary from 20 -38% of total nitrogen present in human breast milk. A portion of this nitrogen is available to the neonate for the synthesis of non-essential amino acids. The ratio of essential to non-essential amino acids stays relatively constant throughout lactation (Chavalittamrong et al., 1981) Non-protein nitrogen also includes nucleotides, many of which perform key roles in various cell processes, such as altering enzymatic activities and acting as metabolic mediators, examples of which include adenine and cytosine (Uauy et al., 1994).

1.3.3 Lipids

The lipids in breast milk are present as an emulsion. The vast majority of lipids secreted are triacylglycerides, contributing towards 98% of the lipid fraction. The remainder predominantly consists of diacylglycerides, monoacylglycerides, free fatty acids, phospholipids and cholesterol. These components are packaged into milk fat lipid globules, with the phospholipids making up the bulk of the membrane of the globules and the triacylglycerols found in the core (Lopez and Menard, 2011).

These globules usually range from 1-10 µm across, with an average diameter in mature milk of 4µm (Michalski et al., 2005).

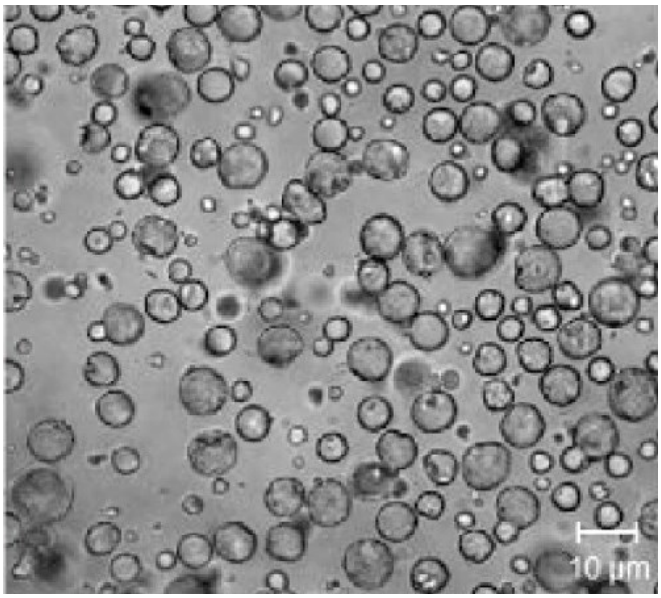


Figure 4: An optical microscopy image of milk fat lipid globules, displaying the structure of milk. Reprinted with permission from (Zou et al., 2012), American Chemical Society.

Analysis of non-polar metabolites in breast milk is essential. Lipids are one of the major energy sources in breast milk, contributing 40-55% of the total energy of breast milk (Koletzko et al., 2001), and are essential for neurodevelopment (Roncada et al., 2013). As well as these, short chain fatty acids (SCFA) are not only an important source of energy (Donohoe et al., 2011), but their presence in breast milk is essential for normal maturation of the gastrointestinal tract (Peng et al., 2009).

The fatty acid profile of breast milk has been studied in many different geographical regions, making it difficult to distinguish the effects of race and diet on the fatty acid composition of breast milk. In The Gambia, a study of 23 rural mothers found that the most abundant fatty acid was oleic acid (18:1), making up almost half of the total fat (Prentice et al., 1989). However this is thought to be exceptionally high as the diet of these women contains a high amount of groundnuts, accounting for around 75% of dietary fat. However, further studies in western populations have also found oleic acid concentrations in breast milk to be high, accounting for 30-40g/100g fat (Koletzko et al., 1988).

De novo synthesis of fatty acids accounts for approximately 17% of the total fat in breast milk (Prentice et al., 1989). A study focusing on well-nourished Sudanese women found that long chain polyunsaturated fatty acids, molecules with a chain length of more than 20 carbon atoms-plus 2 or more double bonds, constitute ~2% of the total fatty acids present in breast milk (Laryea et al., 1995).

Breast milk is known to contain over 200 fatty acids; however, many of these are present in very low concentrations. It has been observed that the positions fatty acids occupy along the glycerol backbone are highly conserved, commonly appearing in preferential positions (Martin et al., 1993). For example, fatty acids present in the highest concentrations in breast milk, oleic, palmitic and linoleic acid were found at the sn-1, sn-2 and sn-3 position respectively, and these distributions were consistent over time (Martin et al., 1993). It appears that the distribution of fatty acids along glycerol influences their availability; palmitic acid at the sn-2 position seems to be absorbed more readily. Significantly, this positional conservation is often not observed in artificial formulas, and was observed to influences plasma lipid profile, including cholesterol concentration (Innis et al., 1993).

Sphingomyelins, present in the milk fat globule membrane, are especially important for central nervous system myelinisation, and have been shown to improve the neurobehavioral development of low-birth-weight infants (Tanaka et al., 2013).

1.3.4 Carbohydrate

There are a huge variety of different carbohydrates present in milk. As well as lactose, various nucleotide sugars, lipid bound carbohydrates and protein bound carbohydrates are also transferred to the infant. Of the carbohydrate content, lactose is by far the most abundant; however oligosaccharides also make up a significant fraction (Coppa et al., 1993). Lactose, a disaccharide, consists of glucose and galactose covalently bound to one another.

1.3.5 Human Milk Oligosaccharides

Human milk oligosaccharides (HMO) are an extremely important component of human milk carbohydrate, together contributing to the third largest component in breast milk, totalling 12.9 ± 3.3 g/L in mature milk and 20.9 ± 4.8 g/L at 4 days post-partum (Coppa et al., 1993). HMO contain from 3 up to 22 saccharide units per molecule, and are made up of 5 different sugars, found in varying different sequences and orientations. The monosaccharide's which make up the oligosaccharides are D-glucose, D-galactose, N-acetylglucosamine, L-fucose and N-acetylneuraminic acid. There are known to be over 200 different types of oligosaccharide in human milk, all of which feature lactose at the reducing end (German et al., 2008).

One known influence on breast milk composition known to be modified by maternal characteristics relates to the oligosaccharides present in milk, as the composition of these is genetically determined. Different profiles of milk oligosaccharides depend on the specific transferase enzymes expressed in the lactocytes.

Two such genes, important for determining the HMO profile a mother produces, are the Secretor, and Lewis blood group genes. The secretor gene encodes for the enzyme α(1,2)-fucosyltransferase (FUT2), responsible for linking fucose in a α1-2 linkage to elongate the HMO chain. The enzyme FUT3 is encoded for by the Lewis blood group gene; this enzyme catalyses the reaction between fucose in a α1-3/4 linkage, creating further fucosylated oligosaccharides. As a result of the different expressions of these enzymes, there are four main phenotypes in relation to HMO profile; Se^+/Le^+, Se^-/Le^+, Se^+/Le^- and Se^-/Le^- (Thurl et al., 1997).

One study investigating breast milk HMO profile demonstrated Se^+/Le^+ mothers produced all types of fucosylated oligosaccharides, whilst Se^-/Le^+ mothers did not produce α1,2-fucosylated structures, such as 2'-fucosyllactose. Se^+/Le^- mothers secreted α1,2- and α1,3-fucosylated oligosaccharides, but not HMO containing α1,4-fucose residues (Pratico et al., 2013). However, it was noted that in Se^-/Le^+ mothers, α1,3-Fucosylated oligosaccharides, such as 3'-fucosyllactose, were between two to fivefold higher than in Se^+/Le^+ mother's breast milk. This suggests there is an increase in FucT3 activity in non-secretor mothers, meaning that the total oligosaccharide production is relatively equal between the different groups (Pratico et al., 2013).

HMO function as prebiotics, encouraging the growth of certain strains of beneficial bacteria, such as *bifidobacterium infantis*, within the infant gastrointestinal tract, protecting the infant from colonisation by pathogenic bacteria (Ward et al., 2006).

Furthermore, another mechanism by which HMO protect infants against gastrointestinal infection is by acting as receptor decoys. A crucial step in the initiation of infection is the binding of pathogens to carbohydrates present on intestinal epithelial cells. HMO inhibit this process due to their analogous shapes to cell surface carbohydrates, which are recognised and bound to by pathogens, which then passes harmlessly from the gastrointestinal tract, i.e. HMO competitively inhibit pathogen binding to the infants epithelial cells. Demonstrating this protective capacity, an observational study found a significant association between low levels of specific 2-linked fucosylated oligosaccharides in human milk and increased rates of *Campylobacter* diarrhoea in breast fed infants. Furthermore, infants who received milk containing a low concentration of lacto-N-difucohexaose had an increased incidence of calcivirus diarrhoea, (Morrow et al., 2004). Breast milk contains relatively equal concentrations of HMO between mothers, but it is the structures of the HMO which are genetically determined, and furthermore it is the specific structures which inhibit pathogen binding as different pathogen receptors have different affinities for specific carbohydrate structures.

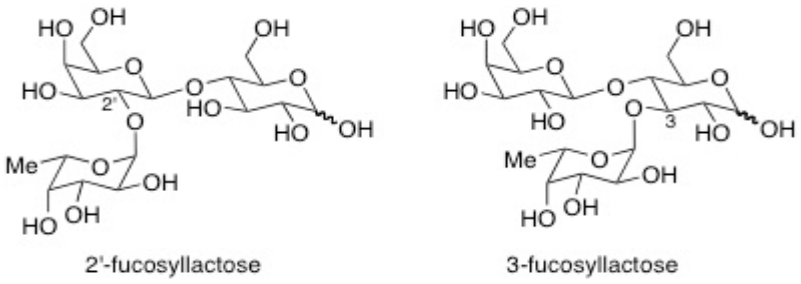


Figure 5: Structure of 2'- and 3'-fucosyllactose. Reproduced from (Pereira and McDonald, 2012).

1.4 Differences In breast milk from overweight/obese mother to lean mothers

1.4.1 Macronutrient differences in breast milk of mothers with differing BMI

The overall macronutrient content of breast milk increases with increased maternal BMI, Table 1 (Michaelsen et al., 1990). Mothers with a BMI of less than 22kg/m^2 have been observed to have a 5% decrease in the protein content of their breast milk compared to mothers of a higher BMI (Michaelsen et al., 1990). This study used an infrared analysis to determine to the concentrations of these nutrients in breast milk.

Table 1: The relationship between nutritional content of breast milk per unit increase in maternal BMI. Standard error is shown in brackets. Adapted from (Michaelsen et al., 1990).

Macronutrient, (g/L)	Maternal body mass index (BMI) (kg/m^2)
Protein	+0.054 x BMI (0.006)
Fat	+0.58 x BMI (0.06)
Carbohydrate	+0.056 x BMI (0.014)

Further research has highlighted differences in the nutritional content of breast milk from Otomi Indian mothers of a high and low BMI. In this study, mothers breast milk fat concentrations were significantly lower in the low BMI group in comparison to the high BMI group at 6 months post-partum, 29.1±8.7 vs 35.7±9.2mg/g, p=0.04, in the low and high BMI group respectively.

Furthermore, breast milk fat concentrations were positively correlated with maternal body fat, as measured by deuterium dilution, at three and six months post-partum, r=0.32 and 0.40; p=0.04 and 0.01, respectively. However, it should be noted that, due to the inverse correlation between breast milk production and fat concentrations in milk, fat secretion into milk did not differ between groups and therefore the growth velocities of the infants did not significantly differ between the low and high BMI groups, (Barbosa et al., 1997). This means that although infants of obese mothers were receiving milk with a higher caloric density they are provided with less volume, therefore on average receiving the same quantity of calories as their peers born to lean mothers.

Furthermore, breast milk from overweight mothers has been observed to have significantly increased glucose concentrations. The mean milk glucose value of mothers with a BMI of below 25kg/m^2 was 32.6±21.8mg/dL, whilst mothers with a BMI above 25kg/m^2 was 51.9±20.5mg/dL (Ahuja S, 2011).

1.4.2 Differences in non-nutritive compounds in breast milk of mothers with differing BMI

Many studies have identified a correlation between maternal overweight/obesity and breast milk appetite regulating hormone concentrations. For example, the concentration of insulin in breast milk has been found to be greater in overweight mothers compared to controls. Milk-borne insulin values of mothers with a BMI of below 25kg/m^2 and mothers with a BMI above 25kg/m^2 were 4.5±7.6μIU/mL and 30.1±56.3μIU/mL respectively, (Ahuja S, 2011). Multiple studies explore the link between maternal BMI and appetite regulating hormones present in breast milk; these are systematically reviewed and investigated in vivo in Chapter 3.

1.4.3 Evidence from animal models suggests differences in milk composition from overweight animals

Previous research investigating the influence of maternal dietary induced obesity and altered diet have previously been undertaken employing animal models. In one such study, changes in milk composition, including protein, lactose and fat content, as well as fat composition were determined for lean and obese rats fed a highly palatable diet. Milk of the obese rats was found to be more calorific, due to an increase in fat content. However, obese rats milk contained less protein than lean rats milk (Rolls et al., 1986).

1.5 Incidence of overweight and obesity

In the United Kingdom from 1990 to 2004 there was seen to be a 60% increase in the incidence of maternal obesity and over 50% of women of reproductive age are now classified as either overweight or obese (Heslehurst et al., 2007). The cost of obesity for the NHS in England is estimated to be approximately £1 billion (Heslehurst et al., 2007).

Worldwide, rates of overweight and obesity have increased dramatically, almost doubling since 1980, (WHO, 2013). In 2008, over 1.4 billion adults were overweight, with half a billion of these individuals classed as obese. Currently, overweight and obesity kill more people than underweight, accounting for at least 2.8 million deaths each year, and is the fifth largest cause of global deaths (WHO, 2013).

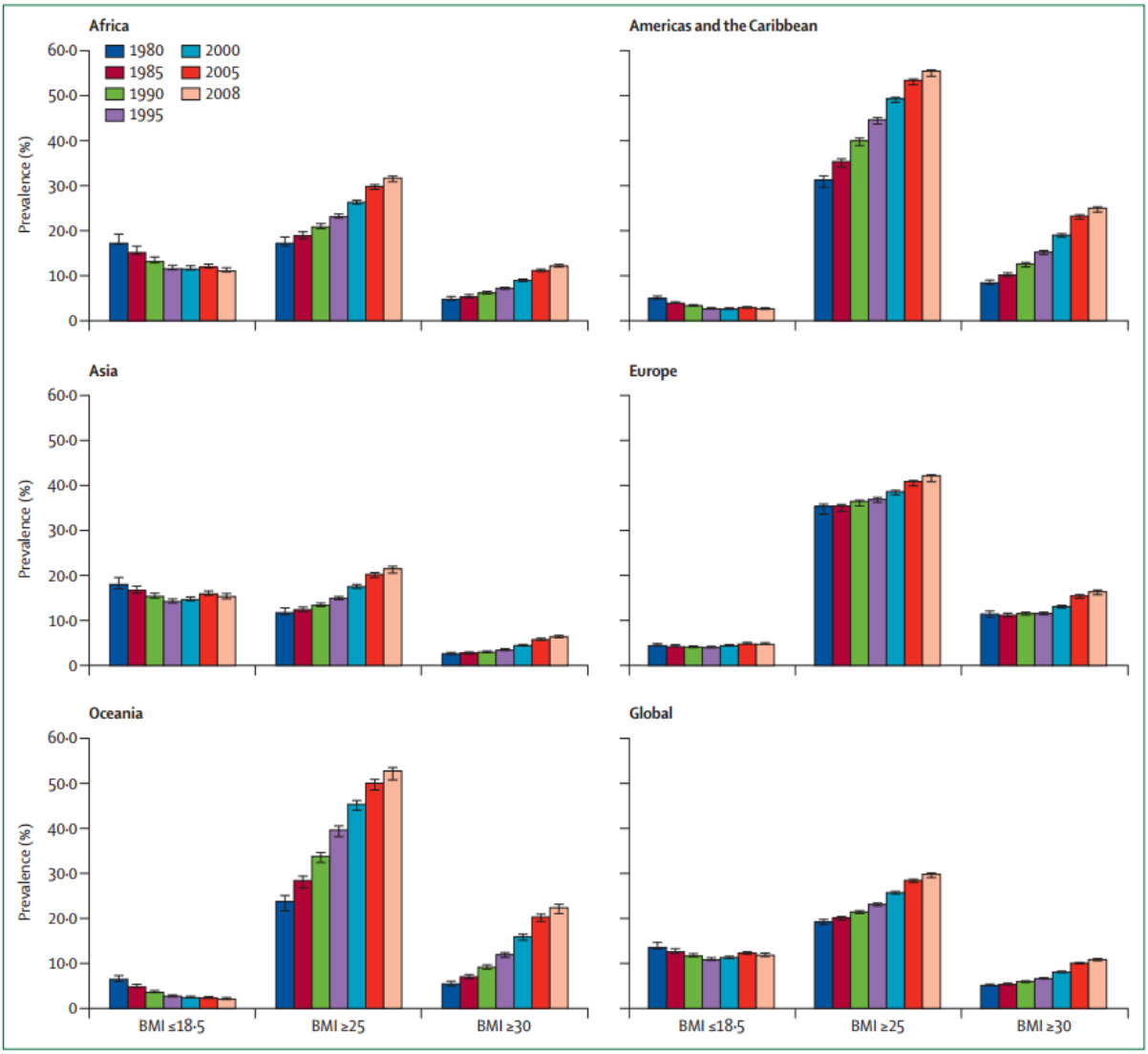


Figure 6: Rates of underweight (BMI <18.5kg/m^2), overweight (BMI >25kg/m^2) and obesity (BMI >30kg/m^2) from 1980 to 2008 in women aged 20-49 years. Figure reproduced with permission from Elsevier, (Black et al., 2013).

1.6 Early life programming

Nutrition received during the period of early development in mammals permanently affects the health of the subsequent adult, as well as the individual's offspring. Sub-optimal maternal nutrition during this time is of major concern due to the long lasting negative effects it is able to exert on offspring well-being (Ferland-McCollough et al., 2012).

Professor David Barker at the University of Southampton first described evidence indicating an association between low birth weight and an increased likelihood of developing coronary heart disease in later life. This association has now been observed for various non-communicable diseases such as stroke, diabetes and obesity (Barker, 1995). These findings were later named in the British Medical Journal as the 'Barker Hypothesis' (Paneth and Susser, 1995); this states conditions in early life program an infant's development, potentially predisposing them to various diseases in later life (Barker, 1995).



Figure 7: Two healthy term infants; the infant on the right is at an increased risk of developing non-communicable diseases in later life compared to its peer due to its low birth weight. Reproduced from http://www.beginbeforebirth.org/in-the-womb/fetal-programming.

Programming is proposed to occur during specific crucial time periods of growth during early life. An insult or stimulus, such as malnutrition, causes a disruption of the normal development of the infant. Because the infant adapts in response to this environmental insult, it 'predicts' the environment it will be raised in. However, this does not always reflect the environment the infant is subsequently exposed to, therefore, if there is a mismatch between the two, this predisposes the infant to disease, as it is poorly adapted to the environment it is raised in (Rogers and Velten, 2011).

This programming of infant metabolism is relevant to both the developed and developing world; as there is often a caloric mismatch; women in the developing world are often malnourished, whilst in

the developed world, women often eat unbalanced diets in regard to macronutrient content and are often deficient in micronutrients, such as folate, with the developing fetus suffering as a result.

Much of the evidence for programming suggests its effects are established *in utero* (Desai and Hales, 1997). However, it is likely that in addition to this antenatal programming, sub-optimal nutrition in the postpartum period, for example receiving formula milk, or breast milk with and altered composition, may further contribute towards the programming of infant metabolism.

A wide range of negative consequences have been observed in infants of obese/overweight mothers, including an increased risk of being either large (Ehrenberg et al., 2004), or small (Rajasingam et al., 2009) for gestational age, both of which are associated with negative outcomes in later life. For example, offspring of obese mothers are at an increased risk of being obese themselves (Parsons et al., 2001), as well as having an increased risk of developing diabetes, (Wei et al., 2003). However, whether this effect is due to the shared environment or genuine programming of infant metabolism is under debate, but mounting evidence shows a strong contribution of this effect is due to the shared environment, (Lustig, 2006).

Nonetheless, evidence suggesting programming does play a role in this phenomenon, having additional effects over shared environmental factors, comes from sibling studies of obese mothers studied before and after maternal bariatric surgery, (Smith et al., 2009). Children born to mothers after bariatric surgery were found to have a lower birth weight (2.9+/-0.1kg after maternal surgery vs. 3.3+/-0.1kg before maternal surgery, p=0.003), and a reduction in the prevalence of macrosomia (1.8 after maternal surgery vs. 14.8% before maternal surgery, p=0.03) was noted, but no difference in the incidence underweight was observed, (Smith et al., 2009).

When these infants were followed up, individuals who had been born after maternal bariatric surgery were shown to have a 3-fold lower prevalence of severe obesity, 11 vs. 35%, p=0.004. Furthermore, these infants received many other metabolic benefits, with beneficial effects on cardiometabolic markers maintained into adolescence, including better insulin sensitivity and lipid profile, than individuals born to mothers before undergoing bariatric surgery. The authors contributed this effect to an improved intrauterine environment (Smith et al., 2009).

Additionally, evidence of metabolic programing during the perinatal period has been proposed to occur through the epigenetic modification of DNA. In this study, mice dams were fed a high fat diet during pregnancy and lactation. This was seen to induce a co-ordinated and long-lasting change in the expression of insulin like growth factor 2, and genes involved in fat metabolism in the offspring (Zhang et al., 2009).

Human milk is known to contain a vast number of nutrients, however, in addition to these nutritional components, breast milk also contains a multitude of hormones and immunological agents, often in greater concentrations than in maternal plasma (Savino and Liguori, 2008). These are potent biological agents, which have been shown to influence the metabolism of the neonate.

It is conceivable that the composition of breast milk may signal satiety to the baby as a feedback mechanism to control milk intake. For example, it has been reported that the concentration of leptin in breast milk of obese women is higher than non-obese women (Houseknecht et al., 1997). Leptin down regulates appetite (Dundar et al., 2005a), so high leptin concentrations in milk may reduce the intake of milk. This may be a positive mechanism for offspring of obese women as their milk is known to be particularly nutrient and energy dense (Michaelsen et al., 1990). Therefore, the higher leptin concentration in breast milk may reduce the intake of excess calories, protecting the baby from early onset of obesity (Miralles et al., 2006).

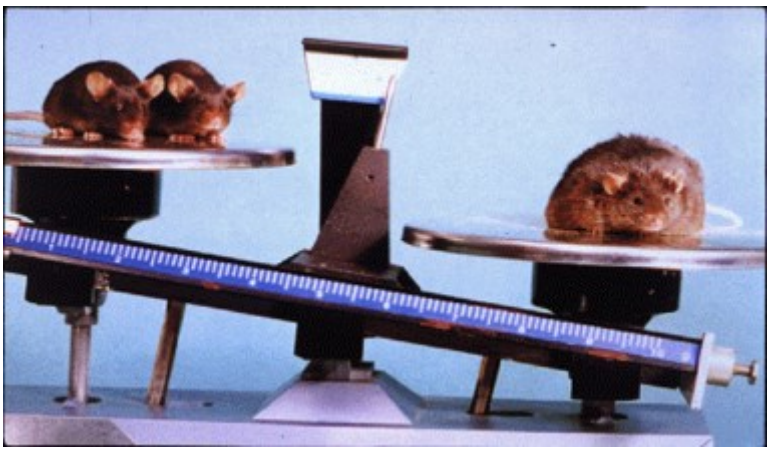


Figure 8: On the left are two wild type mice, whilst on the right is an ob-/ob- mouse, which cannot produce leptin, and is therefore unable to regulate its appetite properly. Reproduced from biocadmin.otago.ac.nz/fmi/xsl/bioc/learnbitslecture.xsl?-db=BIOCweb.fp7&-lay=Lectures&-recid=5207&-find=

If this effect is due to altered breast milk composition then potentially the altered breast milk composition of overweight and obese mothers may influence the metabolism of their infants. Consequently, if this is the case then proper delineation of exactly how breast milk from these mothers differs from lean mothers needs to be established. Further animal models could be employed to establish to what extent altered breast milk composition is able to influence infant and subsequent adult metabolic phenotype.

Evidence supporting this hypothesis has been collected in human studies investigating the hormonal composition of breast milk and its correlation with infant growth. A number of studies have highlighted a negative correlation between infant growth and leptin, an anorexigenic hormone

(Doneray et al., 2009, Miralles et al., 2006). However, in contrast other such similar studies have not found this effect (Uysal et al., 2002).

1.6.1 Animal models supporting the contribution of breast milk towards infant programming

Animal studies have reported programming of appetite regulation due to the type of milk ingested. In one such study, rat pups from control dams were cross-fostered to dams with gestational diabetes. Cross-fostered pups where shown to have mal-programmed hypothalamic orexigenic and anorexigenic neural circuits, which are involved in appetite regulation (Fahrenkrog et al., 2004).

Further research using rodent models have investigated the impact of maternal high fat diet during lactation on the neonate. Here, researchers established that dams fed a high fat diet during lactation predisposed their offspring to obesity in later life. Contributing towards this effect, offspring were shown to have impaired glucose homeostasis, associated with an impairment of the hypothalamic melanocortin circuitry (Vogt et al., 2014).

In order to establish the mechanism behind this effect, the number, and neuropeptide expression of anorexigenic proopiomelanocortin (POMC) - appetite supressing, and orexigenic agouti-related peptide (AgRP) - appetite stimulating, neurons were investigated. The electrophysiological properties of POMC neurons and posttranslational processing of POMC were also studied. These properties all remained unaffected in the neonates of dams fed high fat diets. However, it was observed that the formation of projections from POMC and AgRP neurons to their hypothalamic target sites was impaired in neonates of dams fed a high fat diet (Vogt et al., 2014).

The impaired formation of POMC projections could be prevented by abolishing the action of insulin in the POMC neurons, which simultaneously abolished the effect of abnormal parasympathetic innervation to the pancreas, and impaired glucose stimulated insulin secretion seen (Vogt et al., 2014).

These observations demonstrate the crucial timing in programming of infant metabolism, occurring during the period where neuronal projections are formed, and altered insulin signalling has an important role in mediating this effect (Vogt et al., 2014).

Research by Franco *et al* demonstrated that a maternal isocaloric high fat diet changes milk composition, with increased fat and protein concentrations and hyperleptinaemia. This altered milk composition induced obesity in the male rat offspring of the mothers fed this high fat diet (Franco et al., 2012).

Evidence from these studies suggest that if neonates are subjected to an abnormal hormonal milieu during development, persistent changes in the function of hypothalamic neurocircuits result, responsible for the regulation of energy and glucose metabolism.

Whether these effects would be replicated in humans is unclear. This is because, in humans, it is thought that the development of the hypothalamic neurocircuits is more mature at birth, whist in rodents this process continues until several weeks post-partum, throwing into question the translatability of animal models in regard to these observations (Grayson et al., 2006, Koutcherov et al., 2003).

Nonetheless, programming effects have been observed in primate models during the post-partum period, suggesting that there is still some developmental plasticity during this period. One such study of infant over and under nutrition in baboons tested the hypothesis pre-weaning nutrition is able to influence adult fat cell numbers and adiposity (Lewis et al., 1986). In this study infants were fed formula milk containing different caloric densities, after which they were fed the same diets. Fat cell number and size were then studied in ten different adipose depots. It was observed that females who were overfed as infants were found to have greater fat depot mass, due to fat cell hypertrophy, whilst males overfed as infants had greater fat mass depots in four of the ten depots studied, the different diets did not influence fat cell number (Lewis et al., 1986).

Further evidence supporting the postulation that developmental differences occur for infants fed different diets comes from a study using NMR spectroscopy to investigate the metabolic implications of breast feeding and formula feeding rhesus monkeys. This study investigated urine and serum metabolome, recorded anthropometric measurements, and profiled the infant microbiome and serum cytokines. Formula fed infants were found to be larger than breast fed infants and displayed an altered microbiome; with breast fed infants having higher levels of Lactobacillus genus bacteria, a beneficial strain of bacteria. Additionally formula fed infants had increased concentrations of serum insulin, as well as amino acids. This study gives support to the hypothesis that different diets in early life can influence infant metabolism, gut microbiome and immunity (O'Sullivan et al., 2013a).

1.7 Maternal influences on breast milk composition

Breast milk composition is extremely complex, varying with the time of day, maternal diet and many other factors, outlined below (Lawrence RA, 2005). The change in breast milk composition over lactation is known to reflect the infant's needs, becoming more energy dense over lactation, with increasing carbohydrate and lipid concentrations to provide for the infants increased energy requirements. Total protein is seen to decrease, probably due to the decreasing concentration of factors such as immunoglobulins secreted into breast milk (Mickleson and Moriarty, 1982). This not only reflects the infants decreased requirement for them, as their own immune system becomes functional, but also reflects the increasing inability of the infant gut to absorb proteins, as gut closure occurs and the permeability of the gut to macromolecules decreases over the first 3 days of life (Vukavic, 1984).

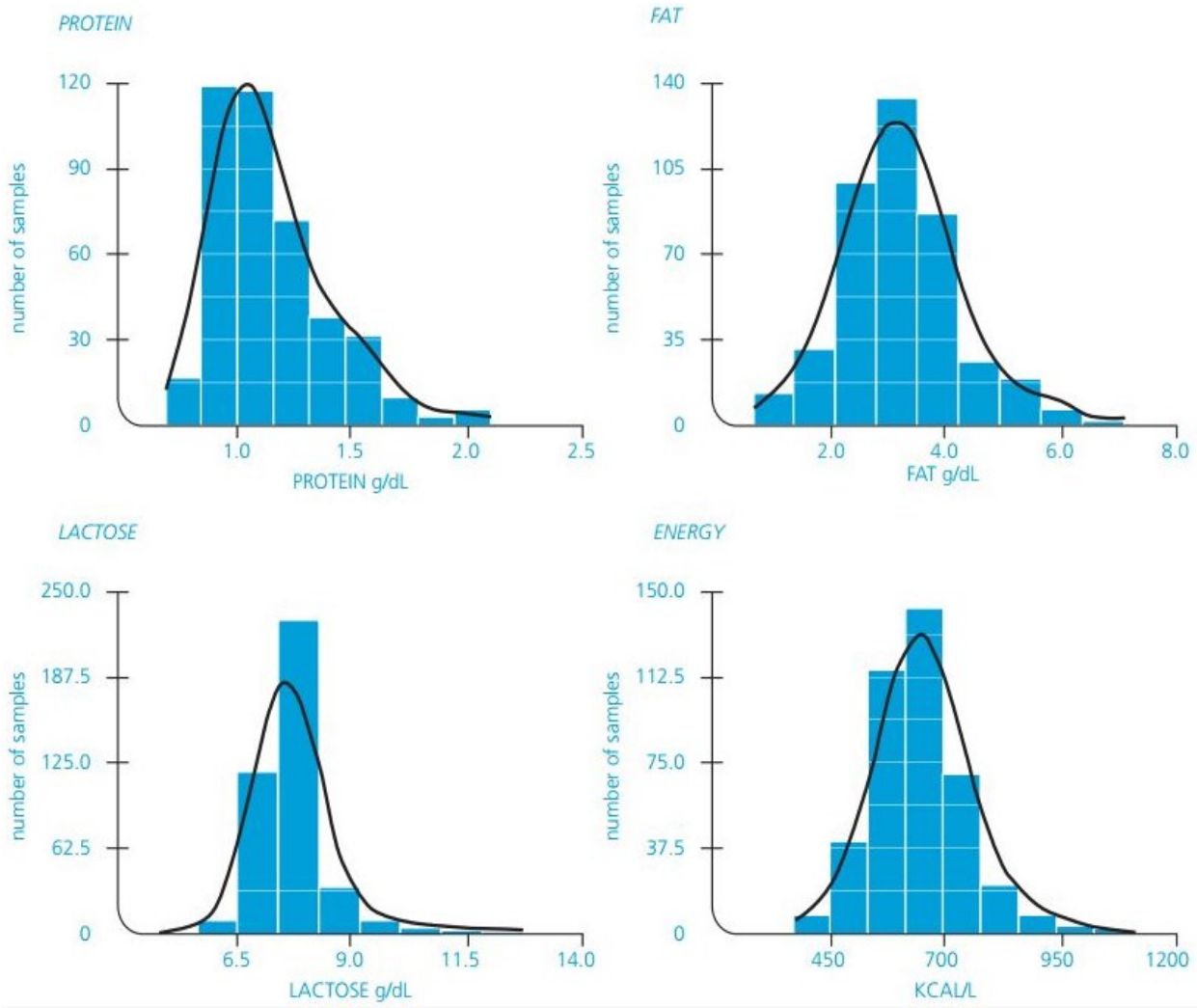


Figure 9: Variation in the content of protein, fat, lactose and total energy of breast milk from different mothers, analysis using a Miris milk analyser. Reproduced from Miris. (Miris, 2013).

1.7.1 Time since last feed

One of the most significant predictors of milk fat concentration is the length of time since the last feed; the longer this interval is, the lower the concentration of fat in the milk. In keeping with this, fat concentrations at the end of the previous feed, as well as the volume of milk received at the previous feed, have been found to be particularly important predictors of milk fat concentration (Jackson et al., 1988).

1.7.2 Stage of the nursing process

The stage of the nursing process is known to result in a large change in the composition of breast milk. There is an increase in the fat content from the beginning, known as fore milk, to the end of a feed, hind milk, whilst lactose shows an inverse correlation to the change in fat content (Hytten, 1954). This effect is responsible for some of the biggest changes seen in the alteration of milk composition. It is a common misconception that there are two kinds of milk, however these distinctions correspond to a gradual change in the composition of milk throughout a feed as the breast milk steadily becomes fattier towards the end of a feed.

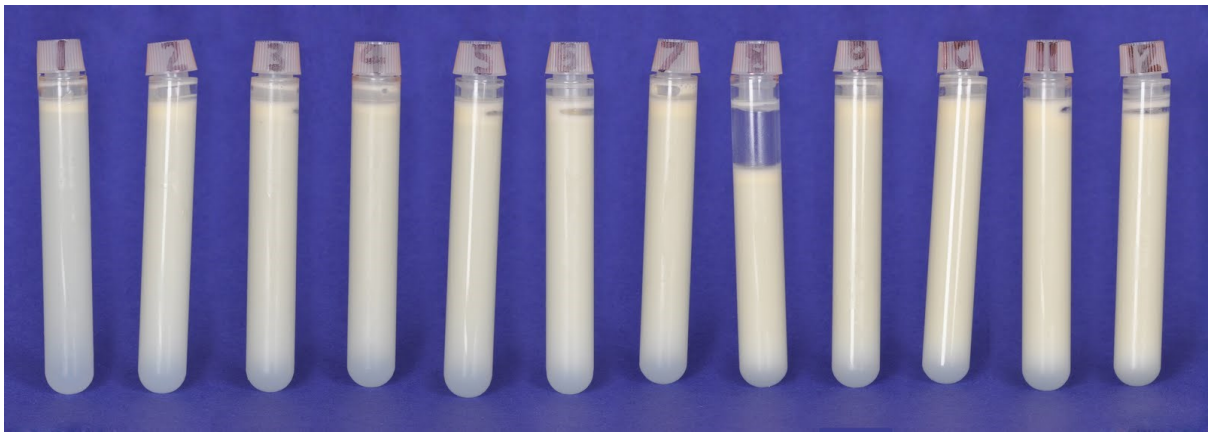


Figure 10: Image displaying the gradual increase in milk fat concentration from the beginning far left (1) to the end far right (12) of a feed. Replicated from thefunnyshapedwoman.blogspot.co.uk /2011/ 05/ foremilk-and-hindmilk-in-quest-of.html

1.7.3 Length of Lactation

There are considerable changes in the composition of milk over the course of lactation. Milk is commonly classified into colostrum, transitional milk and mature milk. Similarly to fore and hind milk, these are not distinct classes of milk, but refer to the gradual alteration in the content of milk throughout lactation (Jenness, 1979). The average content of protein in breast milk gradually decreases from the second month to the seventh month, after which the speed of reduction of protein content levels off. Lactose production is highest in the forth to seventh month, after which it decreases. In contrast to this, HMO production is very high during early lactation, tapering off as lactation proceeds, with one study finding concentrations reducing from ~21 g/L to ~13 g/L from day

4 to day 120 post-partum. Also there is a gradual increase in the concentration of lipid in the milk over lactation (Kader et al., 1972).

1.7.4 Age of Mother

Protein concentration is highest in breast milk of mothers aged 20-30, however, maternal age does not seem to influence lipid or lactose concentrations (Kader et al., 1972). Similarly, another group concluded maternal age did not significantly alter breast milk composition in terms of fat or carbohydrate concentration. This study also found protein levels decreased with increasing maternal age, however the authors note that the influence was very small and unlikely to be of nutritional relevance (Michaelsen et al., 1990).

1.7.5 Birth weight

Milk fat concentration increases if there is a deviation from normal birth weight i.e. there is a u-shaped association between fat content and infant birth weight, with a 20-30% increase observed at the lowest and highest infant birth weights. Concentrations of protein and carbohydrate have not been seen to change significantly in relation to infant birth weight (Michaelsen et al., 1990). However, this study did not collect information on length of gestation; therefore, this influence may simply be a marker of the maturity of the infant.

1.7.6 Weight gain during pregnancy

A correlation between maternal weight gain during pregnancy and breast milk fat content has been reported, however, this was only observed to be significant at four months post-partum not at two months post-partum. The authors hypothesise that this phenomenon may be due to the laying down of fat stores during pregnancy, which are used as an energy reserve during lactation and subsequently more quickly diminished in the low weight gain group of mothers (Michaelsen, 1997). Despite this finding, two further studies were unable to identify an association between maternal weight gain during pregnancy and breast milk fat content (Butte et al., 1984), (Dewey KG, 1986).

1.7.7 Ethnicity

An analysis summarising research on the composition of milk of mothers from seven countries suggests breast milk composition is relatively consistent from mothers across different ethnicities. Of the variation which was observed, fat content was seen to vary by the greatest amount. Importantly, the magnitude of inter-individual variation between mothers of the same ethnicity was as great as that observed between mothers from different ethnicities (Jenness, 1979).

1.7.8 Diet

The influence of maternal diet on breast milk composition is complex. Depending on the type of nutrient, maternal diet can have virtually no impact on a nutrients concentration, whilst for other nutrients, maternal diet can result in large variations in milk composition (Innis, 2014).

Previous research on the macronutrient content of breast milk comparing Swedish, Ethiopian privileged, and Ethiopian underprivileged mothers found little variation based on diet (Lonnerdal et al., 1976). However, this study investigated total macronutrient content, not specific metabolites accounting for macronutrient content, therefore, it was not sensitive enough to distinguish more subtle differences between groups.

The variation in milk lipid concentration appears to be independent of maternal diet (Ballard and Morrow, 2013), however, fatty acids which form the lipid fraction are sensitive to maternal diet. These fatty acids are either endogenously synthesised in the mammary gland, or taken up from the maternal plasma. Both of these fatty acid sources have been observed to be influenced by maternal diet (Innis, 1992). Because triacylglycerides consist of three fatty acids esterified to a glycerol backbone, any increase in one particular type of fatty acid naturally results in a concurrent decrease in concentration of another type of fatty acid.

There have been numerous studies observing the fatty acid profile of breast milk can be altered by manipulating the maternal diet, (Innis, 1992), (Brenna et al., 2007) and (Koletzko et al., 1992), especially of the monounsaturated, n–6, and n–3 fatty acids. In one such study, Insull et al altered the diets of lactating women, providing 40% of their dietary energy from lard, corn or linseed oil. Within 2–3 days of changing the mothers' diet to corn oil (containing ∼52% of 18:2 n–6), the amount of the 18:2 fatty acid increased from 8–10% to ∼42% of the fatty acid present in breast milk. Simultaneously, the relative contribution of arachidonic acid (20:4 n–6) and DHA to total milk composition fell from 0.7% to 0.3%, and 0.3% to trace amounts respectively (Insull et al., 1959). Dietary fatty acids are therefore thought to be transferred rapidly into breast milk, and within 2 to 3 days breast milk changes to mimic that of dietary fat.

The mammary gland is capable of synthesizing the medium-chain fatty acids (MCFAs) 10:0, 12:0 and 14:0. Women receiving a high carbohydrate, low fat diet have increased MCFA synthesis, maintaining the quantity of triacylglycerides in breast milk (Novak and Innis, 2011). This increase in MCFA is accompanied by a decrease in the concentration of mono-unsaturated fatty acids (MUFA), 18:2 n–6, and 18:3 n–3 in the breast milk (Novak and Innis, 2011).

Animal studies investigating the composition of breast milk in relation to maternal diet have reported, in pigs, that the kind of dietary supplementation had little effect on milk composition, but the timing of dietary supplementation appeared to have a significant influence, especially on the percentages of fat and protein in the milk. It was shown that the fatty acid profile of the maternal diet during gestation also reflected the fatty acid profile of milk during lactation. The most dramatic differences in milk fatty acid profile were seen in sows that had received either sunflower oil or fish oil diets during gestation; with changes in milk fatty acid profile again reflecting the fatty acid composition of the diet (Laws et al., 2009).

1.7.9 Diurnal variation

There has been shown to be a typical diurnal variation of fat content in milk, with a peak fat content occurring at midmorning and a low during the night. This variation can be from 3g/100ml to 5g/100ml (Jenness, 1979). Figure 11 displays a summary diagram, indicating the findings from multiple studies investigating the variation fat content of breast milk throughout the day.

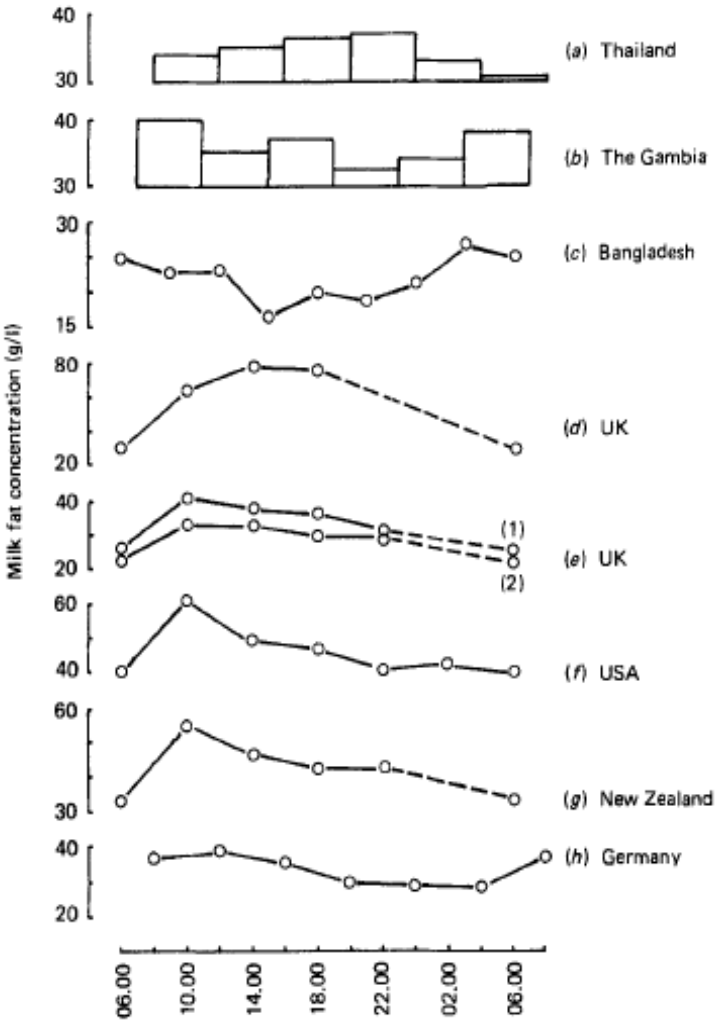


Figure 11: Circadian variation in fat content of breast-milk from previously published studies.
(a) Thailand, demand feeding, fore and hind milk expressed samples, nineteen mothers studied for 24 h, infants aged 1-9 months (Jackson et al., 1988). (b) The Gambia, demand feeding, fore and hind milk expressed samples, sixteen mothers studied for 24 h, infants aged 1-18 months (Prentice et al., 1989). (c) Bangladesh, demand feeding, total breast expression via breast pump, seven mothers studied for 24 h, infants aged 1-9 months (Brown and McDonald, 1982). (d) UK, scheduled feeds, fore and hind milk expressed samples, one mother studied for 72 h, age of infant unspecified (Hall, 1979). (e) UK, scheduled feeds, total breast expression via breast pump, (1) twenty-nine mothers studied for 24 h, infants aged 3-8 d, (2) twenty mothers studied for 24 h, infants aged 21d-4 months, (Hytten, 1954). f) USA, scheduled feeds, total manual breast expression, values given for one mother, studied for 24 h on six occasions and 72 h on one occasion, infant aged 6-60 weeks (Nims, 1932). (g) New Zealand, scheduled feeds, total manual breast expression, 28 mothers studied for 24 h, infants aged 1-8 months (Deem, 1931). (h) Germany, scheduled feeds, total manual breast expression, two mothers studied for 24 h, six mothers studied for 52 h, infants aged 8-11 d (Gunther and Stanier, 1949). Reproduced with permission from (Jackson et al., 1988).

The alterations observed in breast milk lipid concentration do not obviously relate to differences in study design or sampling methods. As many of the studies report different findings in relation to when the peak of breast milk fat occurs, the authors suggest that this disparity may be due to the feeding behaviour of the infant, as this is significantly correlated to variations in fat content, (Jackson et al., 1988).

1.7.10 Colostrum

Colostrum, the first milk produced, is significantly different from mature milk produced later on into lactation. Colostrum differs from mature milk in many ways, containing higher concentrations of protein and lower concentrations of lactose and fat (Jenness, 1979, Saint et al., 1984). As well these macronutrient differences, colostrum is dramatically different to mature breast milk in terms of its bioactive properties, containing high concentrations of secretory immunoglobulin (Castellote et al., 2011). These qualities suggest that the primary role of colostrum is not nutritional, but immunologic, protecting the baby as it emerges from the relatively sterile environment of the womb to being exposed to many environmental pathogens. In accordance with this, the concentration of HMO in colostrum is particularly high, being approximately double that of mature milk.

As well as its immunologic and nutritional roles, colostrum appears to also act as a growth promoter. Colostrum contains many growth factors, again often in greater concentrations than in mature milk, for example, epidermal growth factor (Okada et al., 1991), transforming growth factor-beta (Saito et al., 1993) and colony stimulating factor-1 (Flidel-Rimon and Roth, 1997) are all found in higher concentrations in colostrum in comparison to breast milk.

1.8 Methods previously used to measure breast milk composition

Methods previously used to measure breast milk composition have frequently focused on specific nutritional components such as fat or protein content, or, if a group is interested in a specific component, assays such as Radioimmunoassay (RIA) or Enzyme Linked Immunosorbent Assay (ELISA) are used (Miller et al., 2013). Methods used to measure various macronutrient and peptides are outlined below.

1.8.1 Protein

Protein content can be measured using a number of different techniques. One technique is colorimetric assay, using methods such as bicinchoninic acid, Lowery-Peterson or the Bradford test. However, it has been noted that is important to use a reference method specific for milk protein in order to get a reproducible result (Keller and Neville, 1986).

Another method for determining total protein used is elemental analysis. Elemental analysis of carbon, hydrogen, and nitrogen is achieved by measuring the amount of nitrogen by combustion of a milk sample and measuring the waste NO_2 produced (Power et al., 2002).

However the gold standard of determining protein concentrations is the Kjeldahl method. This method determines total nitrogen content, and is used as the reference technique which other methods are measured against. The Kjeldahl method is extremely precise; however it requires a large (10ml) sample volume (Miller et al., 2013), which is often impractical. All these methods simply quantify the total nitrogen content present in a sample, this leads to an overestimation of actual protein content, due to the non-protein nitrogen found in milk, however attempts are usually made to adjust for this.

Recent analysis, using a combination of electrophoresis and two-dimensional liquid chromatography, has enabled the identification of over 400 proteins present in human breast milk, many of which were identified for the first time (Molinari et al., 2012).

1.8.2 Lipid

Many methods have been established in order to measure the fat content in human milk. One common technique is the creamatocrit method. Milk is first drawn into a standardised capillary tube and centrifuged forming a cream layer at the top of the sample. Callipers are then used to measure the thickness of the cream layer, the ratio of the measurement to the column length allows this to be converted into grams of fat per litre (Lucas et al., 1978)

Quantification of the lipid fraction of milk using solvent extraction is a direct way of determining lipid content. However, specific solvents can be used to influence which species of lipid are extracted. This method allows for the determination of milk fat as a percentage of total weight.

Again more recent methods using liquid chromatography have unveiled the staggering diversity present in the lipid fraction of breast milk. One study, detailing the triacylglyceride content alone, revealed 170 different triacylglycerides (Winter et al., 1993).

1.8.3 Carbohydrate

The predominant carbohydrate in milk is lactose; however Human Milk Oligosaccharides, monosaccharides and other carbohydrates also make up a significant portion of the carbohydrate content in milk. Many methods developed to measure the carbohydrate content of milk only quantify lactose concentration, leading to an underestimation of the true carbohydrate content. Similarly to protein quantification, lactose concentration can also be measured using colorimetric techniques, with comparison to a standard (Miller et al., 2013).

Assessment of total sugar content can be measured using a phenol-sulphuric acid method. This method hydrolyses the bond between sugars using sulphuric acid; sugars released then react with the phenol present, giving a yellow reaction product, which can be quantified using a spectrometer, (Dubois, 1956).

More recent techniques used to investigate the carbohydrate content of milk have used liquid chromatography coupled to mass spectrometry. Using this method, the diversity of human milk oligosaccharide content has been investigated, with around 200 distinct oligosaccharides being identified (German et al., 2008).

1.8.4 Mid Infrared transmission spectroscopy

Mid infrared transmission spectroscopy allows for the quantification of fat, carbohydrate and protein content of milk samples. It has only relatively newly been applied to the analysis of breast milk. The primary use of this technology is for the clinical determination of nutritional content of breast milk, in order to establish whether fortification of breast milk is required for preterm infants nutritional intake (Menjo et al., 2009). This method has more recently been used in standalone research in order to investigate how maternal characteristics influence breast milk composition (Dizdar et al., 2013). This research concluded that higher concentrations of protein could be found in colostrum of mothers who had given birth vaginally. The authors speculate that this could arise from altered hormonal activity induced by labour pains and uterine contractions. Furthermore, this study also found that an increase in maternal age led to a decrease in the protein concentration of breast milk.

1.9 An Introduction to Metabonomics

Using metabonomics, which employs ^{1}H NMR Spectroscopy and/or chromatographic techniques coupled to Mass Spectrometry (MS), it is possible to study a very large number of metabolites simultaneously, covering a range of metabolite classes mostly in the low molecular weight range such as organic acids, amino acids, sugars, phenolics, lipids and carbohydrates. These analytical techniques are used for metabonomic studies, analysing metabolites in a detailed manner and requiring only small sample volumes. Metabonomics is defined as `the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification' (Nicholson et al., 1999).

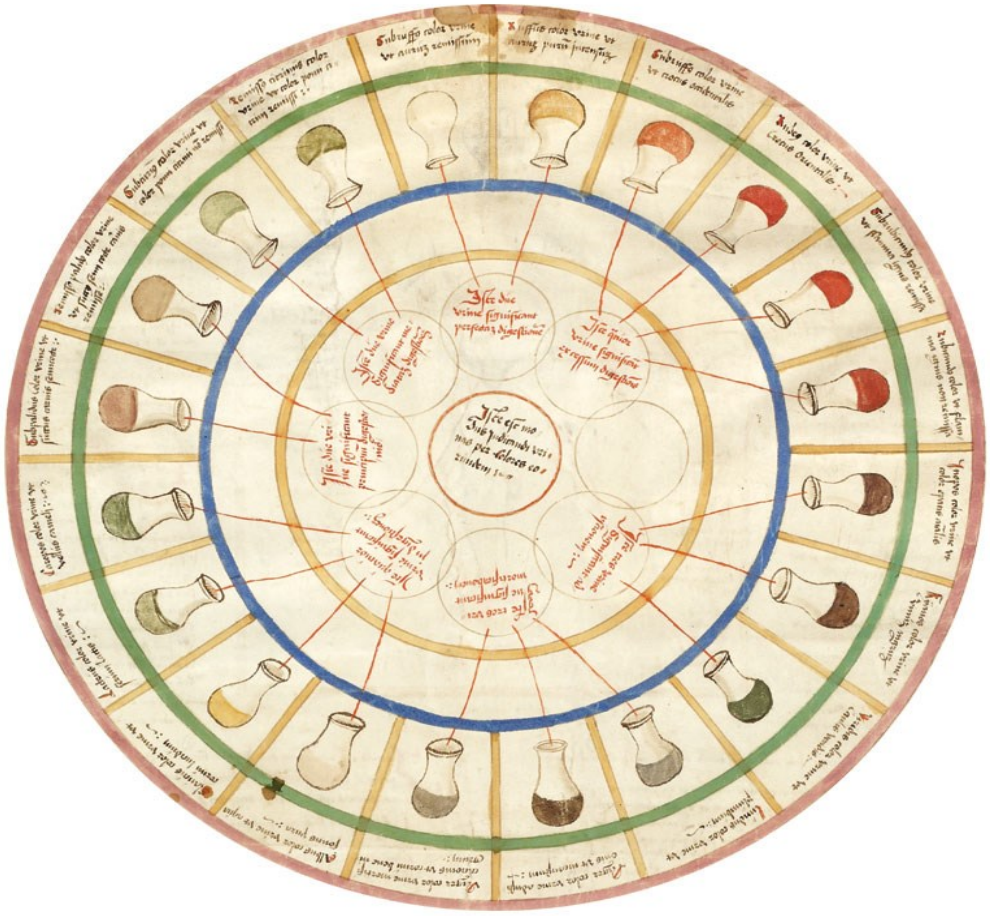


Figure 12: This image displays a urine wheel, published in 1506 by Ullrich Pinder, describing the colour, smell and taste of urine, using them for the diagnosis of diseases. Reproduced with permission from (Nicholson and Lindon, 2008) NPG.

These techniques allow for the examination of many biological processes occurring in complex organisms, or systems. This is because the metabolites found in a sample are present due to the cells which manufacture and secrete them (Lindon et al., 2000). As the composition of biofluids is

frequently altered as a result of disease or metabolic changes, metabonomics provides an excellent insight into the current biochemical status of an individual (Holmes et al., 1994).

Once spectral data are collected, advanced multivariate statistical tools, commonly referred to as chemometrics, are used to extract the maximum amount of information possible concerning complex biological samples (Richards et al., 2010). These techniques provide a metabolic fingerprint of the individual; known as a metabolome; capable of classifying samples, and can also have a diagnostic value, (Lindon, 2005). This classification of samples enables investigation into the metabolic changes which result in the classification obtained, giving an indication of the metabolic alterations which occur as a consequence of a disease or condition, such as obesity.

Since none of the analytical platforms used in metabonomics can extract all the relevant information from a sample, a combination of several techniques such as ^{1}H NMR spectroscopy and chromatographic techniques coupled to MS are employed for a comprehensive understanding for components present in samples.

1.9.1 Summary of previous studies employing metabonomics to investigate breast milk composition

Very few previous studies document the use of metabonomic techniques to investigate human breast milk. A preliminary study using metabonomics to investigate global breast milk composition analysed preterm breast milk, as well as preterm formulas analysed for comparison, using ^{1}H NMR spectroscopy, concluded that there was much biochemical variability between samples, but did not identify which metabolites accounted for these alterations. However, the authors did identify that lactose was present in a higher abundance in breast milk in comparison to formula, and that lactose concentrations increased over time (Marincola et al., 2012).

Further research by Practico et al also used NMR based metabonomics to investigate human breast milk composition, suggesting it could be used for rapid characterisation of breast milk nutritional content. The researchers identified a total of 43 metabolites, but note the difficulty of fully characterising the spectra due to overlapping peaks deriving from sugars and macromolecules. By comparing NMR spectra, three different milk groups were identified, dependent on the pattern of fucosylated oligosaccharides produced (Pratico et al., 2014).

Other studies using NMR spectroscopy or mass spectrometry to investigate human breast milk primarily investigate either a specific breast milk protein, or the structure of HMO.

A previous study using NMR Spectroscopy to investigate breast milk composition and its variation due to maternal phenotype and diet has been undertaken (Smilowitz et al., 2013). Here, milk was collected at 90 days post-partum, from 52 healthy women, allowing for the quantification of 65 milk metabolites, of various biochemical classes.

There was a wide range of biological variation between mothers, with some metabolites ranging from 4–120%; although several metabolites displayed a low variation, less than 20%, such as lactose. The authors identified two groups of mothers who differentiated in their HMO profiles, these women were classified as either secretors or non-secretors of products of the fucosyltransferase 2 (FUT2) gene, which catalyses the production of oligosaccharides such as 2'-fucosyllactose, lactodifucotetraose, and lacto-N-fucopentaose I. Intraindividual concentrations of HMO were conserved, differing by approximately 18%, suggesting tight regulation of oligosaccharide production; however, interindividual concentrations of specific oligosaccharides varied widely from 30.4–84.3%. The authors concluded that the variability observed in certain milk metabolites suggested a possible role in infant gut microbial development (Smilowitz et al., 2013).

This paper also investigated differences in milk metabolites from women with a different BMI, observing lower levels of acetone in women with a higher BMI (ρ= -0.41, P <0.01). No correlation was found between breast milk composition and age, blood pressure, diet or physical activity using multivariate statistical methods. However, using forward logistic regression the authors observed galactose and alanine were increased in post-prandial samples compared to fasting samples, (Smilowitz et al., 2013).

Breast milk is a complex substance, containing compounds with a molecular weight ranging from tens of Daltons to proteins in the KDa, and contains both polar and non-polar metabolites. Breast milk has a unique micellar structure, allowing the transport of lipid metabolites to the infant within a non-polar aqueous environment. When stored outside its natural environment, and particularly post-freezing, breast milk separates into two phases on the basis of polarity, complicating analytical processes. The range of metabolites contained in breast milk mean that no single analytical technique is capable of resolving the entire breast milk metabolome.

Due to a lack of information regarding human milk, a number of different methods to determine the best technique for preparing milk prior to metabonomic analysis were explored. Previous studies report that, for NMR, skimmed milk is the most suitable preparation method for analysis. This is done in order to remove the lipid fraction, which being less dense than water rises to the top of the sample and in this position it is not analysed, therefore an unrepresentative sample is analysed which does

not reflect the true composition of milk. Furthermore, as the lipid fraction is present in a high concentration, and is a very chemically heterogeneous group of molecules, the baseline is lifted making identifying other molecules present around the same chemical shift impossible.

Other studies employing metabonomics to investigate breast milk composition have used solvent extraction to isolate the lipid and aqueous fractions (Pratico et al., 2013). The method development for this thesis is outlined in detail in the Method Development Section 2.10 in Chapter 2.

In order to ensure the profiling techniques selected adequately covered polar and non-polar metabolites, UPLC-MS was chosen for lipidomic analysis, whilst ^{1}H NMR analysis allowed broad coverage of compounds from aromatic to aliphatic compounds and was chosen for analysis of polar compounds.

Separating the lipid and aqueous phases of milk is an important preparation step prior to analysis using mass spectrometry. This is because the columns used in mass spectrometry can become easily blocked with proteins or hydrophobic/hydrophilic solvents, depending on the column used, changing the conditions of the instrument and therefore giving unreliable results.

As well as these considerations, many previous studies examining milk composition using metabonomic techniques only examine the aqueous fraction; however, as it is likely that maternal BMI influences the lipid fraction of breast milk, a method capable of easily and cleanly separating and examining the fractions is therefore important to provide a comprehensive picture.

1.10 Conclusion of analytical methods

All of the analytical techniques outlined have their advantages and disadvantages in accurately quantifying the true composition of human milk. One of the main advantages in the metabonomic techniques described is that metabolites are measured simultaneously, within a single sample. Secondly, earlier techniques outlined measure only the main classes of nutrient, using metabonomics provides a more detailed image, detailing the specific metabolites which contribute to the groups of nutrients, for example, fatty acid identifications within lipids. Therefore, using a metabonomic approach will allow for a more detailed analysis of how maternal characteristics are able to influence breast milk composition.

1.11 Aims and objectives

The principle objective of this thesis is to evaluate the relationship between maternal BMI and the nutrient and endocrine profile of her breast milk. Also the maternal and infant metabolism, assessed by urinary metabonomics, will be evaluated.

The primary hypothesis to be tested is whether there is a statistically significant correlation between maternal BMI and key components present in breast milk.

The secondary hypothesis to be tested is that there is a statistically significant correlation between maternal BMI and the maternal and the infant urinary metabolome.

1.12 Thesis structure

The hypothesis explored throughout this thesis is that breast milk from overweight/obese mothers has alterations in its composition in comparison to breast milk from mothers of normal weight.

Chapter 2 outlines the methods used throughout this work in order to characterise breast milk composition, I establish a prospective cohort study with longitudinal assessments, and describe the range of analytical techniques employed to characterise breast milk composition. I will discuss the method development phase undertaken in order to optimize sample preparation techniques for the metabonomic studies undertaken later.

In Chapter 3, I report a systematic review of published literature undertaken, exploring the relationship between maternal BMI and concentrations of appetite regulating hormones present in breast milk to determine if any significant association has been observed between the two. Also, I employ a laboratory based *in vitro* analysis, using an immunological assay to explore the appetite regulating hormonal content of breast milk.

In Chapter 4, I examine breast milk composition using ^{1}H NMR spectroscopy, Ultra Performance Liquid Chromatography-Mass Spectrometry and mid infrared transmission spectroscopy. Here, I investigate whether any differences occur in the breast milk of mothers with differing BMI, and evaluate other factors influencing breast milk composition.

In Chapter 5, I examine the infant and maternal urinary metabolomes using ^{1}H NMR spectroscopy and explore their association with maternal BMI.

In Chapter 6 I discuss the findings of my research and the potential scientific and societal impacts of the conclusions drawn.

Chapter 2
Methods and Method Development

In order to investigate the association between maternal BMI and appetite regulating hormone concentrations in breast milk, I undertook a systematic review and subsequently carried out a prospective cohort study to examine the effects of maternal characteristics on human breast milk composition and maternal/infant urinary metabolome, having first optimized the sample preparation techniques for metabolic profiling of milk samples using multiple spectroscopic platforms. Additionally, to broaden the phenotypic description I measured the concentration of appetite regulating hormones, and the macronutrient content of breast milk samples collected from the prospective cohort recruited. The experimental methods used, including sample handling, are detailed for each investigation at the beginning of each results chapter or respective section.

2.1 Systematic review

2.1.1 Systematic review of the published literature

A literature search was conducted in PubMed (www.ncbi.nlm.nih.gov) using Medline MeSH (Medical Subject Heading) terms. Relevant studies were identified by evaluating the abstract or by obtaining a full copy of the article. Reference lists of included articles were searched for further relevant studies. Review articles and commentaries were excluded.

Data extracted from each paper included date of publication, number of participants, sample type, sample preparation method, measurement technique, time of sample collection post-partum, mean, standard deviation and range of maternal BMI, hormone concentration and the correlation coefficient between maternal BMI and hormone concentration. A full breakdown of the methods used for the systematic review are detailed in Section 3.3, Chapter 3. This systematic review was published in PLOS ONE, December 2014, shown in Appendix 3A, (Andreas et al., 2014).

2.2 Prospective cohort study

2.2.1 Research ethics

Research Ethics Committee approval was obtained from the West London (Fulham) Research Ethics Committee, 20th February 2012, REC reference number 12/LO/0203, (Appendix 2A). Thanks to Dr Matthew Hyde for helping me set up the study.

2.2.2 Recruitment

Mothers were introduced to the study and the patient information sheet (Appendix 2B) on the postnatal ward at Chelsea & Westminster NHS Foundation Trust Hospital. Formal enrolment took place following informed, written maternal consent on the post-natal ward, once the mother had had sufficient time to consider whether to participate or not. Mothers from across the booking BMI range were recruited, with an attempt to recruit mothers into roughly equal numbers of BMI categories 18.5-24.9 and >25kg/m^2.

2.2.3 Inclusion criteria

Healthy, primiparous, breastfeeding mothers, over 18 years old and their healthy, term infants, aged 37 weeks and above were included in the study.

2.2.4 Exclusion criteria

Smoking mothers, mothers who had multiple births, mothers on any prescription drugs influencing lactation, as well as diabetic mothers were excluded from taking part in this study.

2.2.5 Collection of demographic data

Detailed demographic and clinical data were collected for each participant upon enrolment, using a data collection form (Appendix 2C). Data collected included maternal age, race, BMI (pre-pregnancy, booking and current BMI), medications and diet, amongst other information. Data were also collected on infant characteristics including sex, mode of delivery, and gestational age at delivery. This data collection form was also used to collect further information at one week and three months post-partum.

2.2.6 Anthropometric measurements

Mothers from across the BMI range were recruited. Maternal booking weight and height were obtained from medical records, and mothers were asked to recall their pre-pregnancy weight. Infant and maternal body weights were measured at the time of each sampling using a Seca 877 Floor Scale (Seca, Hamburg, Germany), with Mother/Child Weigh Function, accurate to within 0.01kg.

The classification of overweight and obesity used throughout this thesis are those used by the WHO; individuals with a BMI greater than or equal to 25kg/m^2 were considered overweight, and individuals possessing a BMI value greater than or equal to 30kg/m^2 were considered obese (WHO, 2013). Throughout the thesis the mothers booking appointment was used as the time point for the diagnosis of overweight/obesity. This point was chosen as the maternal BMI was seen to be highly variable over the post-partum period. The mothers booking BMI was considered to be most reflective of the mothers actual BMI.

In order to measure infant length, a measuring board with a sliding footboard was used, measuring the crown to heel length (cm), accurate to within 1mm (Rollametre: Raven Equipment Ltd., Dunmow, Essex, UK). Infants' heads were held against the headboard whilst the legs were extended and the sliding footboard brought into contact with the infant's heels. Head circumference was measured with a measuring tape; also accurate to 1mm. Measurements were taken midway between the eyebrows and the hairline and round the occipital prominence at the back.

2.2.7 Breast milk collection

Breast milk samples were collected at one week postpartum, and a further sample was collected at 3 months post-partum, if the mother was still breast feeding. Samples were collected at the participants' homes by the research midwife (Suzan Jeffries) using a Lactaline breast milk pump (Ameda, Stone, UK), kindly donated by Central Medical Supplies (Central Medical Supplies, Leek, UK), into breast milk collection bottles donated by Sterifeed (Sterifeed, Cullompton, UK).

Mothers were asked not to feed their infants, or express breast milk, from the breast from which they planned to donate from for an hour before the appointment. The baby could be fed during this time from the opposite breast if hungry. For collection, mothers were asked to express a 15ml sample prior to the start of suckling which was marked as foremilk. The baby was then placed on the breast and after feeding, another sample of 5ml was collected at the end of suckling, marked as hind milk. The date and time of day of collection of each sample was noted. To control for diurnal changes in breast milk composition, collection was standardised to the morning, as far as possible.

Once received at the laboratory, the expressed milk was divided into 1ml aliquots and stored in 1.5ml Eppendorf tubes at -80°C. To prevent the degradation of peptide hormones present in samples, a protease inhibitor cocktail (Sigma-Aldrich, UK) was added to a 1ml aliquot from each mother, the components of which are detailed in Appendix 2D. The cocktail was reconstituted as per the manufacturer's instructions. Prior to samples arriving at the laboratory, a 20µl aliquot of the cocktail

was added to an Eppendorf tube and left to dry, after which the sample was added and inverted several times to mix.

Similarly a 10µl aliquot of sodium Azide solution (Sigma-Aldrich, UK) was added to a separate Eppendorf tube and left to dry, ready for the addition of a sample, once added, the concentration of sodium azide in solution was 0.1mM.

2.2.8 Urine collection

Where possible, maternal and infant urine were collected on the ward before discharge and at one week and 3 months post-partum, if the mother was still breast feeding. Urine from infants was collected by placing cotton wool, (NHS supplier), in the baby's nappy, and transferring to a sterile sample collection tube (STARLAB, Milton Keynes, UK), by squeezing the cotton wool into the tube. If there was only a small volume for a sample, the wool was kept and centrifuged later in order to collect any sample at the bottom of the tube. Once collected, samples were stored at -80°C.

2.2.9 Sample collection and storage

An important factor to consider regarding any method used to analyse biological samples is under which conditions samples are stored. Evidence suggests that factors such as freeze thawing, duration and temperature of freezing all influence breast milk composition. Previous research has suggested that freezing at -80°C should be considered as the gold standard for maintaining sample quality, (Silvestre et al., 2010). Also, research has documented considerable losses in lipid and carbohydrate content, due to duration of freezing in regard to the carbohydrate content, or just the process of freezing in respect to lipid content (Lev et al., 2014). The researchers propose that lipid adheres to polyethylene container walls rapidly, once all the available binding sites are occupied further drops in lipid content are not observed, whilst drops in carbohydrate occur more slowly over the duration of freezing suggesting carbohydrate has a weaker adherence to the container walls. These studies suggest that for analytical reproducibility, transferring samples between multiple containers is inadvisable; exposing samples to as few freeze thaw cycles as possible and storing samples at -80°C is best practice.

Samples obtained at participants homes were stored in an insulated transport container containing freezer blocks frozen to -20°C whilst they were transferred to Chelsea and Westminster Hospital. Once samples reached the hospital they were frozen at -80°C. Once samples from all participants had been collected, they were transferred to the South Kensington Campus in an insulated transport container, with freezer blocks frozen to -80°C, to ensure they did not thaw. Also, samples were aliquoted into 1ml volumes to ensure they underwent as few freeze/thaw cycles as possible. I would like to thank

Suzan Jeffries here for all her hard work both recruiting patients and collecting samples from their homes.

2.2.10 Reagents

2.2.10.1 ^{1}H NMR

Deuterium Oxide for NMR (D, 99.9% min) was purchased from Goss Scientific Ltd (UK). All chemicals used for NMR analysis were purchased from Sigma-Aldrich (UK).

2.2.10.2 CE-MS

Methanol, ammonium hydroxide and formic acid were purchased from Sigma-Aldrich (UK). Sodium hydroxide solution (1.0M) was purchased from Agilent technologies (Germany), and purine and HP-0921 were also from the Agilent Reference Mass Mixture. All buffer and standard solutions were prepared with deionized water purchased from Sigma-Aldrich (UK).

2.2.10.3 UPLC-MS

Water (Optima™ LC-MS from Fisher Chemical), acetonitrile, isopropanol, methanol were all LC-MS Chromasolv) formic acid, ammonium formate (LC-MS ultra), leucine enkephalin acetate salt hydrate and sodium formate were purchased from Sigma-Aldrich (UK), and were of analytical grade.

2.3 ^{1}H NMR Spectroscopy

One and two dimensional ^{1}H NMR spectra of urine and breast milk samples were acquired using a Bruker DRX-600 spectrometer (Bruker Biospin, Rheinstetten, Germany), operating at 600.13MHz, at a probe temperature of 300K. At the beginning of each experiment the NMR probe was tuned and matched to the proton transmitter resonance frequency. Samples were automatically delivered to the spectrometer using an automation device (SampleJet, Bruker) operated by IconNMR 4.7 (Bruker BioSpin). Here I would like to thank Dr Anthony Dona for his help in setting up the NMR spectrometer for the analysis of these samples.

2.3.1 Basic concepts of ^{1}H NMR spectroscopy

NMR spectroscopy is used to detect the absorption of electromagnetic radiation in the radiofrequency range by specific nuclei which possess a property known as spin. The property of spin is determined by the number of unpaired protons and neutrons present in an atoms nucleus, if an atoms nucleus possesses spin, then this also means it possesses a magnetic moment.

Due to having the property of spin, the ^{1}H nucleus possesses a magnetic moment, once placed in an external magnetic field, commonly referred to as B_0, this magnetic moment can either align with, or against, the applied external magnetic field. This orientation is either at a lower, or α, energy state; +½, if it is orientated with the magnetic field, or a higher energy state, β, or -½ when orientated against the external magnetic field. Because ^{1}H contains one unpaired proton, its spin number, I, equals ½, as it aligns with or against the external magnetic field. This means it is detectable by NMR spectroscopy, as any nucleus with an I number greater than zero is.

It is possible to excite nuclei from the lower energy state to the higher state using radiofrequency electromagnetic radiation. The frequency necessary to accomplish this is determined by the energy difference between the energy states, also known as the Larmor frequency, or transition energy. Nuclei at the lower energy level absorb radiation, which is at this specific frequency, promoting them to the higher energy level. Once the radiofrequency pulse ends, relaxation occurs and the spins flip as the nuclei revert back to the lower energy level, emitting detectable electromagnetic radiation in the process; this is known as the free induction decay (FID). The change in magnetisation as the protons relax from the higher to the lower energy state is measured by a receiver coil, this is the NMR signal. However, this signal is recorded in the time domain, therefore, in order to visualise the signal in the frequency domain (as peaks along the chemical shift axis) the signal is Fourier transformed (FT), this results in a set of peaks at specific frequencies, comprising the NMR spectrum.

As described so far, each nucleus in the magnetic field behaves in the same manner. However, due to the nuclear shielding phenomenon, whereby surrounding electrons shield nuclei present in different chemical environments to a greater or lesser extent, each nucleus experiences a slightly different intensity of the applied external magnetic field. This means that different nuclei resonate at slightly different frequencies, giving us the different chemical shifts. For standardisation, this shift is measured in relation to a standard compound, Trimethylsilyl-2,2,3,3-tetradeuteropropionoic acid (TSP) in aqueous solutions and Tetramethylsilane (TMS) in lipid solutions which are set to the frequency δ 0.0.

The concentration of a metabolite can be determined by measuring the intensity of the NMR signal arising from that metabolite if a known concentration of internal standard is added to each sample. This is because the signal intensity is proportional to the number of protons which contribute towards a specific peak.

Neighbouring nuclei are able to interact magnetically with one another via spin-spin couplings, or J-couplings. This interaction causes splitting of peaks; this can cause a single type of nucleus to produce multiple peaks, with a predictable intensity and spacing. This splitting provides detail on the chemical environment that particular nucleus is in, providing useful structural information on the molecule. The combination of chemical shift, peak multiplicity and peak intensities for each metabolite provide complementary information allowing structural elucidation.

2.3.2 Breast milk sample preparation for ^{1}H NMR Spectroscopy

Firstly, breast milk samples were defrosted in 1ml aliquots. Once fully defrosted, samples were vortexed to ensure samples were thoroughly mixed, after which 600µl of sample was added to 3ml chloroform:methanol mixture (Chloroform acquired from VWR, Poole, UK, methanol: Sigma-Aldrich, UK), in a 2:1 ratio. This mixture was vortexed, and 900µl of HPLC grade water (HiPerSolv, VWR) added, after which the mixture was vortexed again, and centrifuged at 5000×g at 4°C for 20 minutes. This resulted in two distinct layers, with the aqueous phase located at the top of the vial and the organic phase below. These different phases were split into two 1.8ml aliquots. Once separated, the aqueous phase was placed in a speed vacuum at room temperature until dry, (Vacufuge, Eppendorf, Hamburg, Germany). The aqueous phase was then reconstituted in 600µl of HPLC grade water, containing 60µl buffer (Appendix 2E). This mixture was then placed on a platform rocker until the precipitate was fully dissolved. Once dissolved, the resulting solution was transferred to a 5mm NMR capillary tube (Bruker, Germany), ready for analysis. This same procedure was carried out on 34 aliquots of a quality control (QC) sample, originating from a single large sample, which was analysed every 12 samples to ensure analytical reproducibility.

2.3.3 Urine sample preparation for ^{1}H NMR Spectroscopy

Approximately half of the infant urine samples had been frozen on cotton wool on which they were collected, therefore these samples were defrosted and centrifuged at 3000×*g* in order to remove the urine from the cotton. The other half of the infant urine samples had collected enough volume to be squeezed from the cotton wool directly into sample collection tubes. Occasionally samples did not have the minimum volume required for analysis, in this case samples were made up to the minimum volume using HPLC grade H_2O, which was carefully noted. All infant urine samples were defrosted at the same time in order to aliquot them into 1ml Eppendorf tubes, to ensure that the samples were treated in the same manner throughout, reducing any potential variation due to freeze thaw cycles.

Prior to analysis, infant and maternal urine samples were defrosted in their 1ml aliquots. Once fully defrosted, samples were centrifuged for ten minutes at 5000×*g* to remove any particulate matter. After centrifugation, 540µl of sample was transferred to an NMR capillary tube (Bruker, Germany) and 60µl of buffer (Appendix 2E), containing TSP was added using an Eppendorf pipette. This solution was then mixed thoroughly by inverting the capillary tube several times. Again, an external QC sample was prepared in the same manner as the study samples, also analysed every 12 samples.

2.3.4 Standard one dimensional ^{1}H NMR spectroscopy

A standard one dimensional ^{1}H NMR spectrum was acquired for each sample using a water presaturation pulse sequence [relaxation delay-90°-t1-90°-tm-90°-acquire free induction decay (FID)]. The relaxation delay was 2 seconds; with 90° relating to the 90° radio frequency pulse applied, t1, referring to the interpulse delay, which was set to 3µs. tm is the mixing time of 100ms. The water signal is reduced in intensity using the presaturation technique. This entails using a long, low power radio frequency pulse to selectively saturate the frequency at which water is found during the relaxation delay and mixing time; also known as O1.

Thirty-two scans were collected for each sample, comprising of 32 thousand data points, giving a total spectral width over 20 ppm (parts per million). An acquisition time of 2.73s was used. An exponential weight function corresponding to a line broadening of 0.3Hz was applied to each FID (free induction delay) prior to Fourier transformation. This is done in order to improve the signal to noise ratio.

The parameters used for the standard pulse sequence of the urine samples were as follows; P1=11.47µs, Pl1=12.8-dBW, Pl9=6.0dB and O1=2820.74Hz.

Parameters used for the breast milk samples used were: P1=11.18µs, Pl1=13.49-dBW and O1=2824.40Hz.

The parameters for the standard pulse sequence used for method development samples were as follows; P1=17.1µs, Pl1=0.06-dBW, Pl9=6.0dB and O1=2027Hz. Parameters are different for the different matrices/extractions as they were optimised for each set of samples.

2.3.5 Two-dimensional NMR spectroscopy

Identifying discriminatory metabolites between different groups was achieved by comparison of chemical shifts, peak number and peak multiplicity using assignment tables and online databases such as HMDB (http://www.hmdb.ca/) and ChemSpider (http://www.chemspider.com/).

However, if peaks remained difficult to identify using the one dimensional ^{1}H NMR spectral information due to overlapping signals, then 2D J-Resolved (JRES) and heteronuclear single-quantum correlation spectroscopy (HSQC) experiments also acquired were referred to, as these contain further structural information on metabolites detected.

JRES spectroscopy provides information on metabolite structure by the dispersion of ^{1}H nuclei signals along a second axis, generating further information on peak multiplicity and coupling constants (Aue et al., 1976).

HSQC spectra allow for easier identification of metabolites by measuring the carbon spectrum in addition to the hydrogen spectrum, which also provides valuable structural information. Here correlations between the carbon and hydrogen nuclei can be visualized, if separated by one bond (Kay et al., 1992).

JRES spectra were acquired using a standard JRES pulse sequence, using a spectral width of 78Hz for the F1 domain and 10026Hz in the F2 domain, resulting in a total of 8192 data points.

2.3.6 NMR data pre-processing

Spectra were automatically referenced to TSP at δ 0.0 for urine, whilst for breast milk, the ppm scale was automatically referenced to glucose, since TSP binds to proteins in milk causing chemical shifts. For lipid extracts, spectra are referenced to the resonance of the TMS signal at δ 0.0. Spectra were corrected for any phasing or baseline distortions. Each spectrum in the raw dataset was examined to ensure that it had been acquired correctly using Topspin 3.2 (Bruker Biospin). This was done by checking the water signal and the width of the TSP peak at half height for urine, the threshold used was 1Hz. For breast milk samples the glucose peak at δ 4.68 at half height was examined, the threshold used was 1.6Hz, if the glucose or TSP peak had a greater width than this then the sample was re-analysed.

Data were then exported to Matlab 7.12 (MathWorks Inc. USA), for further processing. In Matlab the portion of the spectra above δ 10 and below δ -0.2 for urine, and δ -0.175 for breast milk and δ 0.35−10.0 for method development samples, was removed. Samples which had been identified to have analytical artefacts were excluded in Matlab at this point.

The region containing the water peak was removed from each spectrum, in the urine spectra this region fell between δ 4.7 and 4.9, and δ 4.75 and 4.84 for breast milk spectra. Removal of the water peak is performed as water is present in samples in such a high concentration it can interfere with peaks, which fall close to this region on the spectrum, due to variability in the effectiveness of water suppression. Also the TSP peak was removed at this point, from δ -1 to 0.07 in urine spectra, and from δ -1 to 0.175 in breast milk spectra.

2.3.7 Alignment

Spectra were aligned using an in house script (Author, Kirill Veselkov, 2010), designed to intelligently align peaks from the same metabolite so that they present at the same chemical shift on the ppm axis, reducing effects seen of peaks shifting due to the different pH or ionic strength of the sample analysed.

2.3.8 Normalisation

Due to some of the samples collected not having sufficient volume, they required dilution to increase the sample volume up to the minimum required for NMR analysis. This was done with ultrapure analytical grade HPLC water. Because of this dilution, as well as the natural variation in sample concentration, normalisation was performed to account for this inter-sample variation.

Normalisation allows for comparison of spectra by removing the different dilutions between biofluid samples. This reduces the variation observed in the data which are unrelated to differences between the groups studied and which would otherwise make interpretation of differences between groups difficult. This is especially important in urine samples due to the large differences in water content observed.

Spectra were normalised using both probabilistic quotient normalisation (Dieterle et al., 2006) and total area normalisation (Craig et al., 2006). The most common way of normalising data is using total area normalisation; this is achieved by normalising each spectrum to the total spectral area, using the sum of the spectral intensity value. However, total area normalisation can occasionally, inappropriately, downscale spectra. If this is the case then probabilistic quotient normalisation is used, which calculates the most probable concentration of a sample based on the majority of metabolites observed.

Data were assessed to establish which was the most appropriate normalisation method; this was done by visualising how tightly clustered the QC samples were. Urine spectra were normalised using probabilistic quotient normalisation, which is less prone to the influence of high concentration metabolites, whilst breast milk spectra were normalised using total area normalisation.

2.3.9 Data reduction

In order to import data for analysis into SIMCA-P+ 13.0.2 (Umetrics, Sweden) the spectral data were converted into a digitized format. This was achieved by automatic data reduction using a script, (courtesy of Rachel Cavill), using Matlab. Once pre-processed, data were imported into SIMCA-P+ 13.0.2 for analysis using multivariate modelling techniques.

2.3.10 Metabolite Identification

Multiple online reference databases of standard compounds exist including HMDB, ChemSpider and others, aiding in the identification of metabolites. As well as these resources, two dimensional NMR experiments were used to further assist in the identification of metabolites, including J-Resolved spectroscopy (Viant, 2003), and heteronuclear single-quantum correlation spectroscopy (HSQC) (Bodenhausen and Ruben, 1980). Two dimensional experiments display correlated nuclei, allowing the identification of which nuclei are structurally related to one another.

2.4 Ultra Performance Liquid Chromatography-Mass Spectrometry

The lipid fraction of breast milk samples collected were analysed using Ultra Performance Liquid Chromatography-Mass Spectroscopy (UPLC-MS). An Acquity UPLC system (Waters Ltd., Elstree, U.K.) was used for chromatographic separation. Mass spectrometry was undertaken using a Quadrupole-Time-of-flight (Q-TOF) Premier mass spectrometer (Waters MS Technologies, Ltd., Manchester, U.K. Columns, inserts and vials also from Waters). For the analysis of these samples I assisted Dr Maria Gomez-Romero in the operation of the UPLC-MS instrument, and I would like to thank her and Kate Leary for their assistance in the data processing steps.

2.4.1 Basic concepts of UPLC-MS

Mass spectrometry is an analytical technique that allows for the determination of the molecular weight, and structural information, of ionisable molecules present in a sample. It can be coupled to UPLC; this involves first separating out metabolites from one another using LC, and the subsequent detection of these metabolites using MS. The separation of the metabolites is achieved by injecting a small volume (typically a few µL) of sample into the instrument; the sample is mixed with a stream of analytical solvent, also known as the mobile phase and then pumped through an analytical column, (the stationary phase) consisting in a bed of packed particles of sizes below 2µm (Swartz, 2005). The metabolites in solution are retarded by specific competing interactions (hydrophobic, dipole–dipole and/or ionic) with the particles in the column. Because the metabolites analysed have different affinities for the column, as well as the mobile phase, they elute from the end of the column at different rates, separating out from one another. Coupling LC to MS reduces the complexity of the mass spectra due to the separation of metabolites in the time dimension, and provides an additional layer of information on the physico-chemical properties of the metabolites, aiding in identification.

Depending on the analytes of interest the solvents and the columns packing material can be altered to better separate out metabolites, based on the analytes polarity or hydrophobicity. In order to efficiently separate out complex lipid mixtures the manufacturers (Waters), have published an optimised method to best achieve this (Isaac et al.). The parameters used are given in detail in Section 2.4.3, Chapter 2.

Once the metabolites elute from the UPLC column they must be converted to gas-phase ions in order to be detected in the mass spectrometer. A number of interfaces are available to accomplish this; throughout this thesis Electrospray ionization (ESI) was used. ESI is particularly useful for the analysis of metabolites found in biological samples as it is a ‘soft’ ionization technique, meaning metabolites are less likely to fragment into smaller ions, making identification easier. In ESI, the main ions observed

are quasimolecular ions created by the addition (positive mode), or the removal (negative mode), of a proton and denoted $[M+H]^+$ and $[M-H]^-$, respectively. Because metabolites have different ionization potentials, samples are analysed in both modes of ionization in order to detect as many metabolites as possible.

ESI involves dispersing the liquid eluting from the chromatograph into a fine aerosol. The mobile phase solvent contained in the droplets of the aerosol is evaporated using nitrogen gas at a high temperature and flow rate, termed desolvation gas. As the solvent evaporates from the aerosol droplets it eventually reaches the Rayleigh limit. Here, the electrostatic repulsion of the charges held within the droplet overcomes the surface tension, causing the droplet to become unstable and undergo coulomb fission, breaking apart. This occurs to the successive new droplets created by this process until no solvent remains, leaving just the charged ions behind. The ions flow into the mass spectrometer, induced by the combined effects of electrostatic attraction and vacuum, until they reach the mass analyser.

The analyser separates out ions based on their mass to charge (*m/z*) ratio and outputs them to the detector where they are converted to a digital signal. There are a number of of different types of analyser, but throughout this thesis a hybrid instrument, which combines two analysers (a quadrupole, Q, and a Time of Flight, TOF), was employed.

The quadrupole mass analyser can be used as a mass filter and in fragmentation experiments. It consists of four oppositely charged cylindrical rods parallel to one another. The quadrupole is able to filter ions passing through the instrument according to their *m/z* ratio. This is achieved by an electric field being applied to the rods; if a particular ion, travelling though the centre of the quadrupole, has a stable trajectory then it is able to pass through the quadrupole to the detector. However, depending on the voltage of the rods, some ions have an unstable trajectory, if so, these ions collide with the rods and are not detected.

The quadrupole is orthogonally arranged with a TOF mass analyser where ions are subjected to a uniform electromagnetic force, at a specific time. This electrical charge accelerates ions along a flight tube at different speeds, according to their *m/z* ratio and kinetic energy. The lightest ions travel the fastest, arriving at the detector first. The m/z ratio is therefore determined by the time of arrival of the ion at the detector. TOF mass analysers are particularly useful as they allow detection of ions within a large mass range.

For mass accuracy, the instrument was calibrated with a sodium formate (NaF) solution before commencing the run; NaF forms a series of regular clusters over the whole mass range, against which

the instrument is calibrated. To perform mass correction during the analysis, a lock mass solution was used (leucine encephalin; m/z 556.2771 in ESI+, m/z 554.2615 in ESI−) with data collected every 30 s.

Structural elucidation of unknown compounds can be achieved using MS/MS experiments using the Q-TOF mass spectrometer. This process involves fragmenting the metabolites of interest and examining the resultant fragments. Metabolites usually fragment in a predictable manner, with the most labile bonds breaking first. The MS spectrum will show head groups and other groups of the molecule, making identification easier. The precursor ion is selected in the quadrupole, it is fragmented inside a collision cell, located between the quadrupole and flight tube, and the product ions are analysed in the TOF, which gives mass accuracy. In the collision cell, ions collide with gas present, using this kinetic energy to break the bonds within the molecule.

UPLC-MS data interpretation presents many challenges

1. Presence of isomers, which are compounds possessing the same chemical formula but different structures. They display an identical mass to charge ratio (*m/z*), making automated annotation difficult and structural elucidation complicated. However, some isomers are able to be distinguished from one another. For example, one can compare the retention times against a standard solution. Because different chemical structures interact with the column to a different extent, this results in some isomers possessing distinct retention times, if they interact with the column differently. If this is found not to be the case, identifications can be made using MS/MS data.
2. Further complicating analysis is the fact that metabolites dimerize and can also form adducts. If dimerization occurs, then, in addition to detecting the molecular ion, an ion at twice the weight can also be detected. However, this effect can aid in identification as, if dimers are observed, then this gives confidence to the identity of the molecular ion. Adducts are also commonly observed in MS spectra, here, instead of the molecular ion being detected, metabolites can become associated with various salts, for example, sodium $[M+Na]^+$ or ammonium $[M+NH_4]^+$ in positive ionization mode, or chlorine $[M+Cl]^-$ in negative ionization mode. This results in the *m/z* spectrum containing ions greater than the calculated molecular mass.
3. In-source fragmentation. Labile molecules fragment through the MS instrument, obtaining a spectrum with un-desired fragments, sometimes at a higher intensity that the precursor ion, making interpretation more complicated.

2.4.2 Breast milk lipid phase sample preparation for UPLC-MS

The lipid extracts for UPLC-MS analysis of breast milk samples were prepared using Folch extraction as described earlier in ^{1}H NMR Spectroscopic analysis of breast milk samples. Once the lipid fraction was separated from the aqueous fraction the chloroform was left to evaporate under a fume hood, leaving behind a lipid residue. This was frozen at -40°C until analysis. Prior to analysis, extracts were defrosted and reconstituted in 200µL isopropanol/acetonitrile/water in a 2:1:1 ratio (isopropanol and acetonitrile were acquired from Sigma-Aldrich, UK, whilst water was from Fisher Scientific, UK). This suspension was centrifuged for 15 minutes at 5000×*g* at 4°C in order to remove any particulate matter which could block the column. Once centrifuged, samples were transferred to an insert placed inside a glass vial, ready for analysis. At the beginning of the run the column was conditioned by injecting ten QC samples, a pool of all the samples analysed and thus representative of the entire sample set. This QC sample was made by pooling 20µL of each of the samples once they had been reconstituted in the 200µL isopropanol/acetonitrile/water mixture. Also, for every ten samples analysed (i.e. at regular intervals) a QC sample was injected to ensure that analytical reproducibility was maintained, and to obtain structural information, using an automated MS/MS method (data-dependent acquisition).

2.4.3 UPLC-MS system acquisition parameters

Samples were analysed using an Acquity UPLC system (Waters Ltd., Elstree, U.K.) coupled to a quadrupole time of flight (QTOF) mass spectrometer, (QTOF Premier, Waters MS Technologies, Ltd., Manchester, U.K.), in positive and negative ion electrospray mode (ESI+ and ESI-). The chromatographic conditions used have been previously described as optimal for the separation of lipids by the manufacturers (Isaac et al.)

Separation was performed using a Charged Surface Hybrid (CSH) C_{18} column (1.7µm, 2.1 x 100mm) (Waters Corporation, Milford, U.S.A.), at 55°C. Two mobile phases were used in different proportions throughout the analysis, mobile phase A consisted of 0.1% formic acid (v/v) and 10mmol/l ammonium formate in acetonitrile/water 60:40 (v/v). Mobile phase B consisted of 0.1% formic acid (v/v) and 10mmol/l ammonium formate in isopropanol/acetonitrile 90:10 (v/v). The flow rate was 0.4ml/min and the injection volume was 5µl.

The MS parameters were; capillary voltage, 1.5kV; sample cone voltage, 30V; source temperature 120°C; desolvation temperature and gas flow, 600°C and 1000L/h, respectively; cone gas flow, 50l/h and scan range 50-1500*m/z*. For mass accuracy, a LockSpray interface was used with a 2ng/µl leucine enkephalin (555.2645 amu) solution at 10µL/min as the lock mass. Data were collected in centroid

mode with lock mass scans collected every 40 seconds and averaged over 3 scans to perform mass correction.

2.4.4 UPLC-MS data pre-processing

Resulting chromatograms were displayed using MassLynx, Version 4.1 (Waters). Within the MassLynx software, Databridge was used to convert the raw data files into NetCDF format. For the pre-processing of UPLC-MS data XCMS was used, which is available as freeware within RStudio (Version 0.98.953). Data were processed using an in house script written by Matthew Lewis and Paul Benton. An outline of this process is included below.

2.4.5 Peak detection

Firstly parameters were chosen to detect peaks present in the chromatograms by studying the chromatograms of the QC samples analysed. Parameters chosen for positive and negative mode data are shown in Table 2. The time range refers to the amount of time a specific mass had to elute for to be considered as a peak (Smith et al., 2006).

Table 2: Parameters used for peak detection in positive and negative mode.

Parameter	Description	Positive mode	Negative mode
Time range (seconds)	Range (min, max) of chromatographic peak width	1-27	4-27
Mass accuracy of the data (ppm)	Maximal tolerated *m/z* deviation in consecutive scans in one file of a single peak	20	20
Signal to noise threshold cutoff	Maximum peak intensity minus estimated baseline value divided by the standard deviation of the local chromatographic noise	3	3
Prefilter (k,I)	Mass traces are only retained if they contain at least k scans with intensity ≥ I.	3, 75	3, 75
Retention time error (seconds)	Needed for proper alignment	12	18

2.4.6 Retention time correction (Alignment)

Next the retention times of eluting peaks were corrected or aligned; this is necessary as the system conditions change slightly over the course of the analysis, with the pressure of the column generally increasing as it becomes increasingly blocked with precipitate, changes in the conditions within the laboratory such as temperature, and the need to replenish the mobile phase solvent throughout the analysis. These changing conditions result in metabolites eluting at slightly different times as their retention times drift. To correct for this, alignment is used, enhancing the ability to compare data

across experiments. An example retention time deviation plot is shown in Figure 13. The alignment method used for the mass spectrometry data throughout this thesis is known as ordered bijective interpolated warping, or OBI-Warp (Prince and Marcotte, 2006). This method of alignment aligns matrices along a single axis using Dynamic Time Warping and a one-to-one (bijective) interpolated warp function. Dynamic Time Warping allows for non-linear, comprehensive alignment of datasets, providing natural interpolants.

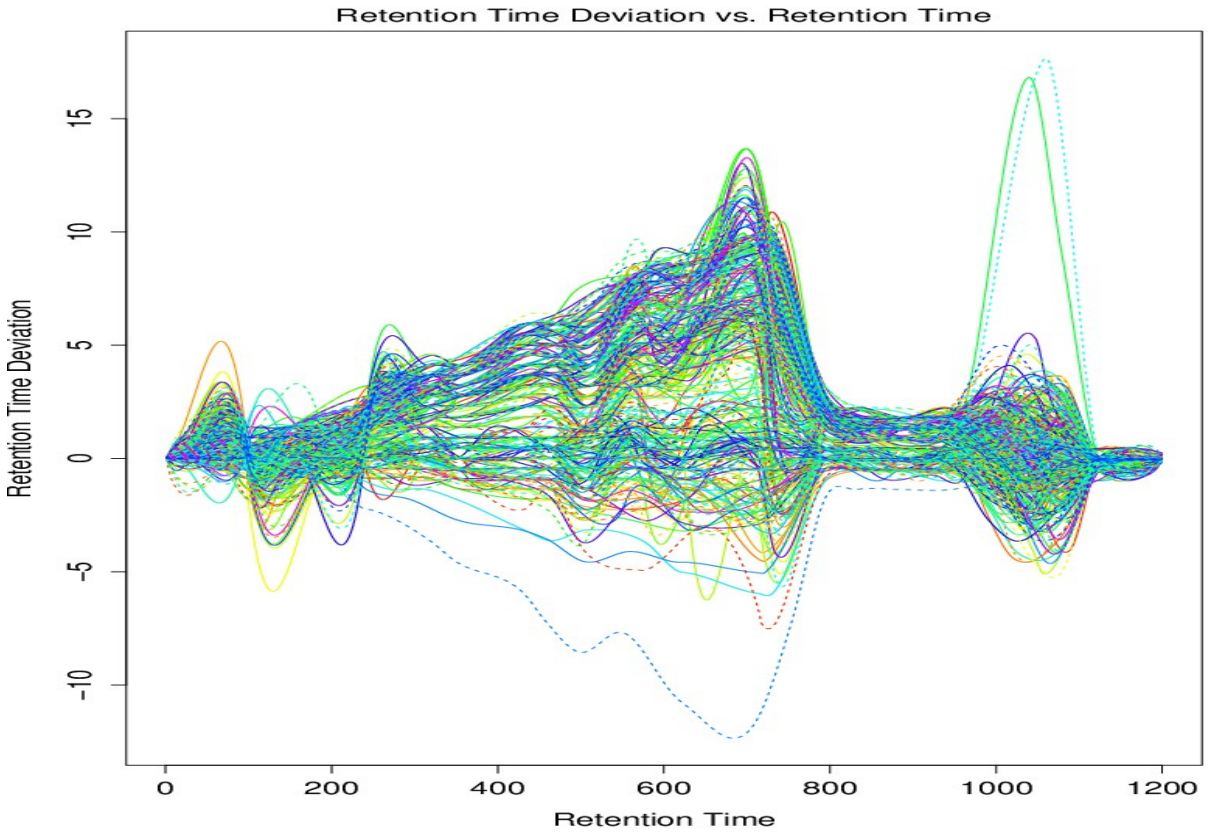


Figure 13: Retention time vs. retention time deviation plot. Each sample is displayed as a line displaying the time in seconds required to align the data to the median retention time. An upwards deflection indicates that that sample eluted after the median retention time, whilst a negative deflection indicates that the sample eluted before the median retention time, at that time point. Samples are coloured by run order, red relates to the first samples analysed and violet being the last samples analysed. Positive ionization mode data. Text adapted from http://metlin.scripps.edu/xcms/xcmsPreprocess.pdf

2.4.7 Filtering of peaks

After the peak detection step, grouping of peaks across the dataset was performed using the density method in XCMS; subsequently each run was checked for the presence of signal for each peak group, and where a peak was not detected, the signal intensity for that region was integrated to create a value for that region. This step is crucial to preserve normality of intensity distribution for statistical analyses.

Filtering of features from the dataset was also carried out using minimum fraction filtering; this involves filtration of noise before data are normalised, improving the results obtained. This process selects for features, or peaks, detectable in at least 50% of at least one sample class, e.g. overweight, and retain them, discarding the others from the dataset. Figure 14 displays the data before and after the filtering process.

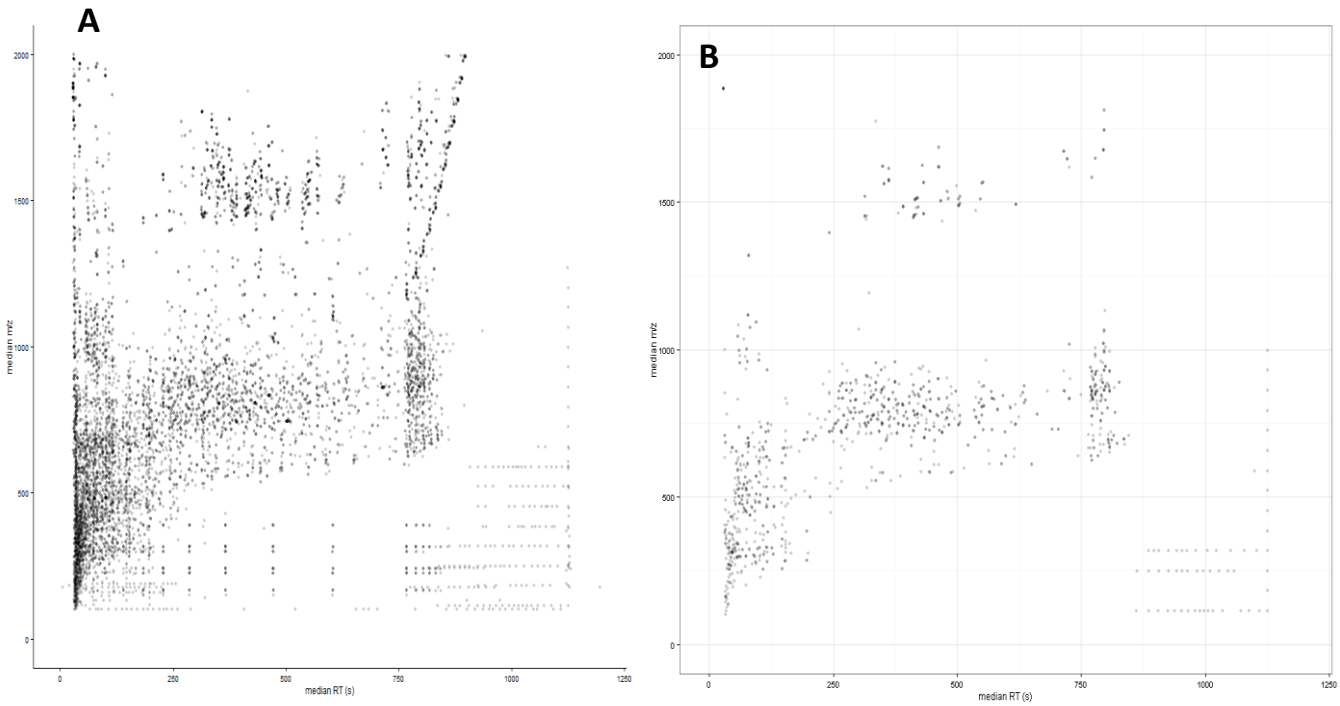


Figure 14: (A) Raw dataset, peaks are viewed from above, plotting the retention time against *m/z* ratio. (B) Dataset after minimum fraction filtering; many fewer peaks are included for further multivariate analysis. Negative ionization mode data.

Furthermore, a coefficient of variation (CV) filter was employed. The CV filter is based on QC samples. The CV is calculated, dividing the standard deviation by the mean, for each peak present in the dataset, and any peak with a CV greater than 30% in the QC sample is discarded from the whole dataset further reducing the noise.

2.4.8 Normalisation

Data were next normalised to remove sources of systematic variation between samples due to factors that are not related to biological processes, such as sample dilution. As samples show differences in concentration, this introduces noise to the dataset by increasing the variance as a function of increased signal intensity, complicating information recovery. Previously, median fold change has been demonstrated to be the most appropriate method of normalisation for UPLC-MS urine data (Veselkov et al., 2011).

Median fold change normalisation adjusts for the median of log fold changes of peak intensities between samples to zero. This method of normalisation is based on the assumption that metabolite peaks are influenced only by dilution, and will display the same fold changes between two samples. This assumption is valid if peak intensities are proportional to the metabolite concentration in solution, any change in intensity is attributed to sample dilution and/or inconsistency of UPLC-MS acquisition; estimated to be equal across each peak, and therefore a fixed scaling factor is used. To estimate the dilution factor, the median value of fold change between a sample and target profile is used. This method of normalisation has been shown to be valid against at least 50% of peak intensities, increasing or decreasing asymmetrically in response to biological factors (Veselkov et al., 2011).

2.4.9 Metabolite ID

UPLC-MS metabolite identification was achieved by searching *m/z* values against online databases including METLIN, Lipidmaps and HMDB, with the mass error set to 10 ppm. In order to identify lipid species, MS/MS data were inspected to identify fragmentation patterns, and the presence of head groups, increasing confidence in the identity of the metabolite identification.

2.5 Capillary Electrophoresis-Mass Spectrometry

An Agilent 7100 CE system coupled to an Agilent 6224 Accurate-Mass TOF MS system (Agilent Technologies, Wilmington, USA) was used to acquire CE-MS data. Ionization was accomplished using ESI, outlined in Section 2.4.1, Chapter 2. Method development samples (described in Section 2.10, Chapter 2) were analysed in a randomised run order, with a QC sample analysed at the beginning of the run, every six samples, and at the end of the sequence. Acquisition parameters and the data processing methods used are described in Appendix 2F. For the analysis of these samples, Dr Isabel Garcia-Perez operated the CE-MS instrument and undertook the data pre-processing, for which I am very grateful.

2.5.1 Basic concepts of CE-MS

Capillary electrophoresis-mass spectrometry (CE-MS), involves firstly separating out metabolites using CE, and the subsequent detection of these metabolites using MS, a description of which is given in Section 2.4.1, Chapter 2.

In capillary electrophoresis, the sample is injected into a capillary possessing a small diameter. An electric field is then applied across the capillary, resulting in both positively and negatively charged ions migrating through the capillary due to the electroosmotic flow established by the electric current.

Ions migrate through the capillary at different rates according to their electrophoretic mobility. This is determined by the charge of the ion, its size, and the viscosity of the solvent, with the smallest most highly charged ions travelling the fastest. Once metabolites pass through the capillary, they are introduced into the mass spectrometer.

2.5.2 Breast milk lipid phase sample preparation for CE-MS

Twenty one individual breast milk samples were prepared in the same manner as the aqueous fraction for ^{1}H NMR analysis, as outlined in Section 2.3.2, Chapter 2. However, 200µl instead of 600µl of breast milk sample was used, with the volumes of the solvents adjusted accordingly. The aqueous extract was re-suspended in 200µl of 20mM of formic acid solution, and centrifuged to remove any particulate matter. The supernatant was transferred to a vial ready for injection into the CE-MS instrument. A QC sample was prepared by pooling 20µl of each of the samples and preparing this in the same manner as the method development samples.

2.5.3 Metabolite Identification

In order to tentatively identify metabolites detected using CE-MS, comparison of the high mass accuracy data (with less than 5 ppm tolerance) was performed against standards analysed in the same manner.

2.6 Multiplex assay of breast milk hormone composition

The concentrations of the hormones leptin, insulin, ghrelin and resistin were determined in a 50µL aliquot of the aqueous supernatant of centrifuged breast milk samples, using a Bio-Plex Pro™ human diabetes immunoassay (Bio-Rad, USA), as per the manufacturer's instructions. Concentrations of milk hormones were calculated using a standard reference curve, constructed from standards provided with the kit. A Bio-Plex MAGPIX multiplex reader (Bio-Rad, USA) was used to determine the concentration of the hormones present. A detailed overview of the experimental design used for the analysis is given in Appendix 2G. Dr Bronwen Herbert was particularly helpful in the analysis of these samples, helping to design the workflow and operating the MAGPIX multiplex reader.

2.6.1 An Introduction to Multiplex assays

Multiplex assays allow for the simultaneous detection of multiple biomolecules within a single sample. This is achieved using fluorescently dyed beads, possessing a distinct colour code, which permits discrimination between the individual tests.

The principle of the assay is essentially that of a sandwich ELISA formatted on magnetic beads. Antibodies against the analytes of interest are covalently bound to magnetic beads; once the sample is introduced the antibody binds to any analytes present in the sample, whilst unbound protein is washed away. A biotinylated detection antibody is then introduced, creating the sandwich complex. Finally streptavidin-phycoerythrin, a fluorescent indicator, is added forming a complex with the detection antibody.

The fluorescent dyes are then illuminated with a red (635 nm) laser which classifies the bead, and therefore the assay identification. A green (532 nm) laser simultaneously excites the phycoerythrin, generating a reporter signal, detected by a photomultiplier tube (PMT). The concentration of the analytes in samples are then extrapolated from a standard calibration curve, simultaneously prepared using multiple dilutions of a standard mixture.

2.6.2 Breast milk sample preparation for multiplex assay

A multiplex assay was employed to measure concentrations of the appetite regulating hormones leptin, insulin, ghrelin and resistin in breast milk. Once breast milk samples had been collected they were aliquoted into an Eppendorf tube containing a protease inhibitor cocktail (Sigma-Aldrich, UK), (Appendix 2D). Breast milk samples were first defrosted in a refrigerator, 1ml aliquots were centrifuged at 20000×*g* at 4°C for 10 minutes. The lipid fraction was drawn off using a sterile pipette tip and 500µl of the aqueous fraction was pipetted into a separate Eppendorf tube and frozen for later analysis.

Prior to analysing samples, three trial samples were analysed in order to establish whether samples required dilution, or addition of Bovine Serum Albumin (BSA), (Sigma-Aldrich, UK), to block non-specific antibody binding. Two trial samples were diluted 1:4 in sample diluent, 1:1 in sample diluent and one was left undiluted. In addition to these dilutions, a parallel set of dilutions were prepared, with the addition of BSA. The results from this trial were evaluated, suggesting addition of BSA was unnecessary, as it did not appear to impact the results obtained, whilst dilution meant concentrations would appear on the lower end of the standard curve, where quantification is less precise.

2.7 Mid infrared (IR) transmission spectroscopy analysis of breast milk samples

Data on breast milk macronutrient content were collected using a Miris Human Milk Analyser, kindly donated by Miris, (Miris, Uppsala, Sweden). The analytical accuracy of the instrument is reported to be <0.1g/100ml, with a repeatability of <0.05g/100ml, (Casadio et al., 2010).

2.7.1 An Introduction to mid IR spectroscopy

Mid Infrared (IR) Spectroscopy measures the nutritional content of breast milk via the absorption of infrared energy. The sample is first drawn into a fluid film 50µm thick in a transparent cuvette and exposed to IR radiation, transmitted from an emitter. Any reduction in the transmission of the different wavelengths transmitted is quantified by a detector, and then correlated to the macronutrient content of the sample. Four functional groups are measured by the analyser; these chemical groups absorb IR radiation at specific wavelengths. The wavelengths of the IR radiation absorbed by chemical groups in lipid are 5.7µm and 3.5µm, absorbed by the CH_2 carbon-hydrogen group and by the C=O carbonyl groups respectively. Also measured is the absorption at 6.5µm by CONH groups, found in amino acids, and 9.6µm absorbed by hydroxyl groups present in carbohydrates. As the hydroxyl group is also found in Human Milk Oligosaccharides (HMO) the analyser gives a reading for total carbohydrate, including lactose and HMO.

The Human Milk Analyser is calibrated against a set of reference samples with different concentrations of macronutrients present. These samples are also measured using approved biochemical reference methods with the results used to fit a nonlinear calibration curve for each milk analyte. The Miris milk analyser gives values for both crude protein and true protein; this is due to a substantial proportion of nitrogen present in milk being non-protein nitrogen, mainly HMO. To account for this the 'true' protein value is derived by applying a factor of 0.8 to the crude protein values.

Various researchers have evaluated the accuracy of the Human Milk Analyser (Silvestre et al., 2012), (Casadio et al., 2010), suggesting that there is a small but statistically significant difference in the concentrations of macronutrients measured between the Human Milk Analyser and reference methods. This is due to the chemical principles the assays are governed by, however, they conclude that with proper sample preparation the analyser is able to provide reliable measurements, as evaluated by Casadio et al, (Casadio et al., 2010).

2.7.2 Breast milk sample preparation for IR spectroscopy

As samples were frozen prior to analysis, a number of preparation steps were undertaken to ensure samples analysed were as homogeneous as possible, meaning representative and reliable results were obtained. Approximately 3ml of sample was first thawed by heating to 40°C in a water bath, and mixed by inverting the aliquot multiple times. Samples were sonicated for a total of 4.5 seconds using the Miris sonicator (Miris, Uppsala, Sweden). This is an ultrasonic device which creates sound waves using clamped piezoelectric discs, causing the probes tip to oscillate in the length direction. This releases energy causing air bubbles to implode, breaking apart fat globules into smaller, more equally distributed globules, diminishing lipid separation. These steps provide a more repeatable, accurate and representative measurement. The ultrasound procedure used, recommended by the manufacturer, involves exposing each ml of milk analysed to 1.5 seconds of ultrasound at 70% amplitude using a micro-tip. The Human milk analyser has two settings, raw and homogenised milk, therefore the homogenised milk setting was used, due to the sample preparation steps used. The sample was then injected into the Miris Human Milk Analyser (Miris, Uppsala, Sweden) for analysis. For every ten samples analysed the Human Milk Analyser was cleaned and the calibration of the instrument was checked; recalibration was performed where necessary.

2.8 Statistical methods for hormonal and macronutrient analysis

2.8.1 General statistical methods

Data from the hormonal analysis and macronutrient analysis were analysed using IBM SPSS Statistics version 22 (IBM, Armonk, NY, USA), unless stated otherwise. In order to assess whether data collected were normally distributed, histograms and Q-Q plots of the data were visualised. If data were found to be normally distributed then parametric tests were used to interpret data. If data were non-normally distributed then natural log transformation was applied to the data, histograms and Q-Q plots were replotted to establish normality of distribution, after which parametric tests were used to investigate the data collected.

2.8.2 Sample size

The group statistician for the section of Neonatal Medicine calculated an appropriate sample size to recruit. This was based on previous literature, and calculated to have sufficient power to observe a difference in the concentration of leptin in breast milk. Using a double sided test, a sample size of 124 would provide 80% power to detect a correlation with an R value of 0.25, and in excess of 90% power for an R value of 0.3, at *p*=0.05, Table 3.

Table 3: Power calculations detailing the number of participants required to have sufficient power to detect a difference in the concentration of leptin in breast milk.

	Number of subjects required to detect a Pearson correlation of:					
	R=0.25			R=0.3		
Power / Significance	70%	80%	90%	70%	80%	90%
0.05	98	124	136	67	86	114
0.01	150	182	236	104	124	158

Determining an appropriate sample size for metabonomic data is complicated due to the data sets produced being highly dimensional, as well as the variables being highly correlated with one another. Because of these reasons, no accepted method currently exists to calculate sample sizes for metabonomic experiments (Nyamundanda et al., 2013). Therefore it was decided that the sample size calculation for leptin would be used, and statistical power would be checked retrospectively for any metabolites showing a correlation with maternal BMI, in accordance with accepted approaches in metabonomics.

2.9 Multivariate data analysis methods of metabonomic data

Metabonomic analysis produces global metabolic profiles of the samples examined. In doing this, each individual sample produces a large amount of raw data (e.g. an NMR spectrum or UPLC-MS chromatogram consisting of many data points, which correspond to the many different metabolites present in the samples analysed). In order to analyse datasets of this size and complexity, multivariate data analysis techniques are required.

As previously outlined, ^{1}H NMR spectroscopic data were pre-processed using Matlab, whilst UPLC-MS data were processed using the XCMS package within R. Once pre-processed, data were imported into SIMCA-P+ 13.0.2 (Umetrics, Sweden) for analysis using multivariate modelling techniques. Matlab was also used for multivariate modelling of some NMR data. An outline of the chemometric strategy used throughout this thesis is given in Figure 15.

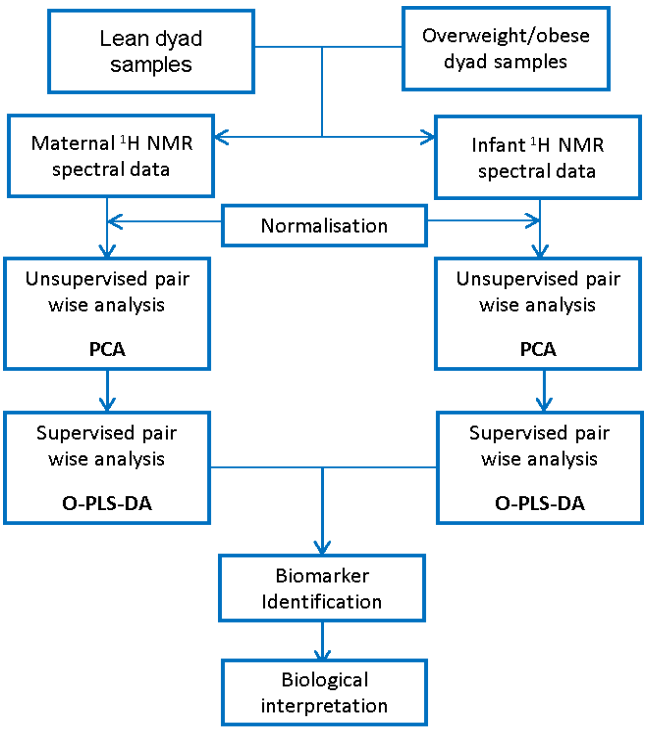


Figure 15: Chemometric strategy for sample analysis. Flow diagram descibing the analysis stategy used througout this thesis in order to identify biomarkers associated with overweight/obese dyads, and their posible biological relevence.

2.9.1 Scaling

Using SIMCA-P+, data are firstly scaled, as metabolites present in the biofluids analysed are present in vastly different concentrations, often many orders of magnitude. Because of this, the resulting data have substantially different numerical ranges. Because the pattern recognition techniques used

project the variables displaying the greatest variance, metabolites present in the highest concentrations dominate the models produced. Therefore, in order to be able to identify metabolites with an abnormal abundance, which discriminate between groups, the variables are scaled. This is so that the variance found in the smaller peaks are not overshadowed by the contribution of larger peaks, providing a more equal contribution towards the models produced, allowing for more meaningful interpretation of models (L. Eriksson, 2006).

Using SIMCA-P+, data were scaled employing various different scaling methods to establish the most appropriate method of scaling for the data collected. The most common scaling methods used are unit variance (UV) scaling, Pareto and mean centering. UV scaling is carried out by dividing each variable by the standard deviation, giving all variables equal variance. However, UV scaling can introduce artefacts as it gives noise the same importance as genuine peaks.

Mean centering is also commonly used for treating data prior to scaling; this is done by subtracting the average value of each variable without altering the variance. This method of scaling is useful for highlighting variables with the largest dynamic range.

If neither of these scaling methods is found to be appropriate then an intermediate method can be used, Pareto scaling. This is achieved by giving each variable a value equal to its standard deviation, by scaling it using 1/√standard deviation.

Once scaled, data are interpreted using chemometric data analysis techniques. The differently scaled models were visualised using Principal Component Analysis (PCA) scores plots, and the method, which gave the tightest clustering of QC samples was selected for further analysis.

2.9.2 Unsupervised modelling of metabonomic data

Because the datasets which result from metabonomic analysis contain so much information, it is not sufficient to merely examine the tables produced, as the data must be expressed in a way which is comprehensible. Data projection methods are most suited to describe and analyse this type of data. Unsupervised modelling first involves characterising the data collected. The data matrix produced consists of n, equivalent to the number of samples in rows, and K, equivalent to the number of variables, in this example, points along the ppm/δ axis in columns. The projection method used throughout this thesis is PCA. Using this technique, data are represented as a cloud of points in multidimensional space, corresponding to the number of variables collected, K. This is then linearly transformed into a lower dimensional plane, providing a representation of the observations in a manageable format, as a scores plot.

PCA is a useful visualisation tool for observing inherent grouping or clustering of samples within a dataset, and in order to understand the pattern of the data. The first principal component calculated is the direction in K dimensional space, which represents the maximum variance. The second principal component calculated is orthogonal to the first and describes the second largest variation found in the dataset. More principal components are calculated, describing less of the data as more are calculated.

Interpretation of the resulting scores plots for all models was then undertaken to identify metabolic outliers. This involves visualising the PCA scores plot, where each spectrum is displayed as a point, either inside or outside of a Hotellings 95% confidence interval (CI), projected onto the scores plot. Spectra lying outside of this circle were investigated to determine the reason for the difference between them and the main body of the data. Once identified these were excluded (if a valid reason was ascertained, such as insufficient water suppression or poor shimming), and the models recalculated to improve reliability.

Also, corresponding to the scores plots are the loadings plots. Loadings plots display the spectral points which are responsible for the distribution of the points seen in the scores plots and allow for the identification of the spectral points which are responsible for the separation of groups seen in the scores plots, an example loadings plot is given in Figure 16.

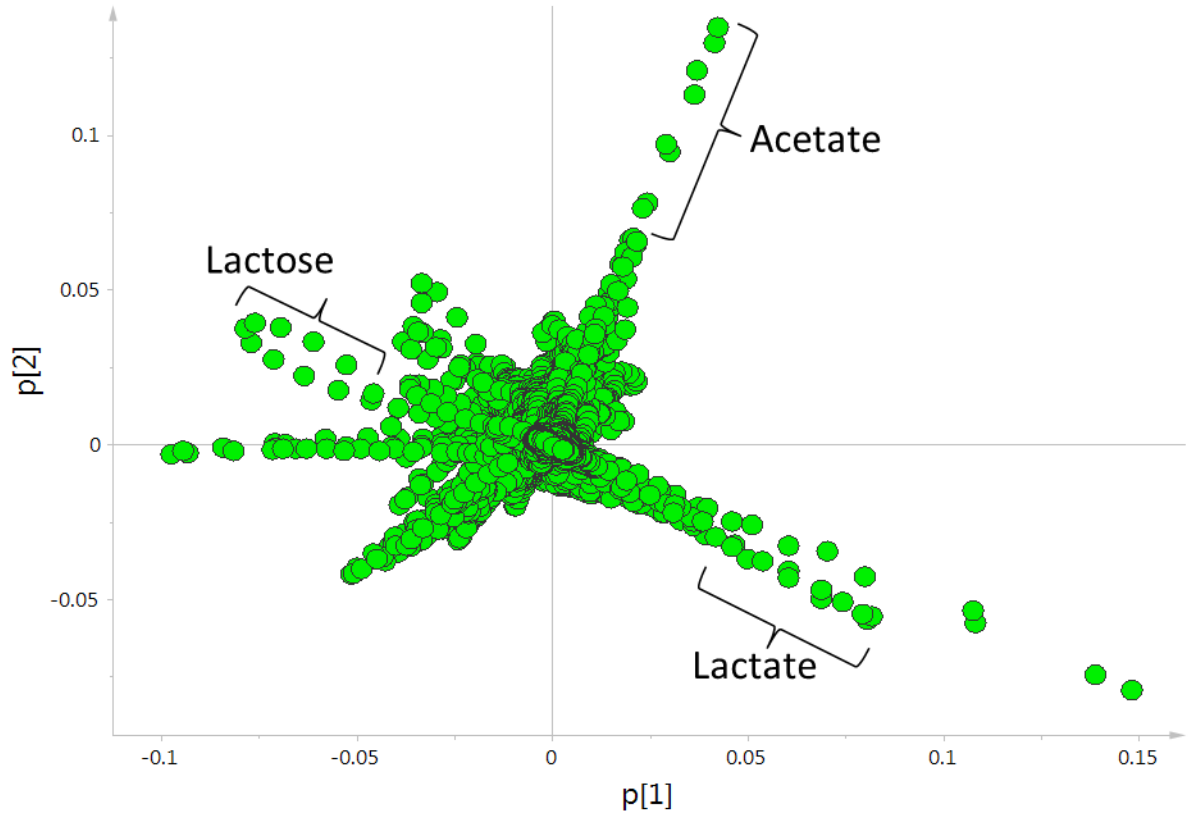


Figure 16: An example loadings plot, displaying the spectral points responsible for the separation of different samples in the scores plot.

The p1 and p2 axis are vectors which display a point for each point along the ppm scale, these loadings allow for the visualisation of any correlation between the data in the matrix. The loadings plots display which data provide similar information (variables close together), if data are negatively correlated (variables on opposite sides of the plot), or if data are unrelated to one another (variables situated at 90 degrees to one another). Variables close to 0 along both the p1 and p2 axis are not able to be explained by the model.

2.9.3 Supervised Orthogonal Projection to Latent Structures-Discriminant Analysis (OPLS-DA) and OPLS modelling of metabonomic data

Matlab and SIMCA-P+ were employed for modelling data using Orthogonal Projection to Latent Structures-Discriminate Analysis (OPLS-DA) and OPLS regression.

OPLS-DA allows the comparison of spectra/chromatograms from different classes of sample (e.g. obese vs. non-obese) using the class information collected over the study period.

OPLS is a regression extension of PCA and is based on Partial Least Squares, or PLS, projection technique and is useful for identifying metabolites which discriminate between individuals based on

their class identity. It includes an internal orthogonal filter, which removes systematic variation from the dataset that is unrelated to the class or property used for differentiation in the model.

The purpose of OPLS-DA is to establish if any relationship exists between the variables measured and class identity of the sample. Re-scaling of variables contributing towards the loadings plots is undertaken to maximise separation between the pre-defined classes. Because differences between classes may not be the most pronounced variation seen in the dataset, OPLS is used to identify and highlight more subtle changes in metabolic profiles. OPLS is also useful for predicting the membership of unknown samples to classes, for example sex.

For NMR spectra analysed throughout this thesis an in-house Matlab code (written by Dr Olivier Cloarec, 2007) was used to compute OPLS-DA models. This method uses UV and mean centred scaled data (Cloarec et al., 2005b). A variable loadings plot is produced, displayed in a similar manner as NMR spectra, allowing easier identification of metabolites contributing to separation in the model. The standard deviation, or covariance, is multiplied onto each respective spectral point, this is then projected onto the spectra. The position of the peak in the loadings coefficient plot indicates whether that metabolite is increased or decreased in the case or control group. Figure 17 gives an example of an OPLS-DA model displayed in this manner. The top region of the loadings coefficient plot shows metabolites which are increased in case class (e.g. obesity) whilst metabolite peaks which are in the lower region identify metabolites which are increased in the control group (e.g. non obese).

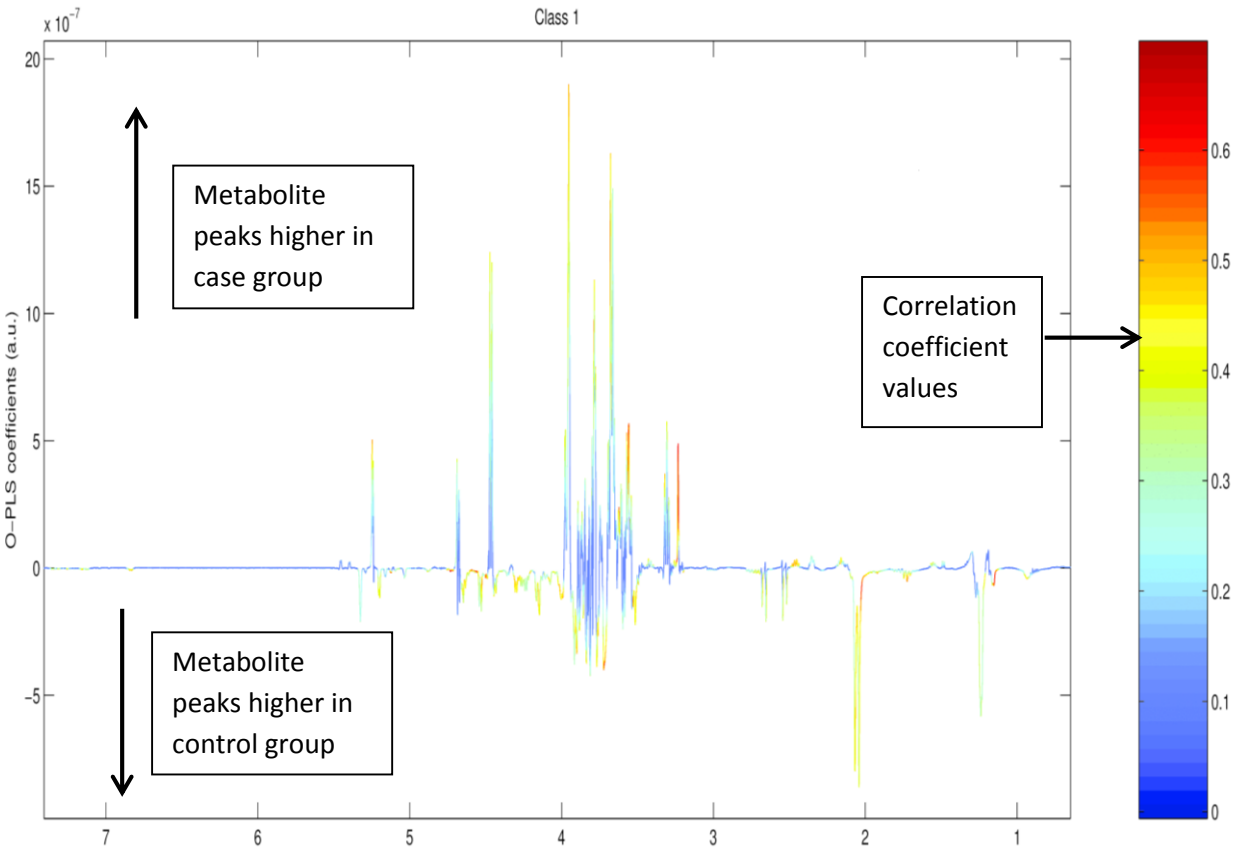


Figure 17: Example OPLS-DA loadings coefficient plot.

Peaks are coloured by the variables which describe the correlation coefficient with the class or Y variable for that peak, R^2, between each variable and the class membership, in order to demonstrate how much each peak contributes to the separation in the plot. This is displayed visually via colouring the peaks from blue to red, reflecting the value of the weight of each coefficient, red indicating a greater R^2 value. The correlation value is displayed by the colour scale to the side of the loadings plot.

For each model, the number of orthogonal components to include to reduce the influence of any non-correlated data by using the maximum R^2 and Q^2 scores obtained was ascertained (Wold et al., 2001), but did not exceed 2 for any model.

2.9.4 Model evaluation

To evaluate models produced, SIMCA-P+ and Matlab calculate two metrics based on both the predictivity and percentage of variation explained by each model, the Q^2 and R^2 values. The R^2 value relates to the percentage of variation explained by the model, describing how well the model fits the data.

The Q^2 value indicates how well the model is able to predict new data, with a higher Q^2 indicating a better predictivity. If the data contain noise or have a few scattered outliers, this results in a poor Q^2

value. The Q^2 value is derived from cross validation by dividing the data into 7 parts and sequentially removing 1/7th of the data. A model is built using the remaining 6/7th of the data and the removed data are predicted from this model, this is repeated 7 times so all of the data has been left out at least once (Wold et al., 1987). The original data are compared against the predicted data and the sum of squared errors is calculated.

In order to assess the number of components required for modelling the acquired data, firstly a number of principal components are calculated. The point before which the ratio of the R^2 value to the Q^2 value starts to decrease is selected as the number of components to include to calculate the model. This ensures that the models produced are valid, and the data not 'over fitted'.

Further validation of multivariate models is achieved by permutation testing, which involves scrambling the class labels and repeating the modelling procedure, for example 100 times, in order to assess the chances of randomly achieving the same R^2 and Q^2 values.

2.9.5 S-plots

S-plots are useful for extracting the identification of metabolites associated with a particular group of participants. S-plots provide a visualisation of the OPLS-DA loadings, using a scatter plot to display the covariance and correlation of the modelled data, displayed in Figure 18. If there is variation in the data, the plot looks like an S. The two axes, p_1 and $p(corr)_1$, describe respectively the magnitude and reliability of each variable in the dataset.

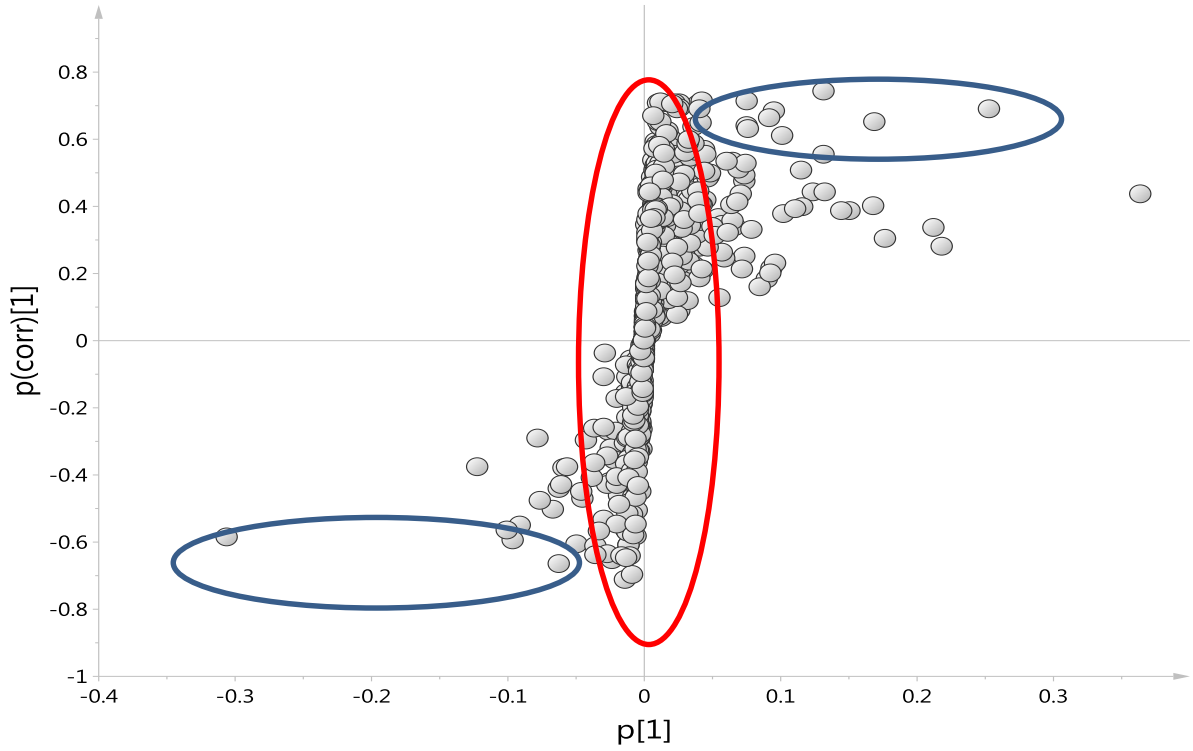


Figure 18: Example S-plot, variables located within the red oval relate to peaks with a low intensity and have a high risk of being a spurious correlation, variables within the blue ovals possess a high intensity and a high reliability, and these are the metabolites of interest in regard to different groupings.

2.9.6 STOCSY

A further tool used in the metabolite identification process is Statistical TOtal Correlation SpectroscopY, or STOCSY, a computational approach for metabolite identification (Cloarec et al., 2005a). STOCSY works on the assumption that peaks present in a spectrum belonging to the same molecule are highly correlated; STOCSY takes advantage of this by generating a pseudo-two dimensional NMR spectrum. The output of STOCSY is a plot which displays the correlation between the intensities of the different variables across the whole spectrum, highlighting peaks belonging to the same molecule (Cloarec et al., 2005a). An example STOSY plot is displayed in Figure 19.

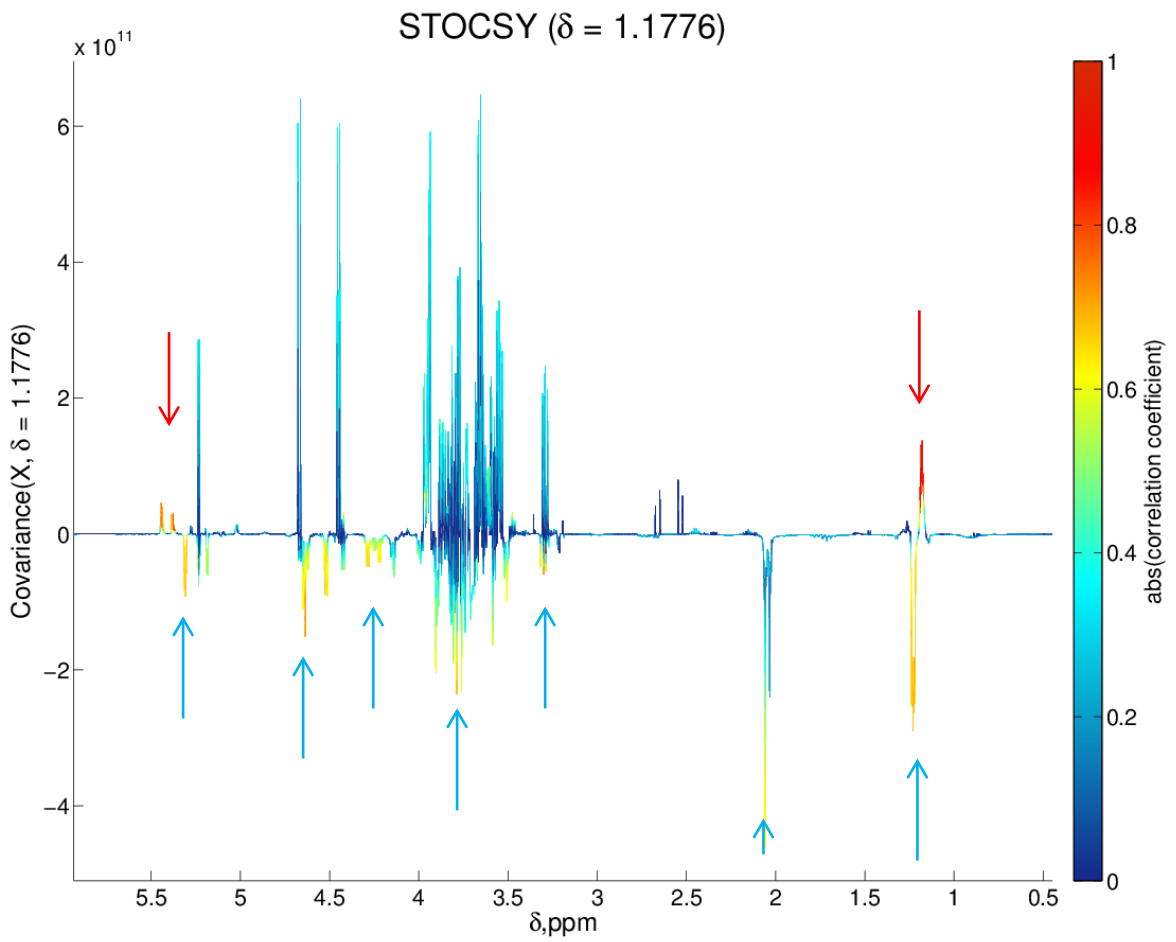


Figure 19: Example STOCSY from the aqueous fraction of breast milk. Peaks highlighted in red pointing up are peaks which are highly correlated with the selected peak at δ 1.17 and belong to the same molecule. Peaks highlighted in red pointing down (blue arrows) belong to another molecule which is negatively correlated with the selected metabolite.

2.9.7 STORM

If metabolites were still difficult to identify using literature or STOCSY identification then SubseT Optimization by Reference Matching (STORM) was used, (Posma et al., 2012). This is a multivariate statistical approach which displays structural information from metabolites, from multiple ^{1}H NMR spectra. STORM works by selecting subsets of ^{1}H NMR spectra containing a spectroscopic signal from a specific metabolite. STORM uses these reduced spectral subsets to display a correlation matrix, containing smaller numbers of samples than the number of variables, which limits the number of false positive associations, making identification easier.

2.10 Method Development

The studies for which these samples were collected were approved by the National Research Ethics Service (ref 08/H07114/3 and 10/H0713/5); research was performed with the mother's written informed consent. These samples were collected separately from the prospective cohort study samples outlined earlier in Section 2.2 of this chapter. These studies had collected a sample volume surplus to requirement, and were used for method development, as outlined below.

2.10.1 Introduction

Analysis of breast milk composition is complicated by the changing nutritional composition of breast milk over the course of a single feed, throughout the day, in response to maternal diet, and over the course of lactation (Michaelsen et al., 1990). Additionally, the presence of large macromolecules and the ultrastructure of breast milk, which is micellar, mean analytical techniques must be preceded by sample preparation to reduce the complexity of breast milk prior to analysis. Therefore samples preparation techniques were optimized prior to metabonomic analysis, using ^{1}H NMR spectroscopy and MS coupled with UPLC and CE.

Very little previous research using metabonomics to analyse breast milk has been undertaken. A search using PubMed revealed only four studies that had used ^{1}H NMR spectroscopy to analyse human breast milk (Smilowitz et al., 2013, Marincola et al., 2012, Pratico et al., 2014, O'Sullivan et al., 2013b). Previous MS studies on breast milk have predominantly investigated the lipid content (Lindholm et al., 2013), or the structure of HMO present (German et al., 2008).

Upon commencement of the method development, no study had employed a combination of metabonomic techniques to widen the global metabolite profile obtainable from breast milk. Since then we have published two articles on this (Villasenor et al., 2014), and (Andreas et al., 2015). The reluctance to implement a multi-platform approach in breast milk analysis may arise from the fact that each analytical technique normally requires different pre-processing steps, thus increasing the amount of sample volume and preparation time required. Therefore a simple sample preparation protocol for the pre-analytical preparation of breast milk for multiplatform metabonomic analysis was established, thus reducing the total volume of milk required to achieve comprehensive coverage of the milk metabolome.

UPLC-MS and ^{1}H NMR spectroscopy were used to characterise hydrophobic metabolites whilst polar metabolites were analysed using ^{1}H NMR spectroscopy and CE-MS. To ensure reproducibility of the extraction protocol and analytical procedures, evaluation of analytical platforms was undertaken

using metabolites chosen to cover the full range of the spectral profiles for each technique, by taking into consideration the relative standard deviation (RSD).

2.10.2 Samples

Seventy human milk samples were obtained from 57 mothers, who delivered healthy term-born infants. Predominantly hindmilk samples were obtained between 2 to 80 days post-partum, by hand expression. Samples were collected into collection bottles and aliquoted into 1.5ml Eppendorf tubes. Samples were frozen as soon as possible after collection and stored at -80°C, until analysis.

2.10.3 Sample pre-treatment

No single technique is able to separate and detect the complete range of metabolites present in such a complex biofluid as breast milk; thus, to achieve a more comprehensive coverage of the metabolome the use of several analytical strategies is required. Furthermore, in the case of UPLC-MS, due to the proteins present in breast milk, a direct injection of a whole milk into the C_{18} column is not possible, as this would block the column; and in the case of ^{1}H NMR, lipid peaks obscure metabolites present in smaller quantities, therefore different sample treatment protocols were tested in order to improve the number of metabolites able to be detected. In order to establish the best method to separate out the aqueous and lipid phases of milk, solvents were employed in liquid-liquid extraction protocols.

Different modifications of the Folch extraction method (Folch et al., 1957) were investigated. A total of 400µL of pooled whole milk was added to 2ml of a chloroform methanol mixture in different ratios (two ratios each of 1:2, 2:1 and 1:1 chloroform:methanol). This mixture was then vortexed and 600µL of demineralised water was added to one set of each of the ratios. The samples with added water were vortexed again; and both sets centrifuged for 10 minutes at 16000×g.

This method produces two phases, a lipid phase at the bottom of the vial, plus an aqueous phase above, with metabolites splitting into the different phases according to their polarity. The phases were transferred to separate glass vials, and aliquoted into smaller volumes for each analytical technique, in order to be able to reconstitute them as needed. Both phases were evaporated to dryness using a speed vacuum concentrator (Eppendorf, Hamburg, Germany). Once dry, the phases were frozen at -80°C until analysis.

The method which produced the cleanest spectra, resolving the greatest number of metabolites was chosen. This was the method using a 2:1 ratio of chloroform:methanol with the addition of 600µL of water after the initial mix. This was the method which we therefore subsequently used to prepare the study samples from the cohort recruited.

2.10.4 Profile characterisation

Each analytical platform generated a set of metabolites, some of which were unique to that platform and some of which were detected by more than one platform. Metabolites present in the profiles of each of these techniques were, as far as possible, identified using online databases and metabolite identification techniques.

2.10.5 ^{1}H NMR Spectroscopy profile characterisation

Analysing the aqueous fraction of breast milk using ^{1}H NMR spectroscopy allowed the identification of ~52 metabolites. In the spectra of the organic extract 14 peaks were identified, however, these peaks do not correspond to specific molecules, but lipid classes or distinct proton environs within the lipid metabolites. Likewise, in the aqueous extract the HMO designations are not definitive, but examples of the type of HMO these peaks may originate from. This is because peaks correspond to proton environments, some of which are shared due to the similar structures of these compounds. Metabolites identified from the two dimensional NMR spectra are presented in Figures 20-22. The one dimensional breast milk aqueous and lipid spectra are displayed in Figure 23 A and B respectively, with identified metabolites defined in Table 4. The main metabolite classes identified from the aqueous fraction included; monosaccharaides, disaccharides, HMO, amino acids and their derivatives, choline containing molecules, intermediates of the tricaboxylic acid cycle, ketones and short chain fatty acids.

Table 4: Peak Assignments for ^{1}H NMR Spectrum from the aqueous and lipid fraction of human breast milk.

#	Compound name	1D /2D	Group	Assign ment	^{1}H (ppm)	^{13}C (ppm)	Multiplicit y	Metabolite class
1	2-Oxoglutarate	2D			2.42, 2.99	33.4, 38.6	(t)	Citrate cycle
2	2'-Fucosyllactose	2D	Fuc(α1-2)	CH-1	5.32	102.3		HMO
				CH-5	4.23	69.9		
				CH_3-6	1.24	18.1		
			Gal(β1-4)	CH-1	4.53	103		
3	3'-Fucosyllactose	2D	Fuc(α1-3)αGlc	CH-1	5.39	101.3		HMO
				CH_3-6	1.19	18.2		
			Fuc(α1-3)βGlc	CH-1	5.44	101.1		
4	3’-Sialyllactose	2D	Neu5Ac(α2-3)	CH-5	3.85	54.5		HMO
				CH-3	2.75	42.7		
				CH-3'	1.78	42.6		
5	6’-Sialyllactose	2D	Neu5Ac(α2-6)	CH-3	2.73	42.9		HMO
				CH-3’	1.73	42.9		
6	Lacto-N-neoDiFucohexaose	2D	Fuc(α1-3)GlcNAc	CH-1	5.19	102.3		HMO
				CH-5	4.3	69.5		
7	Lacto-N-neofucopentaose/ Lacto-N-neotetraose/ Para-Lacto-N-neohexaose/Lacto-N-neooctaose/ Lacto-N-neofucopentaose/ Galacto-N-neoPentaose	2D	Gal(β1-4)GlcNAc	CH-1	4.43	104.6		HMO
				CH-4	4.15	71.34		
8	Lactodifuco-tetraose	2D	Fuc(α1-2)	CH-1	5.29	102.3		HMO
			Fuc(α1-3)αGlc	CH-1	5.4	101.5		
				CH_3-6	1.19	18.34		
			Fuc(α1-3)βGlc	CH-1	5.46	101.21		
9	Lacto-*N*-difucohesaose I		Fuc(α1-2)	CH-1	5.16	102.3		HMO
				CH-5	4.35	69.1		
			GlcNAc(β1-6)	CH-1	4.62	106.17		
10	Lacto-*N*-difucohesaose II		Fuc(α1-4)	CH-1	5.03	100.6		HMO
				CH_3-6	1.18	18.34		
			Fuc(α1-3)αGlc	CH-1	5.38	101.71		
			Fuc(α1-3)βGlc	CH-1	5.43	101.5		
				CH_3-6	1.14	18.34		
			Gal(β1-4)	CH-1	4.52	105.7		

11	Lacto-*N*-fucopentaose II		Fuc(α1-4)	CH-1 CH_3-6	5.03 1.18	100.81 18.34		HMO
12	Lacto-*N*-fucopentaose III		Fuc(α1-3)GlcNAc Fuc(α1-3)GlcNAc	CH_3-6 CH-1 CH_3-6	1.19 5.13 1.19	18.34 101.51 18.34		HMO
13	LNFP V		Fuc(α1-3)αGlc Fuc(α1-3)βGlc	CH-1 CH_3-6 CH-1 CH_3-6	5.39 1.19 5.44 1.19	101.51 18.34 101.22 18.34		HMO
14	Lacto-*N*-neotetraose			CH-1	4.35	104.6		HMO
15	Lacto-*N*-tetraose			CH-1 CH-2	4.35 3.51	104.6 71.5		HMO
16	Acetate	1D			1.92		(s)	Organic acid
17	Acetone	1D			2.22		(s)	Ketone
18	Alanine	1D, 2D			1.48	19	(d)	Amino Acid
19	Aspartate	1D, 2D			2.71, 2.8	39.3, 39.4	(dd), (dd)	Amino Acid
20	Butyrate	1D			0.89, 1.55		(t), (m)	Short chain fatty acids
21	Caprate	1D, 2D			1.27		(m)	Short chain fatty acids
22	Caprylate	1D			1.27		(m)	Short chain fatty acids
23	Carnitine	1D, no 2D			3.25		(s)	Amino acid derivative
24	Choline	1D, 2D			3.18	58.4	(s)	Cholines
25	Citrate	1D, 2D			2.53, 2.66	48.7, 48.7	(dd)	Citrate cycle
26	Creatine	1D			3.04		(s)	Organic Acid
27	Creatine phosphate	1D					(s)	Organic Acid
28	Creatinine	1D			3.048		(s)	Organic Acid
29	Ethanolamine	1D, 2D			3.14	44.18	(t)	Other
30	Formate	1D			8.46		(s)	Organic acid
31	Fucose	1D, 2D			1.18, 1.23, 3.42, 4.15, 4.53, 5.2		(d), (d), (m), (m), (d), (d)	Monosaccharide
32	Galactose	2D			5.28	95	(d)	Monosaccharide
33	Glucose	1D, 2D		CH-1	4.65	98.82	(d)	Monosaccharide

34	Glutamate	1D			2.13, 2.35		(m), (m)	Amino Acid
35	Glutamine	1D			2.13, 2.44		(m), (m)	Amino Acid
36	Lactate	1D, 2D			1.33	22.9	(d)	Organic acid
37	Lactose	1D, 2D			3.3, 3.54-3.97, 4.46, 4.68, 5.24		(t), (multiple peaks), (d), (d), (d)	Disaccharide
38	Isoleucine	1D			0.92		(t)	Amino Acid
39	Leucine	1D			0.96, 1.7		(t), (m)	Amino Acid
40	Lysine	2D			3.01	42.1	(t)	Amino Acid
41	Methanol	1D, 2D			3.37	51.4	(s)	Other
42	Methionine	2D			2.1	17	(m)	Amino Acid
43	*N*-Acetylneuraminic acid	1D			2.06		(s)	Monosaccharide
44	*N*-Acetylglucosamine	1D			2.04, 5.2		(s), (d)	Monosaccharide
45	O-Acetylcarnitine	1D					(s)	Amino acid derivative
46	O-Phosphocholine	1D			3.2		(s)	Cholines
47	sn-Glycero-3-phosphocholine	1D, 2D			3.22	56.7	(s)	Cholines
48	Succinate	1D, 2D			2.4	3.68	(s)	Citrate cycle
49	Taurine	2D			3.41	38.3	(t)	Amino acid derivative
50	Tyrosine	2D			6.83, 7.13	118.3, 133.2	(m), (m)	Amino Acid
51	Urea	1D			5.78			Other
52	Valine	1D			0.99, 1.02		(dd)	Amino Acid

Peak Assignments for ^{1}H NMR Spectrum from the Lipid fraction of human breast milk

Peak number	Proton	Chemical shift(s) (ppm)	Multiplicity
1	Terminal -CH3	0.88	(t)
2	Terminal -CH3 in ω3 fatty acids	0.97	(t)
3	-CH2-	1.25	(s)
4	-CH2-CH2-COOH	1.61	(s)
5	-CH2-CH=CH-	2.01	(q)
6	-CH2-COOH	2.3	(t)
7	=CH-CH2-CH2-COOH	2.34	(t)
8	= CH-CH2-CH=	2.77	(t)
9	-N(CH3)3 Phosphatydylcholine	3.33	(s)
10	Glyceryl, C1, 3, 2	4.14, 4.29, 5.26	(m), (m), (m)
11	-CH=CH-	5.34	(m)

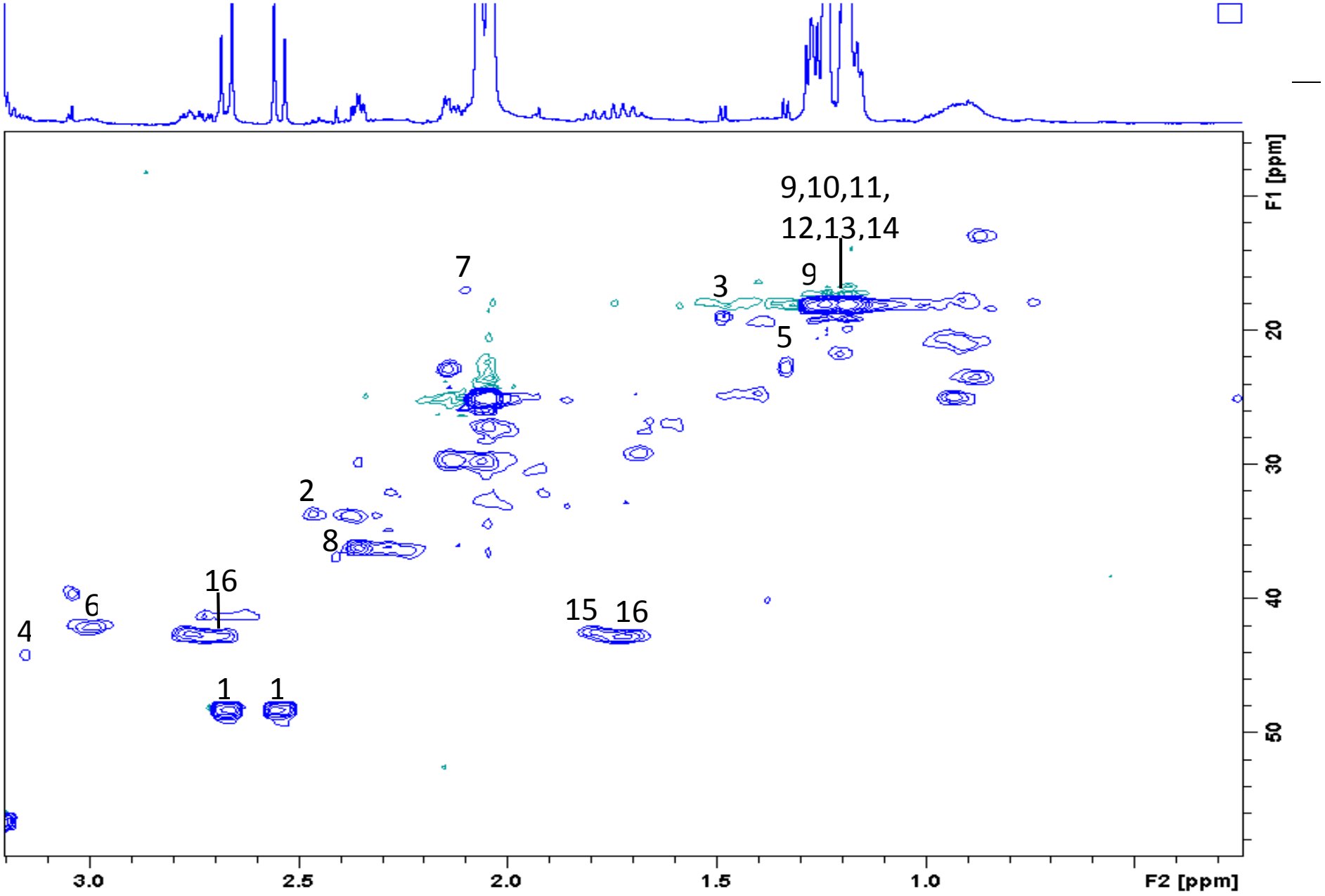


Figure 20: ^{1}H NMR HMDB Spectrum of a pooled milk sample from 0-3 ppm. 1=Citrate, **2**=Oxoglutarate, **3**=Alanine, **4**=Ethanolamine, **5**=lactate, **6**=Lysine, **7**=Methionine, **8**=Succinate, **9**=2'-Fucosyllactose, **10**=3'-Fucosyllactose, **11**=LNFPII, **12**=LDFT, **13**=LNFPIII, **14**=LNFP V, **15**=3'-sialyllactose, **16**=6'-sialyllactose.

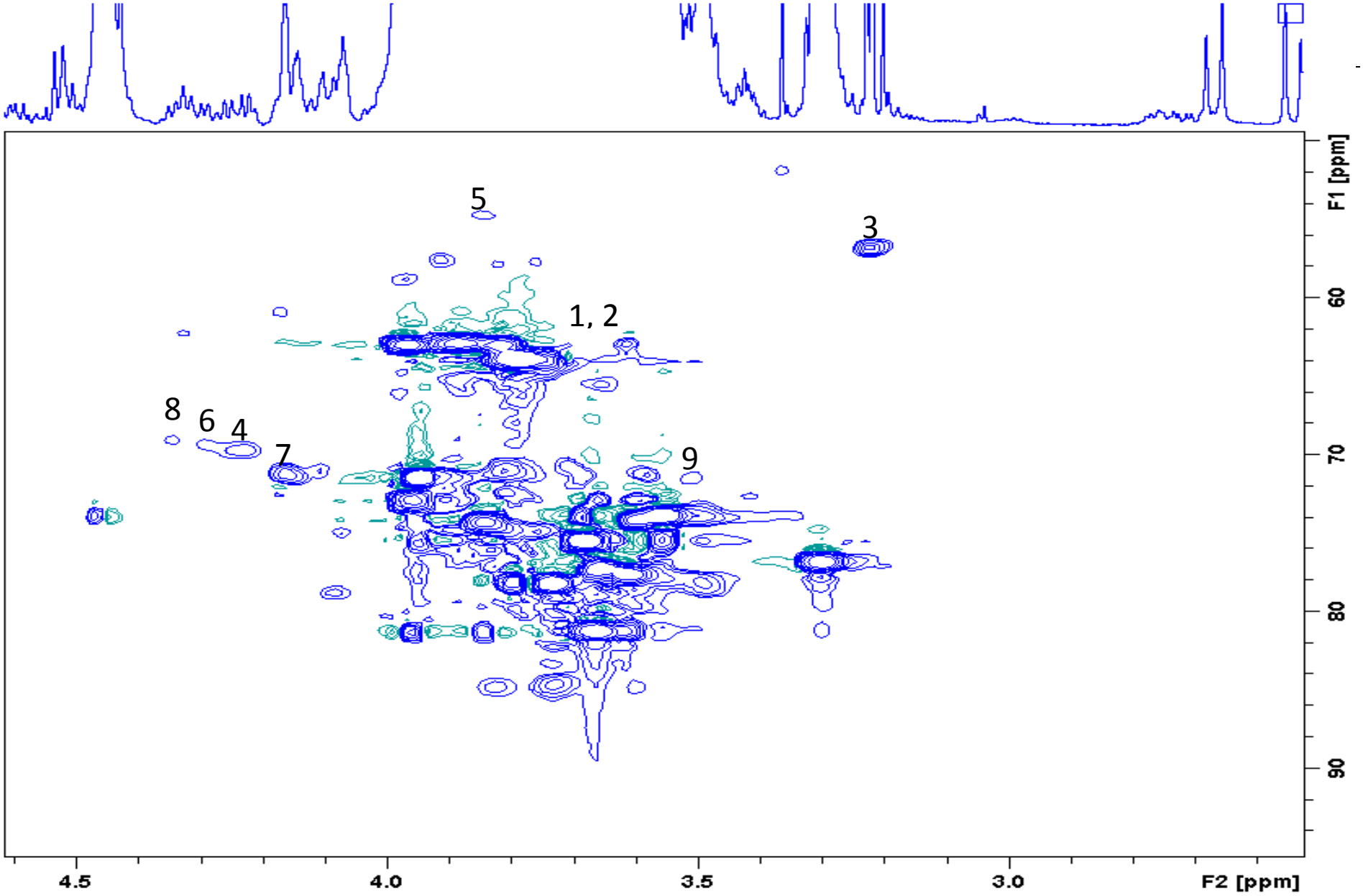


Figure 21: ^{1}H NMR HMDB Spectrum of a pooled milk sample, from 3-4.5 ppm. 1=Lactose, **2**=Galactose, **3**=Glycerophosphocholine, **4**=2'-fucosyllactose, **5**=3'-siallactose, **6**=Lacto-N-neoDiFucohexaose, **7**=Lacto-N-neofucopentaose/ Lacto-N-neotetraose/ Para-Lacto-N-neohexaose/Lacto-N-neooctaose/ Lacto-N-neofucopentaose/ Galacto-N-neoPentaose, **8**=LNDFHI, **9**=Lacto-N-tetraose.

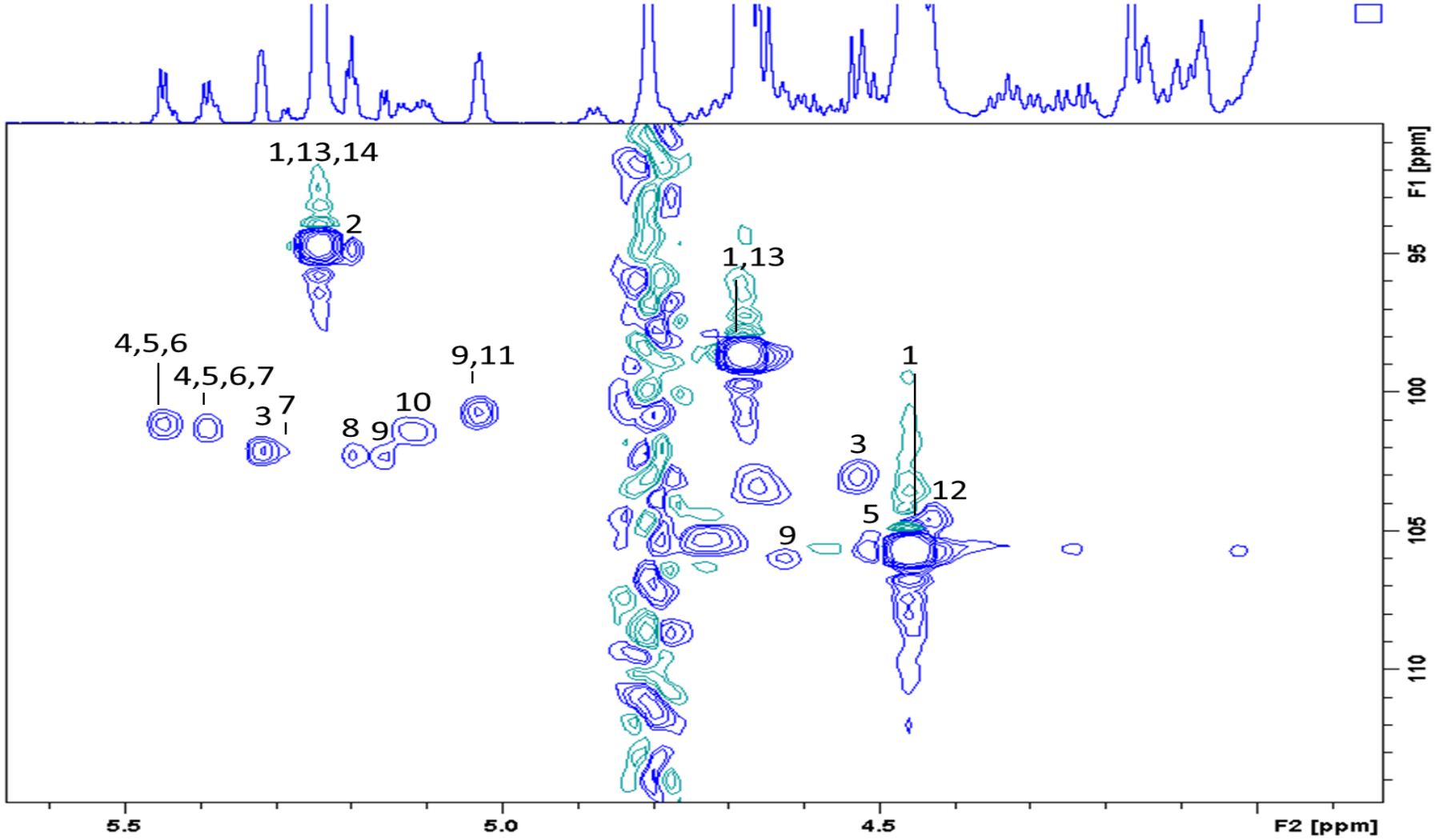


Figure 22: ^{1}H NMR HMDB Spectrum of a pooled milk sample from 4-5.5 ppm. 1=Lactose, **2**=Fucose, **3**=2'-Fucosyllactose, **4**=3'-Fucosyllactose, **5**=LNDFHII, **6**=LNFP V, **7**=LDFT, **8**=Lacto-N-neoDiFucohexaose, **9**=LNDFHI and branched chain oligosaccharides, **10**=Fuc(α1-3)GlcNAc, **11**=LNFPII, **12**=Lacto-N-tetraose, **13**=Glucose,**14**=Galactose.

2.10.6 UPLC-MS lipid fraction profile characterisation

The lipid fraction of breast milk was analysed using UPLC-MS in both positive and negative ionization mode, as outlined in Section 2.4, Chapter 2. The resulting chromatograms were characterised, allowing the tentative identification of 105 metabolites, Table 5. The base peak chromatogram (BPC) displaying a representative sample in both positive and negative ionization mode is presented in Figure 23, D and E respectively, showing the tentative identifications of metabolites with the highest intensities. Metabolite annotation was made using literature, and by searching *m/z* values against online databases including METLIN http://metlin.scripps.edu/, LIPID MAPS, http://lipidmaps.org/, and HMDB, http://hmdb.ca. In negative ionization mode, the main metabolites identified were saturated fatty acids, monounsaturated fatty acids and polyunsaturated fatty acids whilst in positive ionization mode the main classes of lipid identified were triacylglycerols and phospholipids.

Table 5: Metabolites tentatively identified in positive and negative mode using UPLC-MS.

Number	Common Name	Formula	Ion	Experimental mass (Da)	Theoretical mass (Da)	Error (ppm)	RT (min)
ESI(-) Figure 23 D							
1	Lauric acid	$C_{12}H_{24}O_2$	[M-H]	199.1702	199.0698	0.4	1.21
2	Stearidonic acid	$C_{18}H_{28}O_2$	[M-H]	275.2014	275.2011	0.3	1.27
3	Eicosapentaenoic acid	$C_{20}H_{30}O_2$	[M-H]	301.2168	301.2168	0	1.44
4	Linolenic Acid isomer	$C_{18}H_{30}O_2$	[M-H]	277.2171	277.2168	0.3	1.52
5	Docosahexaenoic Acid	$C_{22}H_{32}O_2$	[M-H]	327.2325	327.2324	0.1	1.64
6	Arachidonic acid isomer	$C_{20}H_{32}O_2$	[M-H]	303.2325	303.2324	0.1	1.78
7	Docosapentaenoic acid	$C_{22}H_{34}O_2$	[M-H]	329.2482	329.2481	-0.1	1.85
8	Linoleic Acid	$C_{18}H_{32}O_2$	[M-H]	279.233	279.2324	0.6	1.9
9	Docosapentaenoic acid isomer	$C_{22}H_{34}O_2$	[M-H]	329.2481	329.2481	0	2.05
10	Dihomo-gamma-linolenic acid	$C_{20}H_{34}O_2$	[M-H]	305.248	305.2481	-0.1	2.1
11	Dihomo-gamma-linolenic acid isomer	$C_{20}H_{34}O_2$	[M-H]	305.2479	305.2481	-0.2	2.3
12	Docosatetraenoic acid	$C_{22}H_{36}O_2$	[M-H]	331.2636	331.2637	-0.1	2.33
13	Palmitic acid	$C_{16}H_{32}O_2$	[M-H]	255.2326	255.2324	0.2	2.4
14	Oleic acid	$C_{18}H_{34}O_2$	[M-H]	281.2482	281.2481	0.1	2.48
15	Eicosadienoic acid	$C_{20}H_{36}O_2$	[M-H]	307.2641	307.2637	0.4	2.68
16	Stearic acid	$C_{18}H_{36}O_2$	[M-H]	283.2638	283.2637	0.1	3.21
17	Eicosenoic acid	$C_{20}H_{38}O_2$	[M-H]	309.279	309.2794	-0.4	3.26
18	Docosadienoic acid	$C_{22}H_{40}O_2$	[M-H]	335.2941	335.295	-0.9	3.42
19	Erucic acid	$C_{22}H_{42}O_2$	[M-H]	337.3101	336.3029	-0.6	4.22
ESI(+) Figure 23 E							
1	SM(d32:1)	$C_{37}H_{75}N_2O_6P$	$[M+H]^+$	675.5451	675.5442	0.9	3.98
2	PC(28:0)	$C_{36}H_{72}NO_8P$	$[M+H]^+$	678.5083	678.5074	0.9	4.08
3	SM(d34:2)	$C_{39}H_{77}N_2O_6P$	$[M+H]^+$	701.5605	701.5598	0.7	4.13

4	DG(28:2)	$C_{31}H_{56}O_5$	$[M+H]^+$	509.4195	509.4206	-1.1	4.36
5	PC(32:2)	$C_{40}H_{76}NO_8P$	$[M+H]^+$	730.5397	730.5387	0.9	4.44
6	SM(d33:1)	$C_{38}H_{77}N_2O_6P$	$[M+H]^+$	689.5609	689.5599	1	4.54
7	PC(29:0)	$C_{37}H_{74}NO_8P$	$[M+H]^+$	692.5228	692.5203	-0.2	4.62
8	PC(36:4)	$C_{44}H_{80}NO_8P$	$[M+H]^+$	782.5714	782.5694	1.4	4.84
9	SM(d34:1)	$C_{39}H_{79}N_2O_6P$	$[M+H]^+$	703.5756	703.5754	0.1	5.19
10	PC(30:0)	$C_{38}H_{76}NO_8P$	$[M+H]^+$	706.5397	706.5387	1	5.28
11	PC(32:1)	$C_{40}H_{78}NO_8P$	$[M+H]^+$	732.555	732.5543	0.7	5.43
12	PC(36:4)	$C_{44}H_{80}NO_8P$	$[M+H]^+$	782.5705	782.5694	0.5	5.55
13	DG(30:2)	$C_{33}H_{60}O_5$	$[M+H]^+$	537.4513	537.4519	-0.7	5.65
14	PC(34:2)	$C_{42}H_{80}NO_8P$	$[M+H]^+$	758.5706	758.5699	0.7	5.79
15	PC(36:3)	$C_{44}H_{82}NO_8P$	$[M+H]^+$	784.5859	784.5857	0.2	5.96
16	PS(36:2)	$C_{42}H_{78}NO_{10}P$	$[M+H]^+$	788.5444	788.5442	0.2	6.04
17	PC(36:3)	$C_{44}H_{82}NO_8P$	$[M+H]^+$	784.5857	784.5857	0	6.14
18	PC(36:3)	$C_{44}H_{82}NO_8P$	$[M+H]^+$	784.5853	784.5857	-0.4	6.22
19	PE(36:3)	$C_{41}H_{76}NO_8P$	$[M+H]^+$	742.5389	742.5387	0.1	6.41
20	PE(36:4)	$C_{41}H_{74}NO_7P$	$[M+H]^+$	724.5288	724.5268	0.7	6.77
21	SM(d36:1)	$C_{41}H_{83}N_2O_6P$	$[M+H]^+$	731.6066	731.6067	-0.1	6.86
22	PC(32:0)	$C_{40}H_{80}NO_8P$	$[M+H]^+$	734.5681	734.5700	-1.7	6.93
23	PC(34:1)	$C_{42}H_{82}NO_8P$	$[M+H]^+$	760.5885	760.5856	-0.2	7.12
24	PC(38:4)	$C_{46}H_{84}NO_8P$	$[M+H]^+$	810.6007	810.6009	-0.2	7.31
25	PC(36:2)	$C_{44}H_{84}NO_8P$	$[M+H]^+$	786.6013	786.6013	0	7.37
26	DG(32:2)	$C_{35}H_{64}O_5$	$[M+H]^+$	565.4817	565.4833	-1.6	7.43
27	PC(36:2)	$C_{44}H_{84}NO_8P$	$[M+H]^+$	786.6018	786.6013	0.5	7.64
28	Unknown			663.454			7.82
29	PE(36:2)	$C_{41}H_{78}NO_8P$	$[M+H]^+$	744.5541	744.5543	-0.2	7.95
30	DG(36:4)	$C_{39}H_{68}O_5$	$[M+NH_4]^+$	634.542	634.5404	1.5	8.13
31	PE(36:2)	$C_{41}H_{78}NO_8P$	$[M+H]^+$	744.5541	744.5543	-0.2	8.25
32	DG(38:6)	$C_{41}H_{68}O_5$	$[M+NH_4]^+$	658.5415	658.5404	1	8.61
33	DG(40:7)	$C_{43}H_{70}O_5$	$[M+NH_4]^+$	684.5566	684.5561	0.4	8.84
34	SM(d38:1)	$C_{43}H_{87}N_2O_6P$	$[M+H]^+$	759.6379	759.6380	-0.2	9.1
35	PC(34:0)	$C_{42}H_{84}NO_8P$	$[M+H]^+$	762.6018	762.6013	0.5	9.15
36	DG(32:1)	$C_{35}H_{66}O_5$	$[M+H-H_2O]^+$	549.4875	549.4883	-0.8	9.31
37	PC(36:1)	$C_{44}H_{86}NO_8P$	$[M+H]^+$	788.6169	788.6169	0	9.43
38	SM(d40:2)	$C_{45}H_{89}N_2O_6P$	$[M+H]^+$	785.6535	785.6537	-0.2	9.52
39	DG(34:2)	$C_{37}H_{68}O_5$	$[M+NH_4]^+$	610.5410	610.5404	0.5	9.87
40	DG(36:3)	$C_{39}H_{70}O_5$	$[M+NH_4]^+$	636.5573	636.5567	0.6	10.15
41	PE(36:1)	$C_{41}H_{80}NO_8P$	$[M+H]^+$	746.5707	746.5700	0.7	10.2
42	SM(d41:2)	$C_{46}H_{91}N_2O_6P$	$[M+H]^+$	799.6691	799.6693	-0.3	10.38
43	SM(d39:1)	$C_{44}H_{89}N_2O_6P$	$[M+H]^+$	773.6523	773.6537	-1.4	10.5
44	SM(d41:2)	$C_{46}H_{91}N_2O_6P$	$[M+H]^+$	799.6678	799.6693	-1.6	10.91

45	SM(d42:2)	$C_{47}H_{93}N_2O_6P$	$[M+H]^+$	813.6850	813.6850	0	11.84
46	SM(d40:1)	$C_{45}H_{91}N_2O_6P$	$[M+H]^+$	787.6695	787.6695	0	12.00
47	DG(34:1)	$C_{37}H_{70}O_5$	$[M+H-H_2O]^+$	577.5192	577.5196	-0.4	12.28
48	SM(d42:2)	$C_{47}H_{93}N_2O_6P$	$[M+H]^+$	813.6844	813.6850	-0.6	12.52
49	DG(36:2)	$C_{39}H_{72}O_5$	$[M+H-H_2O]^+$	603.5357	603.5352	0.5	12.62
50	TG (34:0)	$C_{37}H_{70}O_{56}$	$[M+NH_4]^+$	628.5519	528.5516	0.3	12.73
51	SM(d41:1)	$C_{46}H_{93}N_2O_6P$	$[M+H]^+$	801.6851	801.6850	0.1	12.90
52	SM(d42:1)	$C_{47}H_{95}N_2O_6P$	$[M+H]^+$	815.7004	815.7006	-0.2	13.19
53	TG(36:0)	$C_{39}H_{74}O_6$	$[M+NH_4]^+$	656.5832	656.5823	0.9	13.34
54	TG(36:1)	$C_{41}H_{76}O_6$	$[M+NH_4]^+$	682.5983	682.5986	-0.3	13.41
55	TG(40:2)	$C_{43}H_{78}O_6$	$[M+NH_4]^+$	708.6141	708.6136	0.4	13.48
56	TG(42:3)	$C_{45}H_{80}O_6$	$[M+NH_4]^+$	734.6298	734.6293	0.5	13.59
57	TG(44:4)	$C_{47}H_{82}O_6$	$[M+NH_4]^+$	760.6462	760.6449	1.2	13.71
58	TG(38:0)	$C_{41}H_{78}O_6$	$[M+NH_4]^+$	684.6143	684.6142	0.1	13.84
59	TG(40:1)	$C_{43}H_{80}O_6$	$[M+NH_4]^+$	710.6299	710.6299	0	13.88
60	TG(42:2)	$C_{45}H_{82}O_6$	$[M+NH_4]^+$	736.6451	736.6449	0.1	13.94
61	TG(44:3)	$C_{47}H_{84}O_6$	$[M+NH_4]^+$	762.6603	830.7238	-3.6	14.04
62	TG(48:5)	$C_{51}H_{88}O_6$	$[M+NH_4]^+$	814.6915	814.6919	-0.4	14.13
63	TG(40:0)	$C_{43}H_{82}O_6$	$[M+NH_4]^+$	712.6457	712.6455	0.2	14.27
64	TG(42:1)	$C_{45}H_{84}O_6$	$[M+NH_4]^+$	738.6609	738.6612	-0.3	14.29
65	TG(44:2)	$C_{47}H_{86}O_6$	$[M+NH_4]^+$	764.6765	790.6925	-0.8	14.35
66	TG(46:3)	$C_{49}H_{88}O_6$	$[M+NH_4]^+$	790.6917	790.6925	-0.8	14.38
67	TG(48:4)	$C_{51}H_{90}O_6$	$[M+NH_4]^+$	816.7075	842.7238	-0.5	14.42
68	TG(50:5)	$C_{53}H_{92}O_6$	$[M+NH_4]^+$	842.7233	842.7238	-0.5	14.51
69	TG(50:5)	$C_{53}H_{92}O_6$	$[M+NH_4]^+$	842.722	842.7238	-1.8	14.61
70	TG(44:1)	$C_{47}H_{88}O_6$	$[M+NH_4]^+$	766.6918	766.6925	-0.7	14.67
71	TG(46:2)	$C_{49}H_{90}O_6$	$[M+NH_4]^+$	792.707	792.7081	-1.1	14.72
72	TG(48:3)	$C_{51}H_{92}O_6$	$[M+NH_4]^+$	818.723	818.7238	-0.8	14.73
73	TG(50:4)	$C_{53}H_{94}O_6$	$[M+NH_4]^+$	844.7373	844.7394	-2.1	14.74
74	TG(52:5)	$C_{55}H_{96}O_6$	$[M+NH_4]^+$	870.7546	870.7545	0.1	14.86
75	TG(52:5)	$C_{55}H_{96}O_6$	$[M+NH_4]^+$	870.7537	870.7545	-0.8	14.96
76	TG(46:1)	$C_{49}H_{92}O_6$	$[M+NH_4]^+$	794.7239	794.7238	0.1	15.01
77	TG(50:3)	$C_{53}H_{96}O_6$	$[M+NH_4]^+$	846.7549	846.7551	-0.2	15.07
78	TG(52:4)	$C_{55}H_{98}O_6$	$[M+NH_4]^+$	872.7706	872.7707	-0.1	15.11
79	TG(51:3)	$C_{54}H_{98}O_6$	$[M+NH_4]^+$	860.7687	860.7707	-2	15.22
80	TG(54:5)	$C_{57}H_{100}O_6$	$[M+NH_4]^+$	898.7854	898.7864	-1	15.28
81	TG(50:2)	$C_{53}H_{98}O_6$	$[M+NH4]^+$	848.7702	848.7707	-0.5	15.34
82	TG(52:3)	$C_{55}H_{100}O_6$	$[M+NH4]^+$	874.7858	874.7864	-0.6	15.38
83	TG(51:2)	$C_{54}H_{100}O_6$	$[M+NH4]^+$	862.7853	862.7864	-1.1	15.49
84	TG(52:2)	$C_{55}H_{102}O_6$	$[M+NH4]^+$	876.8016	876.8020	-0.4	15.63
85	TG(53:2)	$C_{56}H_{104}O_6$	$[M+NH4]^+$	890.8149	890.8170	-2.2	15.77
86	TG(54:2)	$C_{57}H_{106}O_6$	$[M+NH4]^+$	904.8325	904.8333	-0.8	15.9

2.10.7 CE-MS profile characterisation

CE-MS was used to analyse the aqueous fraction of breast milk, as outlined in Section 2.5, Chapter 2. Using CE-MS, 23 metabolites were identified from the electropherogram; Table 6. Figure 23 C displays an extracted ion chromatogram (EIC) of a representative sample. Metabolites were identified by comparing the mass accuracy and retention times against standards analysed for comparison. Metabolites identified were mainly amino acids and their derivatives, organic acids as well as nicotinamide, glycylglycine and cytosine/dine.

Table 6: Identification of metabolites from the aqueous fraction of human breast milk using CE-MS.

	Metabolite	Metabolite class	Formula	[M+H]+	Experimental mass	Mass error	Standard	Experimental
1	Creatinine	Organic Acid	$C_4H_7N_3O$	114.0662	114.0663	0.9	4.1	2.8
2	Lysine	Amino acid	$C_6H_{14}N_2O_2$	147.1128	147.1127	-0.7	4.2	3
3	Nicotinamide	Vitamin	$C_6H_6N_2O$	123.0553	123.0552	-0.8	4.2	3.1
4	Arginine	Amino acid	$C_6H_{14}N_4O_2$	175.119	175.119	0.0	4.1	3.1
5	Carnitine	Amino acid	$C_7H_{15}NO_3$	162.1125	162.1125	0.0	4.2	3.4
6	Acetyl-L-carnitine	Amino acid	$C_9H_{17}NO_4$	204.123	204.1232	1.0	5	3.7
7	Cytosine	Nucleotide	$C_4H_5N_3O$	112.0505	112.0504	-0.9	3.9	3.7
8	Cytidine	Nucleoside	$C_9H_{13}N_3O_5$	244.0928	244.0929	0.4	4.3	3.7
9	Creatine	Organic Acid	$C_4H_9N_3O_2$	132.0768	132.0769	0.8	5.2	4.2
10	Alanine	Amino acid	$C_3H_7NO_2$	90.055	90.0551	8.7	5.6	4.5
11	5-aminovaleric acid/Valine	Amino acid	$C_5H_{11}NO_2$	118.0863	118.0864	0.8	4.5	4.9
12	n-Methyl-L-Valine/Leucine/Isoleucine	Amino acid	$C_6H_{13}NO_2$	132.1019	132.102	0.8	6.7	5
13	Serine	Amino acid	$C_3H_7NO_3$	106.0499	106.0499	0.0	6.6	5.2
14	Tryptophan	Amino acid	$C_{11}H_{12}N_2O_2$	205.0972	205.0971	-0.5	-	5.2
15	Threonine	Amino acid	$C_4H_9NO_3$	120.0655	120.0654	-0.8	-	5.3
16	Glycylglycine	Dipeptide	$C_4H_8N_2O_3$	133.0608	133.0609	0.8	5	5.2
17	Methionine	Amino acid	$C_5H_{11}NO_2S$	150.0583	150.0582	-0.7	-	5.3
18	Citrulline	Amino acid	$C_6H_{13}N_3O_3$	176.103	176.1027	-1.7	6.1	5.4
19	Phenylalanine	Amino acid	$C_9H_{11}NO_2$	166.0863	166.0861	-1.2	10.8	5.4
20	Glutamic acid	Amino acid	$C_5H_9NO_4$	148.0604	148.0603	-0.7	6.4	5.5
21	Tyrosine	Amino acid	$C_9H_{11}NO_3$	182.0812	182.0811	-0.5	6.1	5.6
22	Cystine	Amino acid	$C_6H_{12}N_2O_4S_2$	241.0312	241.0311	-0.4	-	5.8
23	Aspartic acid	Amino acid	$C_4H_7NO_4$	134.0448	134.0445	-2.2	7.1	6.1

Figure 23: Profiles of breast milk analysed using;

(A) ^{1}H NMR spectrum of aqueous fraction of breast milk, along the X axis is thee ppm scale whilst along the Y axis is intensity. 1=Fucose, 2=Lactate, 3=Alanine, 4=Butyrate, 5=*N*-acetyl glutamine, 6=Acetate, 7=*N*-acetyl neuraminic acid, 8=Glutamine, 9=Glutamate 10=Citrate, 11=Creatine, 12=Creatinine, 13=Choline, 14=Phosphocholine, 15=Glycerophosphocholine, 16=Lactose, 17=Taurine, 18=Glucose, 19=Galactose

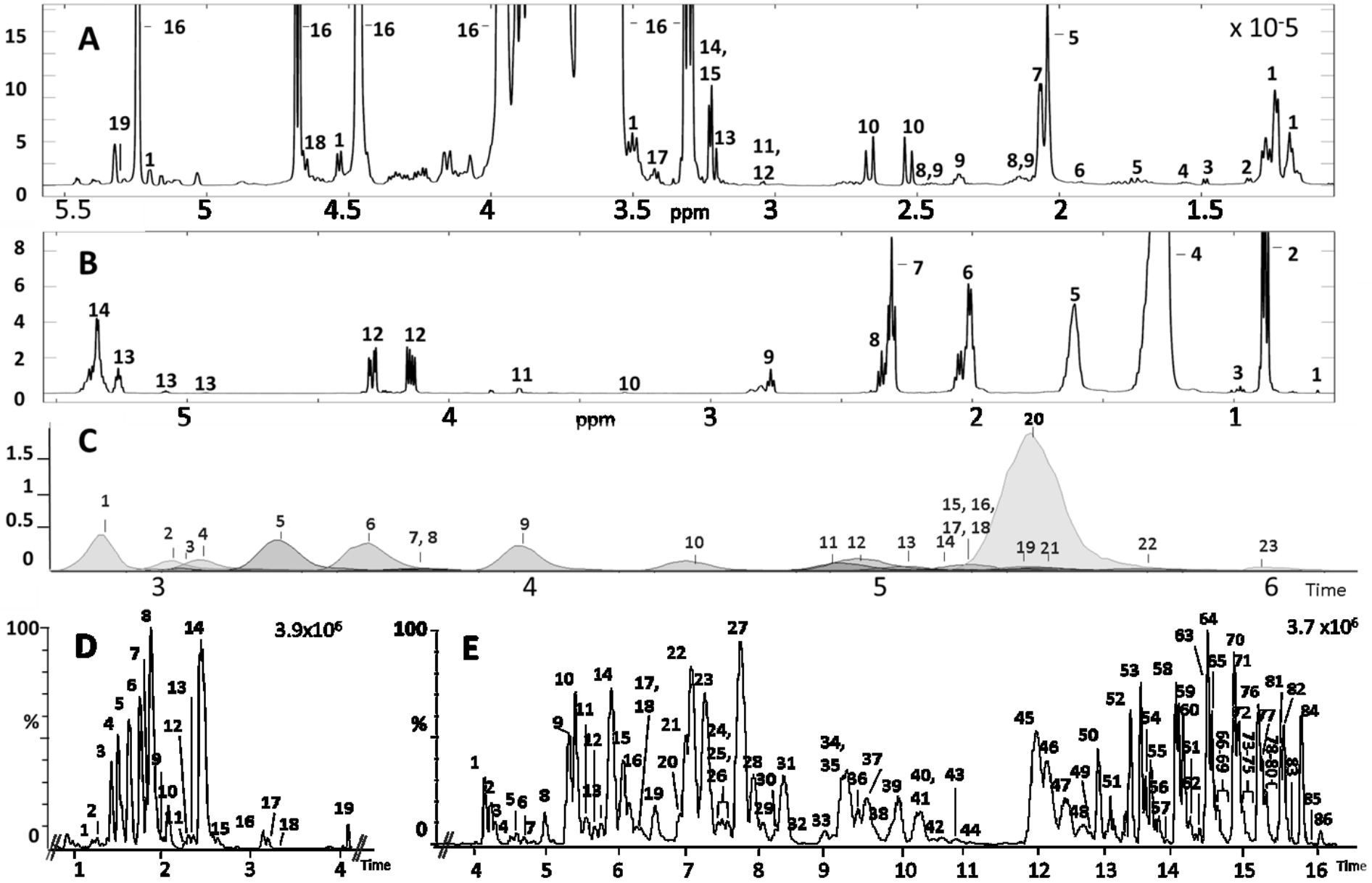
(B) ^{1}H NMR spectrum of lipid fraction of breast milk, 1=Cholesterol, 2=Terminal CH3, 3=Omega 3 terminal CH3, 4=Saturated CH2, 5=CH2-CH2-COOH, 6=Unsaturated fatty acids CH2-CH=CH, 7=CH2-COOH, 8=Docosahexaenoic acid, =CH-CH2-CH2-COOH, 9=Polyunsaturated fatty acid, =CH-CH2-CH= 10=Phosphatidylcholine,-N(CH3)3, 11=Phospholipid, 12=Glyceryl C1,3, 13=Glyceryl C2, 14=Unsaturated fatty acids, -CH=CH-

(C) CE-MS aqueous fraction analysed using positive ionization mode 1=Creatinine, 2=Lysine, 3=Nicotinamide, 4=Arginine, 5=Carnitine, 6=Acetyl-L Carnitine, 7=Cytosine, 8=Cytidine, 9=Creatine, 10=Alanine, 11=5-amino Valeric acid/valine, 12= *N*- methyl-L-Valine/leucine/isoleucine, 13=Serine, 14=Tryptophan, 15=Threonine, 16=Glycyl-glycine, 17=Methionine, 18=Citrulline, 19=Phenylalanine, 20=Glutamic acid, 21=Tyrosine, 22=Cystine, 23=Aspartic acid

(D) UPLC-MS; lipid fraction analysed using negative ionization mode. Identified metabolites are displayed in Table 5.

(E) UPLC-MS; lipid fraction analysed using positive mode. Identified metabolites are displayed in Table 5.

Figure adapted from (Andreas et al., 2014).


A
x 10-5
ppm
B
ppm
C
Time
D
3.9x10⁶
Time
E
3.7 x10⁶
Time

2.10.8 Analytical performance: Relative Standard Deviation

The RSD was calculated for each of the methods, and was used to establish the reproducibility of the methods used, Table 7. All of the methods were found to be robust and reproducible. Of all the methods, ^{1}H NMR of the aqueous breast milk fraction was found to be the most reproducible, with mean RSD value of 6.4%. The UPLC-MS methods also showed good reproducibility across the selected metabolites, with mean RSDs values of 7.95% and 13.56% for positive and negative mode data. The mean RSD for the CE-MS metabolites was the weakest in terms of reproducibility (24.18%), but was still within the accepted cut off value of 30% RSD. CE-MS has a limited reproducibility and analytical robustness in comparison to UPLC-MS and NMR. This is due to the migration time of metabolites in CE-MS being less reproducible, due to modifications in the capillary wall. This was one of the reasons why CE-MS was not employed to analyse the whole sample set of the cohort recruited for this work.

Table 7: The relative standard deviation (RSD) calculated for each of the methods used to establish the reproducibility of the methods. DG; diacylglyceride, PC; phosphocholine, PE; phosphoethanoamine, SM; sphingomyelin, TG; triacylglyceride.

	^{1}H NMR, RSD of Aqueous fraction metabolites, normalised to TSP					^{1}H NMR, RSD of Lipid fraction metabolites, normalised to TMS				
Metabolite	**Alanine (δ1.48)**	**Citrate (δ2.59)**	**Choline (δ3.21)**	**Lactose (δ4.46)**	**Formate (δ8.46)**	**$CDCl_3$ (δ7.26)**	**Choline (δ3.33)**	**$C\underline{H}_2.CH_3$ (δ1.63)**	**CH_3 of fatty acids (δ0.88)**	**Cholesterol (δ0.68)**
RSD %	1.7	1.0	5.4	0.1	24.0	4.72	11.82	9.79	10.13	10.19
	CE-MS Validation parameters for selected metabolites									
Metabolite	**Creatinine**	**Lysine**	**Nicotinamide**	**Carnitine**	**Arginine**	**Acetyl-L-Carnitine**	**Creatine**	**Valine**	**Tryptophan**	**Phenylalanine**
RSD (%)	12.7	21.2	21.0	23.5	22.8	26.4	29.1	29.1	28.3	29.8
	Validation parameters for selected metabolites by UPLC-MS of the Folch extraction, negative mode									
Metabolite	**Lauric acid**	**Eicosapentaenoic acid**	**Docosahexaenoic acid**	**Linoleic acid**	**Palmitic acid**	**Oleic acid**	**Stearic acid**	**Eicosanoic acid**	**Docosadienoic acid**	**Erucic acid**
RSD (%)	4.4	3.8	2.7	14.6	25.7	7.8	4.2	20.3	21.1	26.6
	Validation parameters for selected metabolites by UPLC-MS of the Folch extraction, positive mode									
Metabolite	**SM (d32:1)**	**PC(36:4)**	**PE(36:3)**	**PC(38:4)**	**DG(36:4)**	**SM (d41:2)**	**TG(42:3)**	**TG(38:0)**	**TG(44:1)**	**TG(54:2)**
RSD (%)	5.0	5.0	5.7	5.4	4.8	18.3	22	5	3.2	5.1

2.10.9 Multiplatform assessment of metabolite alterations in breast milk over time

Metabolites were identified which altered in concentration over lactation between the three different groups; days 1-5 post birth (group 1), 6-10 days post birth (group 2) and >10 days post-birth (group 3). In general the models generated for analysis of time-related aqueous components were stronger than those generated for the non-polar fraction, suggesting that changes in the composition of polar metabolites were more systematic than the variation observed in the lipid fraction. The equivalent PCA models provided in Figure 24 show that the differentiation of samples over time according to inherent similarity in chemical composition was apparent only for ^{1}H NMR and CE-MS analysis of the aqueous fraction.

PLS-DA models comparing the three time groups against one another were computed for each of the analytical methods used; these models displayed a gradual change in the composition of breast milk in regard to the time of collection post-birth, Figures 25.

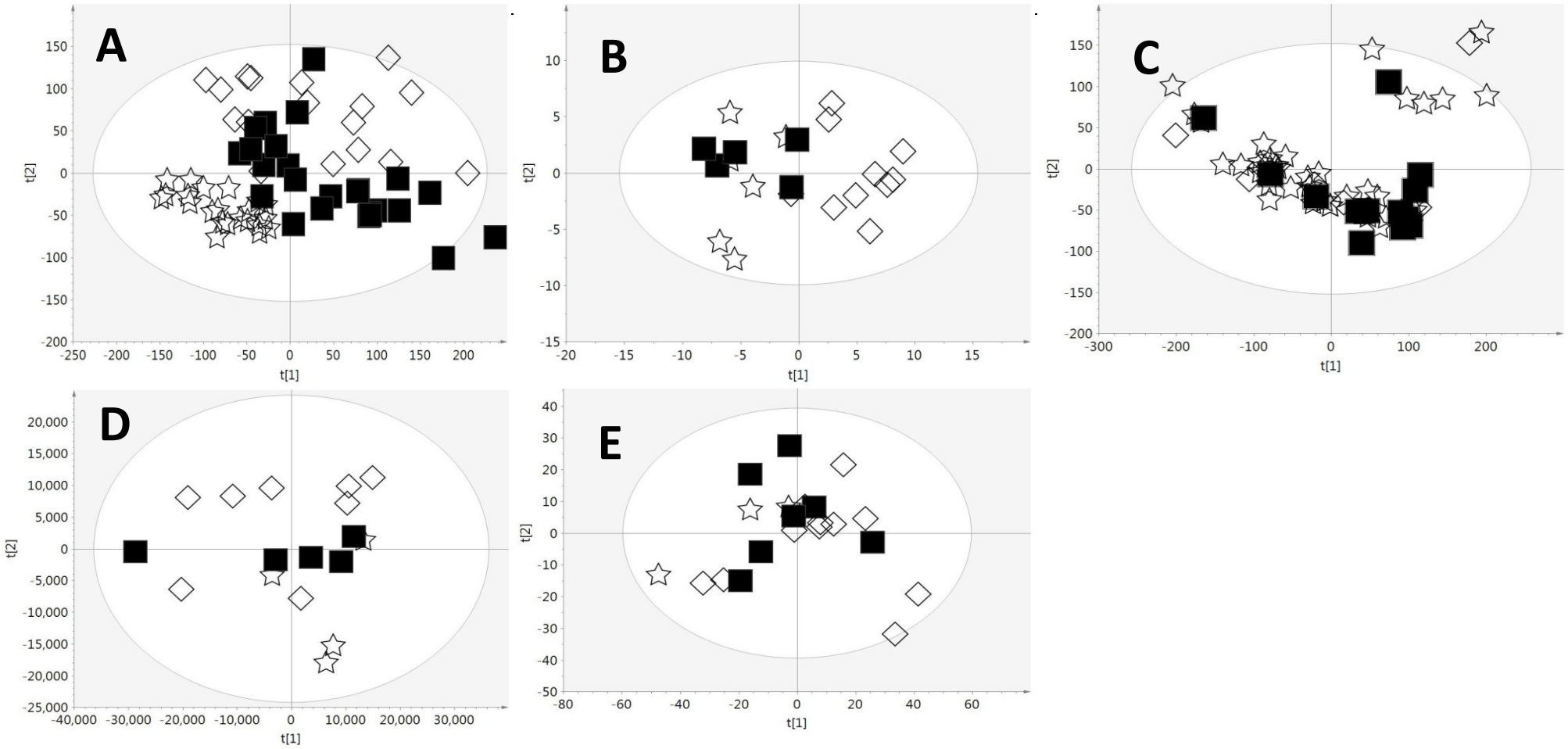


Figure 24: PCA scores plots, separated by time groups. White diamonds represent samples taken during days 1-5 post-partum, black squares show samples taken days 6-10 post-partum and white stars represent samples taken over 10 days post-partum.

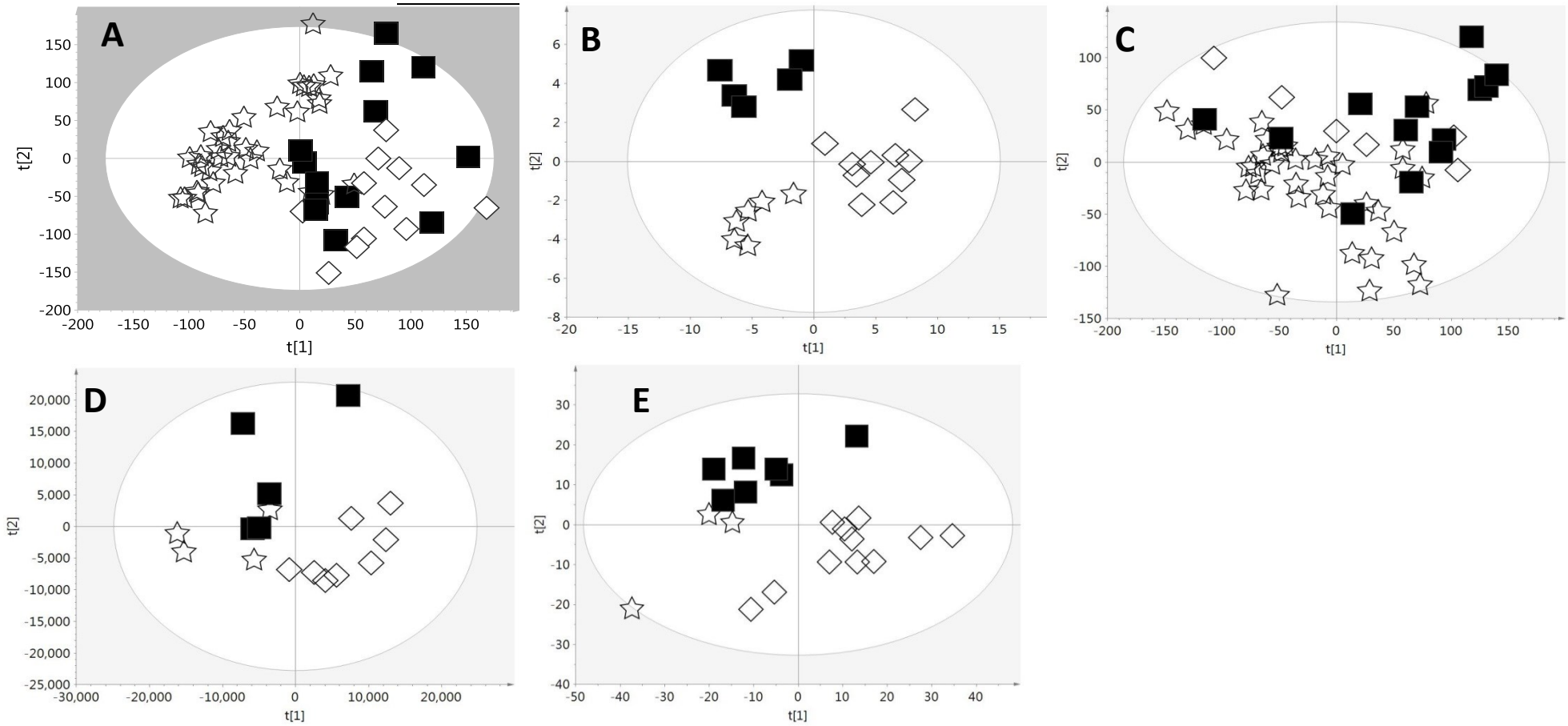


Figure 25: PLS-DA scores plots, separated by time groups; White diamonds represent samples taken during days 1-5 post-partum, black squares show samples taken days 6-10 post-partum and white stars represent samples taken over 10 days post-partum.

None of the models based on analysis of the Folch-extracted organic layer gave valid models. These results indicate that time related compositional changes in lactation are evident in polar metabolites showing a strong systematic change in relative concentration over time.

Pairwise analysis using OPLS-DA was used to establish which metabolites changed over time comparing groups 1 vs 2, 1 vs 3 and 2 vs 3. Identified metabolites are displayed in Table 8. OPLS-DA scores plots comparing these time groups are displayed in Figures 26-28, Appendix 2H gives the R^2 and Q^2 values for these models. Changes identified in breast milk composition over the first few days of lactation include an increase in the relative concentrations of di- and triacylglycerols along with a more sustained increase in lactose, several amino acids, and short and medium chain fatty acids. Conversely a time dependent decrease was observed in multiple HMO, several phosphocholines and energy metabolites such as citrate and pyruvate.

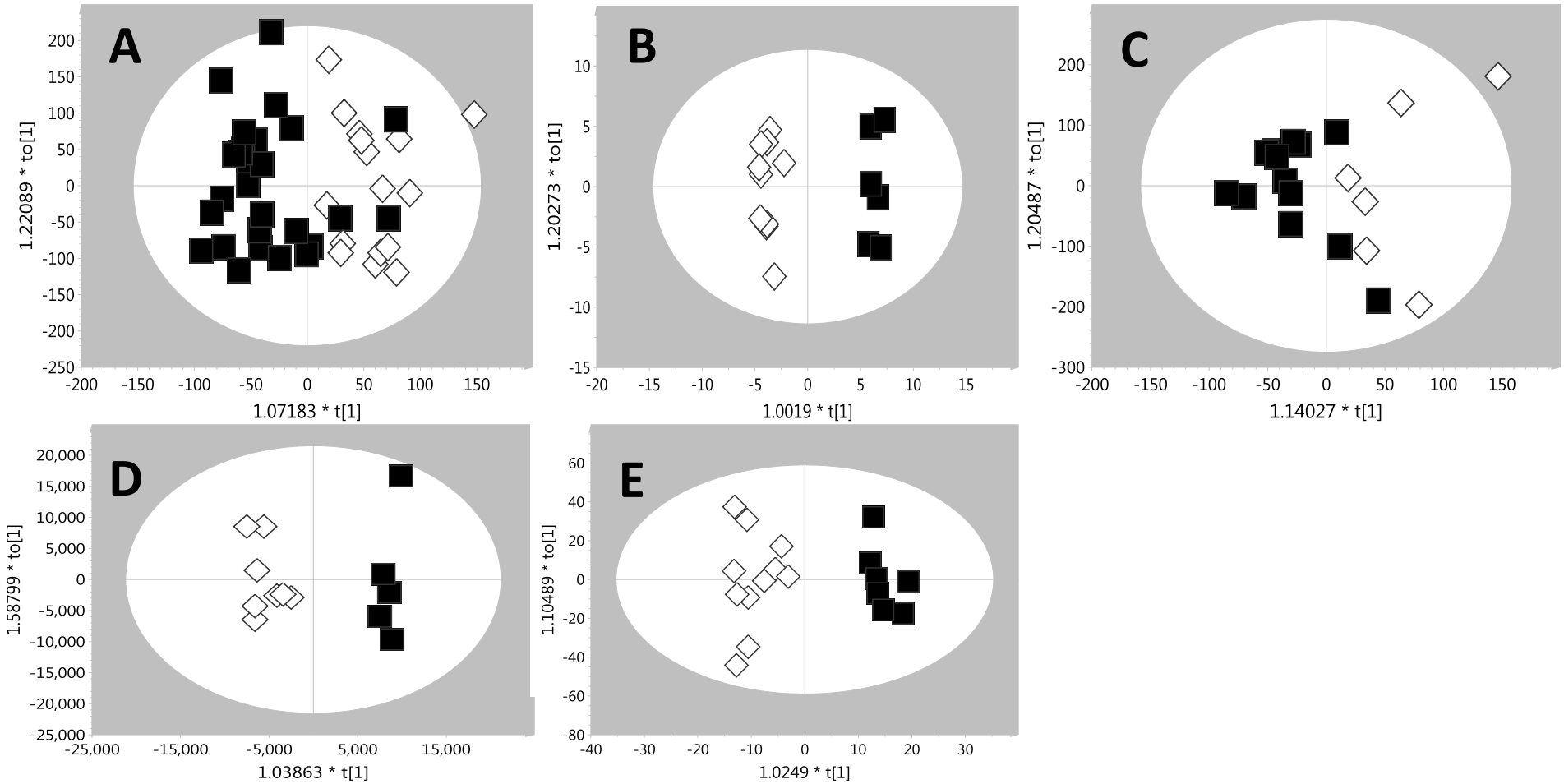


Figure 26: OPLS-DA scores plots separated by time groups. White diamonds represent samples taken during day's 1-5 post-partum, black squares show samples taken day's 6-10 post-partum.

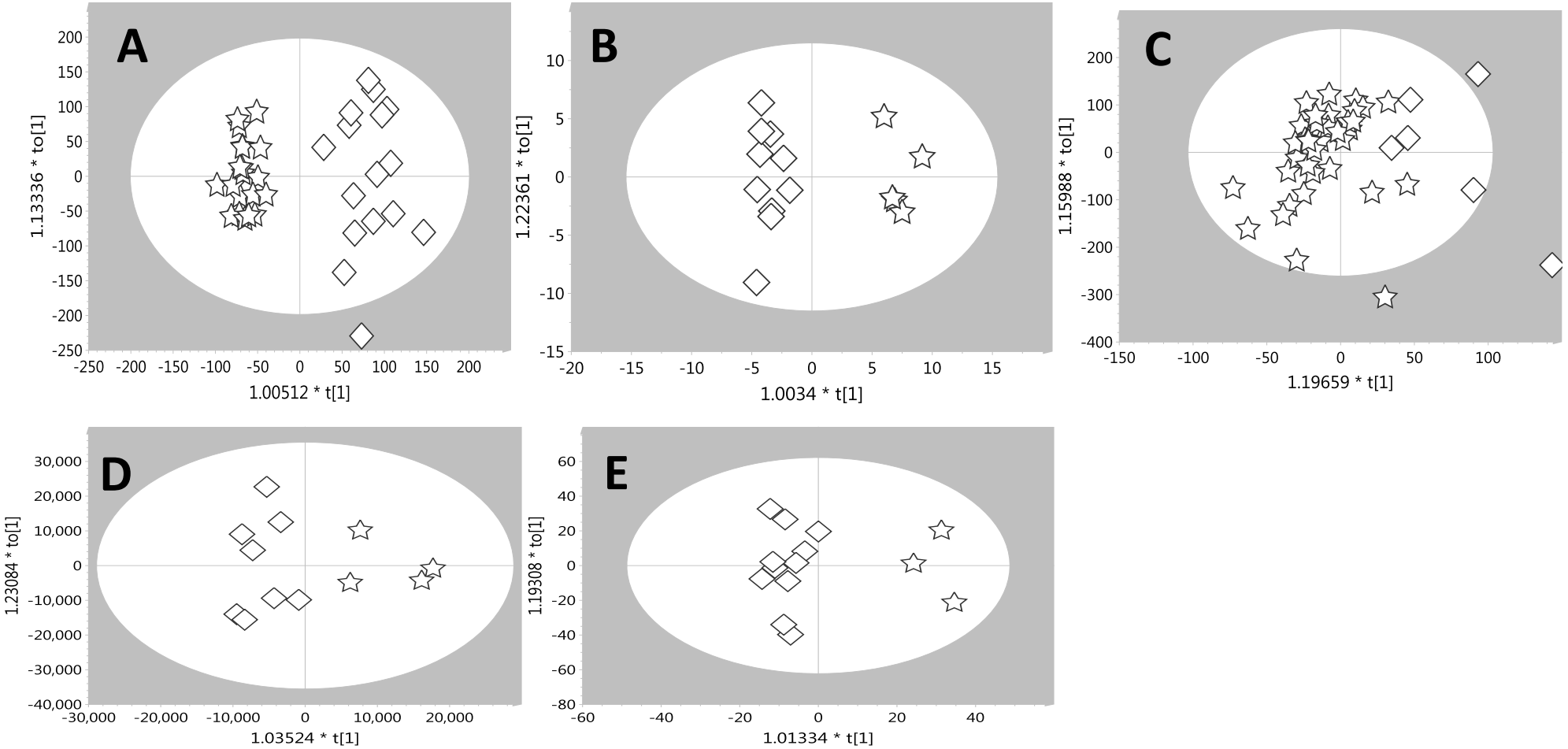


Figure 27: OPLS-DA scores plots separated by time groups. White diamonds represent samples taken during days 1-5 post-partum, white stars show samples taken days +10 post-partum.

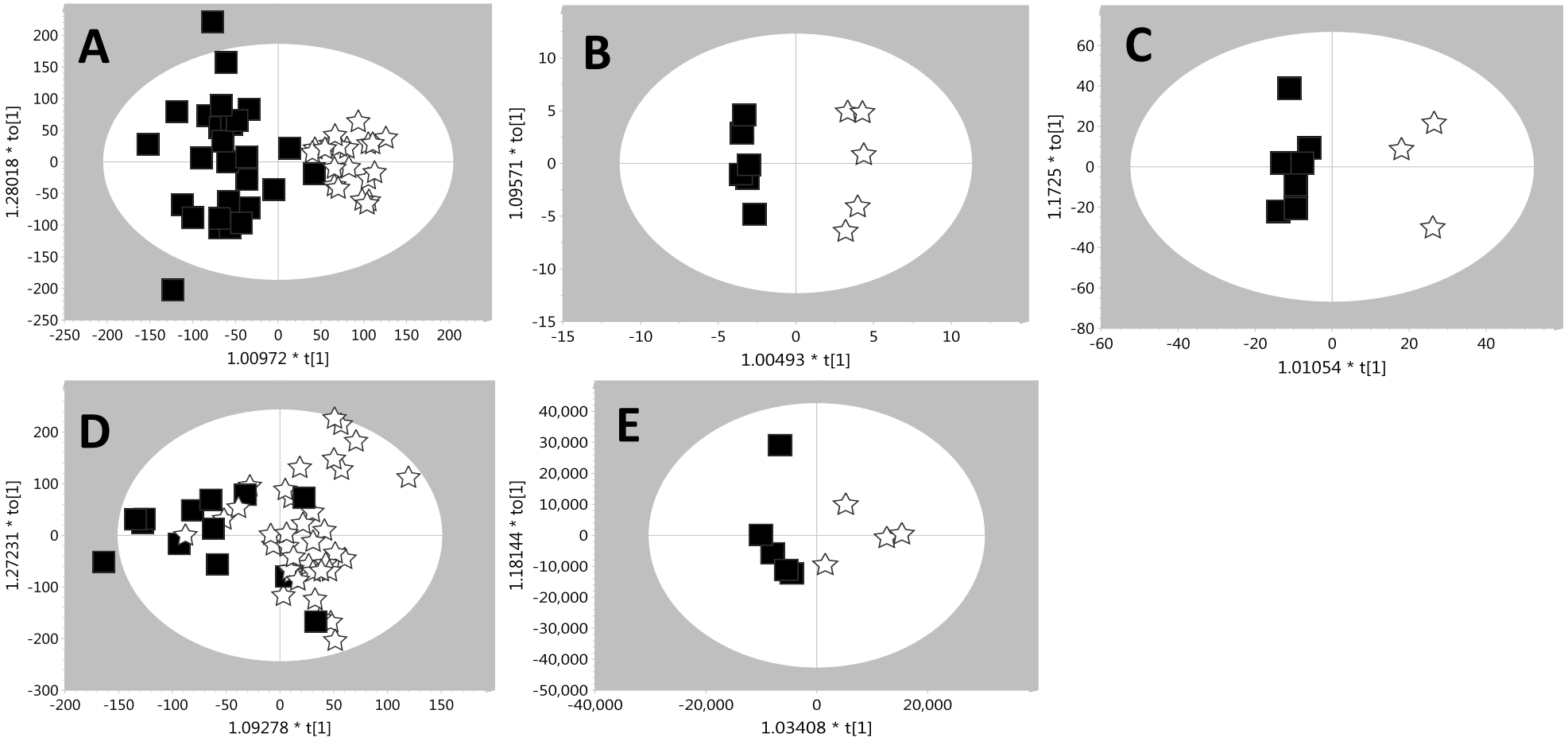


Figure 28: OPLS-DA scores plots separated by time groups. Black squares represent samples taken during days 6-10 post-partum, white stars show samples taken days +10 post-partum.

Table 8: Metabolites identified as changing in abundance in breast milk samples collected at different point's post-partum. Capitals relate to higher concentration in the later time group, whilst lower case letters refer to metabolites which were decreased in abundance in the later time point. NMR aqueous A, a; CE-MS aqueous B, b; HPLC-MS single phase C, c; NMR lipid D, d; UPLC-MS positive E, e; UPLC-MS negative F, f; GC-MS single phase G, g.

Class	Metabolite	1 vs 2	1 vs 3	2 vs 3
Amino acids	Alanine		A	
	Glutamine	A	A	
Acids	3,4 hydroxymandelic acid	g		
	Butyrate	A	A	
	Citrate	a	a	a
	Taurine	f		
	Pyruvic acid		g	
Ceramides	GlucosylCeramide (36:2)			F
	Ceramide (20:4)	c		
	PE-Ceramide (38:2)	f		
	PI-Ceramide (40:0)	f		
	PE-Ceramide (38:2)			
Diacylglycerols	Diacylglycerol (24:0)	C		
	Diacylglycerol (26:0)	C		
	Diacylglycerol (28:2)	C		
	Diacylglycerol (30:2)	C		
	Diacylglycerol (34:0)			E
	Diacylglycerol (34:1)	E		
	Diacylglycerol (34:3)		E	E
	Diacylglycerol (40:7)	C		E
	Diacylglycerol (44:10)		e	e
Prenol Lipids	Tocopherol	g	g	
Pyrimidine Nucleosides	*N*4-Acetylcytidine			B
Amino Acids Derivatives	*N*-acetyl glutamine	a	a	a
Steroids and Derivatives	Cholesteryl stearate	f		
	Cholesterol		d, g	
Sugar alcohols	Glycerol		G	
Phthalic Acid and Derivatives	Dioctyl phthalate	G		
Fatty Acid Esters	Methyl Stearate	G		
Fatty Acids	11S-hydroxy-hexadecanoic acid		F	
	9,12-octadecadienoic acid	G	G	
	Arachidonic Acid		C	
	Caprylic acid	F	F	
	3-Hydroxycapric acid		F	
	Hexadecenoic acid		F	
	Myristic acid	g		
	Oleic acid		G	
	Palmitoleic acid		G	
	Lauric acid		G	
	Linoleic acid	G	G	
	Polyunsaturated FA		d	
	Terminal CH_3 on omega 3 fatty acids		d	
Saccharides	Fucose (from HMO)	a	a, g	a

	D-glucosaminic acid (from HMO)	g	g	
	N-acetyl neuraminic acid (from HMO)	a	a	a
	Glucose	G	G	
	Lactose	A, G	A	A
	Gluconic acid lactone	G		
Glycerophosphocholines	Glycerophosphocholine (18:0)	e		
	Glycerophosphocholine (34:0)		e	e
	Glycerophosphocholine (32:0)	e		e
	Glycerophosphocholine (32:1)	e		
	Glycerophosphocholine (30:0)	e	e	e
	Glycerophosphocholine (38:5)			f
	Glycerophosphocholine (34:2)	e	e	
	Glycerophosphocholine (36:1)		e	c
	Glycerophosphocholine (36:2)	E		
	Glycerophosphocholine (36:3)		e	
	Glycerophosphocholine (36:4)		f	
	Glycerophosphocholine (40:7)		f	
	Glycerophosphocholine	A	A	
Glycerophosphoethanolamines	Glycerophosphoethanolamine (40:6)	F		
	Glycerophosphoethanolamine (36:2)	E		
	Glycerophosphoethanolamine (42:3)		f	
	Glycerophosphoethanolamine (44:2)		f	
Glycerophosphoserines	Glycerophosphoserine (30:3)	F		
	Glycerophosphoserine (18:1)		f	
Glycererophosphoglycerol	Glycerophosphoglycerols (44:7)		f	
Glycerphosphoinositols	Glycerophosphoinositol (35:2)	f		
	Glycerophosphoinositol (32:0)	E		
	Glycerophosphoinositol (37:4)		f	
Monoacylglycerols	Monoacylglycerol (18:3)		b	b
Sphingomyelins	Sphingomyelin (42:0)			F
	Sphingomyelin (34:1)	e	e	
	Sphingomyelin (36:3)			c
	Sphingomyelin (38:1)	e		
Triacylglycerols	Triacylglycerol (34:0)	E		e
	Triacylglycerol (36:0)	E		e
	Triacylglycerol (38:0)	E		e
	Triacylglycerol (42:1)	E		e
	Triacylglycerol (42:2)	E		e
	Triacylglycerol (44:2)	E		e
	Triacylglycerol (45:1)		F	
	Triacylglycerol (48:1)	e		f
	Triacylglycerol (48:4)			e
	Triacylglycerol (48:5)		e	
	Triacylglycerol (50:2)	e		
	Triacylglycerol (51:2)			C
	Triacylglycerol (51:7)	F		
	Triacylglycerol (52:2)	e		E
	Triacylglycerol (55:9)			f
	Triacylglycerol (58:2)		c	
	Triacylglycerol (60:3)	c	c	c

2.11 Discussion

A comprehensive metabolic map of breast milk using different analytical platforms has been generated, tentatively identifying 180 metabolites; previous studies have commonly identified between 50-100 metabolites. Additionally, the utility of multiplatform analytical techniques to investigate questions of clinical and biological importance has been demonstrated, including a report on the changes in abundance of metabolites over the first 3 months of lactation

Each platform contributed a set of unique metabolites to the overall global profile. There was some overlap in metabolites identified by the different platforms, but insofar as this allows corroboration of the data, this may be regarded as a strength of the multiplatform analytical approach adopted. Ultimately, given the complexity of breast milk composition, these results have demonstrated that there is no single analytical platform capable of identifying all metabolites present in milk.

Beyond simply establishing a list of metabolites present in breast milk (although this is not unimportant), in order to answer questions of biological relevance, it is important that these analytical platforms can identify alterations in the abundance of metabolites between different milk samples. It is well known that there is a temporal change in breast milk composition over lactation (Prosser et al., 1984). Based on this metabolites were identified which displayed temporal changes over the first 3 months of lactation, these were in keeping with previous reports using established biochemical assays to investigate breast milk composition.

For example, Ilcol et al demonstrated that glycerophosphocholine concentrations significantly increase in concentration from colostrum to mature milk (Ilcol et al., 2005), which was replicated in this work. One of the most prominent changes observed in the time course analysis of breast milk were the decreasing concentrations of HMO from the 1st time point to the third time point, also previously identified, (Carlson, 1985). Furthermore, this study identified increases in the abundance of lactose (Coppa et al., 1993), and glutamine (Carlson, 1985) in mature milk. In regard to the temporal change in composition of breast milk through lactation, although the data presented here are not novel, they do demonstrate that the analytical techniques utilised have the power to resolve these temporal changes.

Over time, there appeared to be a trend the data towards increased diacylglycerol and reduced triacylglycerols. It is possible that this is a reflection of breast milk lipase activity breaking down the triacylglycerides into diacylglycerides and free fatty acids. Tocopherol was decreased over the first week of lactation, but then remained at constant concentrations; this is in agreement with reports showing lower concentrations of α-Tocopherol in early lactation (Chappell et al., 1985). However, the

decrease noted in citrate over lactation is in contrast to previous studies which have noted an early rise in citrate, causing the lowest point of milk pH at around day 21 of lactation (Morriss et al., 1986).

^{1}H NMR analysis of the polar breast milk fraction provided the most detailed description of the HMO profile, and provided the most reproducible data in terms of stability and capturing systematic compositional changes over time.

Although this analysis provides a comprehensive map of breast milk composition, and shows systematic changes in breast milk over the course of early lactation, the composition of breast milk is complex and subject to the influence of many factors such as time of day, the time during the feed at which the sample is collected, time since the last feed and maternal diet. Ideally these factors require standardisation to increase confidence in results obtained from future studies, however they are often difficult to control.

In summary, this analysis displays the complementarity of various analytical technologies for characterising breast milk composition and identifies the respective strengths and limitations of the different analytical techniques.

Chapter 3
The effect of maternal body mass index on the concentration of appetite regulating hormones present in breast milk: a systematic review and *in vitro* analysis

3.1 Introduction

Early life nutrition is considered a key candidate modulator of feeding behaviour, food intake and energy balance throughout life (Cripps et al., 2007). Evidence from a large number of observational studies identifies the neonatal period as a critical time for the long-term programming of adult health (Lucas, 2005). Meta-analyses indicate that breastfeeding, as opposed to formula feeding, may protect against the development of obesity and metabolic syndrome associated conditions in later life (Owen et al., 2005, Harder et al., 2005, Arenz et al., 2004), though other research suggests that the association between formula feeding and obesity is explained by confounding (Li et al., 2012). Beneficial effects have been hypothesised to derive in part from the action of bioactive components in breast milk such as hormones (Hamosh, 2001). To date, a number of hormones involved in the regulation of energy homeostasis have been identified in breast milk; these include leptin, adiponectin, insulin, ghrelin, resistin, obestatin, peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) (Houseknecht et al., 1997, Martin et al., 2006, Aydin et al., 2006, Aydin et al., 2008, Savino et al., 2012c, Shehadeh et al., 2003, Schueler, 2013, Berseth et al., 1990). In addition to having a direct influence on infant appetite and weight gain, these peptides may also promote gut maturation and the development of neuronal circuits in the central nervous system that control metabolism. In this respect, hormones in breast milk may have long-term metabolic repercussions for the offspring following early life exposure (Agostoni, 2005).

In adults, the circulating concentration of these hormones are linked to an individual's body weight and BMI; leptin is secreted primarily by adipocytes in proportion to the total amount of body adipose tissue, and is therefore positively correlated with BMI (Considine et al., 1996), as is resistin (Degawa-Yamauchi et al., 2003). The concentration of serum insulin also increases with overweight and obesity (Lovejoy and DiGirolamo, 1992). In contrast, adiponectin (Coppola et al., 2009), ghrelin (Cummings et

al., 2002), obestatin (Lippl et al., 2008) and PYY (Alvarez Bartolome et al., 2002) correlate negatively with adiposity and BMI, whilst the association of GLP-1 with BMI is unclear (Madsbad, 2014). Factors controlling breast milk hormone concentrations are also unclear. Whilst leptin, ghrelin, insulin and adiponectin have been speculated to pass from serum into breast milk (Doneray et al., 2009, Savino et al., 2010a, Weyermann et al., 2006, Whitmore et al., 2012), the mammary gland is also capable of synthesising various hormones (Smith-Kirwin et al., 1998, Kierson et al., 2006), potentially contributing to the quantity of hormone detected; the source of the other hormones investigated remains to be established. In this chapter a systematic review of evidence from studies examining the concentration of appetite regulating hormones in breast milk and their relation to maternal BMI is reported in combination with a laboratory based *in vitro* analyses of breast milk appetite regulating hormone content. This systematic review was published in PLOS ONE, December 2014, Appendix 3A, (Andreas et al., 2014), and the in vitro analysis is currently under review at PLOS ONE.

3.2 Aims

- Systematically review the literature regarding the influence of maternal BMI on appetite regulating hormones present in breast milk.

- Evaluate the influence of maternal BMI on appetite regulating hormones present in breast milk using an *in vitro* multiplex assay.

3.2.1 Hypothesis

I will test the following null hypothesis; the concentration of appetite regulating hormones in breast milk does not correlate with maternal BMI.

3.3 Systematic review methods

3.3.1 Literature search

A search in PubMed (www.ncbi.nlm.nih.gov) for studies published before 04/09/2014 in English was carried out using the following search terms and MEDLINE Medical Subject Headings (MeSH) terms (milk, human [MeSH] OR breastmilk OR "breast milk") AND (leptin OR adiponectin OR resistin OR insulin OR ghrelin OR adipokine OR adipokines [MeSH] OR insulin [MeSH] OR ghrelin [MeSH] OR obestatin OR Peptide YY [MeSH] OR Glucagon-Like Peptide 1 [MeSH]). The literature search was conducted by myself, assisted by Dr Matthew Hyde. For inclusion into the systematic review the study must have included a report of breast milk concentrations of any of the hormones; leptin, adiponectin, insulin, ghrelin, resistin, obestatin, PYY and GLP-1, and their relation to maternal BMI, including pre-, in- or post-pregnancy BMI.

3.3.2 Data Extraction from identified papers

Relevant studies were identified by evaluating the abstract, or by obtaining a full copy of the article if the abstract was not available. Review articles and commentaries were excluded. Reference lists of included articles were reviewed by the authors to identify further relevant studies. Data extracted included date of publication, number of participants, sample type, sample preparation method, measurement technique, time of sample collection post-partum, mean, standard deviation and range of maternal BMI, hormone concentration and the correlation coefficient between maternal BMI and hormone concentration. Data were independently verified by myself and Dr Matthew Hyde and checked by Dr Chris Gale.

3.3.3 Quality of included studies

A non-validated but pragmatic score by which to compare and measure the quality of included studies was devised. This involved assessing whether the study disclosed the type of sample analysed, e.g. fore or hind milk, whether maternal BMI was measured at the time of sample collection, if time of sample collection was standardised and whether adjustment was made for confounding factors, and if the study used appropriate statistical analysis. Sample preparation was considered appropriate if there was a centrifugation or sonication step prior to analysis. We considered that some kind of sample preparation was required to get a true reading of the hormone concentration, especially leptin. Previous research suggests that either leptin is associated with milk fat globules (Smith-Kirwin et al., 1998), meaning samples require sonication in order to liberate leptin, or milk lipids interfere with the assays, so centrifugation of samples is required (Lonnerdal and Havel, 2000). Whichever the case, sample preparation appears to be needed in order to acquire accurate and reproducible readings. Sample size of less than 50 participants was defined as small, 50-100 as medium, and above

100 as large. The score was derived by totalling the number of factors the investigators had included and adding half a point for whether the study was small, one point for a medium sized study, and 1.5 points for a large study.

3.3.4 Analysis of statistical methods used in systematic review

As the concentration of hormones present in breast milk is non-normally distributed, non-parametric statistical analysis, or analysis of log transformed data was considered appropriate. Statistical significance was defined as a p value of below 0.05.

3.4 Results: Systematic Review

3.4.1 Literature search

The literature search is outlined in Figure 29. The search strategy identified 313 publications, with two further publications identified from reference lists. Abstracts were screened for suitability; 259 studies were unsuitable and excluded as breast milk hormone concentrations were not investigated. The full texts of 56 articles were reviewed; 30 studies were excluded after full-text review because they did not report the correlation between maternal BMI and breast milk hormone concentration, leaving 26 articles suitable for inclusion to the systematic review. The publications identified used a range of different techniques for quantification of hormone concentration, and reported concentrations over a wide range of time throughout lactation (see Tables 9, 10, 11, 12 and 13 for further details). Table 14 gives an overview of the quality of the studies included. Studies investigating hormone concentrations in both colostrum and mature breast milk are evaluated. There was not a large difference seen in the concentration of hormone between these samples, however infants ingest significantly less colostrum than mature milk, whether there is a different influence of breast milk hormones in colostrum compared to mature milk is not known. Infants will ingest significantly greater quantities of hormone in mature milk; however, it may be that hormones in colostrum are more bioavailable due to gut closure not having completed in colostrum fed infants (Vukavic, 1984).

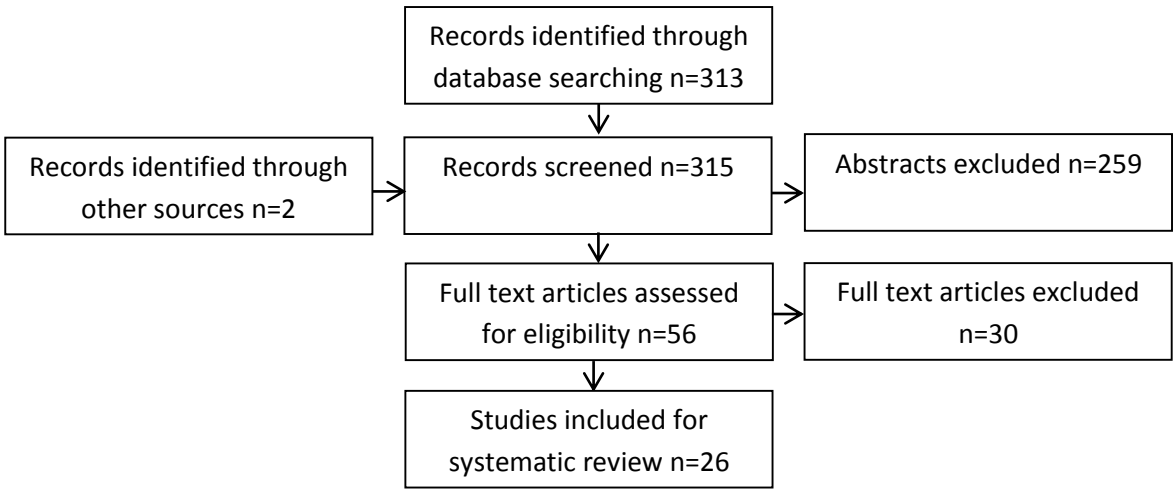


Figure 29: Flowchart of the search strategy used in the systematic review. The relevant number of papers at each point is provided.

3.4.2 Individual study data

3.4.2.1 Leptin

Fifteen studies included a report of the correlation between breast milk leptin concentration and maternal BMI (Bielicki et al., 2004, Bronsky et al., 2006, Bronsky et al., 2011, Dundar et al., 2005b, Eilers et al., 2011, Miralles et al., 2006, Ucar et al., 2000, Uysal et al., 2002, Weyermann et al., 2007, Houseknecht et al., 1997, Fields and Demerath, 2012, Schuster et al., 2011, Savino et al., 2012c, Schueler, 2013, Brunner et al., 2014) (Table 9). In ten studies, a positive correlation between maternal BMI and breast milk leptin concentration was reported at all time points (Bronsky et al., 2006, Eilers et al., 2011, Fields and Demerath, 2012, Houseknecht et al., 1997, Miralles et al., 2006, Uysal et al., 2002, Weyermann et al., 2007, Schuster et al., 2011, Schueler, 2013, Brunner et al., 2014). In an additional study a positive correlation at 2-3 days post-partum was found, but no correlation at six weeks post-partum (Bielicki et al., 2004). In four studies no correlation was found between breast milk leptin and maternal BMI at any time point (Bronsky et al., 2011, Dundar et al., 2005b, Ucar et al., 2000, Savino et al., 2012c). In one study no correlation was identified between maternal BMI and colostrum leptin concentration (Bronsky et al., 2011). In four studies a positive correlation was identified in the first two weeks of lactation between maternal BMI and breast milk leptin concentration (Bielicki et al., 2004, Bronsky et al., 2006, Eilers et al., 2011, Schuster et al., 2011), whilst in one study there was no correlation at 15 days post-partum (Dundar et al., 2005b). In five studies no correlation was found at 1-3 months post-partum (Dundar et al., 2005b, Bielicki et al., 2004, Ucar et al., 2000, Bronsky et al., 2011, Savino et al., 2012c), whilst in six studies a positive correlation was reported over the same period (Eilers et al., 2011, Fields and Demerath, 2012, Miralles et al., 2006, Weyermann et al., 2007, Uysal et al., 2002, Schueler, 2013). In two studies a positive correlation was found up to six months post-partum (Schuster et al., 2011, Brunner et al., 2014), whilst in a further study leptin concentrations were positively correlated with maternal BMI throughout the lactation period, up to nine months post-partum (Miralles et al., 2006). In one study no correlation was found at 12 months of lactation (Bronsky et al., 2011). One study did not specify at which point samples were collected post-partum, but identified a positive correlation (Houseknecht et al., 1997). A negative correlation between maternal BMI and breast milk leptin concentration was not reported by any study (Table 10).

Table 9: Studies examining the association between breast milk leptin concentrations and maternal BMI. AGA: Appropriate for gestational age; EDTA: Ethylenediaminetetraacetic acid; ELISA: Enzyme-linked immunosorbent assay; IQR: Interquartile range; LGA: Large for gestational age; N/A: Not available; RIA: Radioimmunoassay; SGA: Small for gestational age. Data are given as means and standard deviations unless stated otherwise.

Author, Year	Maternal n number	Sample Type, Preparation and Analysis, Protease inhibitor used	Time of day, fasted/fed	Maternal BMI (kg/m2)	Timing of Collection (day/week/month)	Breast milk Leptin (ng/ml)	Statistical Analysis
Housekne cht, 1997	14	Complete breast expression. Skimmed and sonicated whole milk samples prepared. RIA. No protease inhibitor.	08:00am post-prandial	Not disclosed	N/A	Whole milk 10.1±2.6 Skimmed Milk 1.5±0.9	Positive correlation seen with: Whole milk: r=0.5, p<0.06 Skimmed Milk: r=0.66, p<0.001
Ucar, 2000	18	Foremilk and hindmilk. Skimmed. RIA. No protease inhibitor.	Not disclosed	25.9±0.74 Time not reported	40 days post-partum	3.36±11.0	No correlation between breast milk leptin and maternal adiposity. Pearson's correlation using log transformed values
Uysal, 2002	50	Foremilk. Analysis of Skimmed milk by RIA, No protease inhibitor.	08:00-11:00am Not disclosed	BMI at sample collection 25.9±4.4	3 months post-partum	Mothers of: Obese infants 0.27±0.2 Lean infants 0.37±0.4	Leptin concentrations were correlated with maternal BMI; r=0.62, p<0.001 using Spearman correlation
Bielicki, 2004	33	Foremilk. Sonicated. RIA. No protease inhibitor.	Not disclosed. Fed	BMI at sample collection 25.1±0.8 - 23.6±0.85	2-3 days post-partum 4-5 days post-partum 6 weeks post-partum	1.15±0.12 0.79±0.10 -	Linear regression of log values. Correlation with maternal BMI: 2-3 days: r^2 =0.15, p=0.02. No correlation found at 6 weeks
Dundar, 2005	AGA=22 LGA=14 SGA=11	Foremilk. Sonicated, RIA, No protease inhibitor.	10:00-11:00am Not disclosed.	23.8±0.77 Time not reported	15 days post-partum 1 month post-partum 2 months post-partum 3 months post-partum	AGA SGA LGA 13.4±2.2 28.5±4.4 18.2±2.0 17.0±3.4 15.5±4.9 19.4±1.7 11.4±2.3 15.1±2.7 18.3±2.4 9.1±1.8 17.4±3.4 11.8±1.8	No relation between breast milk leptin and BMI. Pearson's correlation

Table 9
(continued)

Bronsky, 2006	59	Hindmilk, skimmed. ELISA, EDTA tubes and protease inhibitor	7:00 am Not disclosed	Pre-pregnancy 21.4±(0.4) Time of delivery 26.8±(0.4)	48 hours post lactation	0.5±0.05	Correlation with: Pre-pregnancy BMI: r=0.397, p=0.003 BMI at delivery: r=0.498, p<0.0001
Miralles, 2006	28	Hindmilk. Whole milk. ELISA No protease inhibitor.	Morning, Not disclosed	Pre-pregnancy 21.6±0.5 Range 16.3-27.3	1±3 days post-partum 3 months post-partum 6 months post-partum 9 months ± 1 week	1 month: 0.156±0.039	Maternal BMI positively correlated with milk leptin concentration at each time point; r=0.387, p<0.01. Stronger association found when concentrations were log transformed (r=0.607). Pearson's correlation
Weyermann, 2007	651	Foremilk. Analysis of skimmed milk samples by ELISA No protease inhibitor.	Not disclosed	Pre-pregnancy 23.6±4.0 (16.7–45.7)	33-71 days post-partum	Median (Range): 0.175 (0-4.12) BMI <20: 0.122±0.129 BMI 20-24.9: 0.234±0.264 BMI 25-29.9: 0.388±0.335 BMI > 30: 0.807±0.885	Leptin concentrations were strongly associated with pre-pregnancy BMI
Bronsky, 2011	72	Hindmilk, skimmed. ELISA. EDTA tubes and protease inhibitor.	Not disclosed	Pre- pregnancy 21.9±0.4	0 day post lactation 1 month 3 months 6 months 12 months	0.3±0.04 0.2±0.03 0.1±0.01 0.1±0.02 0.2±0.04	No correlation throughout the lactation period between pre-pregnancy BMI and leptin using Spearman's correlation
Eilers, 2011	Term=40 Preterm=37	Type of milk not specified. Skimmed milk. RIA. No protease inhibitor.	16:00- 20:00 'No big meals'	Pre-pregnancy 23±3.5	Day 3 lactation Day 28 lactation	Preterm / Term 0.70±0.79 / 0.65±0.67 0.50±0.40 / 0.50±0.40	Spearman's correlation with pre-pregnancy BMI: Term: Day 3: r=0.28, p<0.01; Day 28: 0.45, p<0.0001. Preterm: Day 3: r=0.40, p=0.016; Day 28: r=0.56, p<0.01. Mothers with BMI >25 had higher leptin concentrations compared to those with a BMI <25, p<0.01

Table 9
(continued)

Schuster 2011	23	Sample sonicated and then skimmed. RIA. No protease inhibitor.	Not disclosed	Pre-pregnancy 21.4±2.6 Median (IQR) 20.9 (19.3-22.6)	1 week post-partum 6 months post-partum	0.21±0.19 0.18±0.15	Leptin concentration correlated with maternal BMI; r=0.298, p<0.001. Pre-pregnancy BMI did not correlate with leptin concentration, Spearman's correlation
Fields, 2012	19	Complete breast expression. Skimmed. Immunoassay. No protease inhibitor.	8:00 - 10:00am. Fasted 2h	Pre-pregnancy 26.6±6.6	40±4 days post-partum	0.092±0.05	Positive Spearman's correlation between pre-pregnancy BMI and leptin concentration; r=0.78, p<0.0001
Savino, 2012	23	Sample sonicated and then skimmed. RIA. No protease inhibitor.	Not disclosed	Median (IQR) 27.4 (4.9) Time not reported	87±40 days post-partum	Median (IQR): 2.34 (5.73)	No correlation between maternal anthropometric parameters and leptin using Spearman's correlation
Schueler, 2013	13	Foremilk and hindmilk. Skimmed. RIA. No protease inhibitor used.	07:00-10:00am, fasted 10h	BMI at sample collection 25.9±4.23 Range 20.35-32.9	4-5 weeks post-partum	1.0±0.7 Range 0.2-2.6	Leptin concentrations were positively correlated with maternal BMI, r=0.82, p=0.001. Pearson correlation
Brunner, 2014	6 weeks 152 4 months 120	Complete breast expression. Sonicated then skimmed. RIA. No protease inhibitor used.	After overnight fast	Pre- and during pregnancy BMI. Values not reported	6 weeks post-partum 4 months post-partum	Median (IQR) 0.11 (0.19) 0.09 (0.18)	A strong positive correlation was observed between milk leptin and maternal pre-pregnancy BMI, 15wk and 32nd wk gestation BMI, r=0.49, 052 and 0.57 respectively, p=<0.001 for all, at both time points, (6 week correlations shown). Spearman correlation

Table 10: Summary of findings of studies investigating leptin concentrations in breast milk. Boxes with pluses indicate the study found a positive correlation between breast milk leptin concentration and maternal BMI at this time point. Boxes with zeroes indicate the study found no correlation at this point. NR=Not reported, bold print indicates p value of <0.05.

	~1 day	~2 days	~1 week	~2 weeks	~3 weeks	~4 Weeks	~6 weeks	~2 months	~3 months	~6 months	~9 months	~12 months
Ucar 2000							**0** NR					
Uysal 2002									**+** r=0.62, **p<0.001**			
Bielicki 2004		**+** r^2 =0.15, **p=0.02**	**+** NR				**0** NR					
Brunner 2014							**+** r=0.57 **p=<0.001**		**+** r=~0.57 **p=<0.001**			
Dundar 2005				**0** NR		**0** NR		**0** NR	**0** NR			
Bronsky 2006		**+** r=0.498, **p<0.0001**										
Miralles 2006						**+** r=0.387, **p<0.01**			**+** r=0.387, **p<0.01**	**+** r=0.387, **p<0.01**	**+** r=0.387, **p<0.01**	
Weyermann 2007							**+ NR**					
Eilers 2011		**+** r=0.28, **p<0.01**				**+** 0.45, **p<0.0001**						
Bronsky 2011	**0** NR					**0** NR			**0** NR	**0** NR		**0** NR
Schuster 2011			+ r=0.298, **p<0.001**	+ r=0.298, **p<0.001**	+ r=0.298, **p<0.001**	**+** r=0.298, **p<0.001**		+ r=0.298, **p<0.001**	**+** r=0.298, **p<0.001**	**+** r=0.298, **p<0.001**		
Fields 2012						**+** r=0.78, **p<0.0001**						
Savino 2012									**0 NR**			
Schueler 2013						+ r = 0.82, **p = 0.001**						

3.4.2.2 Adiponectin

Nine studies include a report of the concentration of adiponectin in breast milk and its association to maternal BMI (Dundar et al., 2010, Ley et al., 2012, Luoto et al., 2012, Luoto et al., 2011, Martin et al., 2006, Weyermann et al., 2007, Woo et al., 2009, Bronsky et al., 2011, Brunner et al., 2014) (Table 11). Two studies report a positive correlation between breast milk adiponectin and maternal BMI (Martin et al., 2006, Woo et al., 2009). Woo *et al* (Woo et al., 2009) found a positive correlation between maternal BMI and breast milk adiponectin concentration. Samples were collected longitudinally, and due to a decrease in concentrations of adiponectin in samples collected later on in lactation, adjustment was made for post-partum month and month of the year, after which no correlation was observed. Martin *et al* (Martin et al., 2006) also reported a positive association between maternal BMI and breast milk adiponectin concentration. In seven studies, no correlation between maternal anthropometric measurements and milk adiponectin was found (Dundar et al., 2010, Luoto et al., 2011, Weyermann et al., 2007, Luoto et al., 2012, Ley et al., 2012, Bronsky et al., 2011, Brunner et al., 2014). Many of the studies that found no correlation analysed colostrum rather than mature breast milk. Of the studies in which no correlation was found, six involved the analysis of colostrum (Bronsky et al., 2006, Dundar et al., 2010, Luoto et al., 2011, Luoto et al., 2012, Ley et al., 2012, Bronsky et al., 2011); in three studies samples were collected at 1-4 months post-partum (Weyermann et al., 2007, Ley et al., 2012, Brunner et al., 2014), and in one study samples were collected up to 12 months post-partum (Bronsky et al., 2011).

Table 11: Studies examining the association between breast milk adiponectin concentrations and maternal BMI. EDTA: Ethylenediaminetetraacetic acid; ELISA: Enzyme-linked immunosorbent assay; IQR: Interquartile range; RIA: radioimmunoassay, SEE: Standard Error of the Estimate. Data are given as means and standard deviations unless stated otherwise.

Author, Year	Maternal n Number	Sample Type, Preparation and Analysis, Protease inhibitor used	Time of day, fasted/fed	Maternal BMI (kg/m^2)	Timing of Collection (day/week/month)	Breast milk Adiponectin (ng/ml)	Statistical Analysis
Martin, 2006	22	Whole breast expression Skimmed. RIA. No protease inhibitor	Not disclosed	Taken at sample collection 24.5 (19.5–34.2)	2-242 days post-partum	Median (Range): 17.7 (4.2–87.9)	Maternal post-pregnancy BMI was associated with natural log(milk adiponectin), β=0.08±0.02, P<0.0001. This equates to an 8.33% increase in milk adiponectin concentration with each unit increase in maternal BMI
Weyermann, 2007	651	Foremilk. Analysis of skimmed milk samples by ELISA No protease inhibitor	Not disclosed	Pre-pregnancy 23.6±4.0 (16.7–45.7)	33-71 days post-partum	Median (Range):10.9 (0.8-110) BMI <20: 12.8±9.7 BMI 20-24.9: 12.8±10.4 BMI 25-29.9: 11.8±9.1 BMI > 30: 14.8±12.1	Maternal pre-pregnancy BMI showed no clear association with adiponectin concentrations
Woo, 2009	45	Complete breast expression. Skimmed. RIA. No protease inhibitor	10:00 13:00 Not disclosed	1 month post-partum 25.4±3.5	1 week post-partum 2 weeks post-partum 3 weeks post-partum Then monthly	25.6±8.4	Milk adiponectin was log transformed and positively associated with maternal BMI (0.67±0.30ng/ml increase in milk adiponectin per BMI unit, p=0.02), effect not seen after adjusting for month or $month^2$ (p=0.11)
Dundar, 2010	25	Whole milk collected following an overnight fast. RIA No protease inhibitor.	08:00-10:00 Not disclosed	Pre-pregnancy 23.1±0.8	1 day post-partum	29.5±6.4 Range: 1.26–77.1	No association between adiponectin concentrations and maternal anthropometric measurements using Spearman's correlation

Table 11 (cont.)

Bronsky, 2011	72	Hindmilk. Whole milk, ELISA, EDTA tubes and protease inhibitor	Not disclosed	Pre- pregnancy 21.9±0.4	0 day post-lactation 1 month post-partum 3 months post- partum 6 months post- partum 12 months post- partum	22.8±0.8 22.0±0.6 20.5±0.6 21.4±0.8 25.7±1.4	No correlation using Spearman's correlation between maternal pre-pregnancy BMI and adiponectin
Luoto, 2011	181	Type of sample and preparation not reported. Colostrum samples analysed by dissociation enhanced lanthanide fluoro-immunoassay. No protease inhibitor	Not disclosed	Pre-pregnancy 23.8±3.7	0-3 days post-partum	Median (Range): 18.4 (2.9–317.0)	Pre-pregnancy BMI did not correlate with adiponectin concentration in colostrum (r=0.023, p=0.760). Log values
Luoto, 2011	30	Type of sample and preparation not reported. Colostrum samples analysed by dissociation enhanced lanthanide fluoro-immunoassay. No protease inhibitor	Not disclosed	Pre-pregnancy 23 Range (18.40–28.37)	1-3 days post-partum	Median (Range): 10.5 (3.1–98.9)	Regression analysis revealed no correlation between colostrum adiponectin concentration and pre-pregnancy maternal BMI
Ley, 2012	170	Foremilk. Skimmed. RIA No protease inhibitor	Not disclosed	Pre-pregnancy 24.4±2.9	2 days post-partum 95 days post-partum	Median (IQR) 50.0 (21.9/104.6) 12.3 (9.9/17.2)	Pre-pregnancy BMI was not associated with adiponectin concentration in early or mature milk. General linear model, β±SEE=0.003±0.014, p=0.81, in first week post-partum
Brunner, 2014	6 weeks 151 4 month 120	Complete breast expression. Sonicated then skimmed. RIA. No protease inhibitor	After overnight fast	Pre- and during pregnancy BMI. Values not reported	6 weeks post-partum 4 months post-partum	Median, (IQR) 10.93, (8.34) 10.36, (9.40)	Using Spearman correlation, no correlation was observed between total milk adiponectin and maternal BMI, at either time point measured

3.4.2.3 Insulin

Four studies were identified in which the concentration of insulin in breast milk was measured, and the association with maternal BMI reported (Table 12). In two studies a positive correlation was reported; in two further studies no correlation was reported. Shehadeh *et al* (Shehadeh et al., 2003) found no correlation on day three or day ten post-partum. Fields *et al* (Fields and Demerath, 2012) found no correlation at one month post-partum. Ahuja *et al* (Ahuja S, 2011) reported a positive correlation at six weeks post-partum whilst Ley *et al* reported a positive correlation between maternal BMI and breast milk insulin at three months post-partum, but not during the first week post-partum (Ley et al., 2012).

Table 12: Studies examining the association between breast milk insulin concentrations and maternal BMI. ELISA: Enzyme-linked immunosorbent assay; post-partum: post-partum; IQR: Interquartile range; RIA: radioimmunoassay; SEE: Standard Error of the Estimate. Data are given as means and standard deviations unless stated otherwise.

Author, Year	Maternal n Number	Sample Type, Preparation and Analysis Protease inhibitor used	Time of day, fasted/fed	Timing of Collection (day/week/month)	Maternal BMI (kg/m^2)	Breast milk Insulin (μIU/ml)	Statistical Analysis
Shehadeh, 2003	90	Milk type not reported. Skimmed. RIA. No protease inhibitor	Not disclosed	3 days and 10 days post-partum	23.7±5 Time not reported	Day 3 / Day 10 50.1±34.6 / 41.1±28.5 Median 41.0 / 34.0 Range 7-179 / 12-183	Insulin concentrations were not significantly influenced by BMI on day 3 or day 10 post-partum. Spearman's correlation
Ahuja, 2011	32	Complete breast expression, whole milk analysed by ELISA. No protease inhibitor	09:00-11:00 Not disclosed	6 weeks post-partum	Pre-pregnancy Non obese 21.8±2.34 Range 18.5-24.7 Obese 32.2±3.82 Range 26.4-40.2	Non-obese / Overweight/obese 4.5±7.6 / 30.1±56.3	There was a positive correlation between insulin concentration and maternal pre-pregnancy BMI (r=0.57, p<0.001). Pearson's correlation, non-logged values
Fields,2012	19	Complete breast expression. Skimmed. Immunoassay. No protease inhibitor	8:00 - 10:00am. Fasted 2h	40±4 days post-partum	Pre-pregnancy 26.6±6.6	23.6±18.01	Insulin concentrations were not correlated with maternal BMI. Spearman's correlation.
Ley, 2012	170	Foremilk. Skimmed. Electrochemilum-inescence immunoassay. No protease inhibitor	Not disclosed	2 days post-partum 3 months post-partum	Pre-pregnancy 24.4±2.9	Median (IQR) 24.5 (9.2/57.9) 7.5 (4.9/12.5)	Higher pre-pregnancy BMI was associated with higher [insulin] in mature milk after adjustment for maternal age, ethnicity and time post-partum (β±SEE: 0.053 ±0.014; p=0.0003) at 3 months, but not in the first week post-partum (β±SEE: -0.002±0.02; p=0.91). General linear model

3.4.2.4 Ghrelin

Aydin and colleagues conducted all three studies identified in which the association between maternal BMI and breast milk ghrelin concentration was assessed (Aydin, 2010, Aydin et al., 2007, Aydin et al., 2006). A correlation was not reported in any of these studies (Table 13). In two studies, samples were collected at similar time points during the first two weeks post-partum (Aydin et al., 2006, Aydin et al., 2007); whilst the third neglected to state the time point post-partum in which samples were collected (Aydin, 2010).

Table 13: Studies examining the association between breast milk ghrelin concentrations and maternal BMI. ELISA: Enzyme-linked immunosorbent assay; GDM: Gestational Diabetes; HPLC: High Performance Liquid Chromatography; P-GDM: Pre-Gestational Diabetes; PP: post-partum; RIA: radioimmunoassay
Data are given as means and standard deviations unless stated otherwise.

Author, Year	Maternal n Number	Sample Type, Preparation and Analysis Protease inhibitor used	Time of day, fasted/fed	Maternal BMI (kg/m^2)	Timing of Collection (day/week/month)	Breast milk Ghrelin (pg/ml)	Statistical Analysis
Aydin, 2006	Total study=17 BMI analyses, number not stated "only a few participants examined"	Foremilk. Skimmed. RIA, no protease inhibitor.	Not disclosed	28.98±1.35 Time not reported	1 day post-partum 7 days post-partum 15 days post-partum	Colostrum 73±18 Transitional milk 84±18 Mature milk 97±13	Spearman's correlation between milk ghrelin concentrations and BMI r=0.42, p=0.19
Aydin, 2007	29	Foremilk. Skimmed. RIA and HPLC analysis, no protease inhibitor.	Morning, overnight fast	Post-partum 29.6±1.9	2 days post-partum 15 days post-partum	RIA: 2 days PP / 15 days PP Control 64.1 / 55.5 GDM 26.1 / 54.1 P-GDM 28.5 / 32.1 HPLC analysis Active 23±6 Non-active 548±12 Total 571±128	No correlation observed between ghrelin concentrations and post-partum maternal BMI
Aydin, 2010	20	Type of milk not reported. No sample preparation. ELISA. Protease inhibitor added	Not disclosed	36.3±3.2 Time not reported	Colostrum and mature milk	Colostrum: Active / Non-active Control 39.2±2 / 466.1±52 GDM 27.7±2 / 338.1±49 Mature Milk: Active / Non-active Control 48.2±5.1 / 505.1±52 GDM 27.7±2 / 359.1±51.2	Spearman's correlation between fasting milk ghrelin concentrations and BMI was not significant r=0.46, p=0.6

3.4.2.5 Resistin

A single paper by Savino *et al* in 2012 examined the association between maternal BMI and breast milk concentration of resistin. The median time of collection was 80 days post-partum. Median breast milk resistin concentrations reported were 0.18ng/ml, with an interquartile range of 0.44. The author reported there was no correlation between breast milk resistin concentration and maternal anthropometric measurements (Savino et al., 2012c).

3.4.2.6 Obestatin

No studies were identified in which the association between maternal BMI and breast milk obestatin concentration was examined.

3.4.2.7 Peptide YY

The association between breast milk concentration of peptide YY and maternal BMI was evaluated in a single paper by Schueler *et al* (Schueler, 2013), the researchers also investigated breast milk leptin concentrations, and the study design is outlined in Table 9. Protease inhibitors were added to the aliquot used for peptide YY analysis. The researchers analysed peptide YY in both fore and hind milk, the average concentration in fore milk was 39.5±8.4pg/ml (mean and standard deviation), whilst the hind milk concentration was 38.9±9.1pg/ml at 4-5 weeks post-partum, as analysed by RIA. The authors reported that there was no correlation between maternal BMI and milk peptide YY.

3.4.2.8 Glucagon-like peptide 1

Concentrations of GLP-1 in breast milk were again investigated by Schueler *et al* (Schueler, 2013), in the only study identified to do so. The study design is outlined in Table 9; protease inhibitor was used to preserve samples. In fore milk, the concentration of GLP-1 was 12.7±3.1pM, whilst in hind milk it was 15.4±3.1pM (mean±SD). Milk GLP-1 was analysed using RIA for total GLP-1. The authors reported no correlation between maternal BMI and milk GLP-1.

Table 14: Table assessing the quality of studies included into the systematic review. The scoring system is outlined in Section 3.3.3.

Leptin	Stated what kind of milk	Measured BMI at time of sample collection	Standardised time post-partum of sample collection	Standardised time of sample collection	Adjusted for confounders	Appropriate Statistical analysis	Appropriate sample preparation	Small medium or large study	Score	Positive/ negative/no correlation
Houseknecht, 1997	✔	✔	X	✔	X	X	✔	Small	4.5	Positive
Ucar, 2000	✔	✔	X	✔	X	✔	X	Small	4.5	No correlation
Uysal, 2002	✔	✔	X	✔	X	✔	✔	Medium	6	Positive
Bielicki, 2004	✔	✔	✔	✔	X	✔	✔	Small	6.5	Positive then none
Dundar, 2005	✔	Not stated	✔	✔	X	X	✔	Small	4.5	No correlation
Bronsky, 2006	✔	X	✔	✔	X	✔	✔	Medium	6	Positive
Miralles, 2006	✔	✔	✔	✔	X	✔	✔	Small	6.5	Positive
Weyermann, 2007	✔	X	X	Not stated	✔	✔	✔	Large	5	Positive
Bronsky, 2011	✔	X	✔	✔	X	✔	✔	Medium	6	No correlation
Eilers, 2011	X	X	✔	✔	X	✔	✔	Medium	5	Positive
Schuster 2011	X	✔	✔	X	X	✔	✔	Small	4.5	Positive
Fields, 2012	✔	X	✔	✔	X	✔	✔	Small	5.5	Positive
Savino, 2012	X	Not stated	X	✔	X	✔	✔	Small	3.5	No correlation
Schueler, 2013	✔	✔	✔	✔	X	X	✔	Small	5.5	Positive
Brunner, 2014	✔	X	✔	✔	X	✔	✔	Large	6.5	Positive

Table 14 (continued)

Adiponectin										
Martin, 2006	X	✔	X	✔	X	✔	✔	Small	4.5	Positive
Weyermann, 2007	✔	X	X	Not stated	✔	✔	✔	Large	5	No correlation
Woo, 2009	✔	✔	✔	✔	✔	✔	✔	Small	7.5	Positive, none after adjustment
Dundar, 2010	X	X	✔	✔	X	✔	X	Small	3.5	No correlation
Bronsky, 2011	✔	X	✔	✔	X	✔	✔	Medium	6	No correlation
Luoto, 2011	X	✔	✔	Not stated	X	✔	✔	Large	5	No correlation
Luoto, 2011	X	X	✔	Not stated	X	✔	✔	Small	3.5	No correlation
Ley, 2012	✔	X	✔	Not stated	X	✔	✔	Large	5	No correlation
Brunner, 2014	✔	X	✔	✔	X	✔	✔	Large	6.5	No correlation
Insulin										
Shehadeh, 2003	X	Not stated	✔	Not stated	X	✔	✔	Medium	4	No correlation
Ahuja, 2011	✔	X	✔	✔	X	✔	X	Small	4.5	Positive
Fields, 2012	✔	X	✔	✔	X	✔	✔	Small	5.5	No correlation
Ley, 2012	✔	X	✔	Not stated	X	✔	✔	Large	5	No correlation then Positive
Ghrelin										
Aydin, 2006	✔	Not stated	✔	✔	X	✔	✔	Small	5.5	Not significant
Aydin, 2007	✔	X	✔	✔	X	Not stated	✔	Small	4.5	No correlation
Aydin, 2010	X	Not stated	Not stated	Not stated	X	Not stated	✔	Small	1.5	Not significant

3.5 Multiplex assay of hormone concentrations

Breast milk samples, collected from participants in the prospective cohort study outlined in Section 2.2, Chapter 2, were analysed using a multiplex assay to determine the concentration of leptin, insulin, ghrelin and resistin, as described in Section 2.6, Chapter 2.

3.5.1 Results: Cohort characteristics

Descriptive statistics of the mothers whose samples were analysed using the multiplex array are shown in Table 15. The average maternal age was 34, with a range from 23 to 46 years old, and an average height of 166cm. An attempt was made to recruit approximately equal numbers of participants into the high and low booking BMI groups. However, this proved difficult due to the relatively healthy maternity population served at Chelsea and Westminster hospital. Of the mothers recruited, at booking, 3% were underweight (4), 73% of normal weight (87), 19% overweight (23) and 5% obese (6).

Table 15: Descriptive characteristics of maternal cohort at one week and three months post-partum time points. Data reported are means and standard deviations.

	One week post-partum	Three months post-partum
Number	105	92
Weight, kg	70.3 (12.7)	66.5 (11.3)
BMI	25.4 (4.5)	23.9 (3.6)

The majority of mothers lost weight from the seven day to three month time point, resulting in a drop in the average BMI value of mothers at three months post-partum. In addition to this, there was drop out of several mothers from the study. In total, 25 mothers withdrew from the study, the reasons of which are outlined in Table 16.

Table 16: Reasons given for mothers withdrawing from the study at the one week and three month post-partum time points.

Reasons for withdrawal 1 Week	Number	%	Reasons for withdrawal 3 months	Number	%
Stopped breastfeeding	2	17	Stopped breastfeeding	10	77
Maternal illness	1	8	Maternal illness	1	7
Infant illness	1	8	Infant illness	1	7
Unable to contact mother	2	17	No longer wished to participate	1	7
No longer wished to participate	4	33	Total	13	100
Research midwife illness	1	8			
Recruited in error	1	8			
Total	12	100			

The majority of women withdrew from the study at the one week time point due to no longer wishing to participate in the study, whilst at three months, the majority of women withdrew as they had stopped breast feeding. An analysis of the population who withdrew at three months revealed that these mothers were more likely to be overweight/obese than mothers who did not withdraw. Of the normal weight mothers 15% withdrew (74/87), whilst of the overweight mothers 25% withdrew (18/24) and 66% of obese mothers withdrew from the study (4/6). These findings reflect previous literature showing overweight/obese mothers have more difficulty initiating and maintaining breastfeeding (Lepe et al., 2011).

Of the mothers who were overweight or obese, 46% of women delivered via caesarean section, whilst in the normal weight mothers, this figure was 27%. This puts overweight/obese women at a further disadvantage in successfully initiating early breastfeeding, as demonstrated by a systematic review and meta-analysis, where a negative association between caesarean section and early breastfeeding was observed (Prior et al., 2012). Mothers who withdrew were also less likely to be university educated, Pearson Chi-square=4.4, p value 0.03.

3.5.2 Sample analysis

Samples were analysed in a randomised run order, prepared into 96 well plates (Bio-Rad, USA). A total of 388 samples were analysed, after removal of duplications, and samples which had not analysed correctly, a total of 373 samples were available for further statistical analysis, as well as 3 formula milk samples analysed in singlicate for comparison.

Both fore and hind breast milk samples were collected from mothers at 7 days and 3 months post-partum, as outlined in Section 2.2.7, Chapter 2. The number of samples analysed are given in Table 17.

Table 17: Number of each type of sample analysed in the hormone assay.

	One week	Three months	Total
Number fore milk	105	92	197
Number hind milk	91	81	172
Total	196	173	369

3.5.3 Breast milk hormone concentrations

Concentrations of ghrelin, insulin, leptin and resistin in breast milk were measured simultaneously as outlined in Section 2.6, Chapter 2. The median concentration of these hormones are given in Table 18.

Table 18: Summary statistics for breast milk hormone concentrations. P value derived using repeated measure ANOVA. * refers to results below the 0.05 significance level.

Type of milk	Stage of lactation: Mean (SE) [n]		
	1 week	3 Months	P value
Ghrelin ng/ml			
Fore	123.0 (15.8) [96]	101.1 (11.5) [83]	0.17
Hind	95.8 (16.9) [81]	59.34 (12.3) [66]	0.03*
P value	0.09	0.01*	
Insulin ng/ml			
Fore	515.4 (42.4) [105]	524.8 (30.9) [92]	0.83
Hind	439.5 (45.2) [91]	456.8 (32.9) [81]	0.70
P value	0.08	0.13	
Leptin ng/ml			
Fore	541.7 (161.2) [105]	684.8 (117.4) [92]	0.38
Hind	614.4 (172.1) [91]	464.3 (125.1) [81]	0.38
P value	0.65	0.20	
Resistin ng/ml			
Fore	924.9 (295.9) [105]	792.5 (224.4) [92]	0.65
Hind	967.4 (317.0) [91]	733.9 (238.9) [81]	0.46
P value	0.89	0.85	

For ghrelin, 48 samples were below the limit of detection, whilst three, one and four samples were below the limit of detection for insulin, leptin and resistin, respectively. Concentrations of the hormones were non-normally distributed, as displayed in Figure 30.

Several associations between the hormone concentrations and the time samples were collected could be observed. There was a significant decrease in the concentration of ghrelin in the hind milk samples collected at three months post-partum, similarly, there appeared to be a trend in the same direction in samples collected at one week post-partum, but this did not reach significance. Furthermore, there appeared to be a decrease in concentration of ghrelin in the samples collected at three months post-partum in comparison to samples collected at one week post-partum, but this was only significant for the hind milk samples collected.

There were no significant associations observed between the insulin concentrations of breast milk in regard to time of sample collection. However, there did appear to be a trend towards reduced insulin concentration in hind milk samples in comparison to fore milk at both time points.

There did not appear to be any relationship between time of sample collection and concentrations of either leptin or resistin. Due to data being non-normally distributed, data were log transformed to make the distribution more normal, as shown in Figure 31. Further statistical tests were performed on the log transformed data.

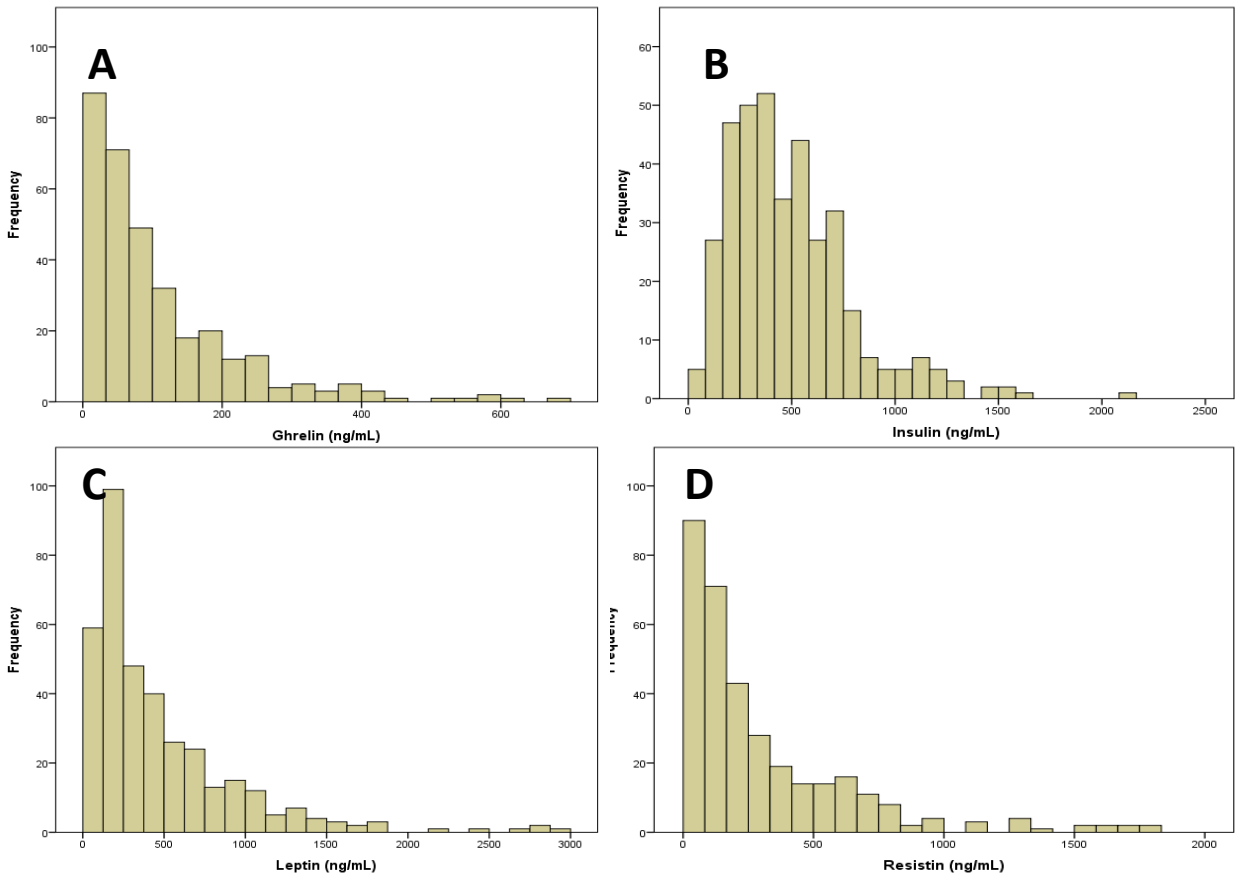


Figure 30: Histograms displaying the distribution of data of breast milk hormone concentrations, at all time points post-partum, in ng/ml (A) ghrelin (B) insulin, (C) leptin, 6 samples lay above the range displayed, up to 16000 ng/ml, and were not plotted for display purposes. (D) resistin, 32 samples are not shown as they lay outside of the plotted range, up to 16000 ng/ml.

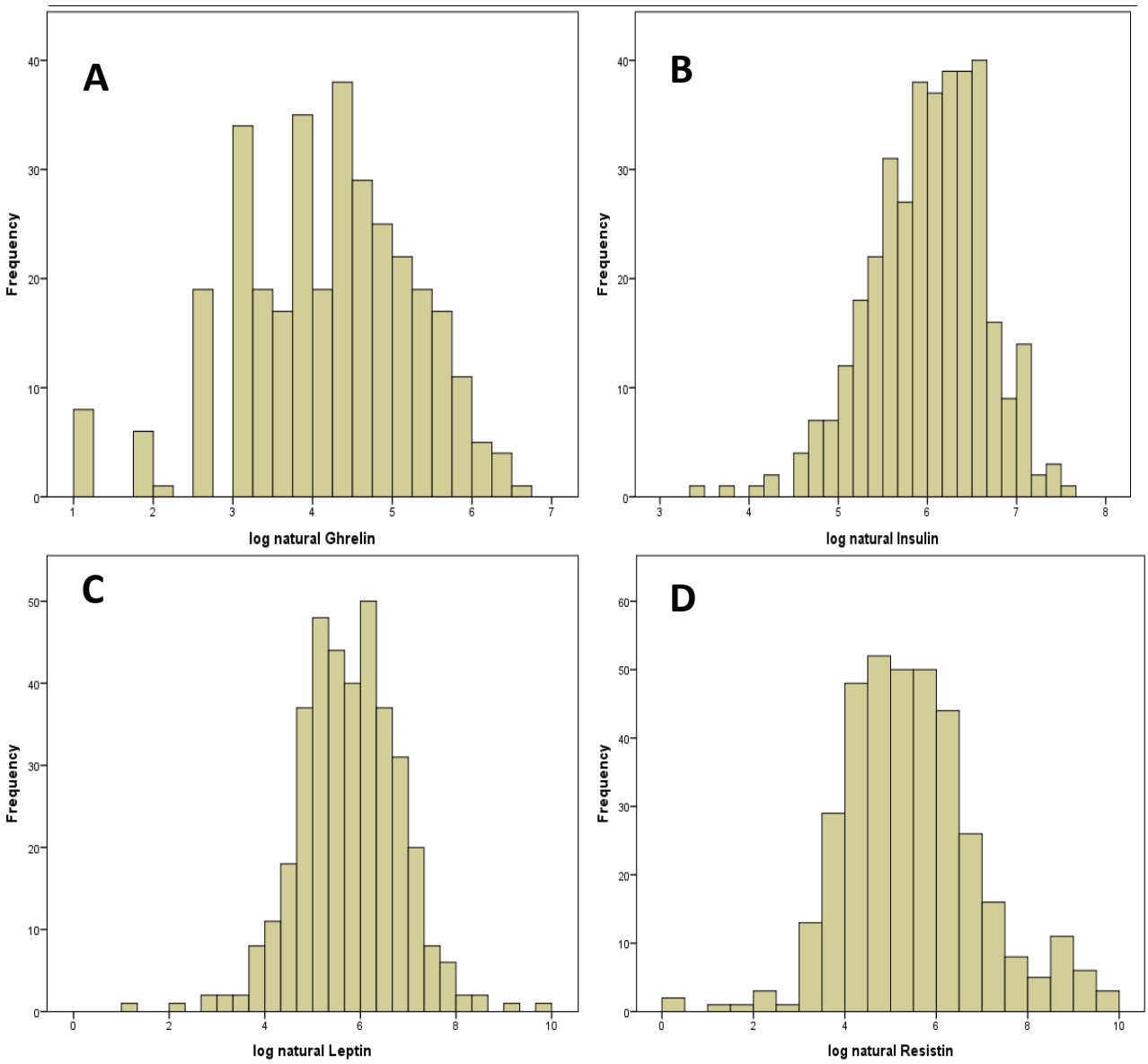


Figure 31: **Histogram (A), (B), (C) and (D) display the distribution of data of natural log transformed ghrelin, insulin, leptin and insulin respectively of breast milk samples, at all time points post-partum.**

3.5.4 Correlation between breast milk hormone concentration and maternal BMI

The log transformed data values were correlated against maternal BMI at each time point, splitting samples into fore and hind subgroups. Two-tailed Pearson correlations of maternal BMI against hormone concentration were computed, as shown in Table 19. One mother was excluded from further analysis as her BMI was extremely high in comparison to the rest of the cohort (52.5kg/m^2), skewing the results obtained, Appendix 3B displays a scatter plot demonstrating this.

Table 19: Correlation between fore and hind milk samples taken at one week and 3 months post-partum against maternal BMI. *Results below the 0.05 significance level.

Fore milk Maternal BMI at 7 days	**Ln ghrelin**	**Ln insulin**	**Ln leptin**	**Ln resistin**
Pearson Correlation	-0.06	0.03	0.20*	-0.05
Significance (2-tailed)	0.55	0.79	0.05	0.64
N	95	103	103	104
Hind milk Maternal BMI at 7 days	Ln ghrelin	Ln insulin	Ln leptin	Ln resistin
Pearson Correlation	0.02	.015	.039	-0.04
Significance (2-tailed)	0.88	0.89	0.72	0.71
N	81	90	90	89
Fore milk Maternal BMI at 3 months	Ln ghrelin	Ln insulin	Ln leptin	Ln resistin
Pearson Correlation	0.20	0.18	0.25*	-0.05
Significance (2-tailed)	0.07	0.10	0.02	0.64
N	83	92	92	91
Hind milk, maternal BMI at 3 months	Ln ghrelin	Ln insulin	Ln leptin	Ln resistin
Pearson Correlation	-0.09	0.01	-0.05	-0.08
Significance (2-tailed)	0.48	0.95	0.69	0.49
N	66	81	81	79

As Table 19 shows, breast milk ghrelin, resistin and insulin were not correlated with maternal BMI, at any of the time points studied. However, leptin concentrations in fore milk, but not hind milk, were seen to correlate to maternal BMI at the one week time point, and more strongly at the three month time point.

Figure 32 and 33 display scatter plots, showing the correlation between maternal BMI and the log breast milk leptin concentration at 7 days, and three months post-partum respectively.

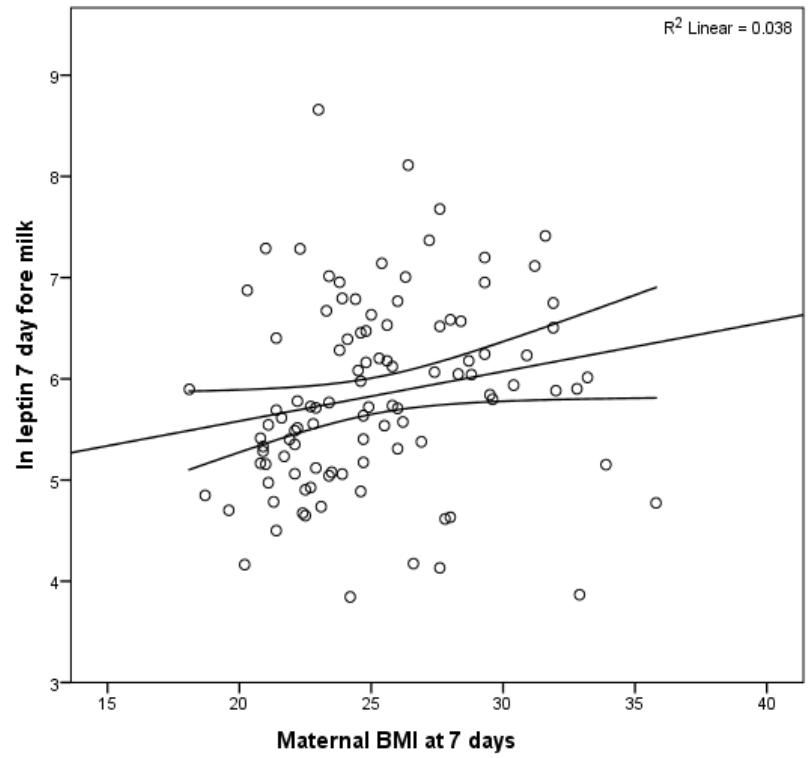


Figure 32: Correlation between breast milk leptin concentrations at 7 days post-partum against maternal BMI. Pearson correlation coefficient 0.19, p=0.04

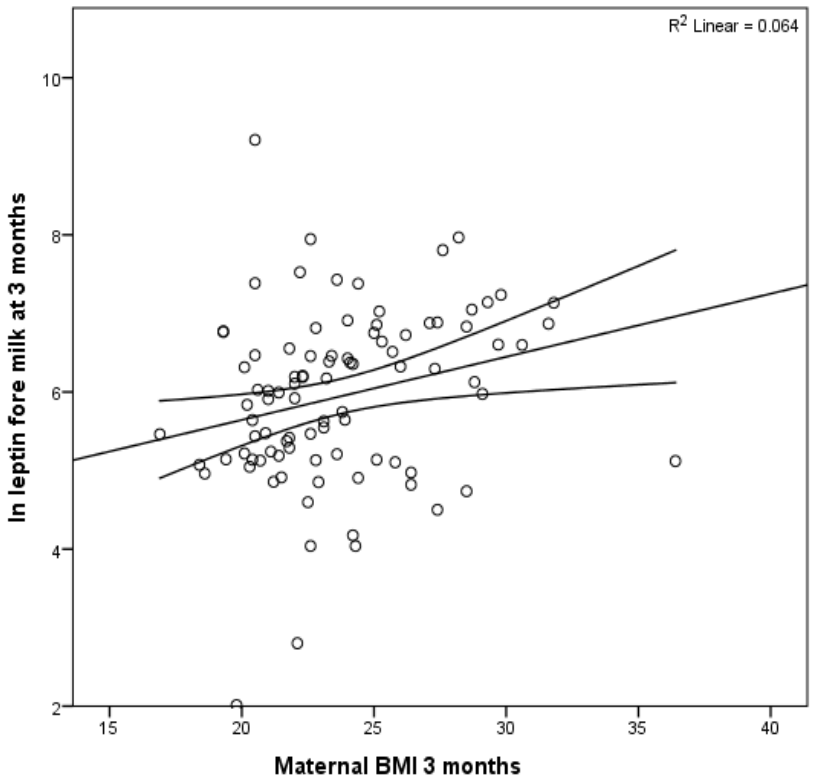


Figure 33: Correlation between breast milk leptin concentrations at 3 months post-partum against maternal BMI. Pearson correlation coefficient 0.25, p=0.01.

3.5.5 Correlations between breast milk hormones

Relationships between the different hormones were also analysed to establish if there were any associations. Each of the time points and the fore and hind milk were grouped for this analysis. Log transformed values of the hormones were compared against one another. Table 20 shows the values relating to this analysis and the correlations identified between these hormones.

Table 20: Pearson correlation coefficient values between the hormones analysed. ** Relates to results below 0.01 significance, *refers to results at the 0.05 significance level.

Ln Leptin vs	Ln ghrelin	Ln insulin	Ln resistin
Pearson Correlation	0.11*	0.24**	0.11*
Significance (2-tailed)	0.04	<0.00	0.04
N	329	371	368

Ln Insulin vs	Ln ghrelin	Ln resistin
Pearson Correlation	0.01	-0.01
Significance (2-tailed)	0.82	0.82
N	329	367

Ln Ghrelin vs	Ln resistin
Pearson Correlation	0.19**
Significance (2-tailed)	<0.00
N	325

There were seen to be multiple correlations between the different hormones, with leptin correlating with ghrelin, insulin and resistin. Also ghrelin and resistin were seen to correlate with one another.

3.6 Discussion

3.6.1 Systematic review

This systematic review, which includes 26 papers and samples from over 1000 participants, indicates that there is an association between the concentration of leptin in breast milk and maternal BMI. Evidence for an association between maternal BMI and other hormones investigated is either contradictory or inconclusive. Inconsistencies in the data reported originate from the quality, design and size of studies. In addition to heterogeneous study design, many studies identified had small sample sizes, and hence limited power to detect differences in the concentration of hormones between mothers with differing BMI.

As BMI is not a direct measure of adiposity, the strength of the correlations between these hormones and BMI may not reflect the true value of this association. The increasing availability of more advanced techniques for evaluating body composition may facilitate such studies in the future. For example, to our knowledge, to date only one study has used dual X-ray absorptiometry to assess maternal adiposity (Schueler, 2013). A further limitation is non-standardised sampling; some studies involved analysis of either fore-milk or hind-milk, some a whole breast expression, and in some the sampling technique was not specified. This is potentially important as the concentration of hormones can change over an expression from a single breast, during a single feed (Schuster et al., 2011) and with increasing duration of lactation (Woo et al., 2009). Though this does not appear to be relevant for leptin (Schuster et al., 2011), it is unknown whether this is the case for other hormones. In the majority of studies, samples were collected at a single time point, and those studies that collected data at multiple time points did not attempt to evaluate or adjust for time (Weyermann et al., 2007).

In regard to the methods of detection used to measure hormone concentrations, predominantly immunoassay methods, RIA or ELISA were used. These techniques resulted in similar values, so it is unlikely that method of detection explains variability in the results obtained. More likely, the method of sample preparation contributes to the different results observed between different studies. In the majority of studies the supernatant of centrifuged breast milk was analysed, whilst in some, whole milk was used without prior treatment. There are conflicting recommendations about the most appropriate method of sample preparation. Some suggest that milk lipid interferes with the assays (Lonnerdal and Havel, 2000), whist others suggest it does not (Houseknecht et al., 1997).

Many studies measured maternal BMI at different times during lactation or pregnancy. There does not appear to be any consistent variation in results obtained from studies measuring BMI at different time points, pre or post-partum. Furthermore, the time of sample collection could have a significant

impact on results obtained. This is because concentrations of hormones change throughout the lactation period (Woo et al., 2009). The majority of studies accounted for this by collecting samples at a specific time point, and those that did not adjusted for this effect (Weyermann et al., 2007).

Table 10 displays the associations observed by different studies over the study period, all but one of these report consistent results over the study period. This could be due to either a consistent biological observation, or the methods of the studies being consistent.

Early nutrition may influence health outcomes in children and adults through metabolic programming; breast feeding is associated with altered adiposity in infancy (Gale et al., 2012) and reduced overweight or obesity in later life compared to formula feeding (Oddy, 2012), but evidence of a causal association is uncertain. In the PROBIT study, a cluster-randomized trial promoting breastfeeding, no difference was found in adiposity measures (weight, triceps and subscapular skinfold thicknesses, waist circumference, percent body fat, fat mass and fat-free mass measured by foot-to-foot bio-impedance) at 11.5 years in two groups fed differing amounts of breast milk (Martin et al., 2013). Nonetheless, it is plausible that breast milk hormones might mediate offspring metabolism and risk of later obesity. This would require the presence of appetite regulating hormones in breast milk which retain their biological activity following ingestion, and affect infant metabolism. Supporting evidence comes from both human and animal models.

Physiological doses of orally administered human leptin have been demonstrated to be absorbed into the bloodstream of nine day old rats (Casabiell et al., 1997). Oral doses of leptin have been shown to have demonstrable biological effects, affecting food preference, body weight, reducing caloric intake and is capable of improving insulin sensitivity (Sanchez et al., 2005, Pico et al., 2007, Sanchez et al., 2008, Priego et al., 2010). Likewise, evidence suggests exogenous insulin is able to be absorbed from the gut into the systemic circulation in rat models (Hirsova and Koldovsky, 1969), retaining its biological activity and potentially mediating effects on infant body composition. In type 1 diabetic humans, all of the insulin present in the milk was artificial, also insulin in human milk is present at comparable concentrations to serum, suggesting there is an active transport mechanism (Whitmore et al., 2012). Further evidence has shown that breast milk leptin may actively influence infant appetite, being associated with the change in infant BMI (Doneray et al., 2009). Thus, current evidence supports the possibility of an association between enteral absorption of breast milk hormones with systemic effects, consistent with the hypothesis that breast milk hormones are an important factor in the programming of infant metabolism in the post-partum period (Shehadeh et al., 2006).

3.6.2 Leptin

The data presented here support a correlation between increasing maternal BMI and increasing breast milk leptin. In the majority of studies a positive correlation between breast milk leptin and maternal BMI was found. In all but two of the studies fewer than 100 participants were recruited. However, a large study with 651 participants (Weyermann et al., 2007) demonstrated a strong association between maternal BMI and breast milk leptin concentration.

Leptin is predominantly synthesised by white adipose tissue, but also by the mammary epithelium (Smith-Kirwin et al., 1998). It is associated with food intake and body weight, being released in proportion to the volume of adipose tissue (Lonnqvist et al., 1995), and displays an anorexigenic effect (Cowley et al., 2001). Obese individuals possess a decreased sensitivity to leptin, described as leptin resistance (Considine, 2011). The concentration of leptin in breast milk correlates with plasma leptin, but is around 5 times lower than that of the plasma (Casabiell et al., 1997, Aydin et al., 2008).

Previously, a positive correlation has been observed between maternal BMI, weight and maternal serum leptin concentration (Savino et al., 2010b). This appears to be reflected in breast milk, due to the positive correlation observed between breast milk leptin concentration and maternal BMI. Infant serum leptin and maternal BMI have also been shown to correlate, lasting for at least 4 months post-partum, suggesting infants breast fed by mothers with a high BMI are exposed to significantly higher quantities of leptin, which is subsequently transferred to the infant bloodstream (Savino et al., 2006). Infants born to obese mothers have been shown to be more likely to become obese themselves (Parsons et al., 2001). Potentially, the infant of the obese mother, who is ingesting an increased amount of leptin, becomes leptin resistant and subsequently has impaired appetite regulation, with an increased risk of obesity. Conversely increased leptin ingestion may enhance satiety, reducing intake and later obesity. However, confounding influences must be considered, such as the shared environment of mother and infant. Leptin present in milk may provide a link between maternal body composition and neonatal growth, development and energy balance. Further human studies have found correlations between the growth of neonates and the concentration of breast milk leptin, (Doneray et al., 2009, Miralles et al., 2006, Uysal et al., 2002).

3.6.3 Adiponectin

Similarly to leptin, maternal serum adiponectin concentrations correlate with maternal weight and BMI (Savino et al., 2012b), also breast milk adiponectin has been reported to be positively correlated with both maternal and infant serum adiponectin concentrations (Savino et al., 2012b, Woo et al., 2009), although it is present at significantly lower concentrations (Weyermann et al., 2006). This

suggests either the mammary epithelial cells are capable of synthesizing adiponectin, or are able to transfer adiponectin from the blood.

The quantity of adiponectin in breast milk may have biological implications for the infant; negative correlations between breast milk adiponectin concentration with infant and childhood adiposity have been found. Evidence demonstrates adiponectin in breast milk is associated with a lower weight of the infant in the first six months of life (Newburg et al., 2010). Furthermore, Luoto *et al* reported that adiponectin concentrations in colostrum were significantly higher in mothers whose offspring were of normal weight at 10 years compared to mothers whose offspring were overweight at 10 years (Luoto et al., 2011). Further supporting evidence is the recent identification of adiponectin receptors in the human intestine (Bronsky et al., 2012). The correlations observed between breast milk adiponectin concentrations and infant adiposity strengthens evidence suggesting there is an association between breastfeeding and a reduced risk of obesity in adulthood. This suggests that breast milk adiponectin is systemically absorbed in human infants, remains biologically active and is capable of programing infant metabolism. However, important confounding factors must be accounted for, as causality has not yet been demonstrated, this association may be a reflection of the increased adiponectin concentrations in normal weight mothers, who are more likely to have normal weight children.

A correlation was not found between adiponectin concentrations in breast milk and maternal BMI. This is unexpected as in the serum of adults, adiponectin concentrations are known to be inversely proportional to adiposity (Coppola et al., 2009). Therefore, it would be logical that overweight and obese mothers would have less adiponectin present in their breast milk. In the study which did find an association between maternal BMI and the concentration of adiponectin in breast milk, a possible explanation is the association between adiponectin, prolactin and adiposity. Adiponectin is negatively correlated with prolactin (Nilsson et al., 2005), as prolactin secretion is reduced in obesity, if adiponectin is produced by the adipose tissue of the mammary gland, negative regulation by prolactin in more adipose women could increase the concentration of adiponectin produced in the mammary tissue, and secreted into breast milk. Although adiponectin in breast milk must be regulated by factors other than maternal BMI, such as duration of breastfeeding (Woo et al., 2009) and smoking status (Weyermann et al., 2007).

3.6.4 Insulin

Evidence of a correlation between maternal BMI and breast milk insulin concentration was inconclusive. This is also unexpected; research investigating the breast milk of diabetic mothers found a direct correlation between serum and breast milk insulin concentrations (Jovanovic-Peterson et al., 1989), and it is well documented that the concentration of insulin in serum increases with increasing

BMI, due to insulin resistance (Mayfield, 1998). One potential explanation is that included studies are underpowered to detect this association.

3.6.5 Ghrelin

With regard to ghrelin, conflicting results are also reported. Ghrelin can be either acylated or de-acylated. The acylated form has caprylic acid attached to a serine residue, and is commonly denoted as the active form, however, de-acetylated ghrelin has also been shown to have appetite stimulating effects (Toshinai et al., 2006). Acylated ghrelin is known to be particularly labile, and therefore it is especially important to either acidify samples at collection, or use a protease inhibitor if attempting to measure acylated ghrelin (Stengel et al., 2009). A positive correlation might be expected as the serum concentration of active ghrelin is increased in obese compared to normal weight patients (Zhang et al., 2011), but this depends on the source of ghrelin found in breast milk. There have been conflicting reports in regard to this, with both maternal serum and mammary gland synthesis being suggested as the source of milk ghrelin (Aydin et al., 2006, Kierson et al., 2006). Correlations have been observed between maternal serum ghrelin concentration and breast milk ghrelin with infant serum concentrations, suggesting breast milk is a source of ghrelin for the infant (Savino et al., 2012a).

3.6.6 Resistin

Resistin concentrations increase with obesity, therefore concentrations of breast milk resistin might be expected to increase with maternal BMI. However, this inference is not supported by the study undertaken by Savino *et al* in which no correlation was found between maternal BMI and the concentration of resistin in breast milk (Savino et al., 2012c). However, the small number of women included means the study may be underpowered to examine this association.

3.6.7 Peptide YY and GLP-1

Concentrations of both Peptide YY and Glucagon-like peptide 1 and their association with maternal BMI were evaluated in a single study. Neither of these hormones was found to have an association with maternal BMI. In regard to GLP-1, no clear association between BMI and serum concentrations of this hormone are yet to be established, therefore it is unsurprising an association was not identified in milk, where the association is likely to be less pronounced. In regard to PYY, where in serum a negative association with maternal BMI has been identified, extrapolation to breast milk suggests that concentrations would be decreased in obese mothers, unless other factors with a stronger influence on the concentration of this hormone in breast milk are present.

3.6.8 Multiplex assay conclusions

In agreement with previous work, outlined in the systematic review undertaken, leptin was seen to correlate with maternal BMI in fore milk samples at both time points. However, leptin was not seen to correlate in the hind milk samples analysed at either time point. Ghrelin, insulin and resistin were not found to correlate with maternal BMI at any of the time points analysed, in either the fore or hind milk.

Also, exploration of associations between hormone concentrations and the time samples were collected was undertaken. Ghrelin was observed to decrease from fore to hind milk samples collected at three months post-partum, similarly, a trend in the same direction was noted for samples collected at one week post-partum. This association has not previously been reported in the literature. Furthermore, a decrease in concentration of ghrelin in the samples collected at three months post-partum in comparison to samples collected at one week post-partum was noted, but this only reached significance in the hind milk samples collected.

No significant associations between insulin concentrations in regard to time of sample collection were seen, although a trend towards reduced insulin concentration in hind milk samples in comparison to fore milk, at both time points, was observed. No relationships between time of sample collection and concentrations of either leptin or resistin were seen.

There were seen to be multiple correlations between the breast milk hormone concentrations, with leptin positively correlating with insulin, resistin and ghrelin. Also ghrelin and resistin were seen to correlate with one another.

3.6.9 Conclusions

In summary, increasing maternal BMI is associated with an increase in the concentration of leptin in breast milk. No evidence of a correlation between maternal BMI and other hormones (adiponectin, insulin, ghrelin, resistin, obestatin PYY or GLP-1) was found, but published data are limited and interpretation problematic due to heterogeneity of study design, sample collection and preparation, and the small number of participants. Improved understanding of the potential of breast milk to influence offspring health requires that future studies address these important methodological issues.

Chapter 4
Breast milk metabonomic and nutritional analysis

4.1 Introduction

Breast milk composition is known to be influenced by many factors, but less well understood is the influence of maternal phenotype.

Many of the constituents of breast milk are taken up from maternal plasma, via transcytosis. In conditions such as obesity, where dysregulation of the concentration of various metabolites in the systemic circulation is often observed, it may be expected that this will be reflected in the composition of breast milk. The objective of the work described in this chapter is to investigate whether overweight/obesity exerts a measurable effect on breast milk composition; explored using ^{1}H NMR spectroscopy, UPLC-MS and mid infra-red spectroscopy.

In this chapter metabonomic techniques are applied to human milk in an attempt to characterise its composition comprehensively. There are however, many challenges in measuring the multitude of components of breast milk both accurately and reliably. Metabolic profiling brings its own analytical challenges such as overlapping resonances in spectra, and metabolites being present in dramatically different concentrations, often of many orders of magnitude.

Furthermore, attempting to ascribe a certain effect on the composition of breast milk to a specific maternal factor, such as obesity, is complicated by the significant background variation in breast milk due to effects from differences in time of collection, time since last feed and maternal genotype. Additionally, the individuals participating in the study are free living individuals, and may have dramatically different diets. These factors all potentially have an unknown impact on the composition of breast milk.

This chapter describes the analysis of the aqueous fraction of breast milk using ^{1}H NMR spectroscopy. The aqueous fraction of breast milk contains many important and interesting metabolites, including lactose, human milk oligosaccharides (HMO) and other compounds important for infant health and development, such as amino acids. Prior to starting this work, no published literature existed investigating the composition of human milk using ^{1}H NMR spectroscopy. A number of papers have

since been published, one preliminary study investigating the composition of breast milk of mothers delivering prematurely. In this study it was noted there was an increase in the abundance of lactose as lactation proceeded, but did not undertake a detailed analysis of sample composition (Marincola et al., 2012).

Another previous paper describing a study using ^{1}H NMR spectroscopy to investigate the aqueous fraction of breast milk has examined the effect of maternal obesity on breast milk composition (Smilowitz et al., 2013). Samples were first centrifuged and then filtered to remove lipids and protein and ^{1}H NMR spectra were acquired using a standard 1D pulse sequence. In this study, it was observed that concentrations of acetone were lower in breast milk from women with a higher BMI ($r=20.41$, $p<0.01$), the authors did not note any other correlations between BMI and breast milk composition.

The lipid fraction of breast milk is of particular interest with regard to its varied biological functions, being involved in neurodevelopment, immunodevelopment as well as acting as an important source of energy, accounting for approximately half of the calories found in breast milk. The lipid fraction of breast milk varies in concentration over a single feed, in relation to time of day, as well as over lactation, meaning it has the most variable content of the macronutrients found in breast milk (Hytten, 1954).

Previous research has investigated the differences in fatty acid composition of fore and hind milk, finding no differences in the profiles of fatty acid secreted pre- and post-feed. This suggests that it is simply the quantity of lipid which increases throughout a feed, rather than the species of lipid secreted (Insull and Ahrens, 1959, Hall, 1979).

Indeed, it does appear that the profile of fatty acids produced is remarkably well conserved between mothers. Evidence for this stems from an investigation collecting samples from women at different times post-partum, there were large differences in the quantity of lipid present, but a remarkable similarity in the profiles of fatty acids was observed. Specifically, in regard to the short-chain fatty acids, up to α-linolenic acid, profiles were seen to be virtually identical. The variation which did occur was observed in the long chain fatty acid derivatives of linoleic acid and α-linolenic acid, which only account for around 1.5% of the total fatty acids present (Hall, 1979).

However, the lipids present in breast milk are known to be impacted by maternal diet, especially pre- and during pregnancy diet, demonstrated by both animal (Laws et al., 2007), and human investigations. For example, when the fatty acid profile of a woman receiving dietary supplementation of unsaturated fats was investigated, significantly higher concentrations of linoleic acid in her milk

were seen, with reduced concentrations of stearic and palmitic acid, in comparison to unsupplemented mothers (Hall, 1979).

In this study, the lipid fraction of breast milk was investigated using lipidomics, specifically, UPLC-MS analysis. The lipidome, a subset of the metabolome, refers to the complete lipid profile of a biological sample (Han and Gross, 2003). UPLC-MS is an extremely sensitive technique for identifying metabolites, capable of detecting concentrations in the picomolar range and is ideally suited to exploring the lipidome of individuals

Previous research using capillary gas–liquid chromatography has examined the effect of obesity on breast milk fatty acid concentrations. Lindholm *et al.* demonstrated that the concentration of long-chain omega 3 fatty acids were lower, and the ratio n-6/n-3 fatty acid higher, in samples of breast milk taken from obese mothers, in comparison to normal weight mothers (Lindholm et al., 2013). Obese mothers who participated in an intervention program which gave general dietary advice were found to have fatty acid concentrations approaching that of the normal-weight mothers.

Further investigated was the breast milk macronutrient content, using mid IR spectroscopy. Previous research using this technique, associating macronutrient content with maternal BMI, has suggested there is a concurrent increase in protein, fat and carbohydrate as maternal BMI increases (Michaelsen et al., 1990). Another group has also identified increases in breast milk fat concentration in relation to increased maternal BMI (Barbosa et al., 1997).

The primary objective described in this chapter is to investigate whether overweight/obesity exerts a measurable effect on breast milk composition. To examine the association between maternal BMI and breast milk metabolome and macronutrient content breast milk samples from a prospective cohort of mothers were acquired, as described in Chapter 2. Longitudinal measures of BMI and other clinical characteristics were recorded, and attempts made to describe the causes of the variability observed in the breast milk samples collected.

4.2 Aims

- Investigate the impact of maternal BMI on maternal aqueous breast milk metabolome, using ^{1}H NMR spectroscopic analysis.

- Investigate the impact of maternal BMI on breast milk lipidome, using UPLC-MS.

- Evaluate the effects of maternal BMI on macronutrient content using mid infrared spectroscopy.

- Examine changes in composition of breast milk samples taken at different times post-partum, and investigate other causes of variability.

4.2.1 Hypothesis

There will be a statistically significant difference in the metabolome/lipidome and nutritional content of breast milk from mothers with a high BMI compared to mothers with a low BMI.

4.3 Methods

Breast milk samples were analysed using NMR spectroscopy, UPLC-MS and mid IR spectroscopy, as explained in detail in Sections 2.3, 2.4 and 2.7, Chapter 2. In all these techniques samples were analysed in a randomised run order.

4.3.1 ^{1}H NMR spectroscopy analysis

Breast milk samples were prepared into 96 place SampleJet racks (Bruker, Biospin). Acquired data were processed and analysed using Matlab 7.12 (MathWorks Inc. USA). For further analysis using multivariate modelling techniques, data were imported into SIMCA-P+ 13.0.2.

4.3.2 UPLC-MS analysis

Breast milk samples were prepared into 48 well plates. Samples were analysed in both positive and negative ionization mode. Once acquired, data were processed using the XCMS package within R. Again, data were imported into SIMCA-P+ 13.0.2 for multivariate analysis.

4.3.3 Macronutrient analysis

Breast milk samples were analysed according to the manufacturers' guidelines, as described in Section 2.7, Chapter 2. Samples which failed analysis were not available for reanalysis, as when a sample is injected it forces the previous sample from the instrument, resulting in the mixing of samples. Data acquired using the Miris macronutrient analyser were analysed by traditional statistical methods in SPSS, Version 22 (IBM).

4.4 Results

4.4.1 Cohort Characteristics

Descriptive characteristics for the maternal cohort studied are presented in Table 21. The average age of the mothers was 34 years (range from 23 to 46), and with an average height of 166cm.

Table 21: Descriptive characteristics of the maternal cohort at one week and three months post-partum. Data are reported as means, standard deviations are given in brackets.

	One week post-partum	Three months post-partum
Number	108	95
Weight, kg	70.4 (12.7)	69 (10.7)
BMI	25.5 (4.5)	24.9 (3.4)

Maternal BMI was seen to drop from the one week time point to the three month time point, as displayed in Figure 34. This was in part due to the mothers losing weight between study visits, as well as participants dropping out of the study who were more likely to have a higher BMI, as described in Section 3.5.1, Chapter 3. This was an unfortunate, as it reduced the power of the study to detect differences in the composition of breast milk between overweight/obese and non-overweight/obese women. Also complicating the analysis and interpretation was the observation that several mothers appeared to increase the BMI over this period.

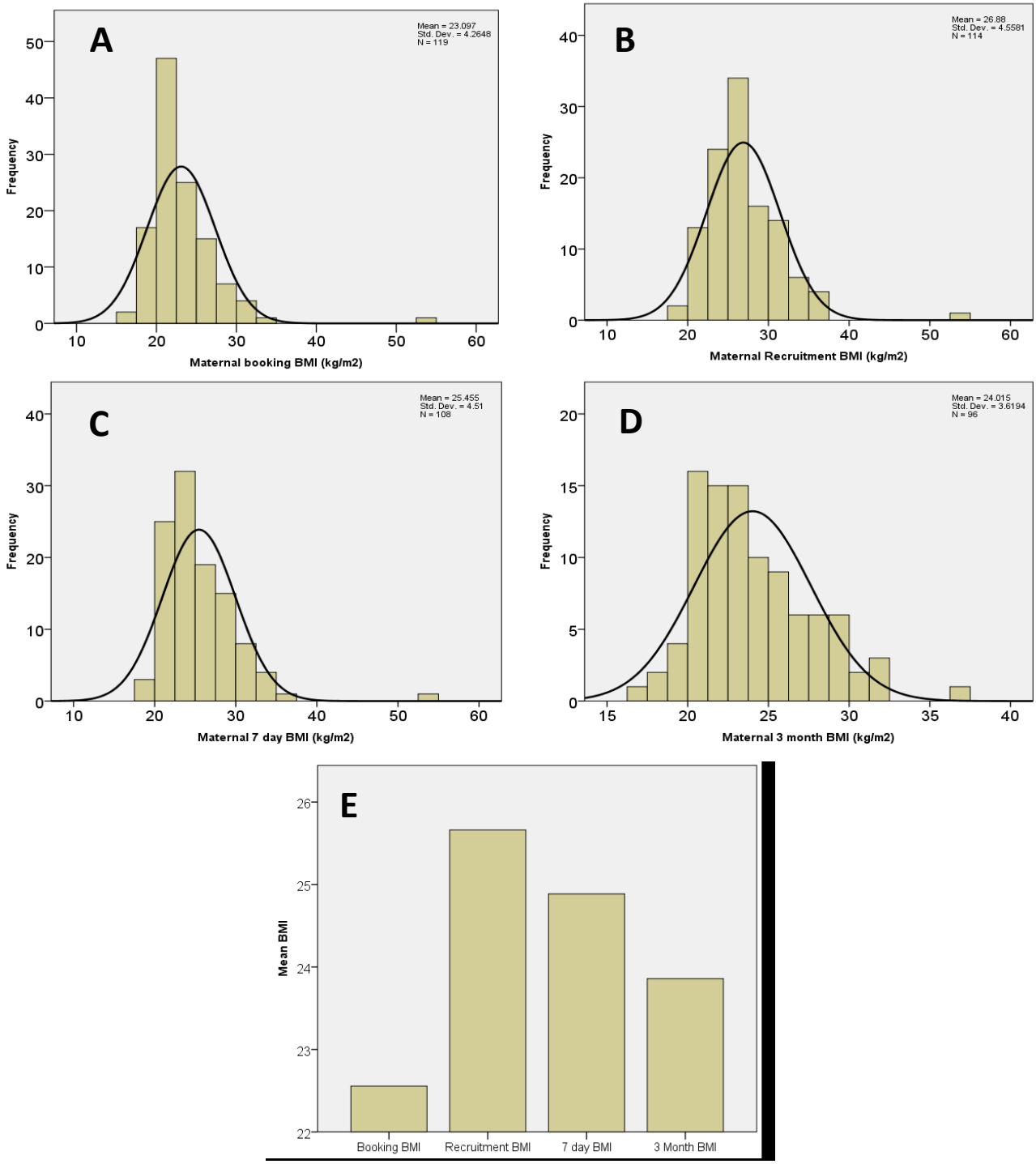


Figure 34: Histograms displaying the distribution of maternal BMI, measured at (A) mothers booking visit (B) at recruitment on the post-natal ward, (C) at 7 days post-partum (D) three months post-partum. (E) Histogram showing the average BMI of the cohort over the study period.

4.4.2 ^{1}H NMR of aqueous fraction of breast milk

A total of 424 samples were analysed, including 34 QC samples, originating from a single large sample, analysed every 12 samples, as well as three formula milks (Cow and Gate, Aptamil and SMA). Not including QC samples and formula, 386 maternal samples were available for analysis. Table 22 shows a breakdown of the number of samples collected at each time point.

Table 22: Samples analysed using ^{1}H NMR, (excluding QC and formula samples).

	One week	Three months	Total
Number fore milk	107	93	200
Number hind milk	98	88	186
Total	205	181	386

Data were processed using Matlab and spectra were visualised for inspection, as displayed in Figure 35. Similarly to the method development samples, spectra were dominated by extremely high concentrations of lactose, running from δ 3.3 to δ 5.3, with the next highest peak intensities belonging to the various HMO species, predominating at δ 1.2 and δ 2.0.

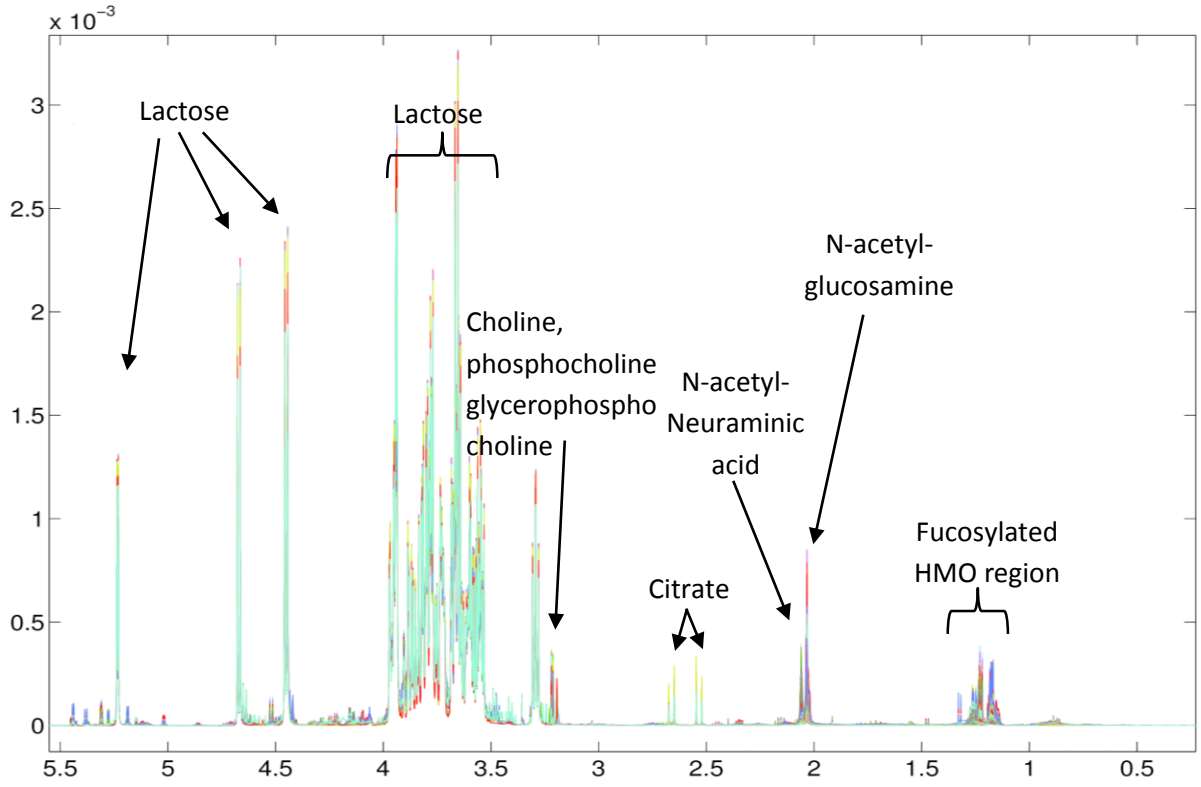


Figure 35: All breast milk spectra overlaid. Spectra as they appear after alignment, normalisation to the total spectral area, and after removal of the water resonance, (n=424).

Once processed, data were imported into SIMCA P+ for multivariate analysis. PCA was performed in order to give an overview of the data, and to establish which method of scaling would be most appropriate, as well as to identify any outliers; samples which were significantly different from the rest of the cohort.

In order to scale the NMR dataset, Pareto scaling was selected, as this method was found to be most appropriate due to the tight clustering of the QC samples displayed in the scores plot. Of the 424 ^{1}H NMR spectra analysed to this point four were excluded, as they had been mislabelled, hence, 420 spectra were available for multivariate analysis.

4.4.2.1 Analysis of length of storage on milk metabolome

In order to ensure the quality of the data, an assessment was made to identify any differences in sample composition based on the length of time samples were stored for at -80°C. Data were modelled using PCA and OPLS-DA, comparing samples stored for less than and greater than 300 days. Percentage of variation and predictivity values for this OPLS-DA model were R^2X=0.61 and Q^2Y=-0.04. This demonstrates that the length of time samples were frozen did not affect the metabolomic profiles, giving confidence that results obtained were not biased by storage time.

4.4.2.2 Investigation of run time effects

To further check the quality of the data aquired, an assessement was made to identify whether there were any run time effects due to the length of analysis, which occured over several days. This was done by assessing the scores plot by the number rack on which samples were analysed, to identify whether there was any obvious drift in the data. This was to ensure any changes seen were not due to analytical artifacts but genuine alterations in the composition of samples.

To do this an OPLS-DA model was computed to investigate whether there were alterations in sample composition due to the sequence of analysis, using the rack number as the class variable.

Figure 36 displays samples analysed on rack 1 against those analysed on rack 5, resulting in a highly predictive model, with a Q^2Y value of 0.78.

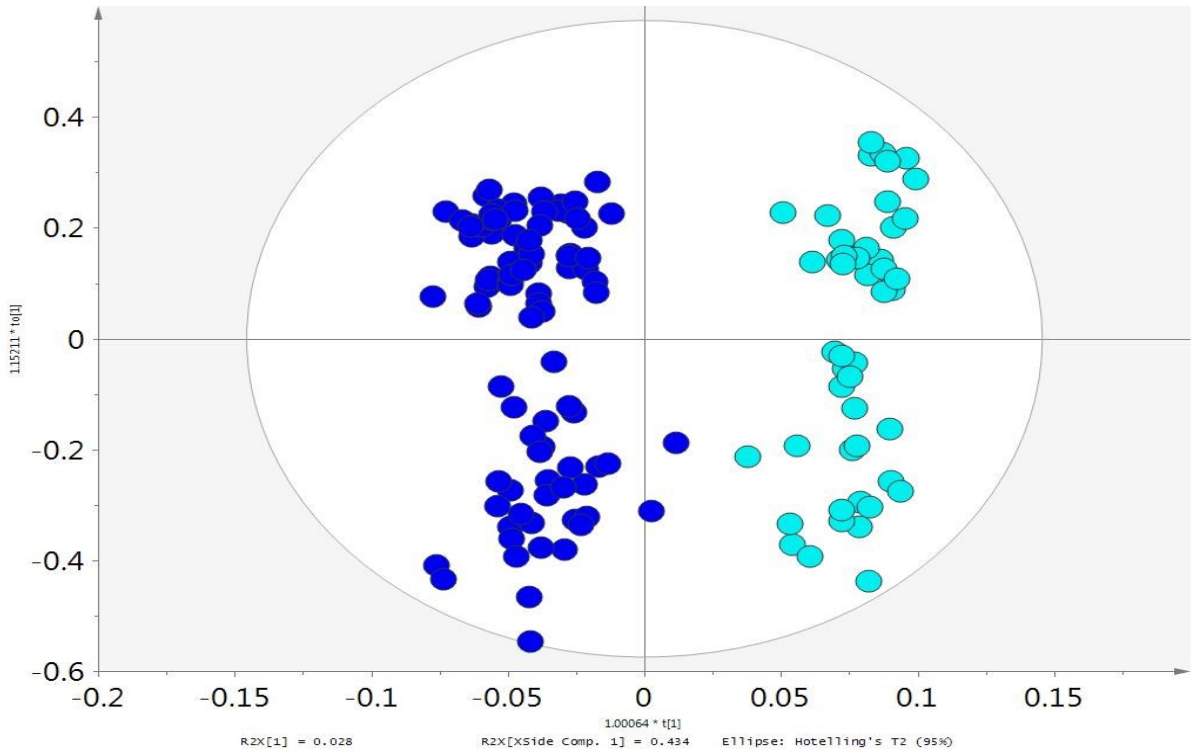


Figure 36: OPLS-DA scores plot of samples analysed on rack 1 in dark blue, vs those analysed on rack 5, turquoise, $R^2X=0.9$, $Q^2Y=0.78$. This plot indicates the presence of systematic differences relating to sample run order.

Spectra were investigated using the loadings plot to identify the cause of this separation. This indicated that the abundance of lactose was decreased on the rack run later, rack 5, shown in Figure 37.

ose

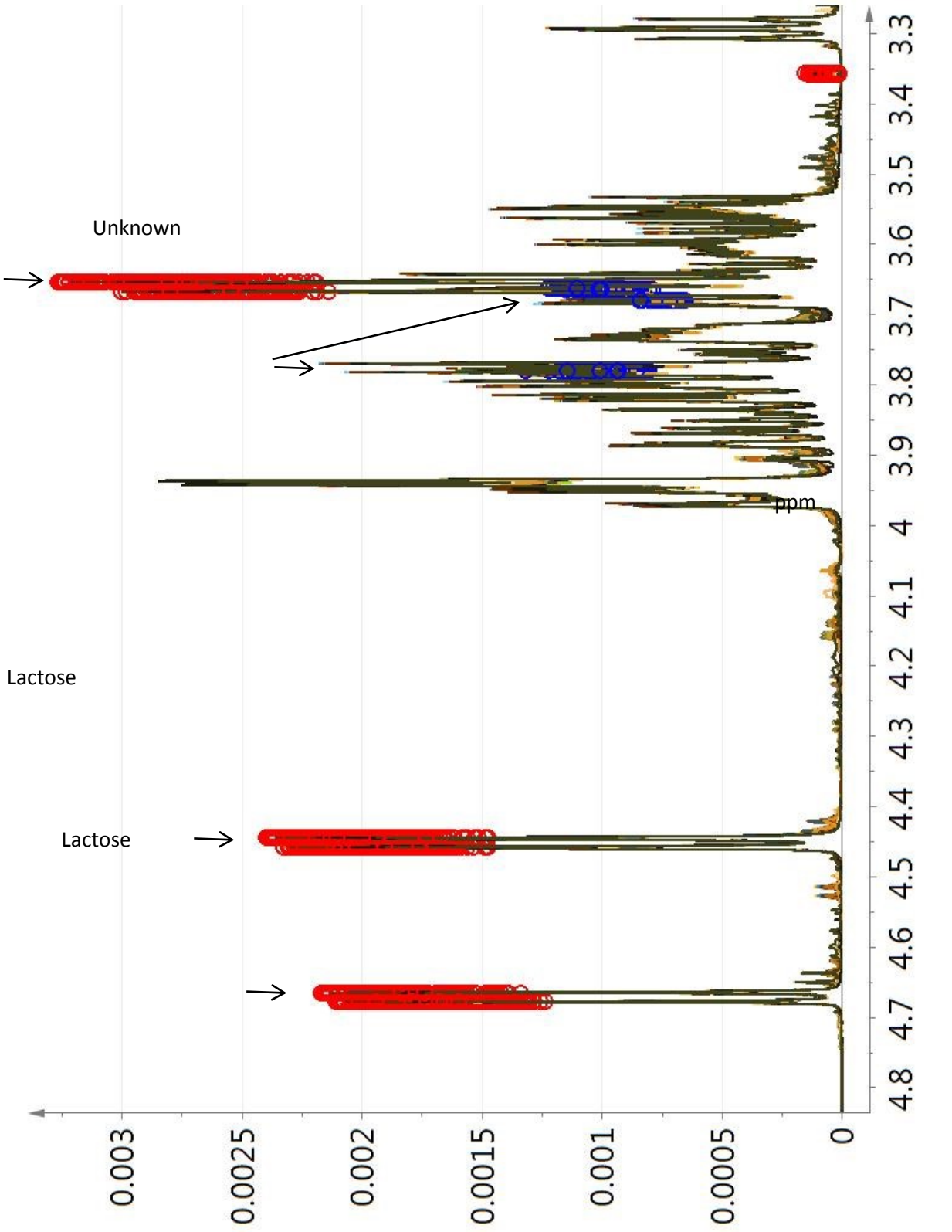


Figure 37: Spectral changes observed due to run time between samples run on rack 1 and rack 5. Peaks highlighted in red were increased in rack 1, which correspond to signals produced by lactose, in blue are signals which were increased in rack 5.

Because lactose was decreasing in abundance inthe racks run later on in the analysis, it was decided to remove the lactose signals from the spectra, as displayed in Figure 38.

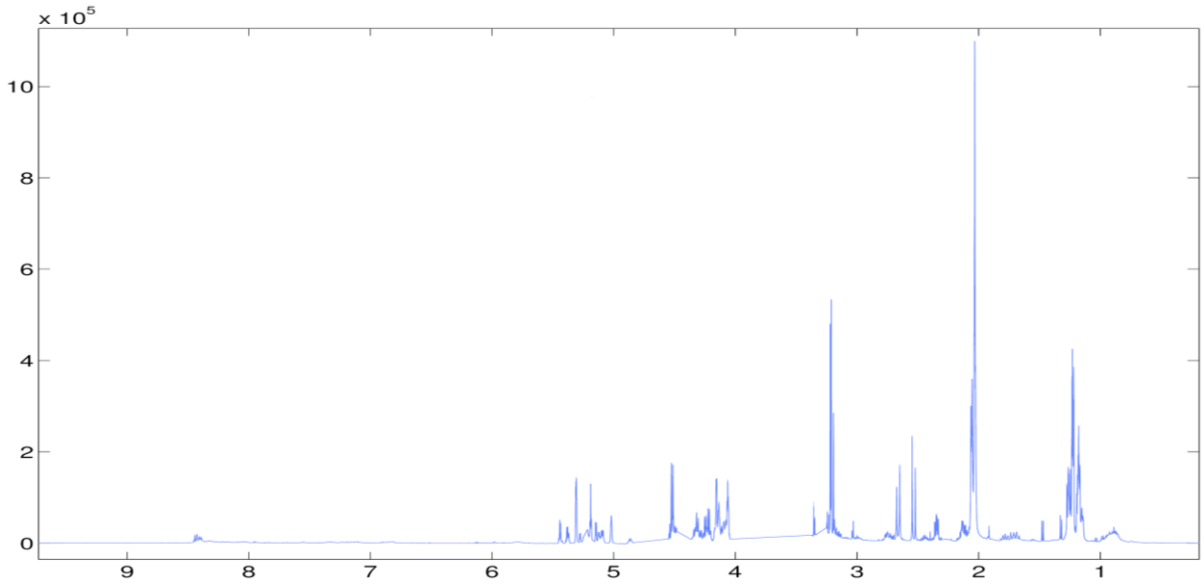


Figure 38: Median spectra of all breast milk collected. TSP and lactose signals have been removed, spectra were normalised to the total area.

Once the lactose peaks were removed, spectra were renormalised, and the resulting data used for subsequent data modelling and analysis, an overview PCA using this data is displayed in Figure 39.

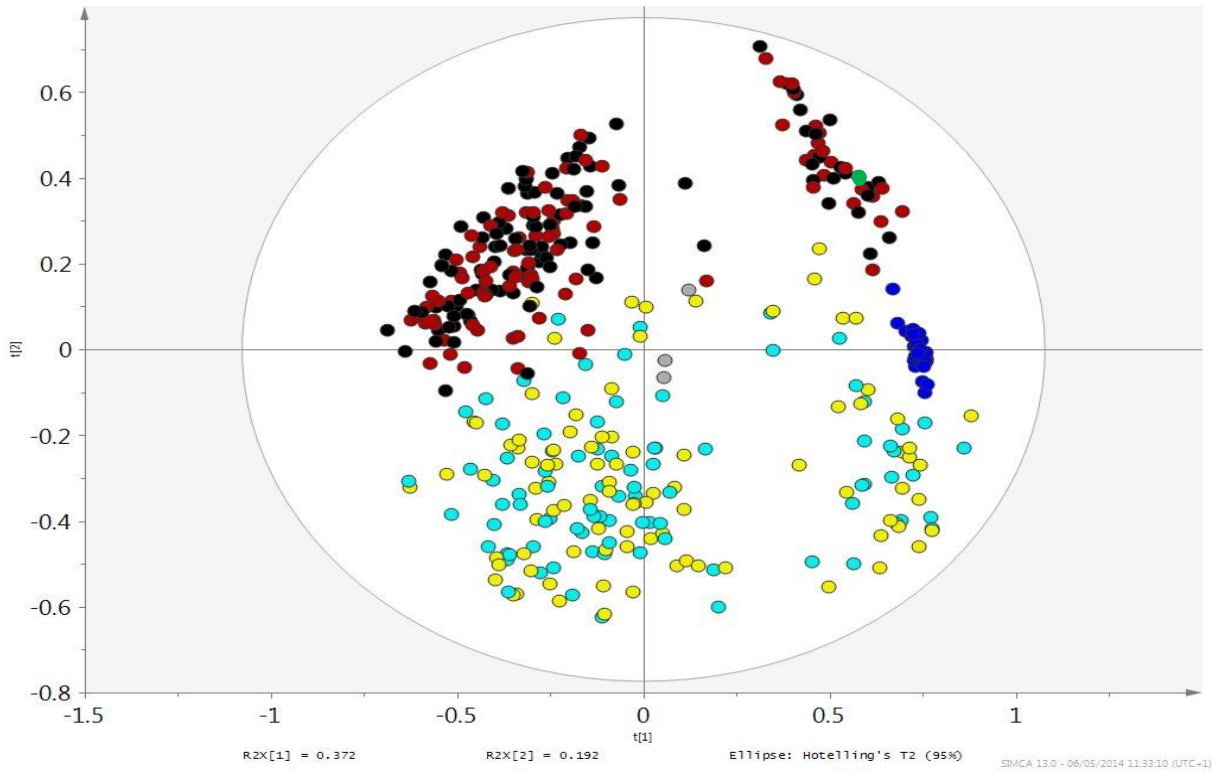


Figure 39: PCA scores plot showing all breast milk samples collected with lactose signals removed. In black and red are fore and hind milk samples collected at one week post-partum whilst in yellow and turquoise are fore and hind milk samples collected at three months post-partum. Blue circles represent QC samples. In grey are formula samples, whilst the green circle at the top right is a colostrum sample. R^2=0.858, Q^2=0.837, indicating that the first two components accounted for the majority of the variation in the data set. All models are normalised to the total area and scaled using Pareto scaling.

Individual PCA scores plots were plotted for each of the time points and for fore and hind milk, as displayed in Figure 40.

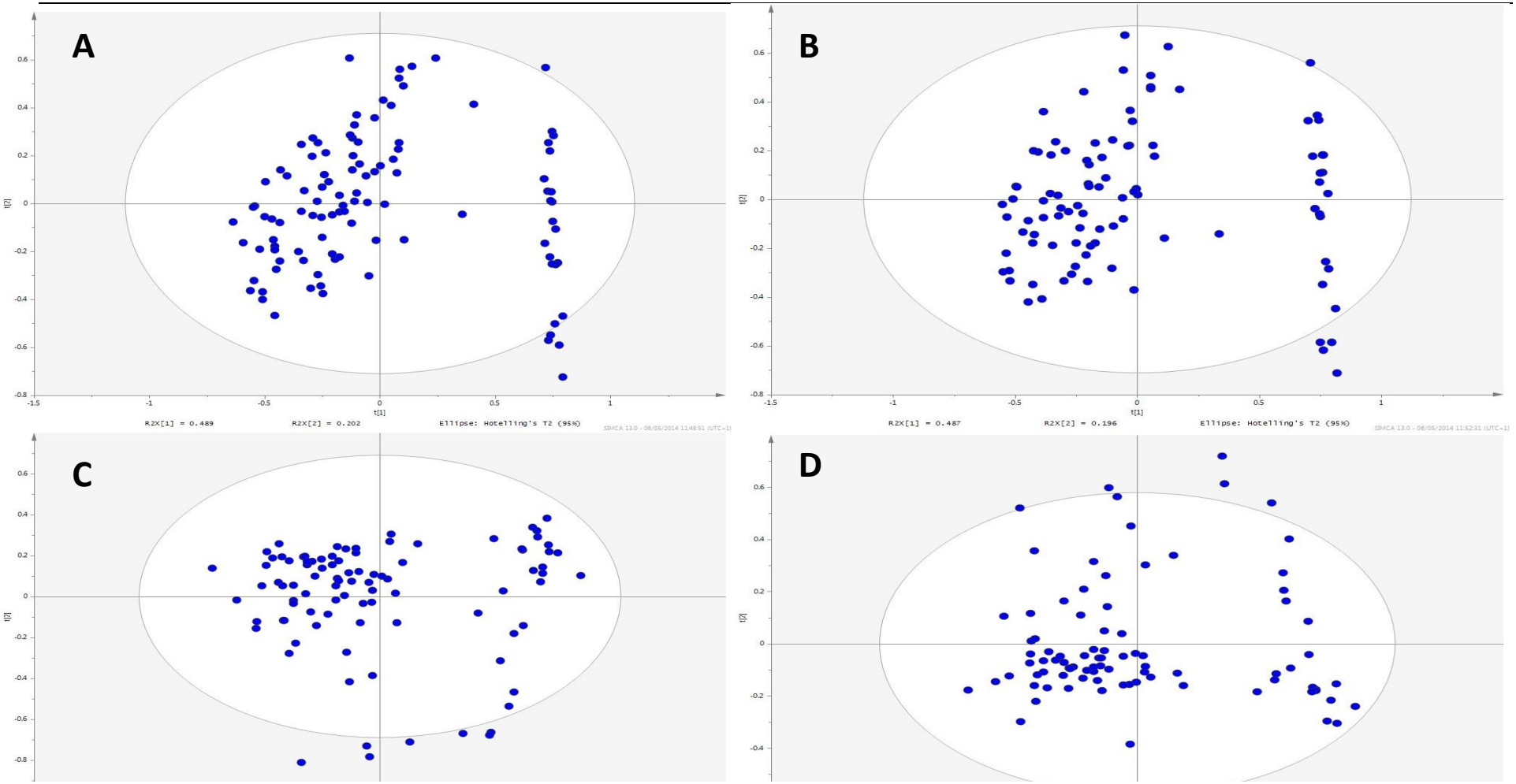


Figure 40: PCA scores plot of; **A** Fore milk samples collected at one week post-partum, R^2=0.806, Q^2=0.773. **B** Hind milk samples collected at one week post-partum, R^2=0.96, Q^2=0.886. **C** Fore milk samples collected at 3 months post-partum, R^2=0.926, Q^2=0.817. **D** Hind milk samples collected at 3 months post-partum, R^2=0.901, Q^2=0.775.

4.4.2.3 Analysis of fore and hind milk

The median spectra of fore and hind milk at each time point were plotted against one another in order to identify differences between these samples. Figure 41 displays the median spectra of fore and hind milk, collected at one week post-partum, overlaid. This figure suggests the composition of the aqueous fraction of breast milk is highly regulated, as there are no obvious systematic differences between these spectra. Results for fore and hind milk samples collected at three months post-partum were similar, with no clear differences between the fore and hind milk samples.

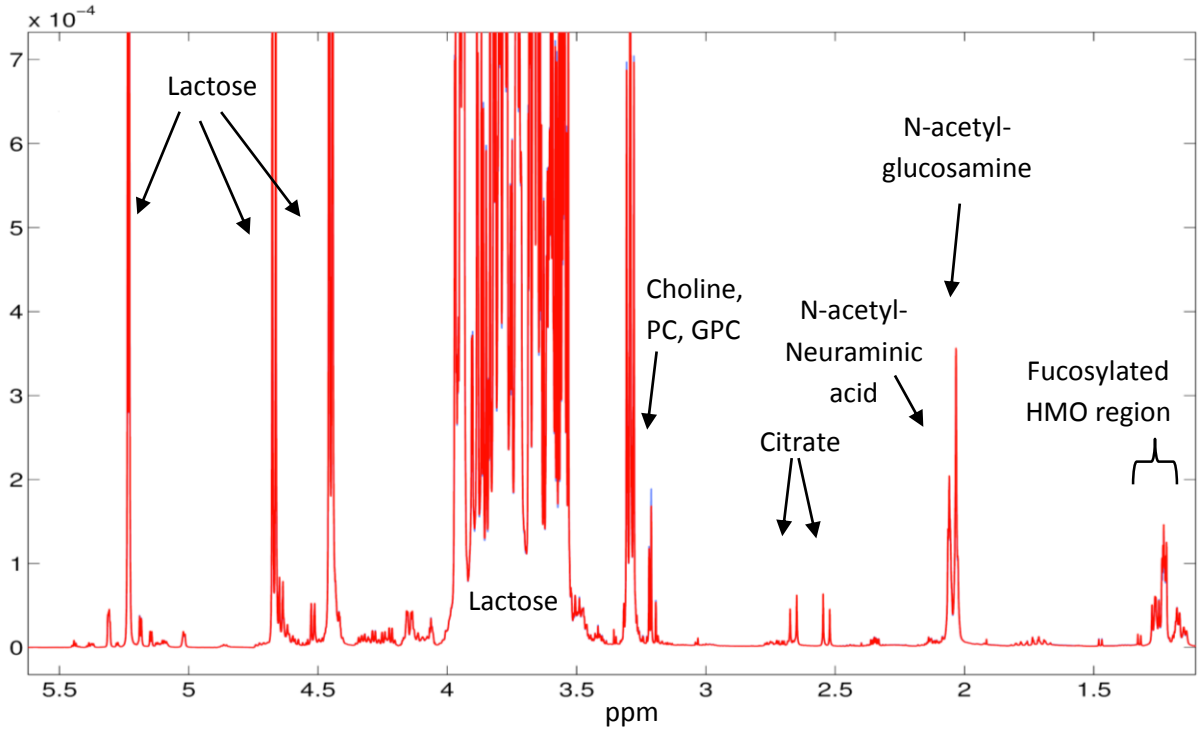


Figure 41: Median spectra of the aqueous fraction of fore and hind milk overlaid. Samples collected at one week post-partum, fore milk is shown in blue (behind) whilst hind milk is shown in red.

Using PCA and OPLS-DA, models were created to compare fore and hind milk samples against one another to see if more subtle differences between these samples could be identified. Similarly to the overlaid spectra in Figure 41, there were no obvious differences between fore and hind milk identified. No separation was observed in either the PCA or OPLS-DA models at either time point, and both OPLS-DA models possessed negative Q^2Y values. Corresponding figures are displayed in Figure 42.

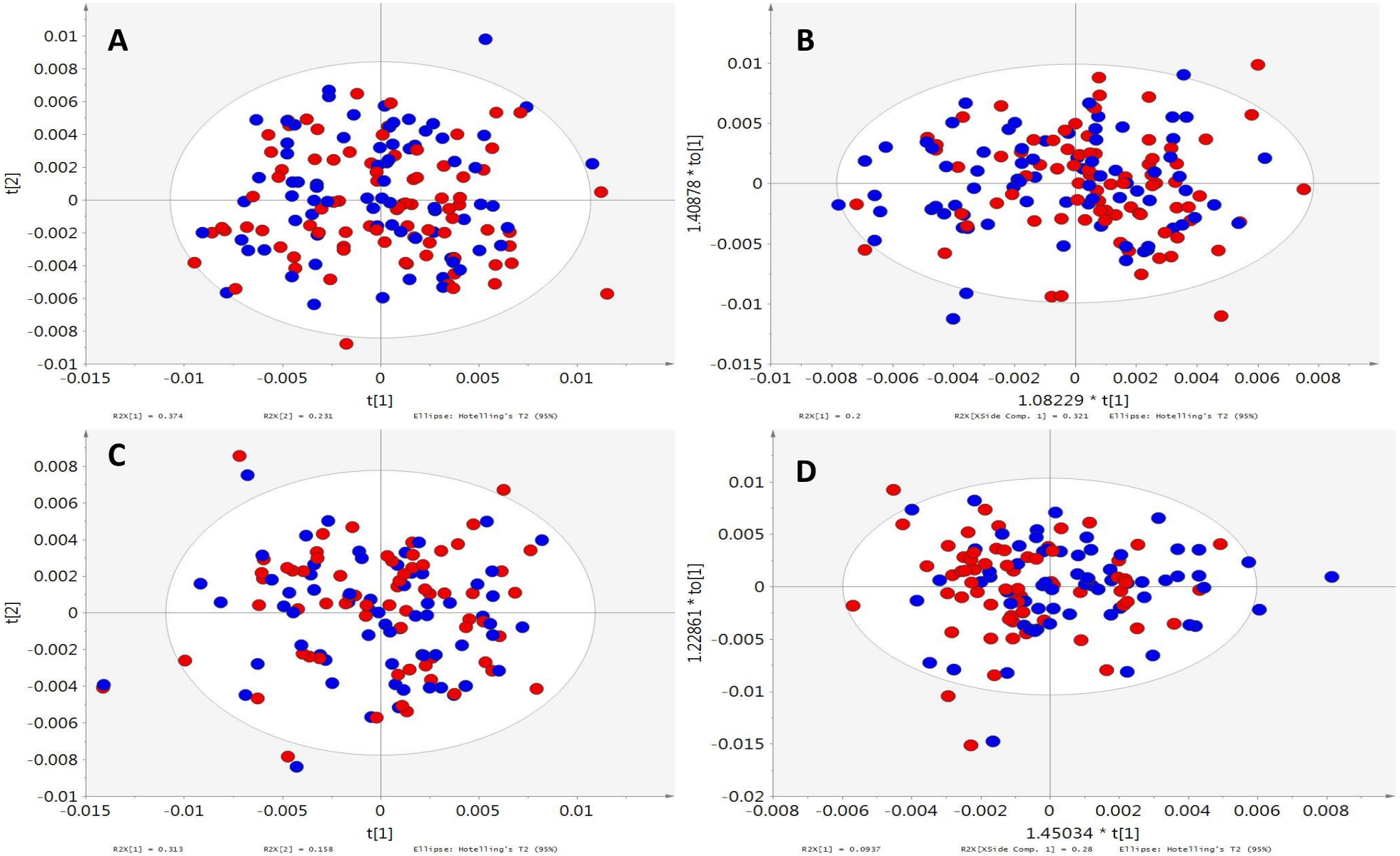


Figure 42: A PCA scores plot comparing fore milk samples (red) against hind milk samples (blue) collected at one week post-partum. R^2=0.605, Q^2=0.586. **B** OPLS-DA scores plot comparing fore and hind milk samples collected at one week post-partum, R^2X=0.521, Q^2=-0.06. **C** PCA scores plot comparing fore milk samples against hind milk samples collected at three months post-partum, R^2X=0.471, Q^2=0.435. **D** OPLS-DA scores plot comparing fore and hind milk samples collected at three months post-partum, R^2=0.374, Q^2=-0.183.

These models suggest the aqueous fraction of breast milk changes very little between fore and hind milk, as analysed using ^{1}H NMR. Because of this finding, it was decided further analyses of the ^{1}H NMR spectroscopic data from the breast milk would be carried out only on fore milk samples. This was done so as not to duplicate the analyses, as the fore and hind milk samples were virtually identical, and in order not to include multiple observations from the same participants.

4.4.2.4 Comparison of samples taken at one week post-partum against three months post-partum

The median spectrum of fore milk samples collected at one week postpartum was plotted against the median spectrum of samples collected at three months post-partum, displayed in Figure 43.

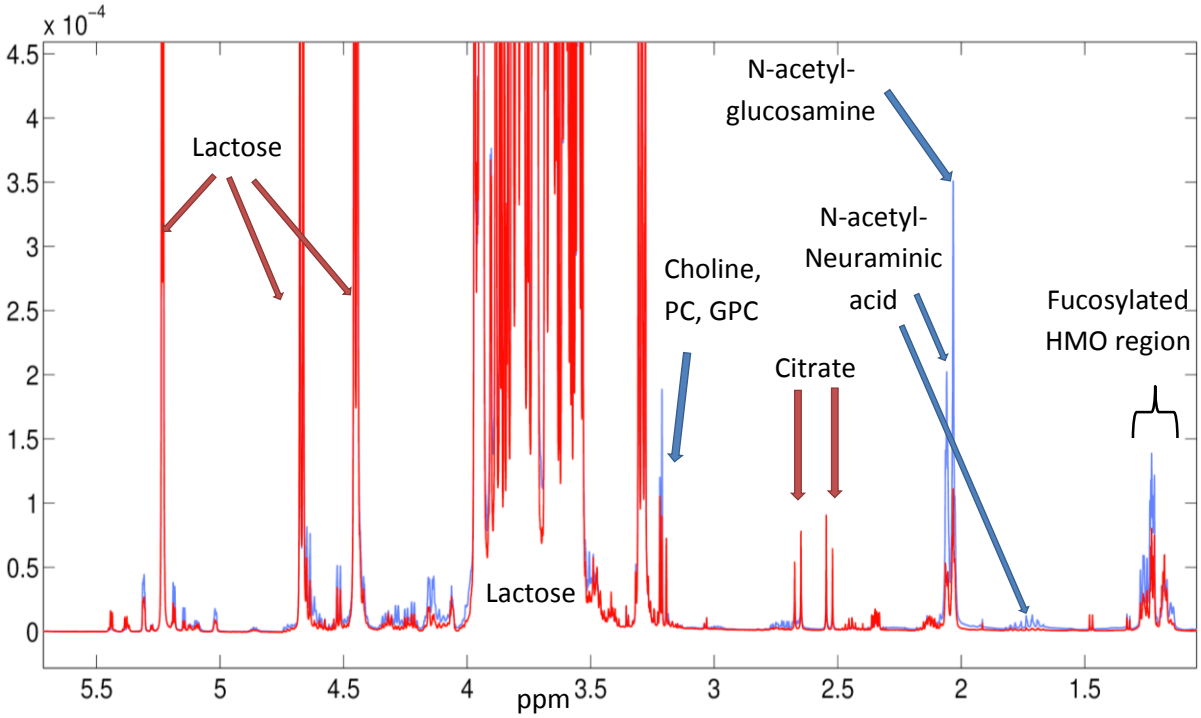


Figure 43: Median spectrum of fore milk. Samples collected at one week post-partum are displayed in blue, sample collected at three months post-partum are displayed in red.

In this figure, clear differences are visible between the sample groups, including a major decrease in the signals at ~ δ 1.2 and δ 2. The decrease in these resonances corresponds to the known decrease in concentration of HMO as lactation proceeds, further described in Section 4.4.2.5, Chapter 4. Furthermore, a large drop in the abundance of certain cholines, particularly glycerophosphocholine is detectable in the later samples, in agreement with the method development samples and previous literature. Comparison between hind milk samples collected at different time points post-partum displayed similar results to Figure 43.

Samples were compared based on the time of collection post-partum using PCA and OPLS-DA. In both scores plots of these models, there was clear separation based on the time of collection post-partum, shown in Figure 44.

In the loadings coefficient plot, Figure 45, there were a number of metabolites seen to alter in abundance in relation to the time the sample was taken post-partum. These metabolites were identified using the online data base HMDB and literature. At three months post-partum, the concentrations of lactose and glutamine were greater compared to those in samples obtained one week post-partum. Also a decrease in the concentration of HMO was noted at 3 months post-partum.

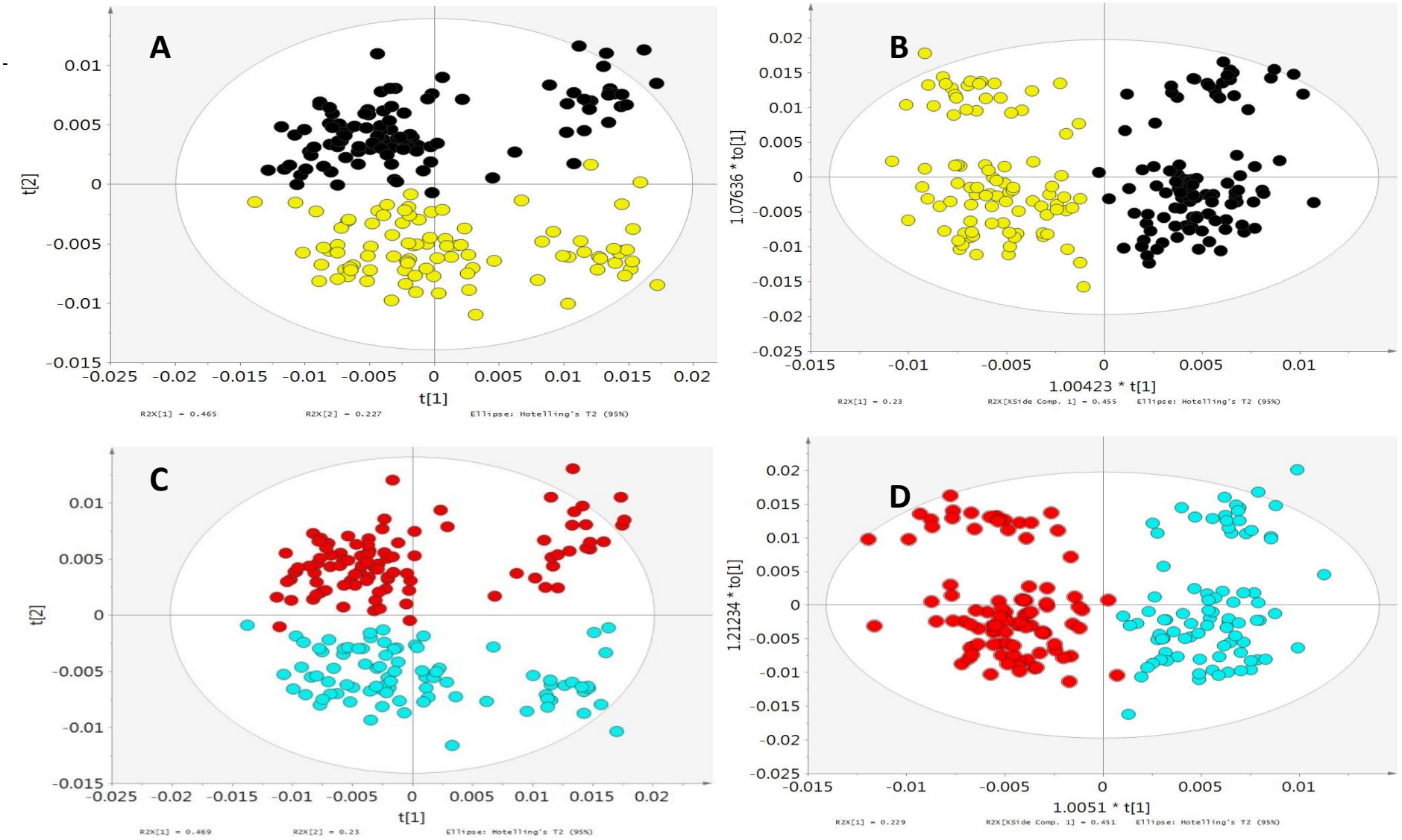


Figure 44: PCA scores plot comparing, A fore milk samples (Black) taken at one week post-partum against fore milk samples (Yellow) collected at three months post-partum. R^2X=0.691, Q^2=0.683. **B** OPLS-DA scores plot comparing fore milk samples taken at one week post-partum against fore milk samples collected at three months post-partum, R^2X=0.684, R^2Y=0.846, Q^2 =0.842. **C** PCA scores plot comparing hind milk (red) samples at one week against hind milk samples (turquoise) collected at three months post-partum, R^2X=0.698, Q^2=0.687. **D** OPLS-DA scores plot comparing hind milk samples taken at one week (red) and hind milk samples collected at three months post-partum, R^2 = 0.68, R^2Y=0.842, Q^2 =837.

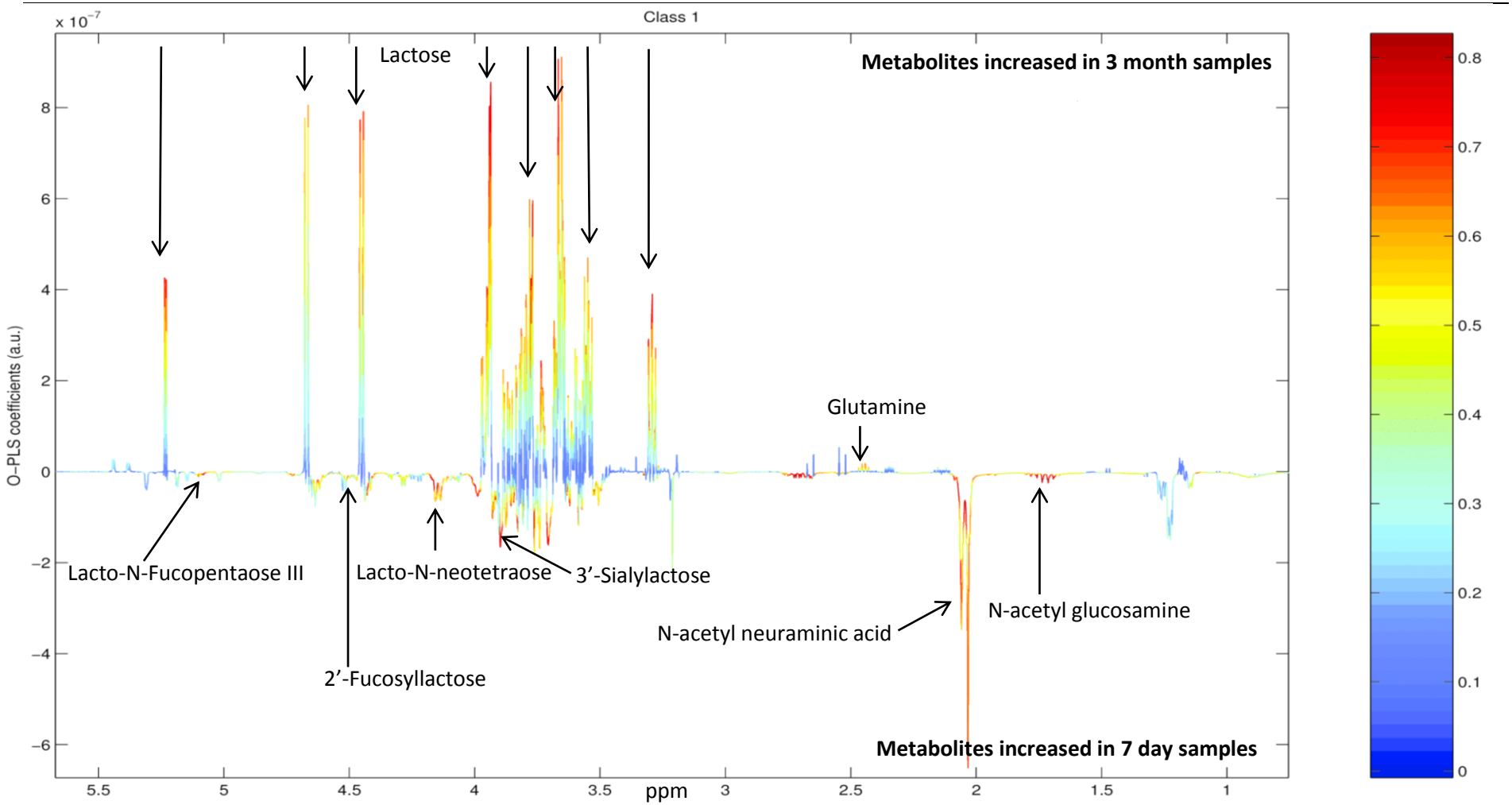


Figure 45: OPLS-DA loadings coefficient plot. Fore milk samples collected at one week post-partum are compared to samples collected at three months post-partum, R^2X=0.619, R^2Y=0.881, Q^2Y=0.871.

4.4.2.5 Differences in HMO profile from different mothers

Following PCA analysis two clear groups were clearly observed in the scores plots, as shown in Figure 39, this separation was most distinct in samples collected at one week post-partum, displayed in Figure 40. This difference accounted for the largest variation between samples, as the separation was in principal component 1. Further analysis was undertaken to determine which metabolites were responsible for this separation. The median spectrums of the two groups were plotted against one another, revealing peaks at δ 2.04, δ 2.06, as well peaks at multiple other regions in the spectra, discriminated between these two groups, as displayed in Figure 46.

These discriminatory peaks were identified, and attributed to the monosaccharaides which compose HMO. Further identification revealed some of these resonances belonged to 2'-Fucosyllactose (2'-FL), and other HMO containing the 2'-FL structure. A portion of the mothers contained very high concentrations of 2'-FL in their milk, whilst other mothers contained none. This was attributed to some mothers possessing an inactive copy of the gene FUT2, encoding for the enzyme 2'-fucosyllactotransferase, outlined in 1.2.5, Chapter 1. This was determined via the ^{1}H NMR spectra as no genetic analysis was undertaken on these mothers. Mothers who possess an inactive version of this gene are unable to synthesize 2'-FL, and HMO containing this structure, and termed 'non-secretors', in accordance with the literature. Mothers who have an active copy of this gene, are able to produce 2'-FL and similar HMO are termed secretors. At one week post-partum the ratio of secretors to non-secretors was 83/23.

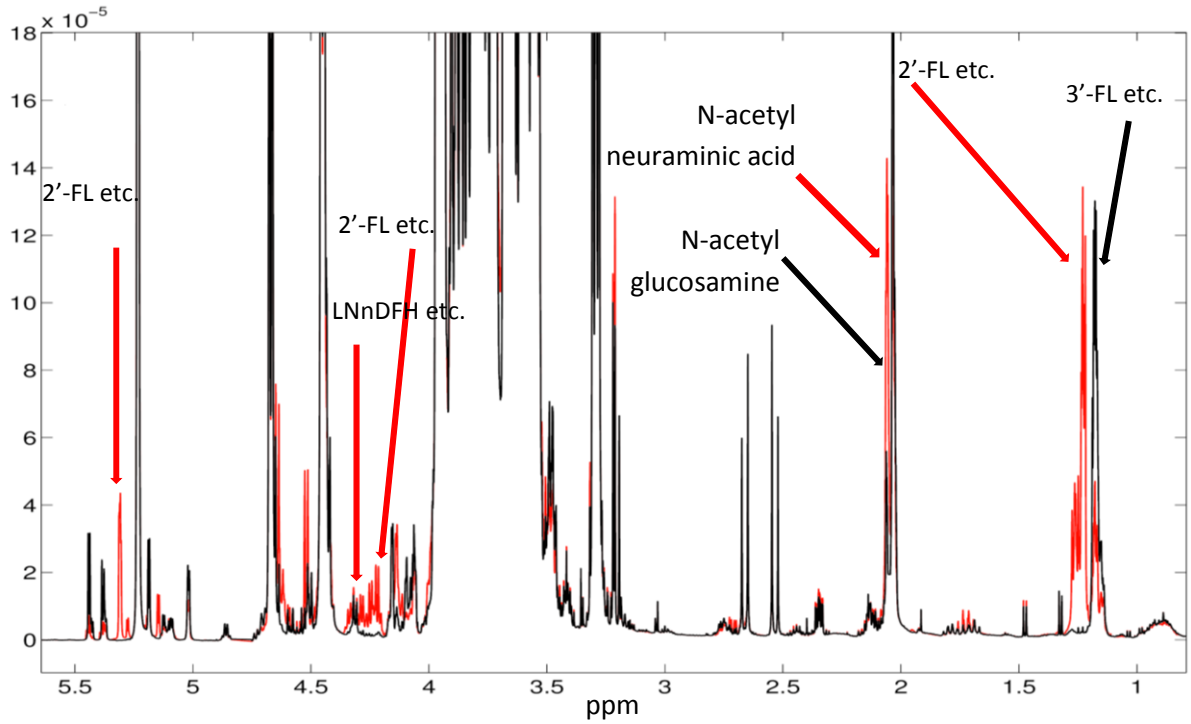


Figure 46: Black shows the median spectrum of non-secretor mothers whilst red displays the median spectrum of secretor mothers. At δ 1.2 a doublet from fucose is found, whilst at δ 2.04 N-acetyl glucosamine is seen; the doublet at δ 1.17 is increased in non-secretor mothers, again from fucose in a different structure to 2'-FL.

Further investigation of the region where the fucosylated oligosaccharides were found also revealed a group of mothers whose milk did not contain HMO with a α1,4-fucosyl linkage, according to previously published literature, these mothers were designated as Lewis blood group negative mothers. These different spectral profiles from different groups of mothers are displayed in Figure 47.

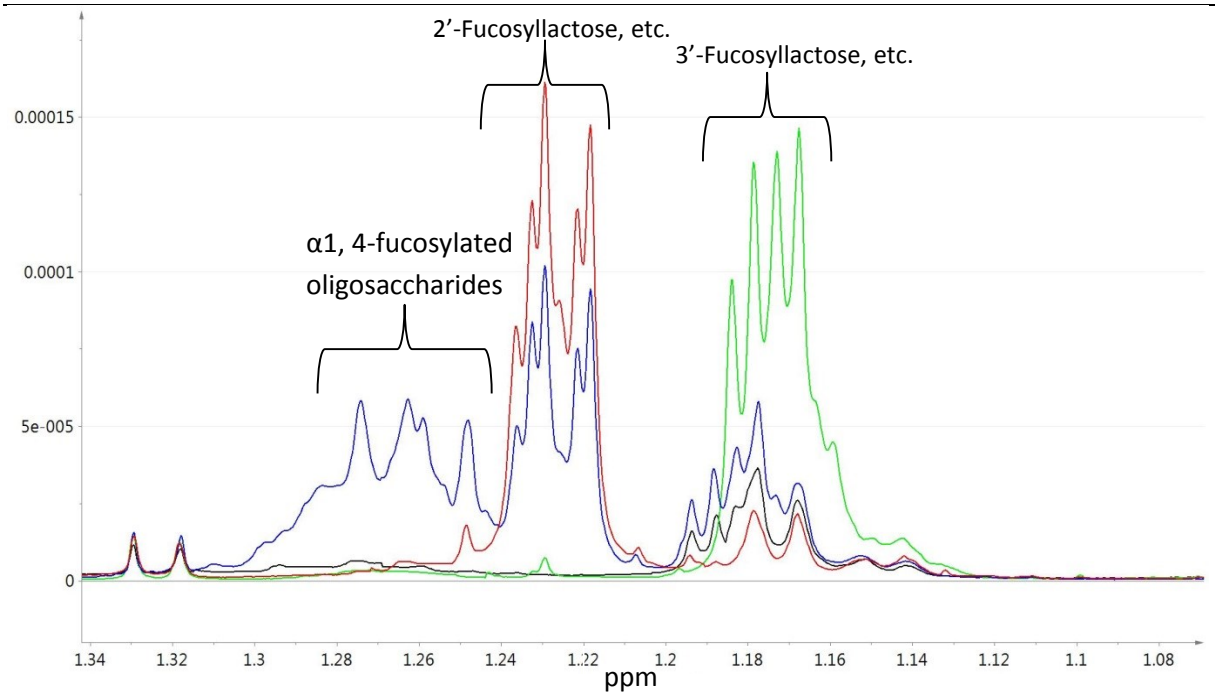


Figure 47: Spectra from four different mothers, displaying divergent HMO profiles. Blue displays a spectrum from a mother whose milk contains all classes of fucosylated oligosaccharides, designated as Secretor^{+}/Lewis^{+} (Se/Le). In green, a mothers whose milk does not contain Fucose(α1-2) Galactose units, designated as Se^{-}/Le^{+}. Displayed in red is a mother whose milk does not contain α1, 4-fucosylated oligosaccharides, (Se^{+}/Le^{-}) whilst in black is a mother who is Se^{-}/Le^{-}.

4.4.2.6 Regression of breast milk metabolome against maternal BMI

Analysis was undertaken to investigate whether maternal BMI had an influence on the breast milk metabolome in a dose dependent manner. Models were computed using data from fore milk spectra. OPLS was used, a regression technique, employing the values of maternal BMI at the booking visit. These models had negative Q^2Y values, indicating maternal BMI had no systematic effect on the composition of the aqueous fraction of breast milk, as analysed by ^{1}H NMR spectroscopy, Table 23.

Table 23: OPLS models computed comparing mothers with different BMI, using booking BMI values.

N	R^2X	R^2Y	Q^2Y	Outliers	t[1]	t[2]	Components	Model
105	0.633	0.163	-0.02	2	0.16	0.46	2	Week
92	0.437	0.258	-0.14	3	0.06	0.37	2	3 Month

4.4.3 UPLC-MS analysis of breast milk lipid fraction

A total of 383 samples were analysed, as well as three formula milks; Cow and Gate, Aptamil and SMA, a breakdown of the samples analysed is given in Table 24. A QC sample was analysed throughout the run, injected every 10 samples, to ensure analytical accuracy was maintained.

Table 24: Samples analysed using UPLC-MS (not including QC and formula samples).

	One week	Three months	Total
Number fore milk	106	93	199
Number hind milk	98	86	184
Total	204	179	383

Chromatograms from the lipid fraction of the breast milk QC sample, analysed using UPLC-MS in positive and negative ion mode, were overlaid, Figure 48. This clearly displays how different metabolites are selectively ionized in different polarities. Retention times were observed to be very accurate at the beginning of the run, with increasing drift in retention time during the middle of the run, and then improving again towards the end of the run, as displayed by the width of the peaks.

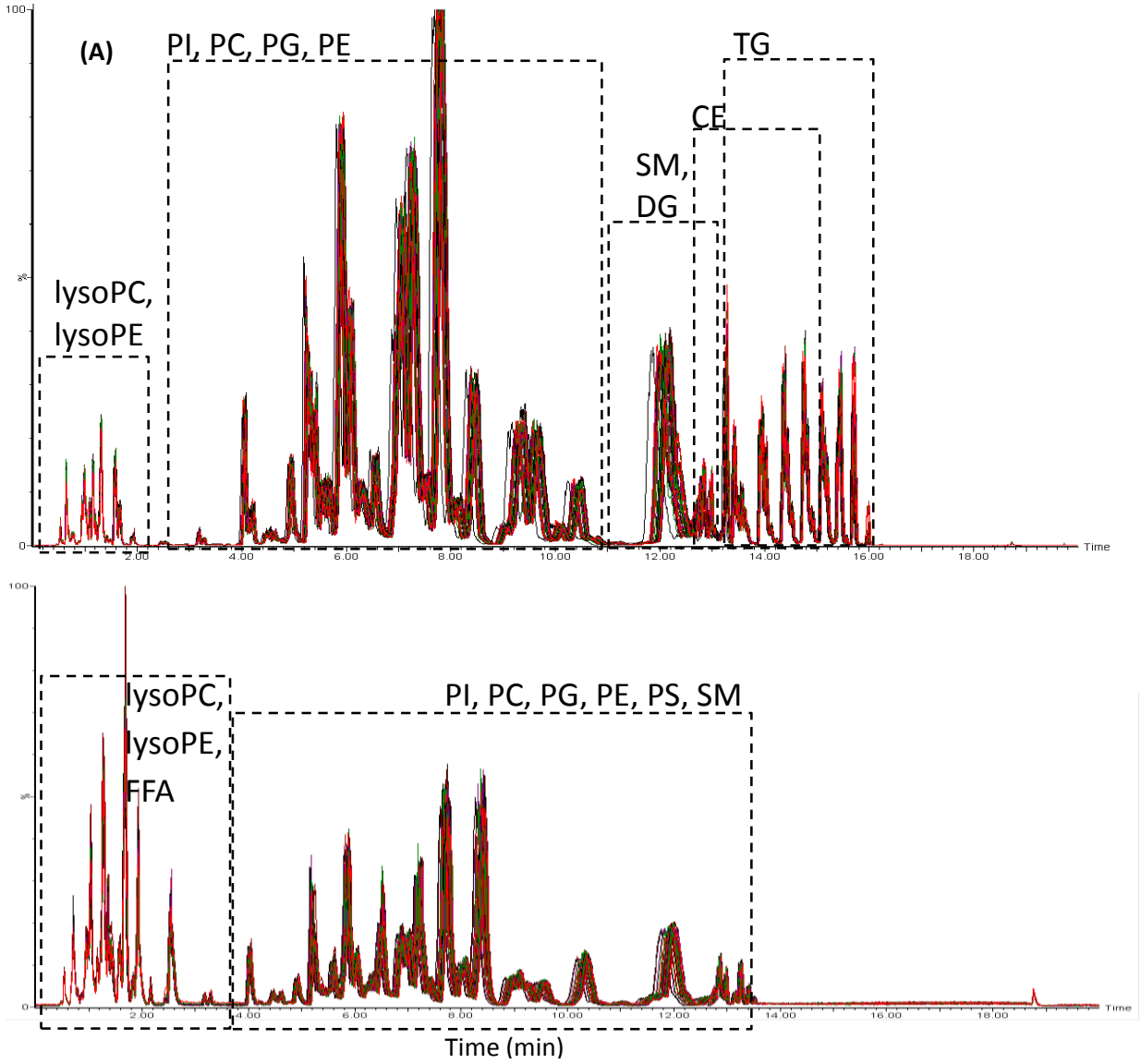


Figure 48: (A) Base Peak Chromatogram (BPC) of all lipid sample QCs overlaid, analysed in positive ionization mode. (B) BPC of lipid sample QCs overlaid, analysed in negative ionization mode. CE, cholesterol esters; DG, diacylglycerols; FFA, free fatty acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PG, phosphatidylglycerols; PI, phosphatidylinositols; PS, phosphatidylserines; SM, sphingomyelins; TG, triacylglycerols.

Data were pre-processed using RStudio, as outlined in 2.1.26, Chapter 2. Pre-processing involved filtering of noise from the data, alignment; to reduce the retention time drift seen in the above chromatograms, and a further filtering step to remove peaks with a high coefficient of variation. Once this processing was complete, data were imported into SIMCA-P+ for multivariate analysis. For both positive and negative ionization mode, data were scaled using Pareto scaling, as this was found to be the most appropriate method of scaling the MS data. UV scaling of MS data results in too much importance given to background noise which may not have been filtered out.

4.4.3.1 UPLC-MS multivariate analysis

PCA scores plots were visualized for all data in order to observe grouping of data, and exclude any outliers. The PCA scores plot of the positive and negative mode data are shown in Figure 49 and 50 respectivley.

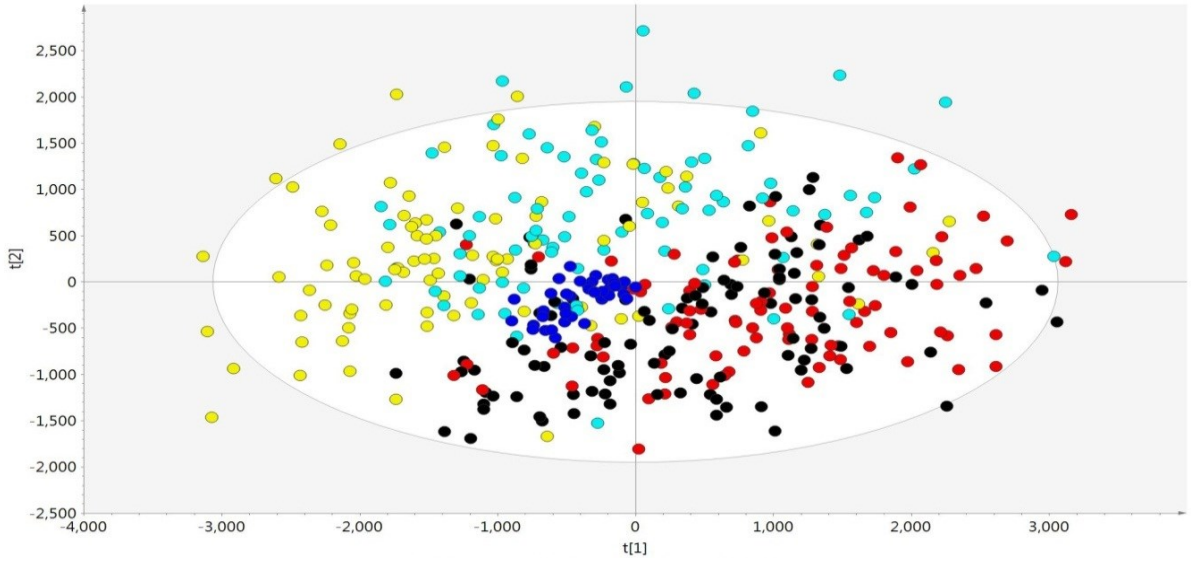


Figure 49: PCA scores plot showing all breast milk lipid samples analysed in positive ionization mode. Black and red display the one week fore and hind milk samples collected. In yellow and turquoise are the fore and hind milk samples collected at three months post-partum. Blue circles represent QC samples. R^2X=0.314, Q^2X=0.255.

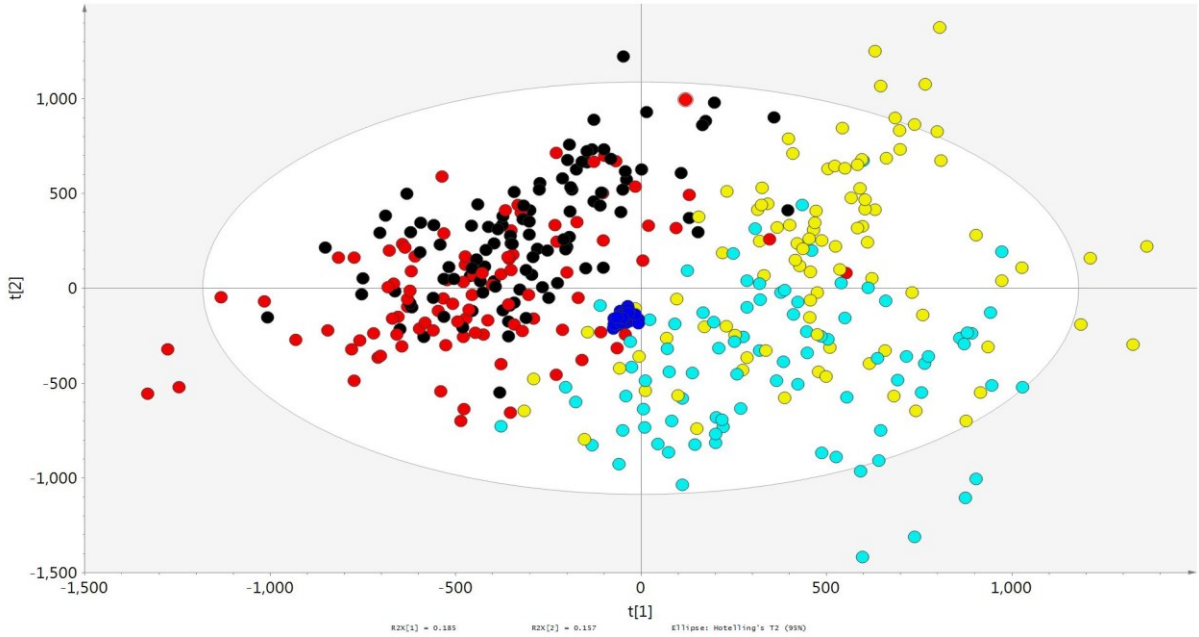


Figure 50: PCA scores plot showing all breast milk lipid samples analysed in negative ionization mode. Black and red display the one week fore and hind milk samples. Yellow and turquoise display the fore and hind milk samples collected at three months post-partum. Blue circles represent QC samples. R^2X=0.607, Q^2X=0.507.

For both positive and negative ionization mode, 2 samples we excluded as they were mislabelled. A further 6 samples were excluded in the outlier analysis, as they were distant from the Hotellings 95% CI.

On investigation of the chromatograms corresponding to these samples, it was observed that, for four of these samples, the abundance of triacylglycerides was much reduced, with a concurrent increase in the concentration of diacylglycerides, potentially as the triacylglycerides had either been enzymatically digested, or had degraded. Another outlier also had an incredibly high abundance of diacylglycerides, but no decrease in triacylglycerides. One more outlier was identified as having very high concentrations of triacylglycerides, but not diacylglycerides. Four of these were matched fore and hind milk samples.

The scores plot show the QCs clustering tightly in the middle of the scores plot, indicating that the data collected were of good quality. PCA scores plots for the individual time points in positive and negative ionization mode are shown in Appendix 4A and B respectively. Also seen in the scores plot is a clear separation between the two time points and differences between fore and hind milk, analysed in more detail in the Sections 4.4.3.4 and 4.4.3.5 below.

4.4.3.2 Analysis of length of storage on milk lipidome

Alterations in sample composition based on length of storage were investigated. Data were modelled using OPLS-DA, comparing samples stored for less than, and greater than 300 days. The Q^2 value for the first component in this OPLS-DA model was negative in positive ionization mode, whilst for negative ionization mode the Q^2 value was 0.04 ; suggesting length of storage did not affect milk lipidome.

4.4.3.3 Investigation of run time effects

To investigate the quality of the data acquired in terms of the length of analysis, as this occurred over several weeks, an OPLS model was computed, using the run order number as the Y variable. The scores plots for these models are shown in Figure 51 and Figure 52, for positive and negative ionization mode respectively.

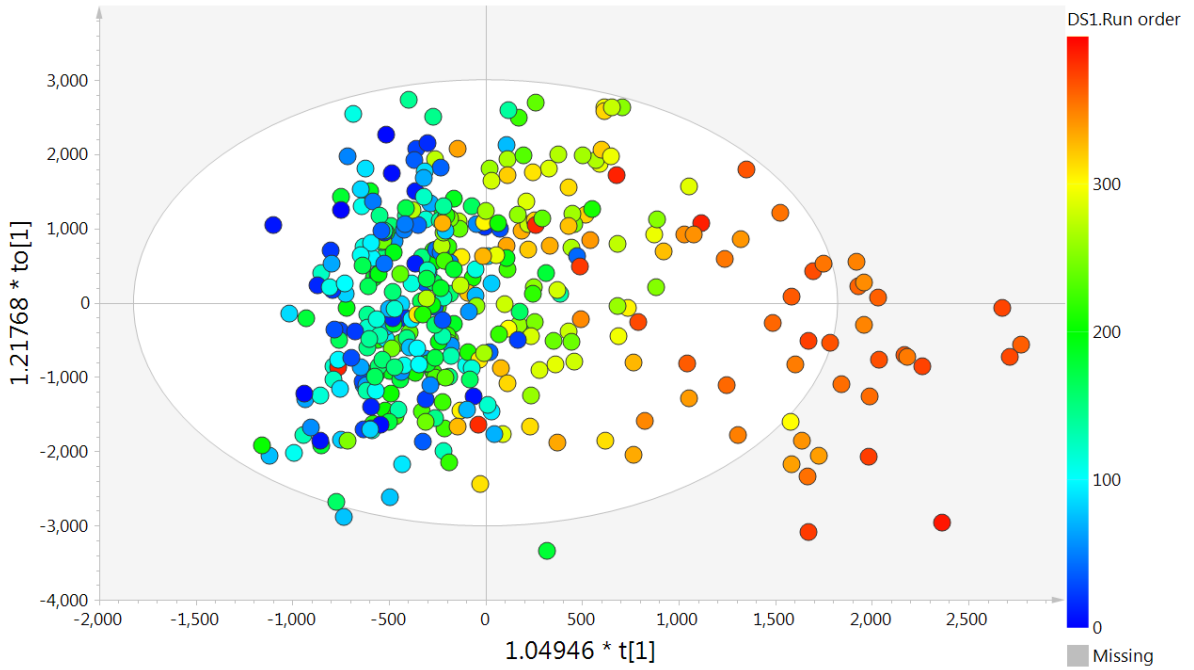


Figure 51: OPLS scores plot of all breast milk lipid samples, classified and coloured by the run order, blue being the first samples analysed with red the last. R^2X=0.285, R^2Y=0.508, Q^2=0.451, positive ionization mode.

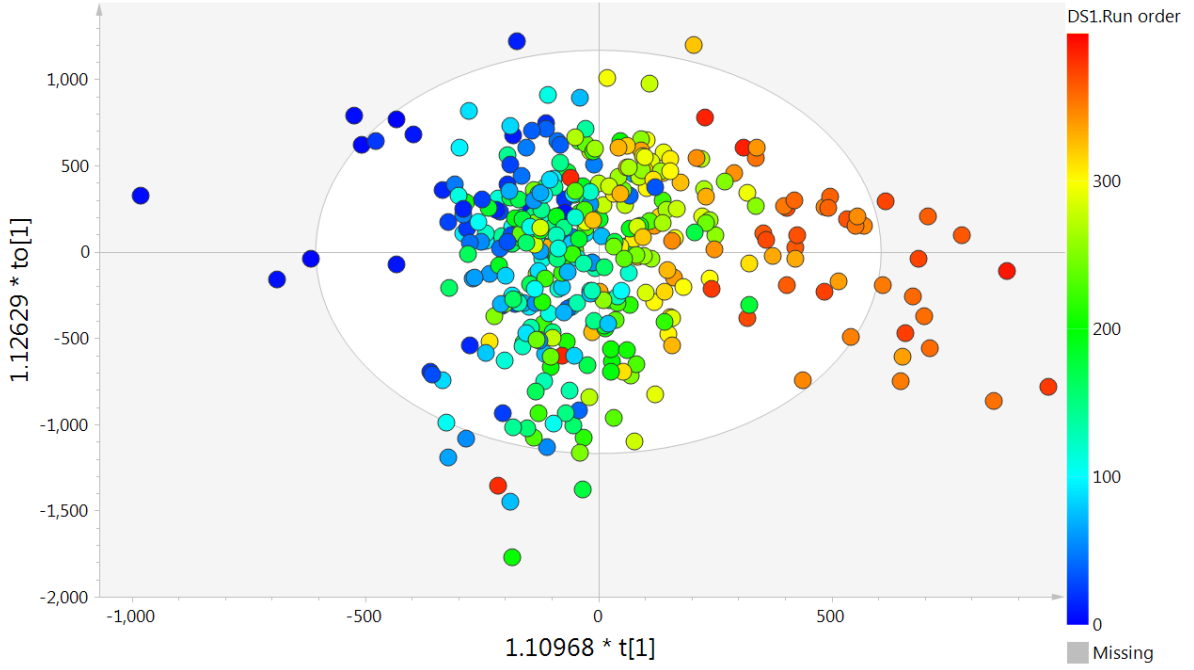


Figure 52: OPLS scores plot of all breast milk lipid samples, classified and coloured by the run order, blue being the first samples analysed with red the last. R^2X=0.224, R^2Y=0.556, Q^2=0.499, negative ionization mode.

As Figures 51 and 52 show, highly predictive models were produced; meaning the run order of samples produced a systematic change in sample composition over the length of the run, which occurred over

a week. This effect appeared to affect predominantly samples run in the last rack, in the positive mode data with other samples being relatively comparable to one another.

This effect is most likely due to the changing conditions of the column, as the pressure was seen to increase throughout the run, effecting the time at which metabolites eluted. It was hoped that the data processing involving retention time correction would account for this, but some effect of length of analysis remained.

4.4.3.4 Analysis of fore and hind milk

PCA and OPLS-DA comparisons of fore and hind milk in positive and negative ionization mode are displayed in Figure 53 and 54 respectively.

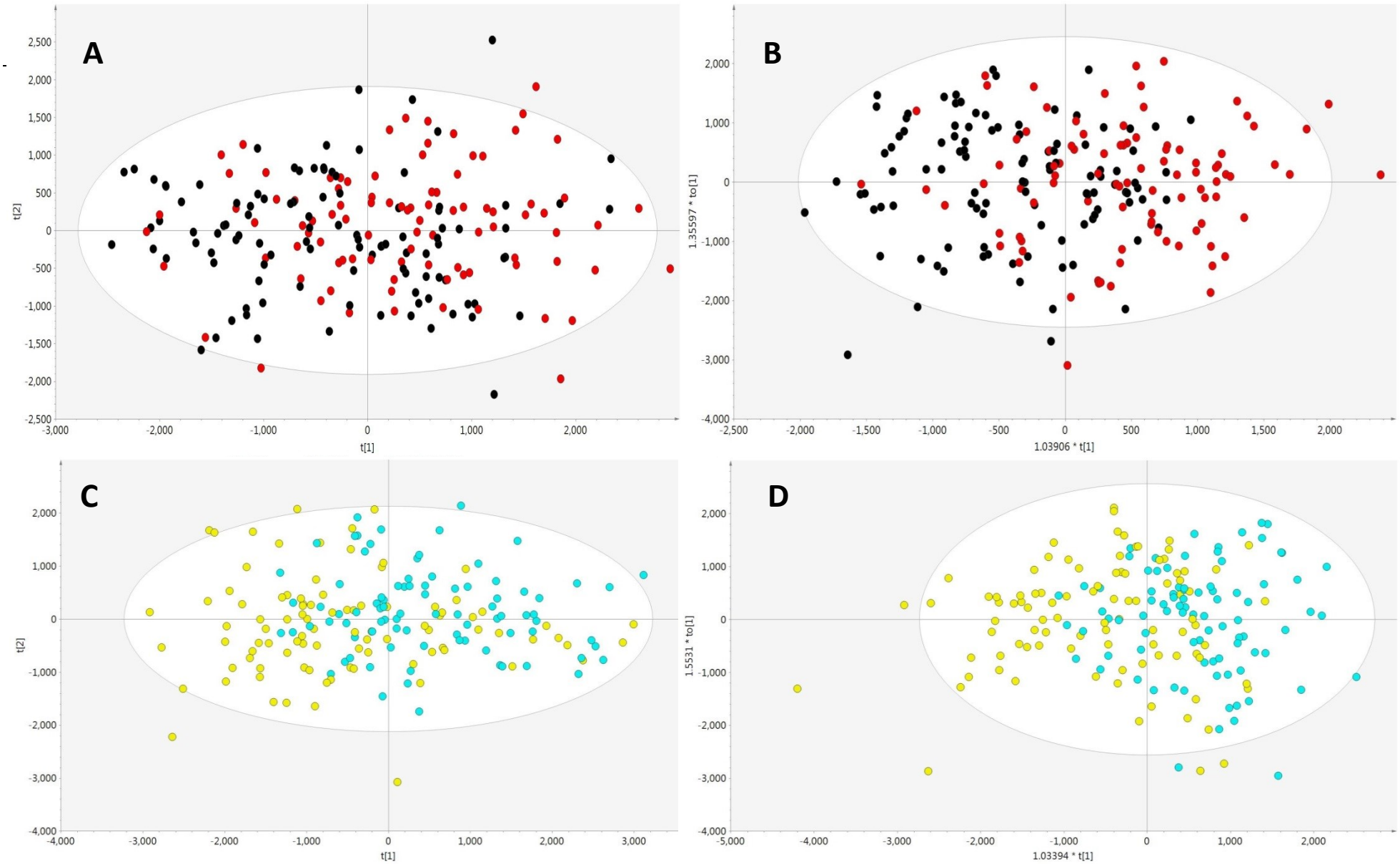


Figure 53: A PCA scores plot comparing fore milk samples (black) against hind milk samples (red) collected at one week post-partum, R^2=0.409, Q^2=0.283. **B** OPLS-DA scores plot comparing fore (black) and hind milk (red) samples collected at one week post-partum, R^2X=0.257, R^2Y=0.277, Q^2=0.08. **C** PCA scores plot comparing fore milk samples (yellow) against hind milk samples (turquoise) collected at three months post-partum, R^2=0.354, Q^2=0.304. **D** OPLS-DA scores plot comparing fore (yellow) and hind milk (turquoise) samples collected at three months post-partum, R^2X=0.332, R^2Y=0.298, Q^2=0.167 in positive Ionisation mode.

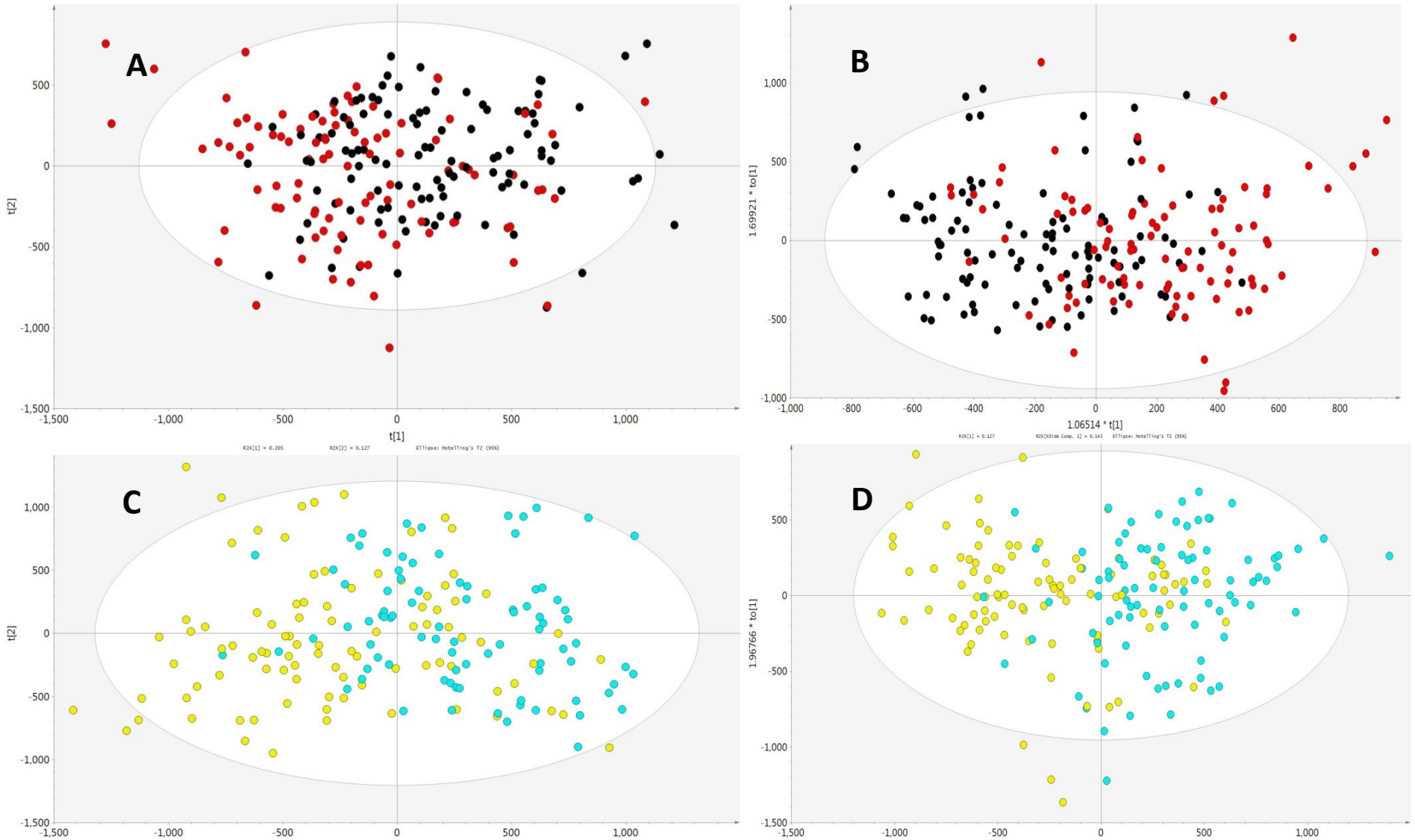


Figure 54: A PCA scores plot comparing fore milk samples (black) against hind milk samples (red) collected at one week post-partum, R^2=0.332 Q^2=0.293. **B** OPLS-DA scores plot comparing fore (black) and hind milk (red) samples collected at one week post-partum, R^2X=0.269, R^2Y=0.312, Q^2=0.08. **C** PCA scores plot comparing fore milk samples (yellow) against hind milk samples (turquoise) collected at three months post-partum, R^2=0.422, Q^2=0.383. **D** OPLS-DA scores plot comparing fore (yellow) and hind milk (turquoise) samples collected at three months post-partum, R^2X=0.31, R^2Y=0.375, Q^2=0.104 in negative ionisation mode.

There appeared to be some separation of the fore and hind milk samples in the PCA models of samples collected at each time point, in both positive and negative ionization mode. However, unexpectedly, the OPLS-DA models were not very strong in relation to their Q^2Y values, typically being around 0.1, meaning they were not very predictive of class designation.

4.4.3.5 Differences in breast milk lipid fraction taken 7 days post-partum compared to 3 months post-partum

Scores plots of PCA and OPLS-DA models comparing samples collected at different time points post-partum, in positive and negative mode, are displayed in Figure 55 and 56 respectively. As these models possess positive Q^2Y values, this indicates the duration of lactation causes a systematic difference in milk composition.

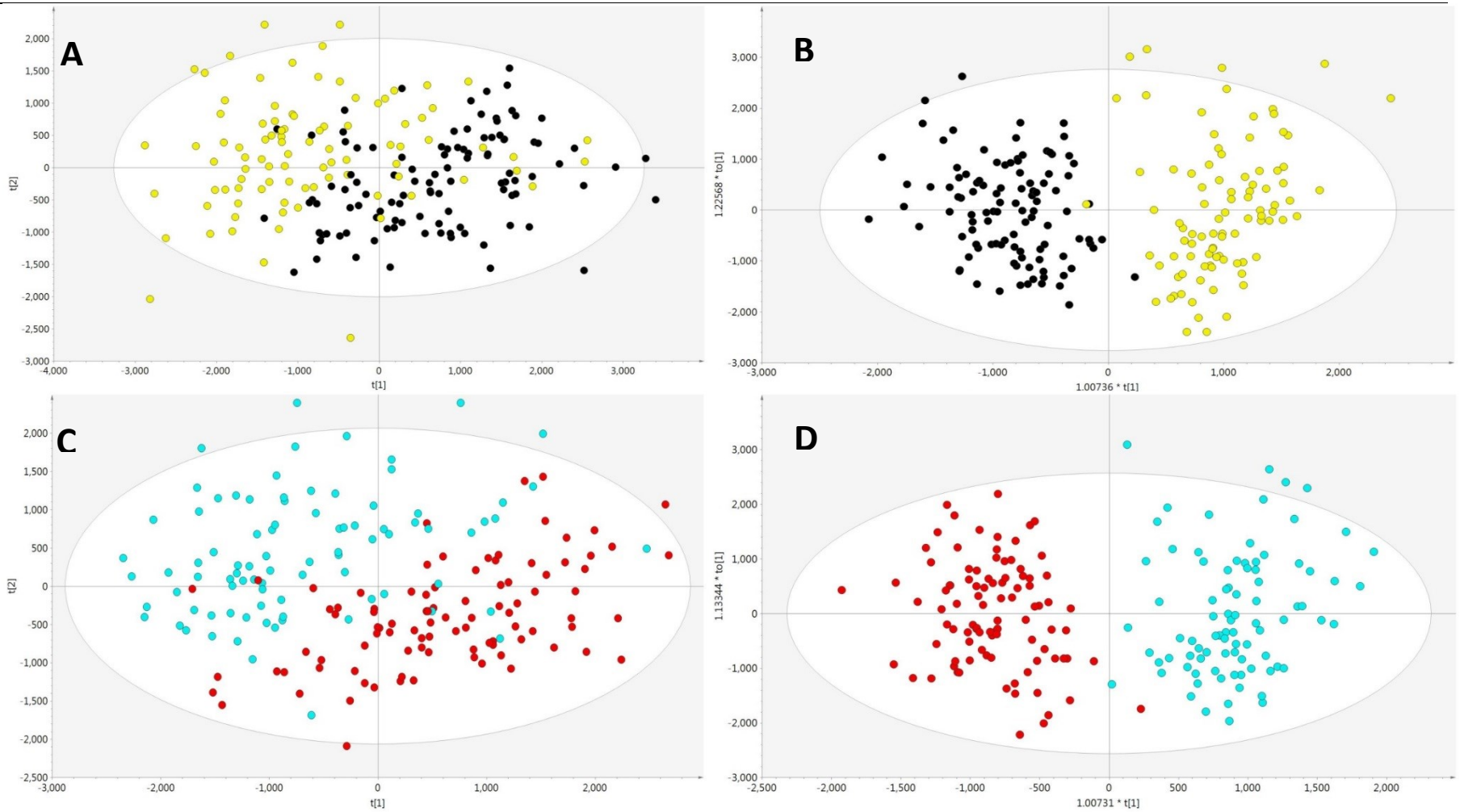


Figure 55: A PCA scores plot comparing 7 day fore milk samples (black) against fore milk samples collected at 3 months post-partum (yellow), R^2=0.342 Q^2=0.293. **B** OPLS-DA scores plot comparing fore milk at 7 days (black) and 3 months post-partum (yellow), R^2X=0.324, R^2Y=0.824, Q^2=0.801. **C** PCA scores plot comparing 7 day hind milk samples (red) against three month hind milk samples (turquoise), R^2=0.219, Q^2=0.212. **D** OPLS-DA scores plot comparing 7 day hind milk samples (red) against three month hind milk samples (turquoise), R^2X =0.277, R^2Y=0.854, Q^2=0.833, positive ionisation mode.

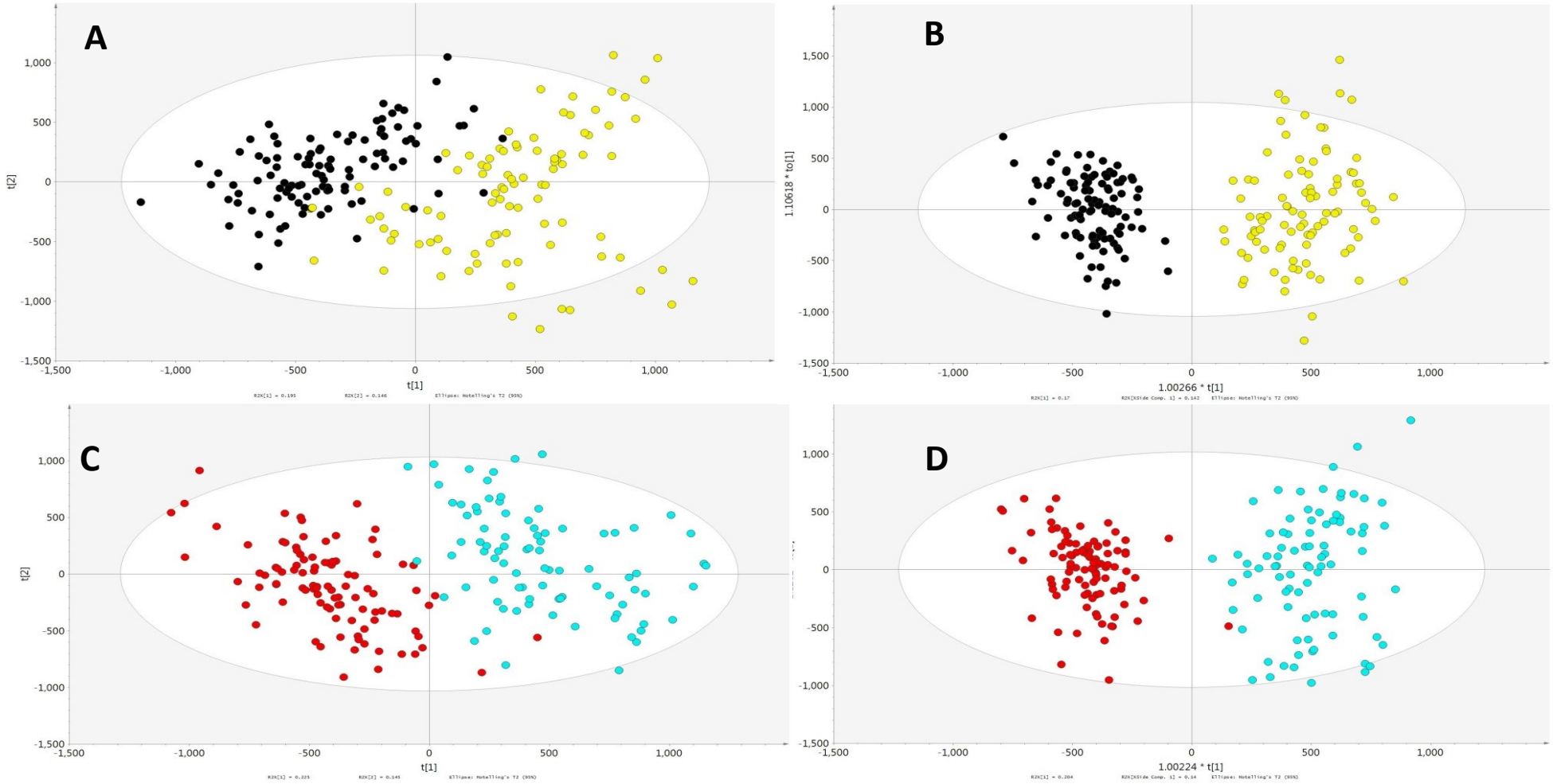


Figure 56: **A** PCA scores plot comparing 7 day fore milk samples (black) against fore milk samples collected at 3 months post-partum (yellow), R^2=0.342 Q^2=0.288. **B** OPLS-DA scores plot comparing fore milk at 7 days (black) and 3 months post-partum (yellow), R^2X=0.312, R^2Y=0.905, Q^2=0.894. **C** PCA scores plot comparing 7 day hind milk samples (red) against three month hind milk samples (turquoise), R^2=0.369, Q^2=0325. **D** OPLS-DA scores plot comparing 7 day hind milk samples (red) against three month hind milk samples (turquoise), R^2X =0.345, R^2Y=0.9, Q^2=0.89 in negative ionisation mode.

To investigate the features or peaks responsible for differences between samples taken at different time points post-partum, S-plots were analysed, an example from this analysis is displayed in Figure 57. S-plots identify variables which most highly contribute to the separation of groups, in this case, fore milk collected at 7 days against fore milk samples collected at 3 months post-partum. This was done for both the positive and negative ionization mode models, and the responsible variables identifications were ascertained.

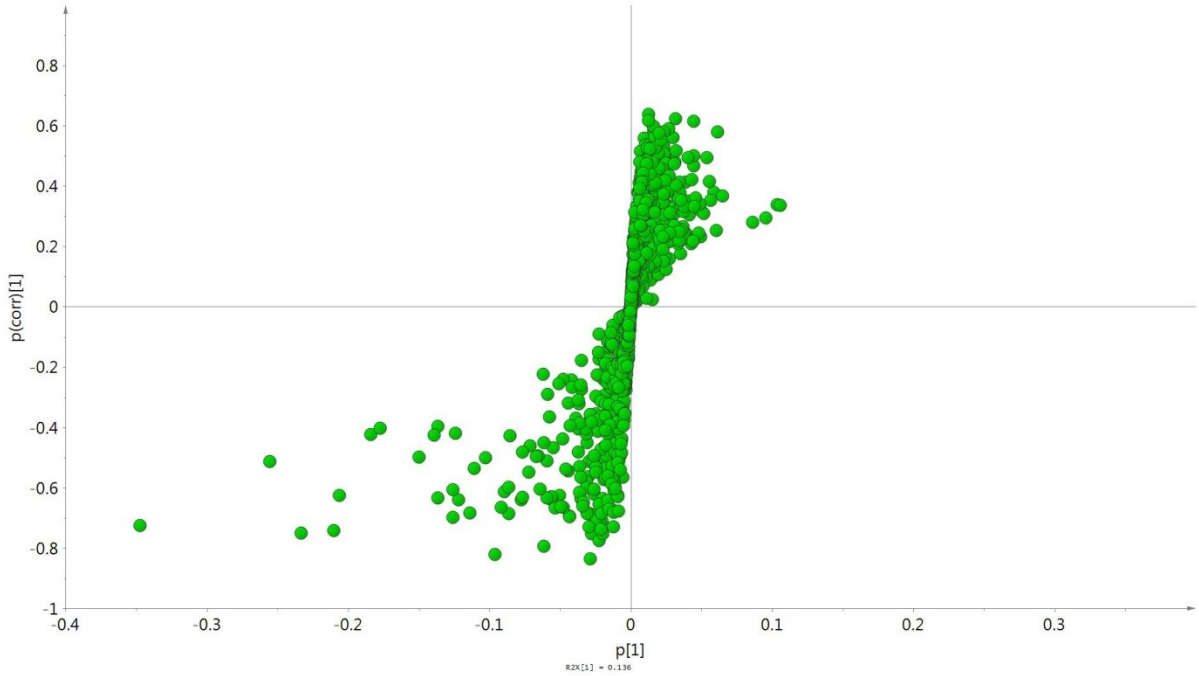


Figure 57: S-plot displaying data from an OPLS-DA model. Comparison of fore milk samples taken at one week against fore milk samples taken at three months post-partum, in negative ionization mode.

The *m/z* ratio and retention times were extracted for the variables located at the end of the tails of the plots. These *m/z* values were then searched for using METLIN, using all possible adducts, removing peptides from the search results, using an accuracy within 5 ppm.

Results were considered plausible if the fatty acid chain was an even number. Frequently, a large number of search results were returned, in order to better identify the most likely metabolite the original chromatogram was visualised, as well as the MS/MS chromatogram. In the MS/MS chromatogram the fragments of the original chromatogram are displayed, MS/MS is described in more detail in Section 2.4, Chapter 2. For MS/MS analysis, a greater collision energy is applied in the quadrupole; as the metabolites fragment in a predictable manner, the head groups of the different phospholipid were searched for to more confidently identify metabolites present in the chromatogram. Because different phospholipids possess different head groups, fragmenting from the remainder of the metabolite, the species of phospholipid can be inferred. For example, if a

phosphatidylcholine is detected, the head group fragment presents in the mass spectrum with a *m/z* value of 184.07, whereas a phosphotidylethanolamine produces a head group fragment with a mass of 141 atomic units.

Due to the difficulty in identification, outlined previously in Section 4.1, the following are considered to be tentative identifications. In order to definitely identify a metabolite using mass spectrometry, the compound of interest must be compared against a standard of sufficient purity, using identical experimental conditions. If the retention time, *m/z* value and fragmentation pattern are identical, only then it can be considered as the same metabolite (Sumner et al., 2007). Due to the impracticality and expense of running thousands of standards, only tentative identification was undertaken.

Table 25 and 26 display the identifications of metabolites altered in concentration from the 7 day time point to the 3 month collection.

Table 25: Metabolites increased in fore milk samples taken at one week against fore milk samples taken at three months post-partum. Analysed in positive and negative ionization mode. PC; phosphatidylcholine, PE; phosphatidylethanolamine, PI; phosphatidylinositol, SM; sphingomyelin.

Experimental *m/z*	Theoretical *m/z*	Retention Time (min)	ppm	Ion	Examples of potential metabolites	Formula
722.513	723.5203	6.87	0	$[M-H]^-$	PE (36:4)	C41H74NO7P
778.562	733.5622	7.00	2	$[M+FA-H]^-$	PC(22:0)	C40H80NO8P
804.578	759.5778	7.20	2	$[M+FA-H]^-$	PC(24:1)	C42H82NO8P
857.678	812.6771	11.93	3	$[M-H]^-$	SM(42:2)	C47H93N2O6P
885.552	886.5571	5.60	2	$[M-H]^-$	PI (38:4)	C47H83O13P
706.541	705.5309	5.37	4	$[M+H]^+$	PC (30:0)	C38H76NO8P
734.573	733.5622	7.04	4	$[M+H]^+$	PC (32:0)	C40H80NO8P
758.572	757.5622	5.88	3	$[M+H]^+$	PC (34:2)	C42H80NO8P
760.588	759.5778	7.25	3	$[M+H]^+$	PC (34:1)	C42H82NO8P
762.605	761.5935	9.29	5	$[M+H]^+$	PC (34:0)	C42H84NO8P
813.688	812.6771	12.03	4	$[M+H]^+$	SM (42:2)	C47H93N2O6P

Table 26: Metabolites which were increased in hind milk samples taken at three months against hind milk samples taken at one week post-partum. Analysed in positive and negative ionization mode. PE; phosphotidylethanolamine, SM; sphingomyelin.

Experimental *m/z*	Theoretical *m/z*	Retention Time (min)	ppm	Ion	Examples of potential metabolites	Formula
279.229	280.2402	1.96	14*	$[M-H]^-$	Linoleic acid	C18H32O2
281.245	282.2559	2.55	12*	$[M-H]^-$	Oleic acid	C18H34O2
476.276	477.2855	1.04	4	$[M-H]^-$	PE (18:2)	C23H44NO7P
478.292	479.3012	1.27	3	$[M-H]^-$	PE (18:1)	C23H46NO7P
740.524	741.5309	6.51	0	$[M-H]^-$	PE (36:3)	C41H76NO8P
742.541	743.5465	8.39	2	$[M-H]^-$	PE (36:2)	C41H78NO8P
803.629	758.6302	9.17	0	$[M+FA-H]^-$	SM (38:1)	C43H87N2O6P
831.66	786.6615	12.10	0	$[M+FA-H]^-$	SM (40:1)	C45H91N2O6P
731.608	730.5989	6.95	2	$[M+H]^+$	SM (36:1)	C41H83N2O6P
744.556	743.5456	8.39	3	$[M+H]^+$	PE (36:2)	C41H78NO8P
759.64	758.6302	9.24	3	$[M+H]^+$	SM (38:1)	C43H87N2O6P
787.672	786.6615	12.21	4	$[M+H]^+$	SM (40:1)	C45H91N2O6P

* Mass accuracy was compromised, possibly due to calibration of the lower m/z range. In previous analysis (Chapter 2), the same two features were tentatively identified as linoleic and oleic acid, with a small ppm error value.

4.4.3.6 Regression of breast milk lipidome of mothers with different BMI

OPLS regression models were computed to investigate differences in the lipid fraction of breast milk from mothers with a different BMI. Table 27 outlines models computed using maternal BMI at the mothers booking visit.

Table 27: OPLS models computed comparing mothers with different BMI using booking BMI values.

N	R^2X	R^2Y	Q^2Y	Outliers	t[1]	t[2]	Components	Time samples taken	Mode
105	0.171	0.399	-0.07	3	0.5	0.11	2	1 Week	-
92	0.249	0.371	-0.20	4	0.04	0.2	2	3 months	-
105	0.25	0.332	-0.223	2	0.05	0.19	2	1 week	+
91	0.295	0.384	-0.211	5	0.04	0.24	2	3 months	+

As displayed in Table 27, all models possessed a negative Q^2Y value, meaning that maternal BMI had no discernable influence on the lipid fraction of her breast milk composition, as measured using MS. Models outlined in Table 27 display results for the fore milk samples, similar results were obtained with hind milk. Furthermore, similar results were observed using maternal weight, and weight change.

4.4.4 Mid IR analysis of macronutrient content

A total of 296 samples were analysed. After removing duplications and samples which had not analysed correctly (due to reading zero, as air was present in the instrument), 284 samples were available for statistical analysis, Table 28.

Table 28: Number of samples analysed for macronutrient content.

	One week	Three months	Total
Number fore milk	95	90	185
Number hind milk	40	59	99
Total	135	149	284

The total number of samples available for analysis using mid IR was less for that of the other techniques as this technique required a larger sample volume (3ml). As well as this, mid IR spectroscopy was used after metabonomic analysis, meaning some sample volume had already been used for the other techniques.

Histograms displaying the distribution of macronutrient content data in fore and hind milk samples collected at 7 days post-partum are displayed in Figures 58 and 59 respectively. Data corresponding to fore and hind milk samples collected at 3 months post-partum are shown in Appendix 4C and D respectively. As these figures display, the data were normally distributed.

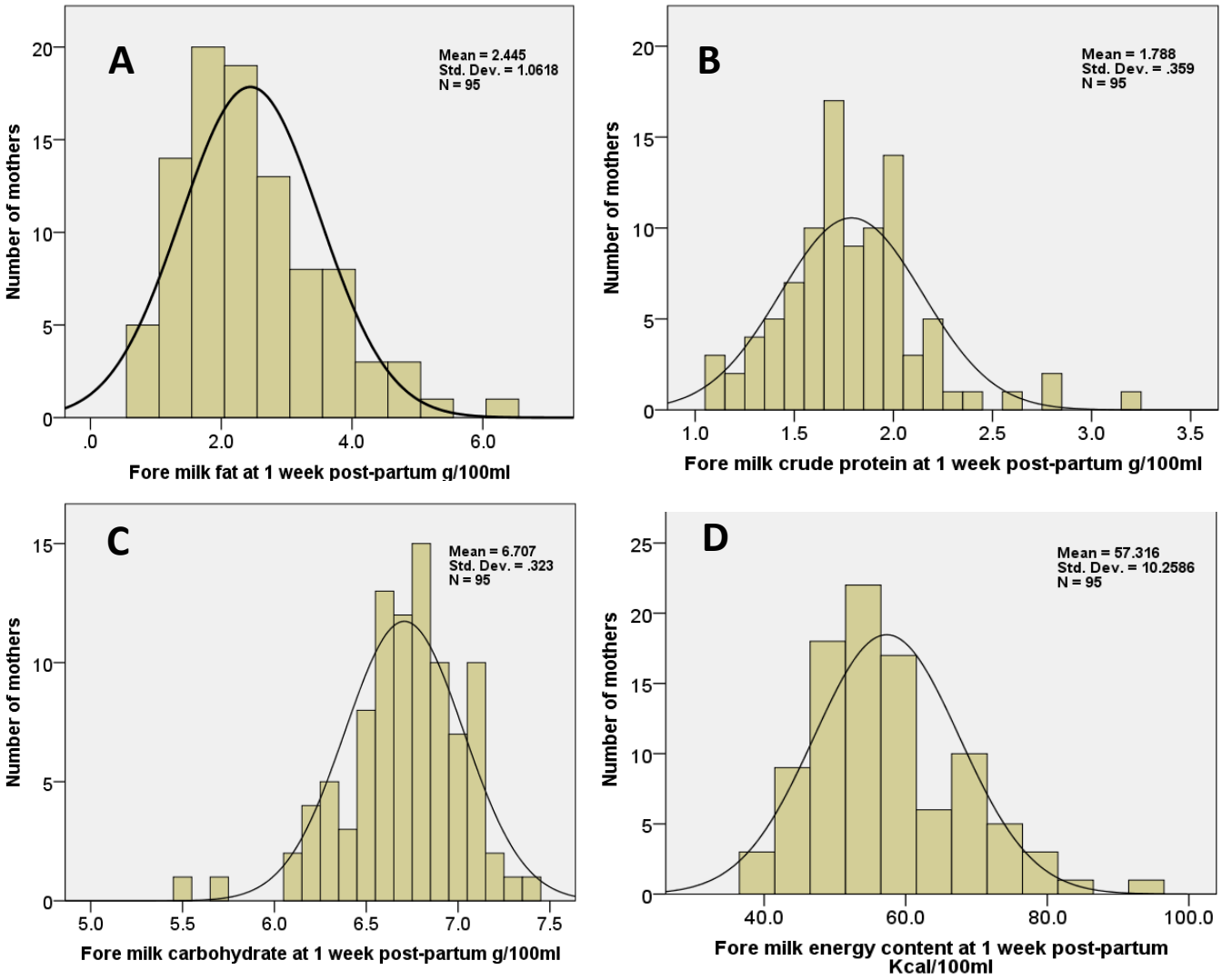


Figure 58: Histograms displaying the distribution of macronutrients in fore milk samples collected at one week post-partum. (A) Fat, (B) crude protein, (C) carbohydrate in g/100ml and (D) energy content Kcal/100ml.

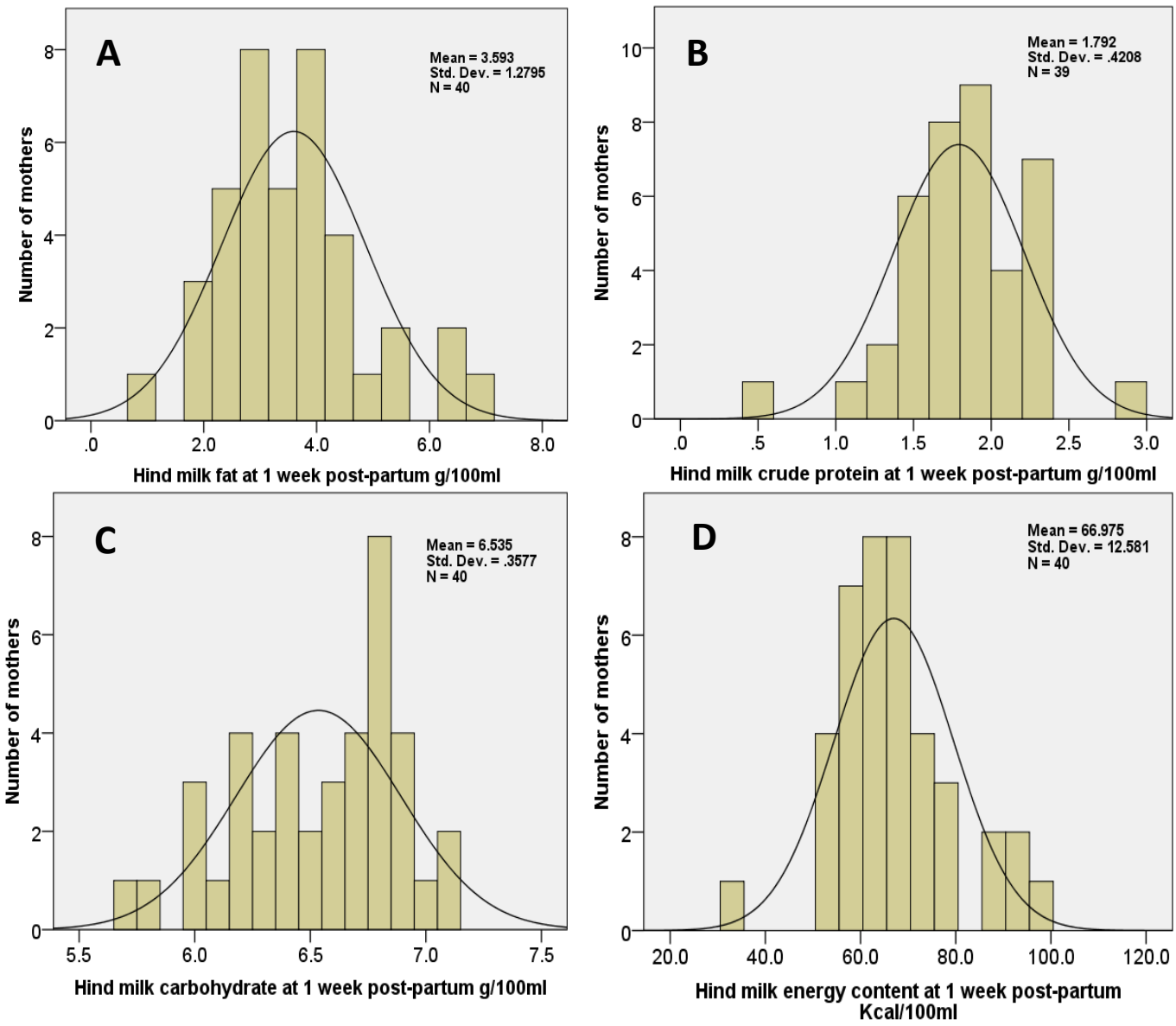


Figure 59: **Histograms displaying the distribution one week post-partum.** (A) Fat, (B) crude protein, (C) carbohydrate in g/100ml and (D) energy content Kcal/100ml.

4.4.4.1 Analysis of fore and hind milk collected at different times post-partum

Table 29 displays the mean concentration of the different macronutrients for these different samples, as well as whether there was a statistically significant difference between them, as established by a repeated measures ANOVA.

Table 29: Descriptions of the means, standard error of the macronutrient content of the milk samples collected at different times post-partum. Based on a repeated measures ANOVA.

Type of milk	Stage of lactation: mean (Standard Error) [n]		
	1 week	3 Months	p-value
Fat g/100ml			
Fore	2.4 (0.2) [95]	2.4 (0.1) [90]	0.85
Hind	3.6 (0.3) [40]	4.3 (0.2) [59]	0.01
p-value	<0.01	<0.01	
Carbohydrate g/100ml			
Fore	6.7 (0.04) [95]	7.9 (0.03) [90]	<0.01
Hind	6.5 (0.06) [40]	6.8 (0.04) [59]	<0.01
p-value	<0.01	<0.01	
True Protein g/100ml			
Fore	1.4 (0.05) [95]	0.8 (0.04) [90]	<0.01
Hind	1.4 (0.07) [40]	1.0 (0.05) [59]	<0.01
p-value	0.86	<0.01	
Crude protein g/100ml			
Fore	1.8 (0.1) [95]	1.1 (0.04) [90]	<0.01
Hind	1.8 (0.1) [40]	1.3 (0.1) [59]	<0.01
p-value	0.71	<0.01	
Total solids g/100ml			
Fore	11.1 (0.2) [95]	10.6 (0.2) [90]	0.02
Hind	12.1 (0.3) [40]	12.6 (0.2) [59]	0.09
p-value	<0.01	<0.01	
Total energy kcal/100ml			
Fore	57.3 (1.9) [95]	54.7 (1.4) [90]	0.18
Hind	67.2 (2.7) [40]	72.8 (1.8) [59]	0.04
p-value	<0.01	<0.01	

As Table 29 shows, hind milk was observed to contain significantly more fat, and less carbohydrate than fore milk, regardless of the stage of lactation. However, protein concentration in fore and hind milk differed with stage of lactation: at 7 days post-partum, there was no difference between fore and hind milk; but at 3 months post-partum there was a statistically significant increase protein concentration in hind milk, p=<0.01. Between 7 days and 3 months of lactation, in fore milk, the concentration of fat remained stable, whilst carbohydrate content increased and protein decreased; in hind milk there were significant increases in fat and carbohydrate, but a significant decrease in the concentration of protein.

4.4.4.2 Macronutrient correlations

Further investigation of the data revealed various correlations between the different macronutrients, as displayed in Figures 60.

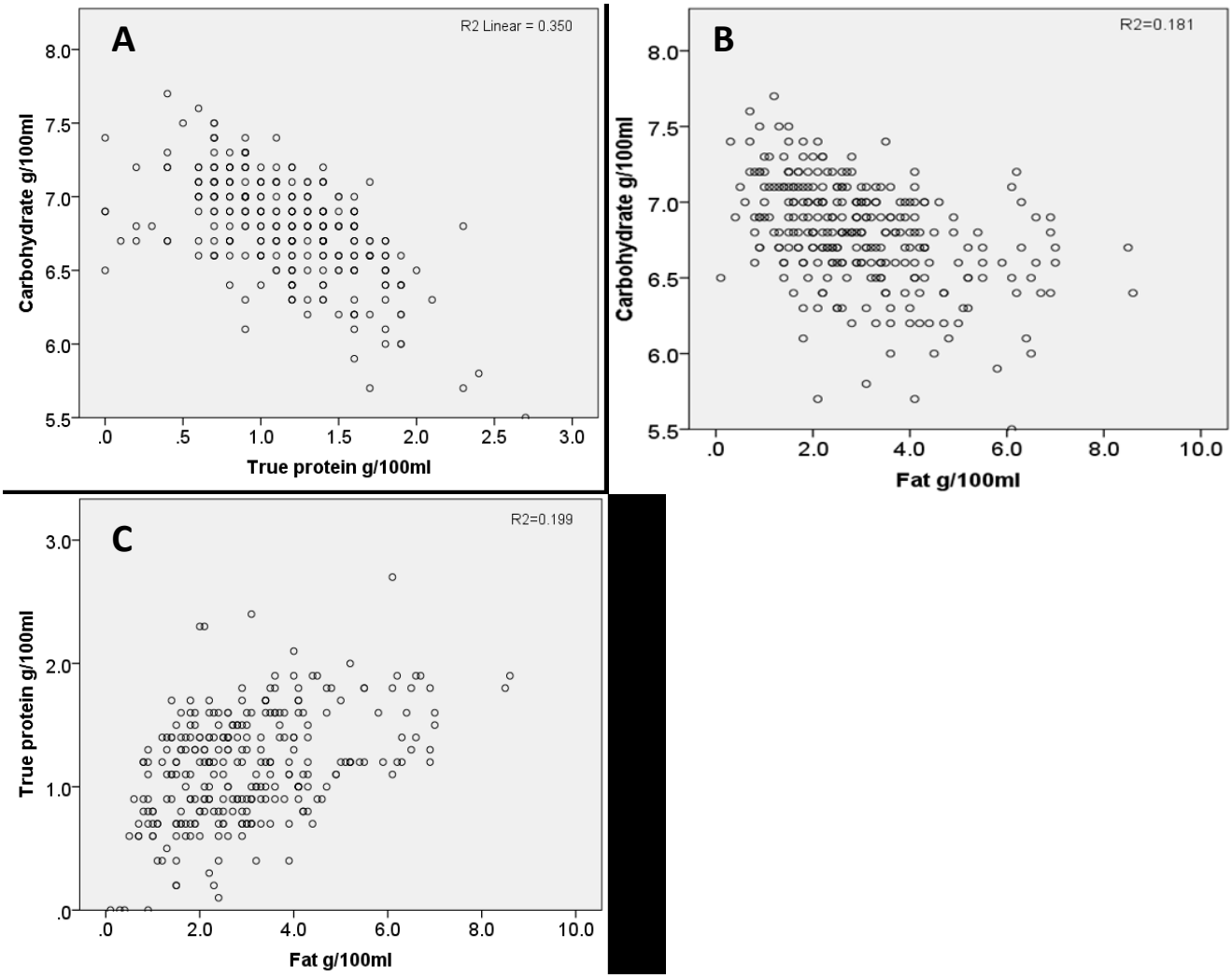


Figure 60: Scatter plots displaying correlations between breast milk macronutrient content in fore milk samples collected at one week post-partum. (A) True protein concentration plotted against carbohydrate, (B) Fat concentration against carbohydrate concentration, (C) Fat concentration against true protein concentration.

This analysis revealed a negative correlation between breast milk carbohydrate content and fat and protein concentration, and a positive correlation between fat and protein concentrations, at one week post-partum Figure 60. Similar results were obtained for samples collected at three months post-partum.

4.4.4.3 Association between breast milk macronutrient content and maternal BMI

Scatter plots were produced to investigate whether there was a correlation between maternal BMI and the macronutrient content of breast milk in fore milk samples collected at 7 days post-partum. These plots are displayed in Figure 61.

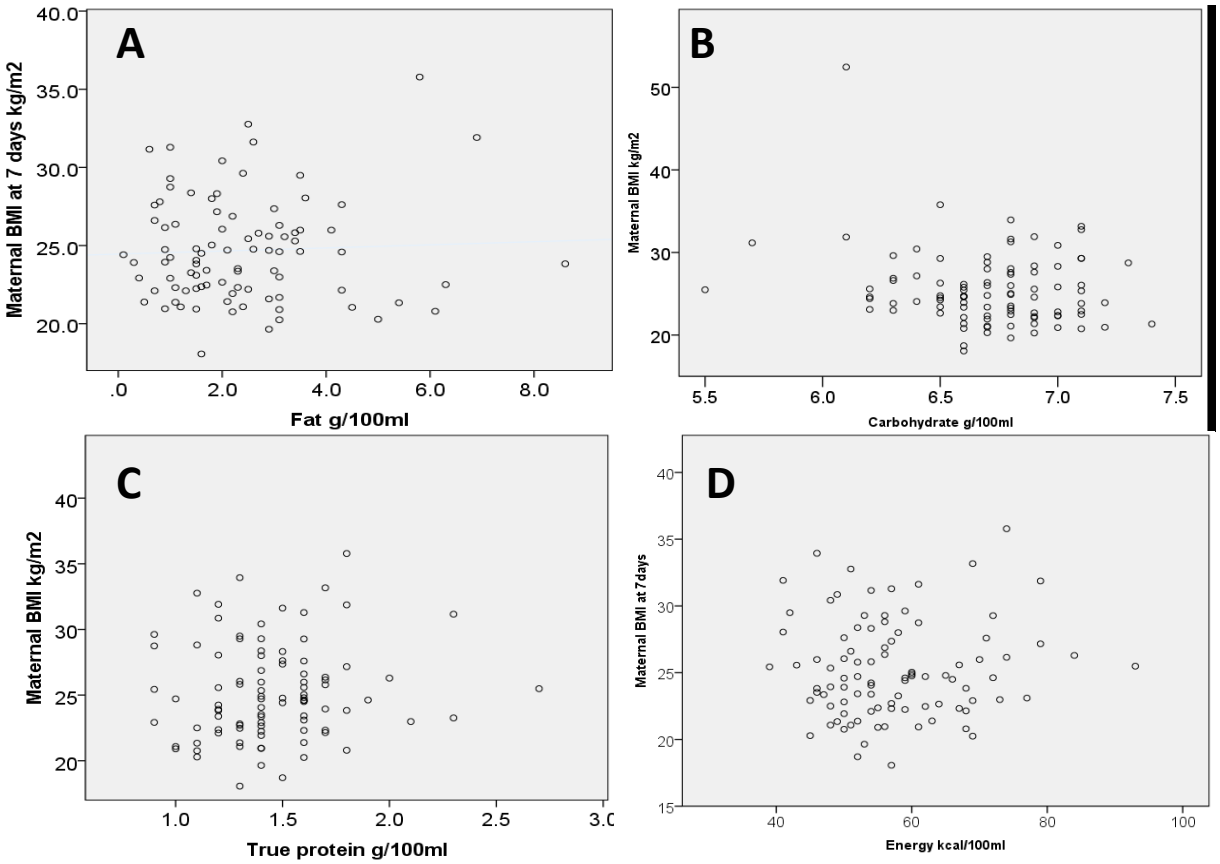


Figure 61: Macronutrient content in fore milk samples, taken at one week postpartum, plotted against maternal BMI at one week post-partum. Maternal BMI at sample collection plotted against (A) Fat, (B) Carbohydrate, (C) True protein and (D) Energy.

These figures demonstrate that there was no correlation between maternal BMI and the macronutrient content of the breast milk. This lack of correlation was also observed for hind milk samples, and the fore and hind milk samples collected at three months post-partum. Further analysis also revealed there was no correlation between the macronutrient content of breast milk using other measures of BMI, including booking and recruitment BMI, as well as maternal weight.

To try and control for confounding factors which were stable over time, maternal weight change was plotted against the change in the fat content of milk, from one week post-partum to three months post-partum. However, there was no relationship obeserved between these two factors, Figure 62.

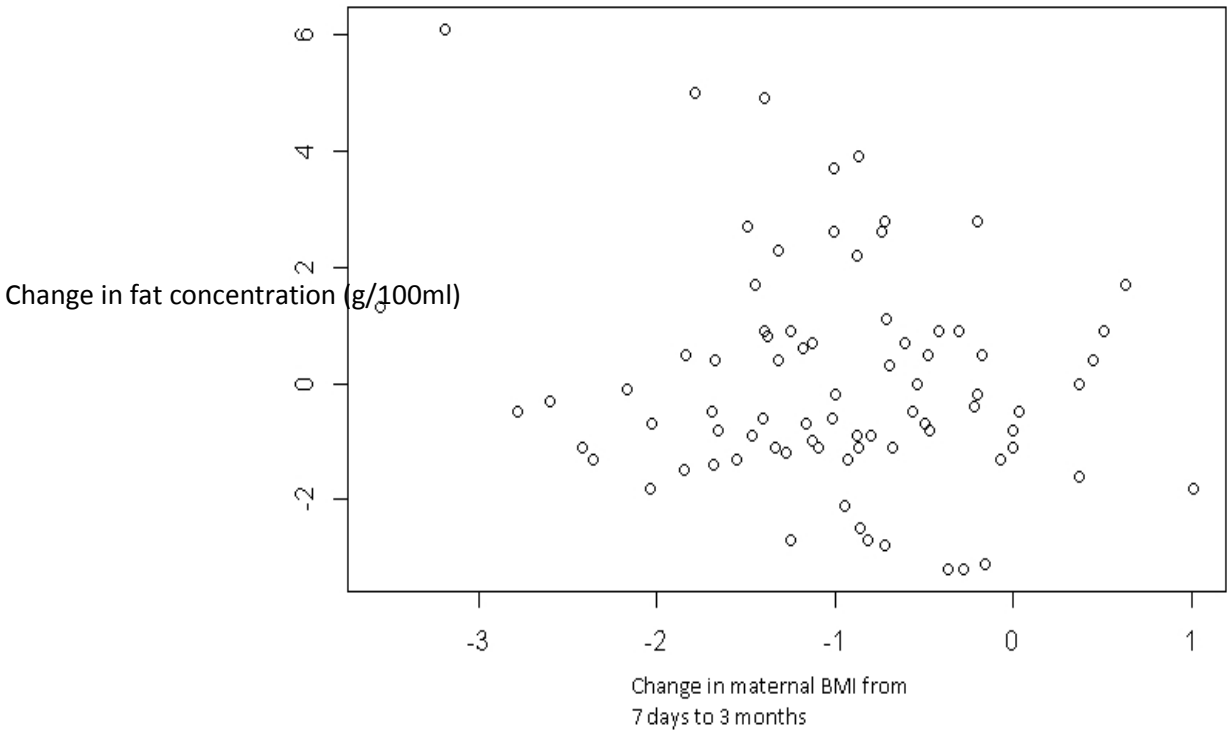


Figure 62: Scatter plot displaying the change in breast milk fat concentration from 7 days to 3 months post-partum plotted against the change in maternal BMI over this time, in kg/m². R^2 linear=0.01.

4.5 Discussion

The primary aims of this chapter were to establish whether maternal BMI exerted an influence on the aqueous, lipid or macronutrient content of breast milk, analysed using ^{1}H NMR spectroscopy, UPLC-MS and mid IR spectroscopy.

No differences were observed in the aqueous fraction of breast milk in relation to maternal BMI, analysed using ^{1}H NMR spectroscopy. This finding is in contrast to the only previous study investigating this relationship using the same technique. This study, undertaken by Smilowitz et al., reported a decrease in the concentration of acetone in mothers of a higher BMI, which was not observed in this study, (Smilowitz et al., 2013). However, Smilowitz et al. only investigated the breast milk of 52 women, and arguably did not have sufficient power to detect differences in the breast milk metabolome.

Furthermore, samples were prepared differently in these two studies, which must be taken into account as sample preparation can have a large impact on the composition of resulting samples. In the study by Smilowitz et al., samples were first centrifuged, and the aqueous layer was removed and filtered through a 3000 molecular weight cut-off filter to further remove lipids and proteins. To prepare samples for ^{1}H NMR spectroscopy throughout the present study, samples underwent solvent extraction, rather than these centrifugation and filtration steps, which may have contributed to differences in the findings.

No correlation was found between maternal BMI and breast milk macronutrient content in this study using mid IR spectroscopy. Again, this is in contrast to previous findings. One such study, undertaken by Michaelsen et al., noted that each of the macronutrients, protein, fat and carbohydrate increased in conjunction with maternal BMI (Michaelsen et al., 1990). However, the Michaelsen et al. study recruited twice the number of participants than the present study, and analysing over 2000 samples, and therefore had more power to detect whether there was a correlation between these parameters.

In agreement with results obtained from both ^{1}H NMR spectroscopy and mid IR spectroscopy, the lipid fraction of breast milk was not seen to have any predictable relationship with maternal BMI, at any time point measured, as analysed by UPLC-MS. Again this suggests that breast milk composition is regulated independently of maternal BMI

One issue encountered throughout this study was the withdrawal of mothers who had a higher BMI, reducing the power of the study to detect a difference in the breast milk composition of mothers of a

high BMI. This should be accounted for in future studies by ensuring the overweight/obese population is over sampled in order to address this issue.

Even though no differences in breast milk composition in regard to maternal BMI were observed, the sample set and metadata collected allowed for the analysis of multiple other influencing factors on breast milk composition, revealing several interesting associations.

In agreement with previous research, one of the largest influences on breast milk composition observed was the time samples were collected post-partum. Using ^{1}H NMR spectroscopy, it was found that samples collected later on in lactation displayed an increase in the abundance of lactose and glutamine and an associated decrease in the abundance of a number of different species of HMO.

These changes are most likely so highly conserved because they bestow a survival advantage to the infants receiving them, and are likely to be timed to provide key nutrients and other factors to the infant at specific times during development.

Again in agreement with the NMR and macronutrient analysis, differences in the lipid profile of samples collected at different times post-partum were also identified, with the responsible metabolites tentatively identified. The most variable of the metabolites based on time of collection were the phospholipids, which are the constituents of the milk fat lipid globule membrane. In general, in fore milk samples collected at one week post-partum, there was an increase in several phosphocholine species, whilst in the hind milk samples, an increase in several phosphoethanoamines and sphingomyelins was noted, in the samples collected at three months post-partum.

The macronutrient analysis revealed that between 7 days and 3 months of lactation, in fore milk, the concentration of fat remained stable, whilst carbohydrate content increased and protein decreased; in hind milk there were significant increases in fat and carbohydrate, but a significant decrease in the concentration of protein.

Protein concentrations in fore and hind milk were observed to differ based on time of collection post-partum, at 7 days post-partum there was no difference between the two, but at 3 months post-partum there a statistically significant increase protein concentration in hind milk. This may be explained by the infants removing more breast milk at this age, therefore having a greater impact on the composition of the milk (Neville et al., 1988).

Another prominent influence on breast milk composition appeared to be maternal genetic makeup; this was not tested, but was extrapolated from the results obtained, based on the classes of HMO present in mothers breast milk. In the NMR spectroscopic analysis, the largest differences in breast

milk composition related to HMO content, predominantly whether the mother was a secretor or non-secretor of 2'-FL, and other related HMO. Further investigation revealed a great deal of variation observed in the profiles of fucosylated HMO produced, the production of which is under genetic control. Large differences in the type and quantity of HMO found in breast milk were seen, known to be dependent on whether the mother was able to express specific fucosyltransferase enzymes.

Previous research has investigated the relationship between the concentration of various HMO and the composition of infant faecal microbiota. For example, on day two, *Streptococcus* was observed to be negatively correlated with 2'-FL (r=–0.94), whilst at day ten *Bacteroides* was positively correlated with 2'-FL, (r=0.73) (Williams et al., 2013). Similarly, *Lactococcus*, *Leuconostoc* and *Weissella* positively correlated with DSLN, (r=0.98, 0.92, and 0.93 respectively). These observations imply that the types and concentrations of HMO produced by a mother impact the species and diversity of her infants microbiota (Williams et al., 2013).

No differences were identified between fore and hind milk samples using ^{1}H NMR spectroscopy. No published literature exists to compare this data with. A possible explanation for this observation may be this analysis was undertaken on the aqueous fraction of breast milk. Because the largest differences between fore and hind milk are due to the increase in lipids in hind milk, this is potentially not reflected in the aqueous fraction. Furthermore, due to the sampling technique (hind milk samples were taken once the infant had fed), different volumes of breast milk will have been removed, acting as a confounding factor as if less milk was removed then the milk will have less lipid content, this factor is difficult to control. However, giving confidence in the analysis techniques used, alterations in lipid content were able to be distinguished between fore and hind milk samples using both UPLC-MS and mid IR spectroscopic analysis.

In regard to the comparison of fore and hind milk using data acquired from the UPLC-MS analysis, the models created did not have especially high Q^2Y values, in either ionization mode. This may be explained by the composition of fore and hind milk being similar in terms of species of molecule present, but the concentrations of the macronutrients changing, i.e. the types of lipid secreted do not alter within a single feed, just the concentration of the lipid, meaning the models produced were not particularly strong. Complicating this analysis was the fact that infants fed for differing amounts of time, meaning that it is difficult to compare these samples.

In regard to the macronutrient analysis, hind milk was observed to contain significantly more fat, and less carbohydrate than fore milk, independent of the stage of lactation. The macronutrient analysis also revealed a negative correlation between breast milk carbohydrate and fat and protein

concentration, whilst a positive correlation between fat and protein concentrations at one week post-partum was observed.

One difficultly experienced in the analysis of these samples was the length of time required to analyse the whole sample set. This introduced analytical artefacts in relation to run order, observed in both the ^{1}H NMR spectroscopy and mass spectrometry analysis. This was most likely identified due to the extremely high sensitivity of these techniques. In the ^{1}H NMR analysis, the abundance of lactose was seen to decrease from the beginning to the end of the analytical run. It is hypothesised that the lactose may have precipitated out of solution. In order to correct for this, the lactose peaks were removed from the spectra, and the resulting data were used for the multivariate analysis. However, clearly this is undesirable, as this treatment removes much information from the spectra, which may contribute towards differences between participants.

A number of outliers in the MS analysis were excluded. The majority of the excluded samples were observed to have much higher abundance of diacylglycerides than the QC samples, and a corresponding decrease in the abundance of the triacylglycerols. This observation is potentially due to degradation of these samples, due to the length of the analytical run. Alternatively, these samples may have been enzymatically digested, releasing the component parts of the triacylglycerides.

Likewise, for the UPLC-MS analysis run time effects were observed, it was hoped that the data pre-processing steps would account for this, via the correction of retention time deviation, but some effects clearly remained. However, giving confidence to the analysis, the scores plots produced suggested only the samples analysed on the last rack were statistically significantly different from the rest of the run. As well as this, the QC samples were relatively well clustered.

In relation to how these findings may impact the infant, each technique employed demonstrated that sample composition was conserved across mothers of a different BMI. Where alterations in sample composition did occur, it was in agreement with previous literature.

Analysis using NMR spectroscopy revealed increasing lactose concentrations and decreasing HMO concentrations from one week to three months post-partum. This may reflect the primarily protective effect of early milk for the infant, as early milk contains large quantities of antibodies and other immune factors, whilst at three months the increase in lactose may reflect the increased energy demands at this time point.

Tight regulation of breast milk composition has been demonstrated previously, for example, iron, zinc and copper concentrations in breast milk are independent of maternal mineral status (Domellof et al., 2004). Similarly, breast milk production and composition from women possessing a low BMI is

comparable to that of well-nourished mothers (Nazlee et al., 2011) (Soliman et al., 2014). This tight regulation is most likely accounted for by evolutionary pressures, and maintained in order to provide the infant the best possible chance of survival through infancy, meaning optimal nourishment aiding in infant survival via breast milk is likely to be highly conserved, as is observed.

4.5.1 Conclusions

In conclusion, it was observed maternal BMI had no detectable influence on breast milk composition, suggesting breast milk composition is regulated independently of maternal BMI.

There were large, but predictable, alterations in breast milk composition based on the time samples were collected post-partum, detected using each analytical platform.

No detectable differences were observed between fore and hind milk analysed using ^{1}H NMR spectoscopy, but differences were detected in fore and hind milk using both UPLC-MS and mid IR spectroscopy.

Maternal genotype resulted in large differences in the species of HMO produced, as detected using ^{1}H NMR spectroscopy.

The length of analysis posed some analytical challenges, as sample composition was observed to be unstable over the analytical run.

Chapter 5
Infant and maternal urinary metabolome

5.1 Introduction

Metabolic profiling of urine provides a unique insight into the current metabolic state of an individual. Overweight and obesity are conditions which are intimately linked to metabolism, and dysregulation of many metabolic processes occurs as a result. Consequently, metabonomics is uniquely placed in its ability to determine these metabolic changes, generating hypotheses about potential causal mechanisms, and determining the consequences of these alterations, suggesting new avenues of research to pursue.

Previous research has investigated the effect of obesity on the human urinary metabolome; using ^{1}H NMR spectroscopy. A study of fifteen morbidly obese patients, assessed against ten age-matched controls revealed metabolites differing between these two groups were predominantly metabolites of intestinal microbial origin. Hippuric acid, trigonelline and xanthine were decreased in the urine of obese individuals, whilst 2-hydroxyisobutyrate was increased in the urine of obese individuals. These observations suggest that gut microbial metabolism is intimately associated with obesity (Calvani et al., 2010).

Much previous research using animal models to investigate the effects of obesity on the urinary metabolome has been undertaken. One such study, assessing Zucker rats, concluded that the different phenotypes of rat studied, (obese (fa/fa,), lean (fa/-), and lean (-/-)), could be easily identified from one another, using ^{1}H NMR spectroscopy. It was observed that the lean individuals possessed relatively lower hippuric acid and creatinine concentrations, but higher levels of isoleucine, leucine and acetate than the obese animals studied (Waldram et al., 2009).

The authors further concluded, via the analysis of the animal's microbial communities, selection of specific microbial species in each phenotype, and that the microbiota was associated with the animals' metabolic phenotype (Waldram et al., 2009). This study again highlights the close link between obesity and the intestinal microbiota.

To examine the association between maternal BMI and infant and maternal urinary metabolome, urine samples and longitudinal measures of BMI from a prospective cohort of mothers and their

infants were acquired, as described in Chapter 2. To the best of my knowledge this is the first study to examine the effect of maternal BMI on infant urinary metabolome.

As discussed in the previous chapter, the maternal secretor status was capable of being determined analysing breast milk samples using ^{1}H NMR spectroscopy. In order to further investigate this interesting observation, comparison of secretor/non secretor mother's urine, as well as the infants of these mothers, was undertaken. This was done in order to establish whether secretor status could be determined using urine samples, a much more accessible biofluid.

Interpretations of the results from this thesis are complicated by many confounding factors, such as large variations in the environments, and backgrounds, of the participants, which are difficult to control. For example, the different dietary habits of individuals (Walsh et al., 2006), caesarean section, classes and doses of drugs individuals were exposed to, and the different excretion rate of these, as well as any background physiological variation in individuals' metabolism are all factors contributing to the variation expressed in the spectrum of an individual's urinary metabolome. A strategy is developed to accommodate the confounders and to focus on the investigation of the effect of obesity on the metabolic profiles of the offspring.

5.2 Aims

- Investigate the impact of maternal BMI on maternal urinary metabolome, using ^{1}H NMR spectroscopic analysis

- Investigate the impact of maternal BMI on infant urinary metabolome, using ^{1}H NMR spectroscopic analysis

5.2.1 Hypothesis

The following null hypotesis will be tested; there is no significant difference in the urinary metabolome of mothers with a high BMI compared to a normal BMI.

Secondly: There is no significant difference in the urinary metabolome of infants born to mothers of a high or normal BMI.

5.3 Methods

Maternal and infant samples were analysed in a randomised run order to moderate any effects seen in the spectra due to order of collection. Samples were run on 96 place racks (Bruker, Biospin). A total of 590 samples were analysed, including 50 quality control samples, originating from an external control (urine collected from another adult of sufficient volume), analysed to ensure analytical reproducibility was maintained. This QC was analysed after every 11 study samples. Urine sample collection is outlined in Section 2.2.8, sample preparation in 2.3.3, data collection and processing in 2.3.4-9, and metabolite identification procedures in 2.3.10, Chapter 2.

5.4 Results

5.4.1 Cohort Characteristics

Descriptive characteristics for the infant and maternal cohort are presented in Table 30-32.

Table 30: Descriptive characteristics for the infant cohort at recruitment, one week and three months post-partum. Data are reported as means, standard deviations are shown in brackets.

	Recruitment	One week post-partum	Three months post-partum
Number	47	82	91
Post-partum age, days	1.3 (0.7)	7.3 (0.7)	87
Weight, kg	3.45 (0.5)	3.37 (0.5)	6 (0.85)
Length, cm	52.5 (3.1)	51 (2.3)	61 (2.6)
Head Circumference, cm	34.9 (1.3)	35.3 (1.3)	40.4 (1.2)

The mean gestational age of these infants was 40.5 weeks, with a standard deviation of 1.2 weeks. The mean weights, weight for age percentile and ponderal index for this cohort of infants is shown in Table 31.

Table 31: Average weights and weight for age percentiles of male and female infants. Calculated using www.infantchart.com, which employs WHO charts.

	Weight in kg (percentile) [ponderal index]*		
	Recruitment	**One week post-partum**	**Three months post-partum**
Male	3.48 (60.6) [24]	3.42 (45.4) [25.3]	6.18 (41.1) [26.3]
Female	3.32 (57.5) [24]	3.3 (45.8) [25.7]	5.62 (39.5) [25.2]

*Ponderal index calculated using (Mass in kg)/(Height in m)^3

Table 32: Descriptive characteristics of the maternal cohort at recruitment, one week and three months post-partum. Data are reported as means, standard deviations are given in brackets.

	Recruitment	One week post-partum	Three months post-partum
Number	107	103	97
Weight, kg	73.8 (12)	70.3 (12.4)	66.5 (11.2)
BMI	26 (4.5)	25.3 (4.4)	23.9 (3.6)
Height, cm	166 (6)		

The median age of the mothers was 34 years old, with a range from 23, to 46 years old. More samples were available for the maternal cohort due to the difficultly of collecting infant urine samples, especially at recruitment before participants were discharged home from hospital.

5.4.2 ^{1}H NMR spectroscopy data processing and analysis

^{1}H NMR data were acquired for samples collected from participants outlined in Table 36 and 38, as described in Section 2.3, Chapter 2. Acquired spectra were visualised and samples which had not run correctly were excluded, with a total of thirteen samples excluded. Twelve samples were excluded of which eight were maternal samples, and four were infant samples as the water peak had not been supressed properly due to highly diluted samples, therefore influencing the region of the spectrum around it. An additional infant sample was excluded, as it had been contaminated with stool.

Signals arising from TSP δ 0.0, water, δ ~4.7, and urea, δ 5.4-6.1 were excluded. Spectra were aligned to correct for peak shifting, and normalised using probabilistic quotient normalisation, as this has been demonstrated to be a robust and accurate method of normalisation for metabonomic studies (Dieterle et al., 2006). The median maternal and infant urine spectra, after these processing steps, at each of the time points collected, are displayed in Figure 63 and 64 respectively.

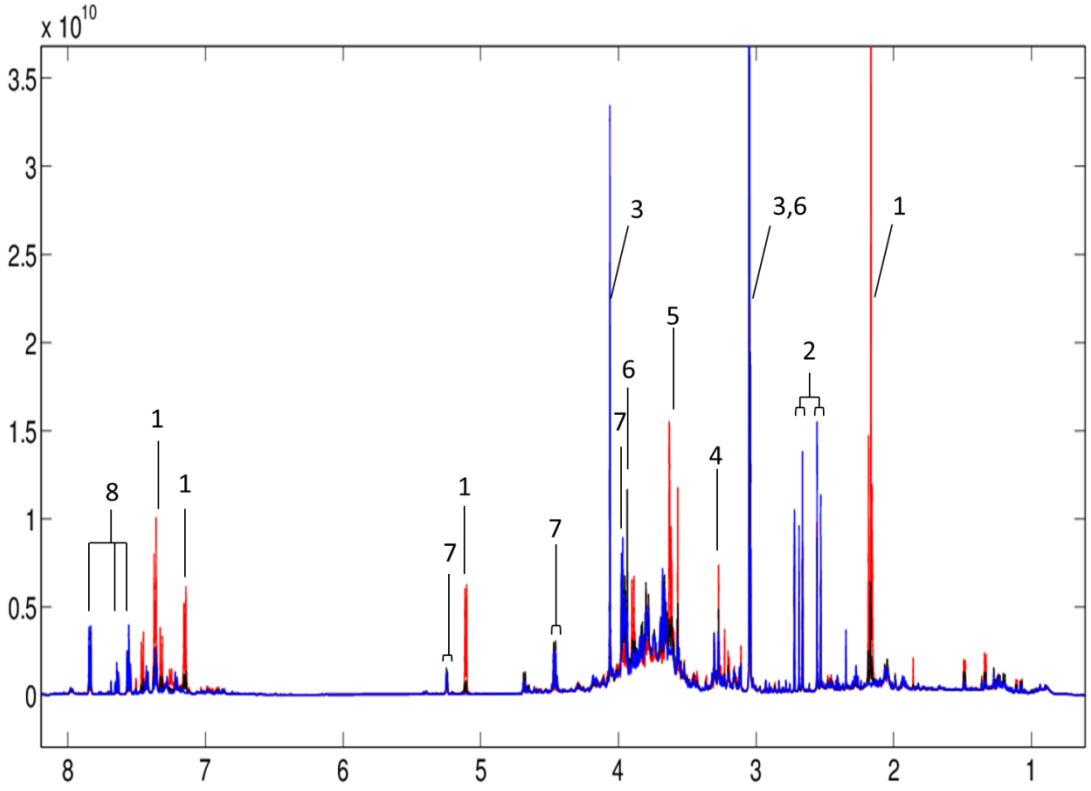


Figure 63: Median maternal urine spectra overlaid after normalisation and alignment. In red, black and blue are maternal urine samples collected at recruitment, one week post-partum and 3 months post-partum respectively. 1, Paracetamol/sulfate/glucuronide; 2; Citrate; 3, Creatinine; 4, Trimethylamine-*N*-oxide; 5, Glycine; 6, Creatine; 7, Glucose; 8, Hippuric acid.

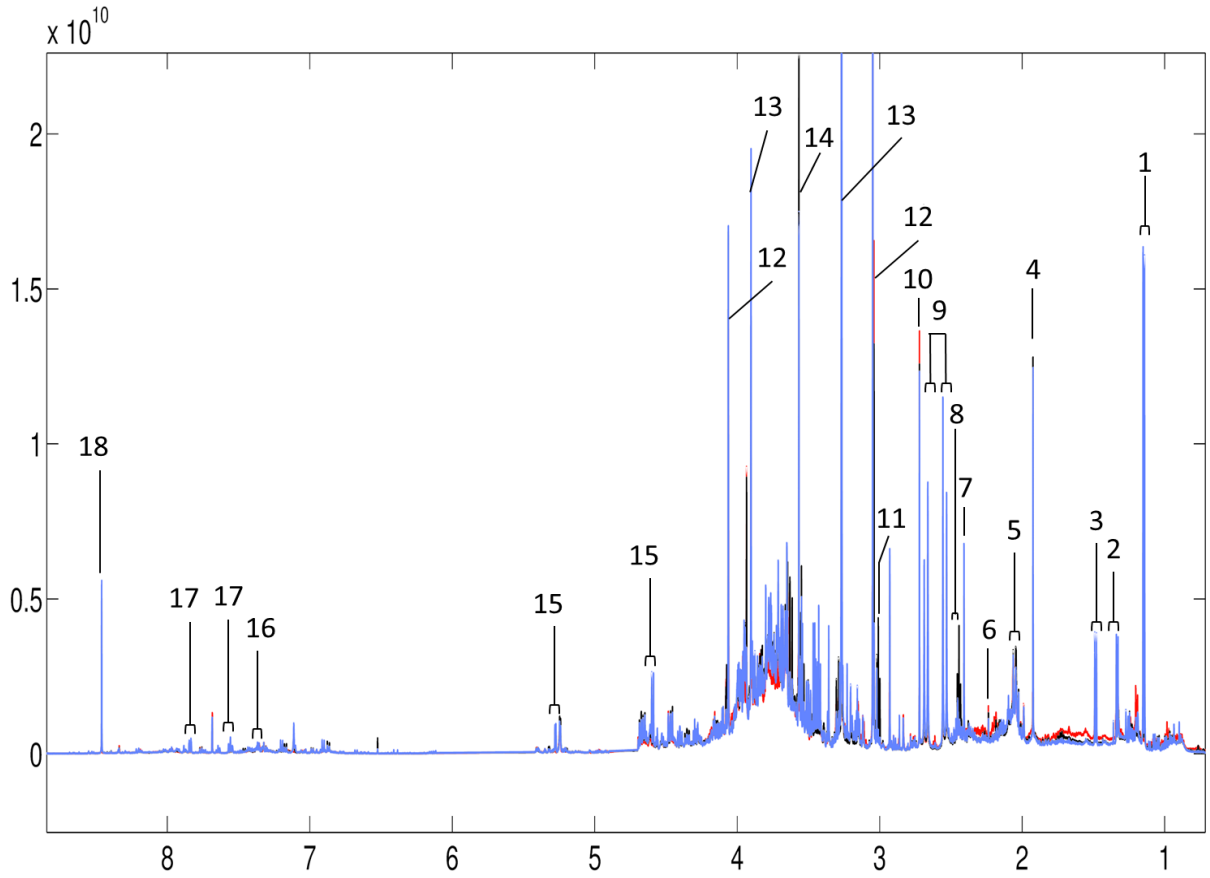


Figure 64: Median infant urine spectra overlaid after normalisation and alignment. Samples at recruitment (red), one week post-partum (black) and 3 months post-partum (blue).
1, 3-D-Hydroxybutyrate; 2, lactate, 3, unknown; 4, acetate; 5, *N*-acetyl groups; 6, acetone; 7, succinate; 8, beta-Hydroxybutyrate; 9, citrate; 10, trimethylamine; 11, creatine; 12, creatinine; 13, betaine; 14, glycine; 15, glucose; 16, phenacetylglutamine; 17, hippuric acid; 18, formic acid.

Once processed, data were then imported into SIMCA-P+ 13.0.2 (Umetrics, Sweden), and PCA performed in order to give an overview of the data. At this point data were also scaled, the most suitable method of scaling for this dataset was Pareto scaling as the QC samples were most tightly clustered using this method.

Following PCA outlier analysis, described in Section 2.9.2, Chapter 2, a further six spectra (three maternal and three infant samples) were excluded. The three infant spectra were excluded due to two being very dilute, and one having extremely high concentrations of propylene glycol (thought to be a chemical contaminant). The maternal samples were excluded as two had extremely high concentrations of paracetamol, and one had an extremely high concentration of hippuric acid.

An overview PCA containing maternal samples is displayed below in Figure 65, and infant samples in Figure 66. PCA was also performed separately for all six groups of urine sample, grouped by time point (recruitment, one week and three moths post-partum), and by whether the sample was maternal or infant urine, displayed in Appendix 5A. This was done in order to assess general reproducibility and stability of the data and to identify outliers.

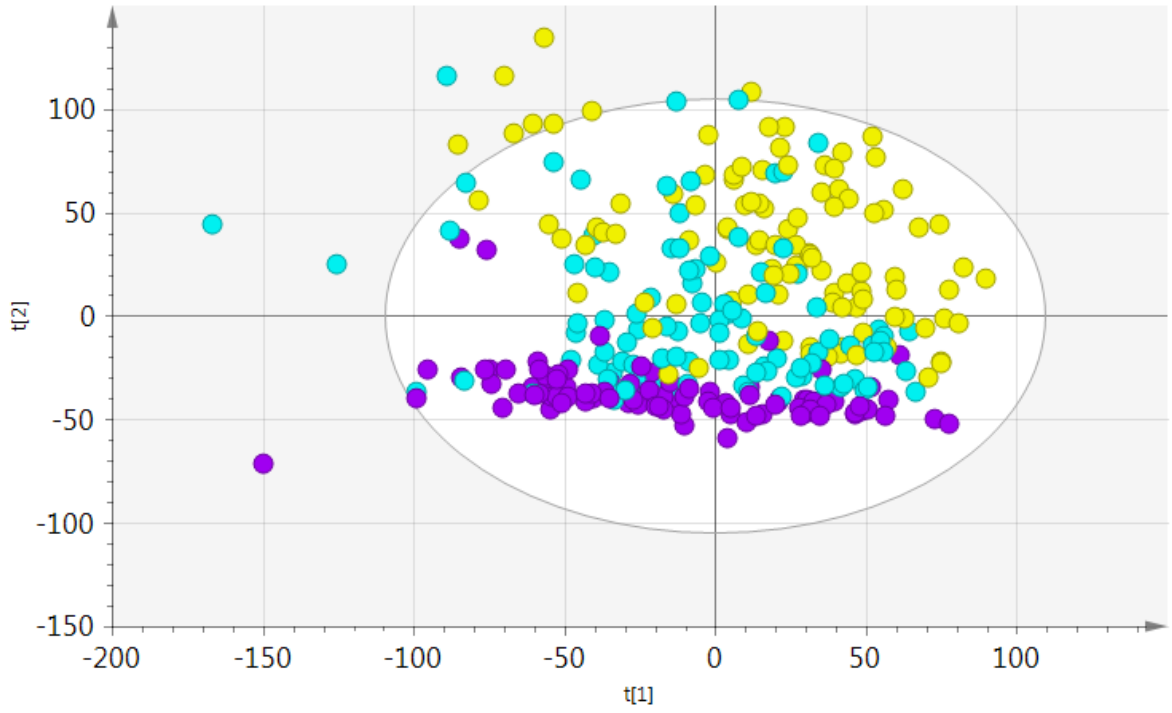


Figure 65: PCA scores plot of maternal urine samples, coloured by time of collection. Maternal urine samples collected at recruitment (yellow), one week post-partum (turquoise) and 3 months post-partum (purple). R^2X=0.143, Q^2X=0.122 (UV scaling).

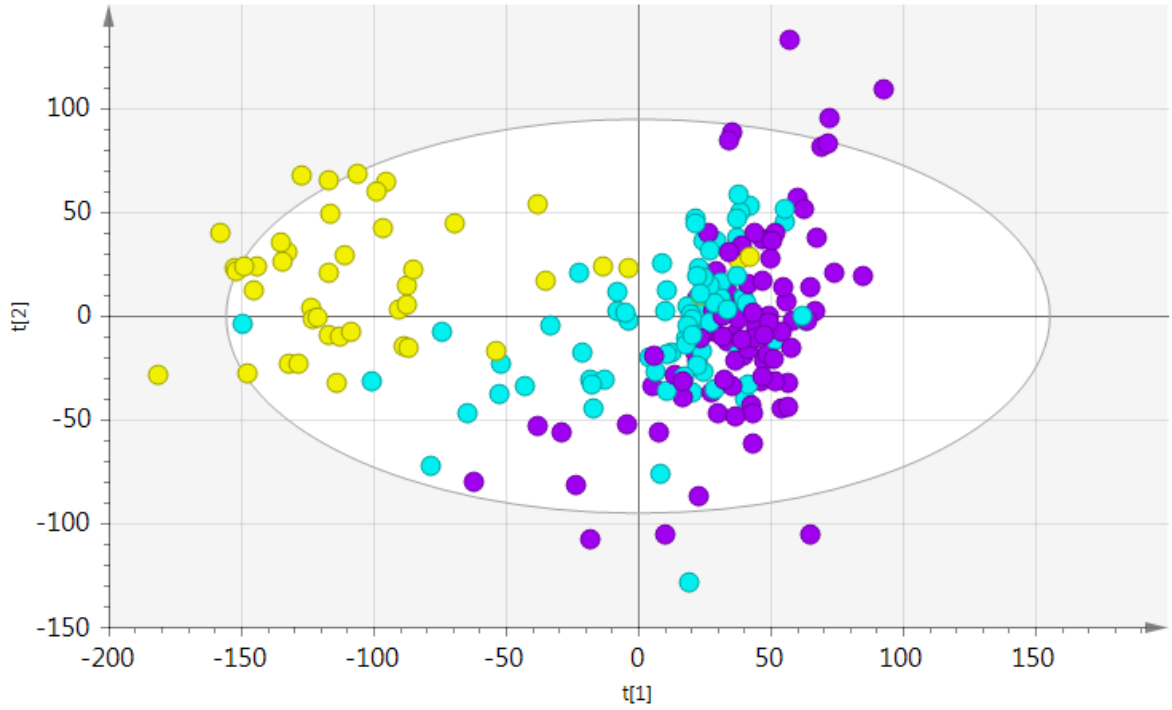


Figure 66: PCA scores plot of infant urine samples, coloured by time of collection. Infant urine samples collected at recruitment (yellow), one week (turquoise) and three months post-partum (purple). R^2X=0.205, Q^2X=0.182 (UV scaling).

5.4.3 Investigation of run time effects

To ensure the quality of data collected, data were assessed to establish whether there were any run time effects, due to the length of analysis, as this occurred over several days. Samples in the PCA scores plot were coloured by the number rack on which samples were analysed, to identify any drift in the data, displayed in Figure 67. This plot did not show any obvious differences in sample composition due to the time of analysis.

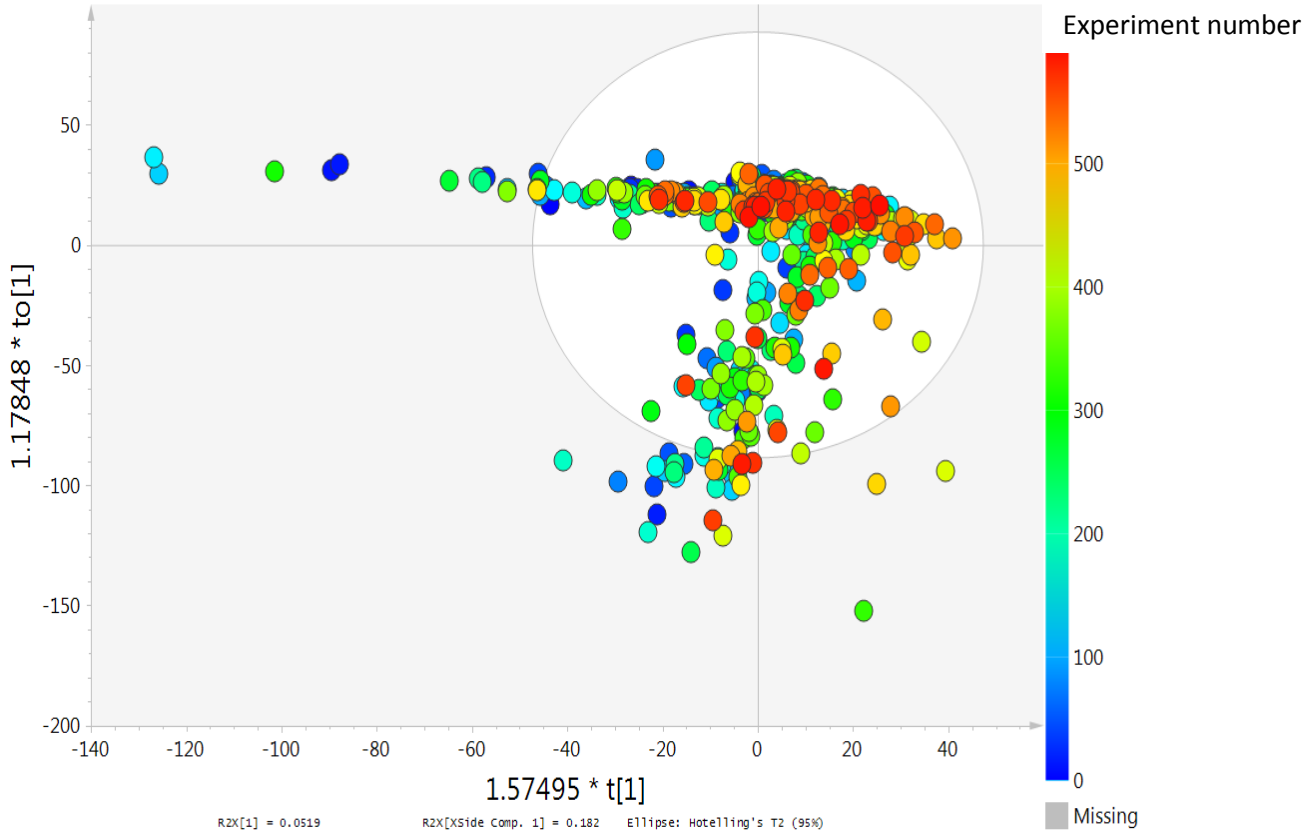


Figure 67: OPLS scores plot of urine NMR spectra classified by run number. Blue to red relate to samples analysed early on in the run to samples run later on, R^2X=0.234, Q^2Y=-0.06.

As the OPLS model shown in Figure 67 has a negative Q^2Y value, it was concluded that length of analysis did not affect sample composition, and therefore variability seen should be due to biological variation, rather than analytical variability.

5.4.4 Regression of maternal urinary metabolome against maternal BMI

OPLS analysis was undertaken to investigate whether maternal BMI had an influence on the maternal urinary metabolome when a continuous measure of BMI was used. Here, values of maternal BMI were used instead of classifying mothers as obese/non-obese; using maternal BMI at booking visit. Values for these models are displayed in Table 33. A progressive increase in the predictive capability of the models was found, as shown by the Q^2Y values.

Table 33: OPLS models computed for maternal urinary metabolome, comparing mothers of a different BMI using booking BMI values.

N	R^2X	R^2Y	Q^2Y	Outliers	t[1]	t[2]	Components	Sample type
102	0.238	0.574	-0.329	2	0.02	0.21	2	Recruitment
98	0.273	0.324	0.05	5	0.06	0.20	2	One week
91	0.205	0.484	0.05*	5	0.08	0.12	2	3 months

*1st component of this model had a Q^2 value of 0.15.

As the final model in Table 33 appeared to have a small predictive capacity in the first component, scores and loadings plots were plotted to investigate the discriminatory metabolites, Figure 68.

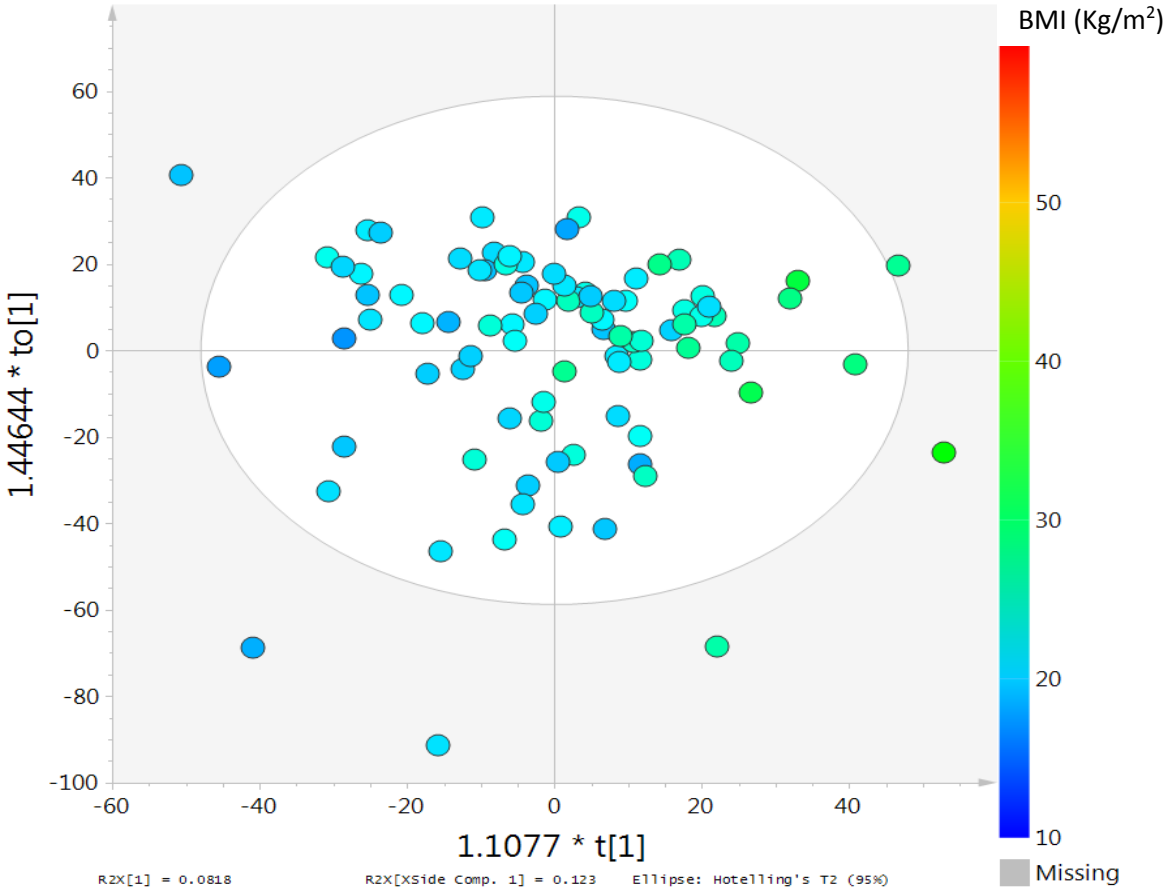


Figure 68: OPLS scores plot of maternal urine collected at three months post-partum against maternal booking BMI value. R^2X=0.205, R^2Y=0.485, Q^2Y=0.05, (1st component had a Q^2Y value of 0.15).

In order to assess whether this was a valid model, as the Q^2Y value was not very high, permutation testing was undertaken. Here, in order to ensure that there is not a spurious correlation between the X and Y matrices, the Y matrix is randomly permuted and new models produced. In Figure 69, the result of this testing is displayed; the original models values are shown on the right, whist the values from the permuted values are shown on the left.

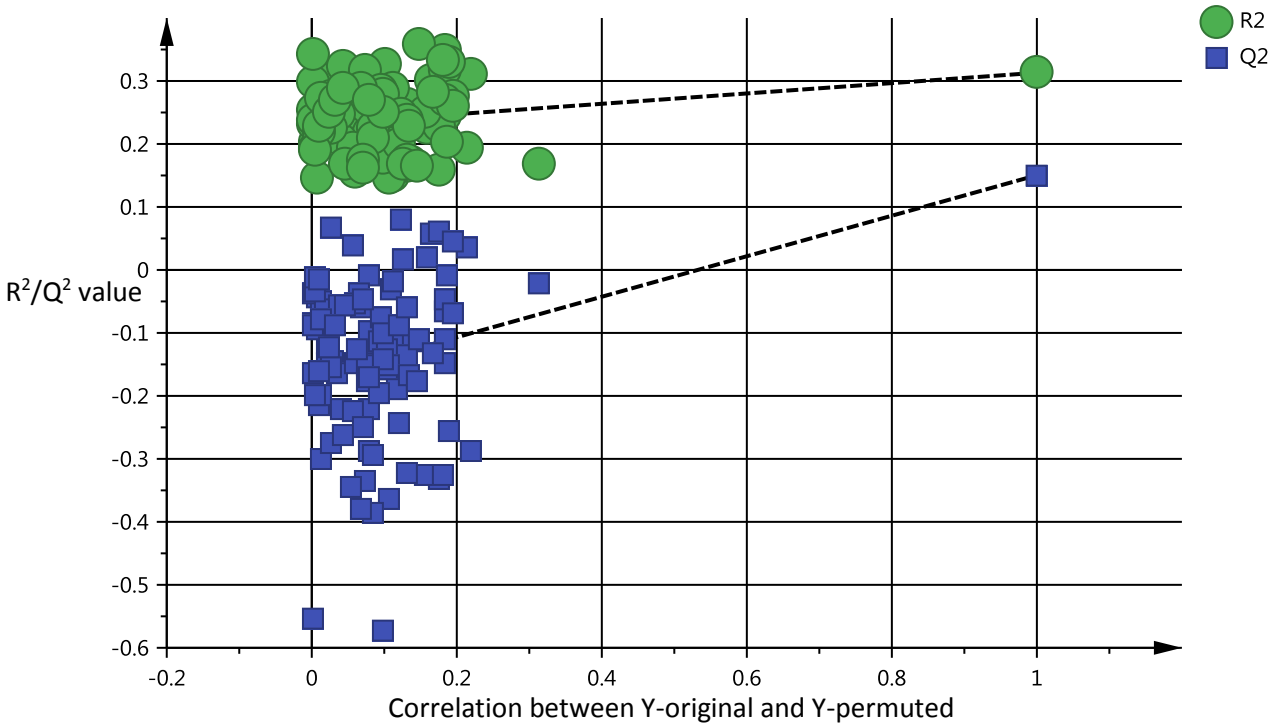


Figure 69: Permutation test of the model displayed in Figure 67, using 100 permutations on the first component.

Figure 69 indicates that this model is valid as all the Q^2 values are below the original (far right), and the blue regression line meets the Y axis below zero.

The loadings plot corresponding to this model was investigated in order to establish the metabolites responsible for this separation. This analysis revealed that the abundance of creatine (singlet at δ 3.02 and 3.92), creatinine (singlet at δ 3.03 and 4.05) and trimethylamine-*N*-oxide (TMAO) (singlet at δ 3.27) were increased in mothers possessing a higher BMI, whilst mothers possessing a lower BMI had an increased abundance of hippuric acid (triplet at δ 7.55), the corresponding loadings plot is displayed in Appendix 5B.

5.4.5 Comparison of NMR spectra from infants fed formula and breast milk

To establish whether any there were systematic differences in infant urinary metabolome for infants fed different kinds of milk, i.e. formula vs breast milk, OPLS-DA was used, by classifying infants as to whether they had ever been fed formula or not. The scores plot corresponding to this model is displayed in in Figure 70.

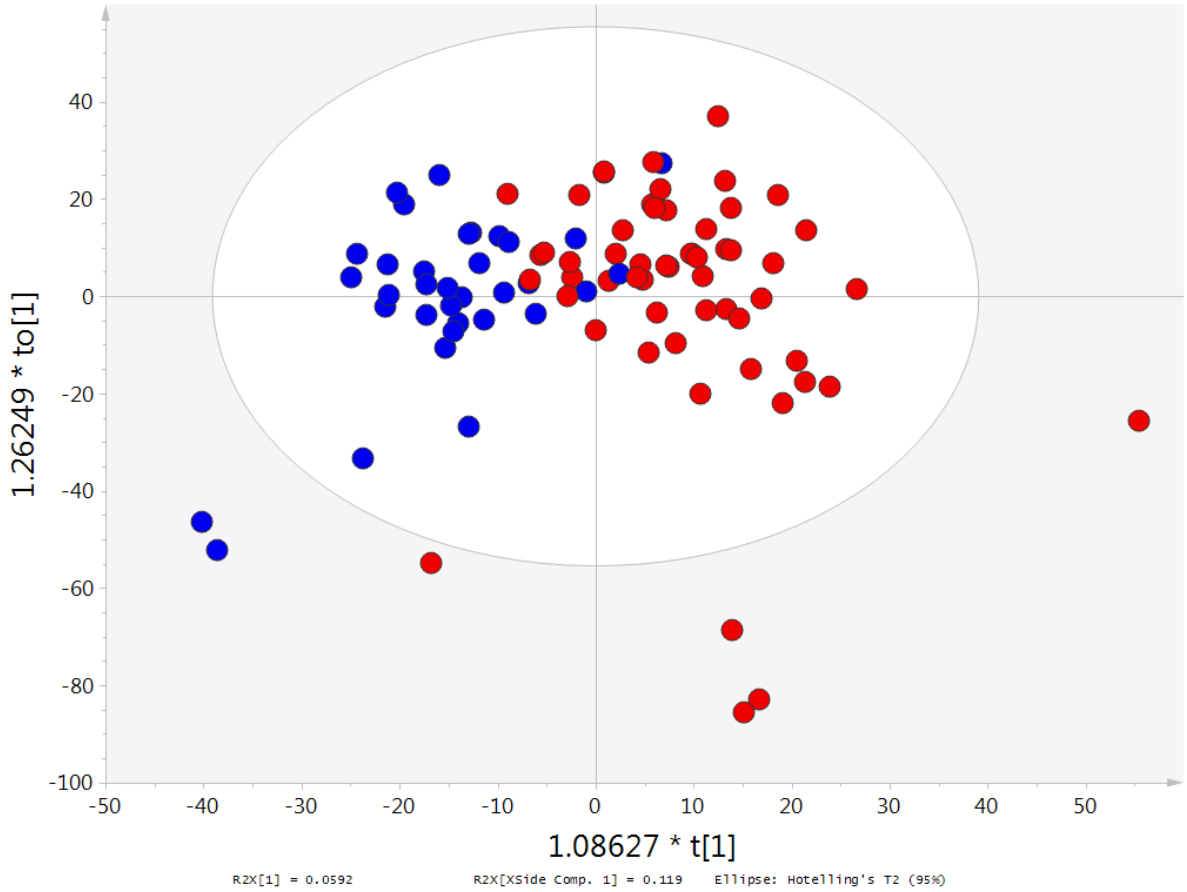


Figure 70: OPLS-DA scores plot comparing spectra from infant urine samples from formula fed infants to infants solely fed breast milk, at 3 months post-partum. Red circles are infants exclusively fed breast milk; blue circles indicate infants fed any amount of formula. R^2X=0.178, R^2Y=0.561 Q^2Y=0.276.

The separation of formula fed/breast fed infants was not seen in infant urine samples collected at one week post-partum, (R^2X=0.15, Q^2Y=-0.24). This is potentially due to the infants being fed less formula at this point, and/or the formula had not yet been administered for long enough to have an effect on the infant urinary metabolome.

The model displayed in Figure 70 possesses a relatively good Q^2Y value, meaning it has a positive predictive ability, demonstrating that formula feeding impacts infant metabolism, and systematic differences exist in the urine of breast fed and formula fed infants.

Therefore, when modelling infant urine samples, models were computed both including and excluding formula fed infants to assess whether there were any differences in the models produced, as formula feeding is potentially a confounding factor.

OPLS-DA was used to identify peaks systematically changed in these two groups, Figure 71.

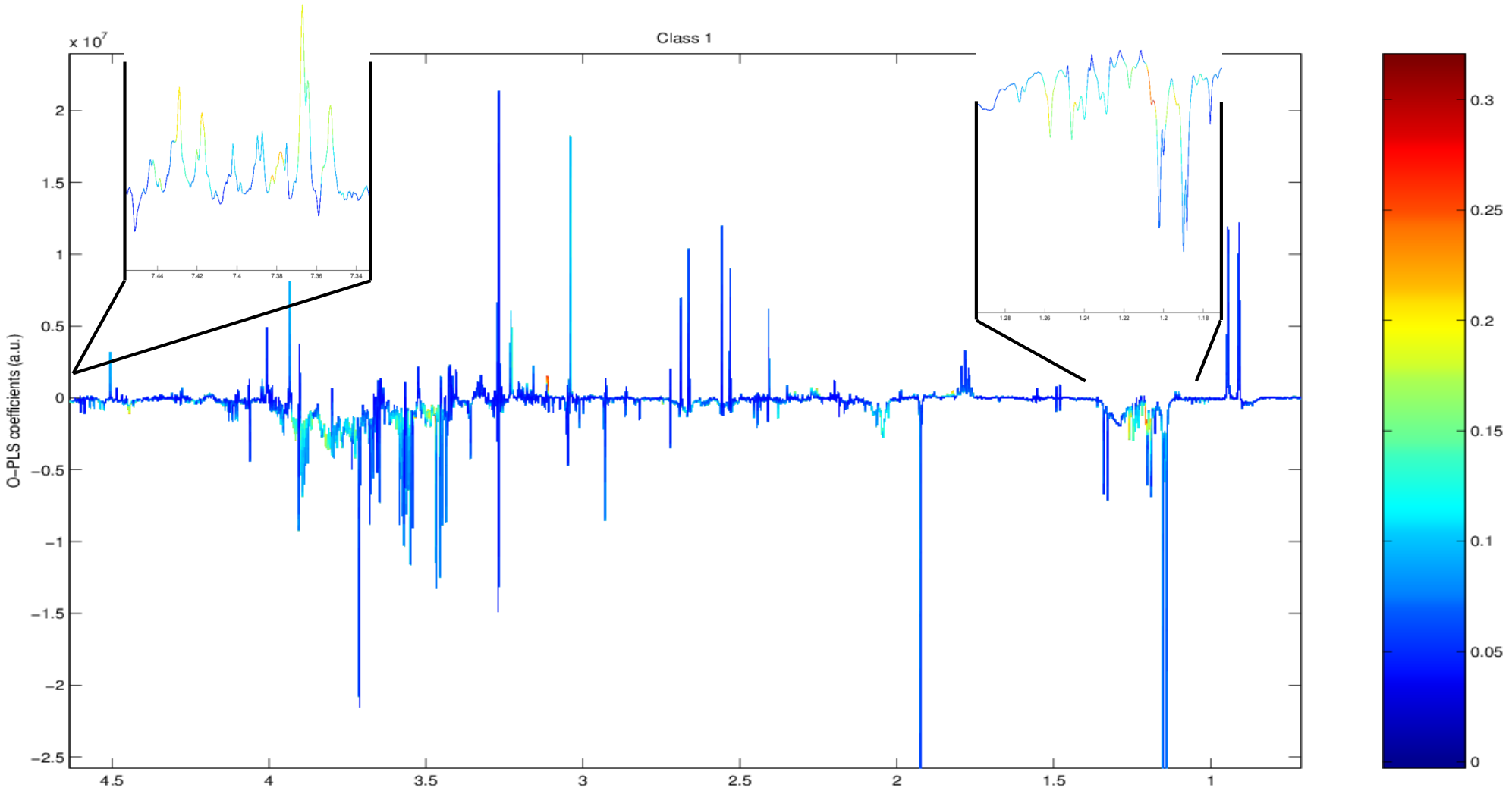


Figure 71: OPLS-DA loadings plot comparing infants exclusively fed breast milk (upper section) against infants fed formula (lower section) at three months post-partum. Peaks coloured red and pointing either up or down are metabolites which are significantly associated with that group of infants, UV scaling.

An attempt was made to identify the metabolite at δ 7.37, a discriminatory peak in the OPLS-DA model shown in Figure 71, using STOCSY, outlined in Section 2.9.6, Chapter 2. However, as there were several overlapping signals this was difficult. Therefore STORM analysis was used in order to identify this metabolite, (method outlined in Section 2.9.7, Chapter 2).

This method is useful for identification as it reduces the incidence of false positive results. In this case, using STOCSY displayed many correlations with unrelated metabolites in the loadings plot due to the overlapping signals in the spectra; this effect is reduced in STORM analysis.

STORM analysis revealed the main metabolite contributing to the separation of the formula fed, versus breast fed infants, was phenylacetylglutamine, or PAG, displayed in Figure 72.

Also increased in the breast fed infants was 3-Aminoisobutyrate, as confirmed by STOCSY. Peaks identified included a doublet at δ 1.2, a multiplet at δ ~2.61 and two doublet of doublets at δ 3.00 and δ 3.10.

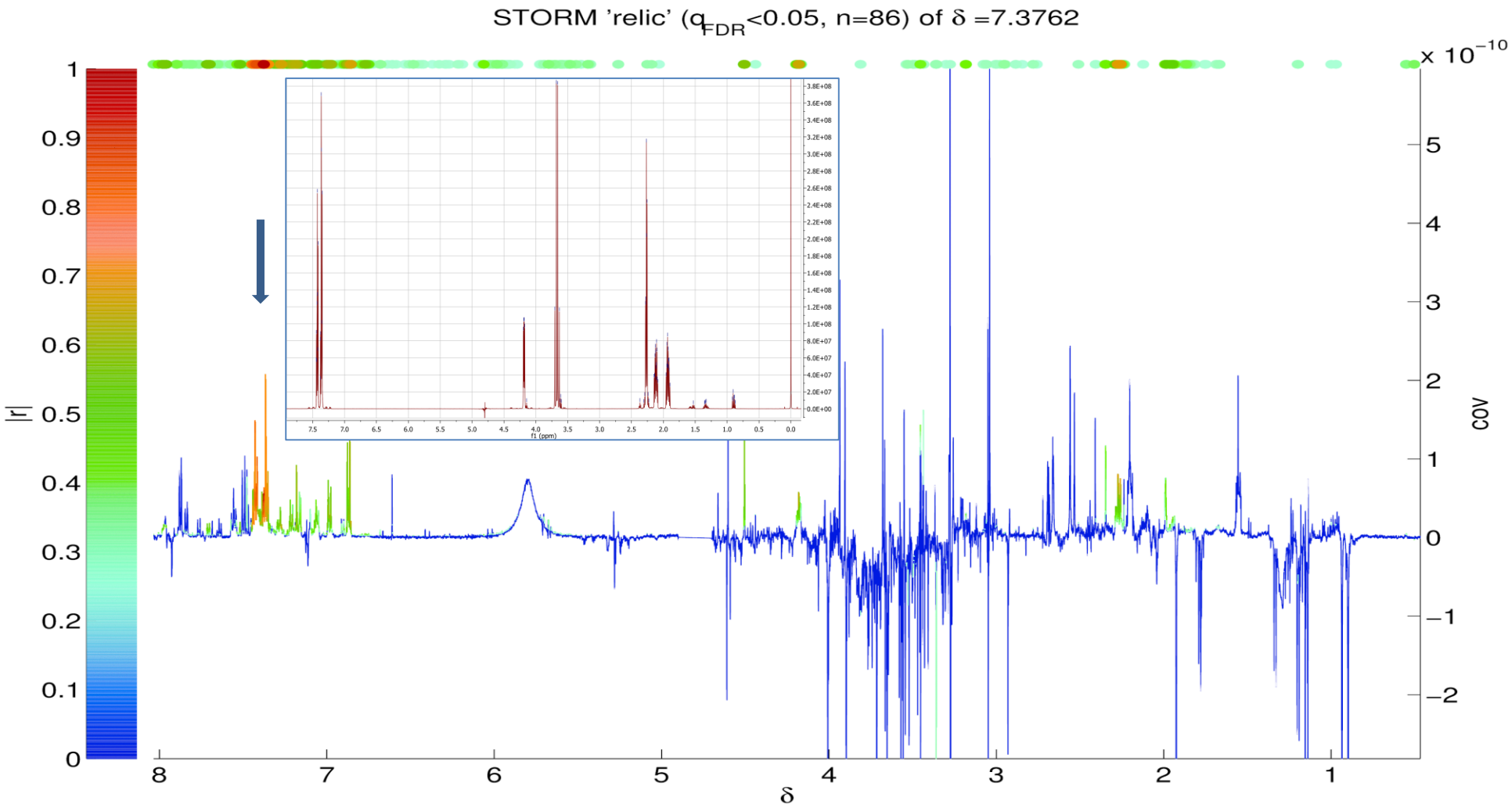


Figure 72: STORM analysis identifying PAG as a discriminatory metabolite between FF and BF infants. Peaks coloured red/orange are significantly associated with the peak presenting at 7.37, and agree with database assignments of the other peaks belonging to the PAG molecule. The peaks present in both the spectra and spectral database include a multiplet at 1.93, 2.12, 2.27, 3.67, 4.19 and triplets at 7.36 and 7. 43. Inset displays a standard spectrum of PAG (kindly given by Joram Posma). Arrow shows position of peak used for correlation.

5.4.6 Regression of infant urinary metabolome against maternal BMI

A regression of maternal BMI using the maternal booking BMI values was performed against the infant urinary metabolome. Values for these models are shown in Table 34.

Table 34: OPLS models computed for infant urinary metabolome from infants born to mothers of a different BMI.

N	R^2X	R^2Y	Q^2Y	Outliers	t[1]	t[2]	Components	Model
44	0.203	0.773	-0.04	1	0.05	0.15	2	Recruitment
81	0.15	0.479	-0.26	3	0.03	0.11	2	Week
91	0.149	0.419	-0.28	7	0.03	0.11	2	3 month

These models all possess a negative Q^2 value suggesting maternal BMI has no detectable influence on the infant urinary metabolome immediately post-partum, at one week or at three months post-partum.

5.4.7 Investigation of gestational effects on infant urinary metabolome - Regression of infant urinary metabolome against gestational age

The impact of infant gestational age on the infant urinary metabolome was also investigated. This was achieved by decimalising infant gestational age and computing OPLS models, using gestational age as a continuous variable. The OPLS models produced are outlined in Table 35. All the infants recruited were born at term, with a range from 37 weeks postpartum to 42 weeks post-partum.

Table 35: OPLS models computed for infant urinary metabolomes comparing infant born with different gestational ages.

N	R^2X	R^2Y	Q^2Y	Outliers	t[1]	t[2]	Components	Model
45	0.142	0.818	0.29*	2	0.04	0.09	2	Recruitment
81	0.114	0.616	-0.02	3	0.03	0.07	2	Week
91	0.172	0.207	-0.16	7	0.04	0.12	2	3 month

*The Q^2 value for the first component in this model was 0.01.

As can be seen from the models in Table 35, the model of the infant urine collected at recruitment against gestational age had a positive predictive value suggesting there are systematic differences in

urine from infants born at different gestational ages. The model validation using permutation testing is shown in Appendix 5C.

These alterations were identified by visualising the loadings and scores plot for this model, shown in Figures 73 and 74.

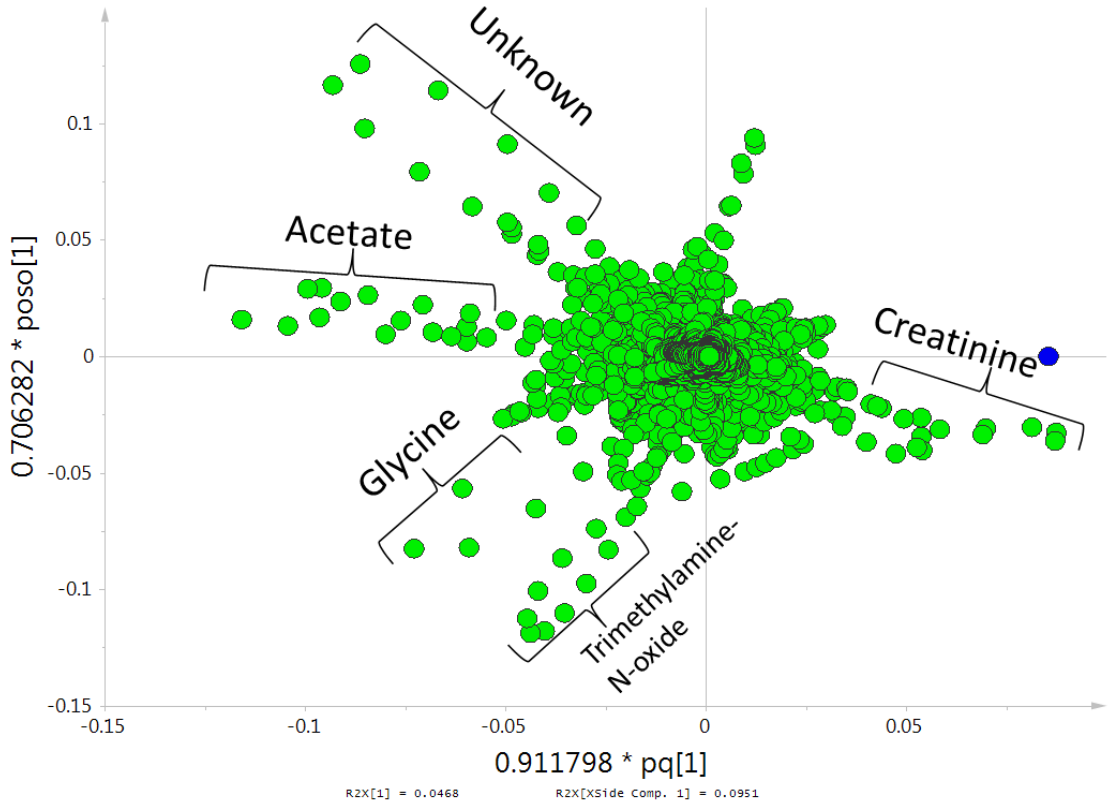


Figure 73: OPLS loading plot of infant urine collected at recruitment against infant gestational age. Points on the right of the plot indicate metabolites which are increased in infants with a later gestational age, whilst points to the left indicate those metabolites increased in infants with a lower gestational age. R^2X=0.142, R^2Y=0.818, Q^2Y=0. 292.

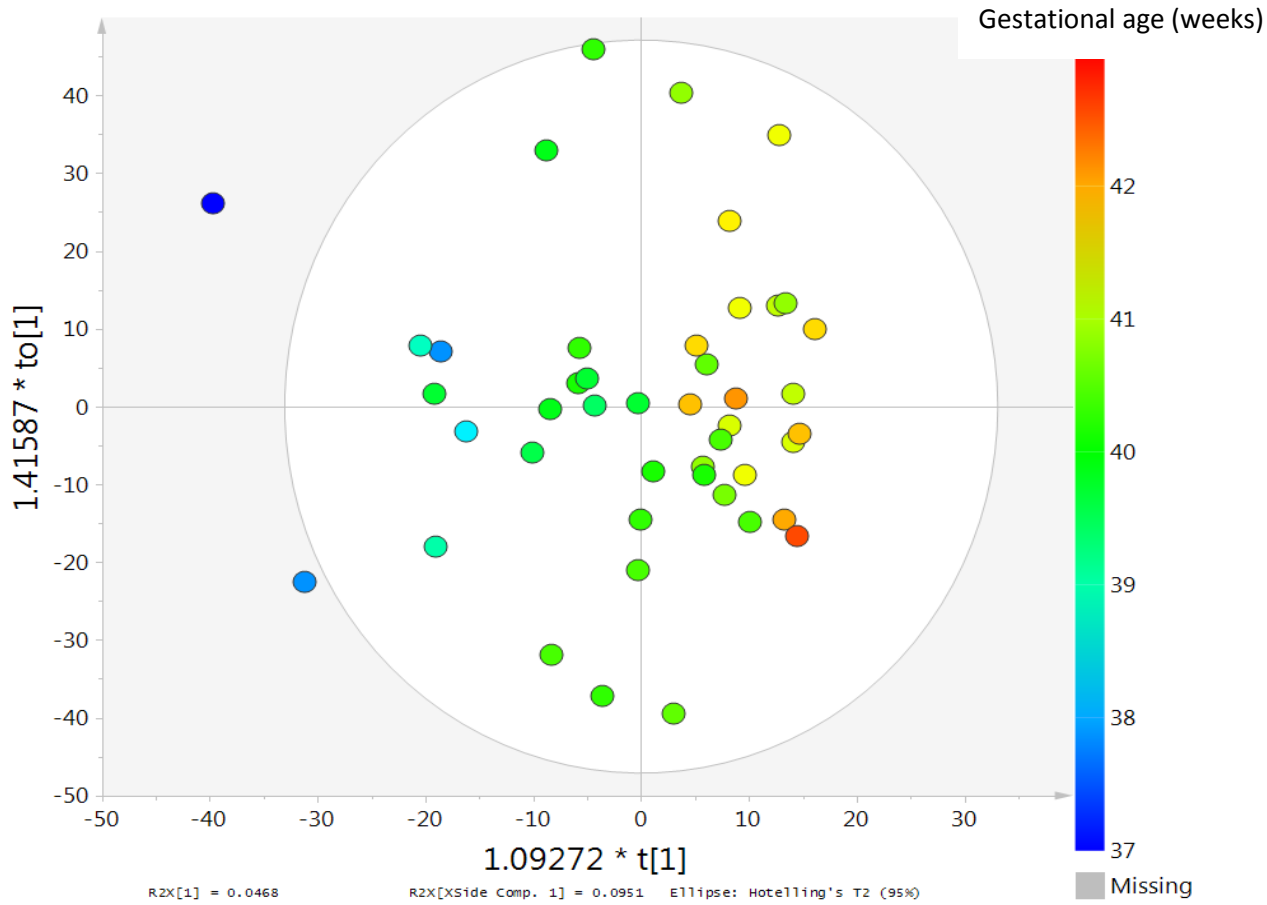


Figure 74: OPLS scores plot of infant urine collected at recruitment against infant gestational age. R^2X=0.142, R^2Y=0.818, Q^2Y=0.292.

The metabolites contributing towards this separation in the scores plots were identified using the loadings plot. Increased in abundance in infants born later term was creatinine, whilst metabolites increased in abundance in infants born in early term were acetate (singlet at δ 1.92), trimethylamine-*N*-oxide (singlet at δ 3.27) and glycine (singlet at δ 3.56). The chemical shift of the unknown signal was δ 4.00 (singlet).

5.4.8 Comparison of maternal urine samples comparing secretor mothers against non- secretors of 2'-FL and related HMO

Also investigated was the influence of maternal secretor status on the maternal urinary metabolome. This was achieved by classifying mothers as either secretors or non-secretors based on the profile of HMO she produced, as determined by the NMR spectroscopic analysis of breast milk. OPLS-DA models were then produced in order to determine whether mothers could be discriminated into these two groups based on their urinary metabolome. Using Pareto scaling, the OPLS-DA models produced had negative Q^2Y values, however when rescaled using UV scaling, giving greater importance to low abundance metabolites, a good model was produced, shown in Figure 75. The difference in the model validity is a function of the HMO being present at relatively low concentrations compared to metabolites such as lactose, and are largely either present or absent, therefore they are down weighted by Pareto scaling which is expressed relative to the median spectrum.

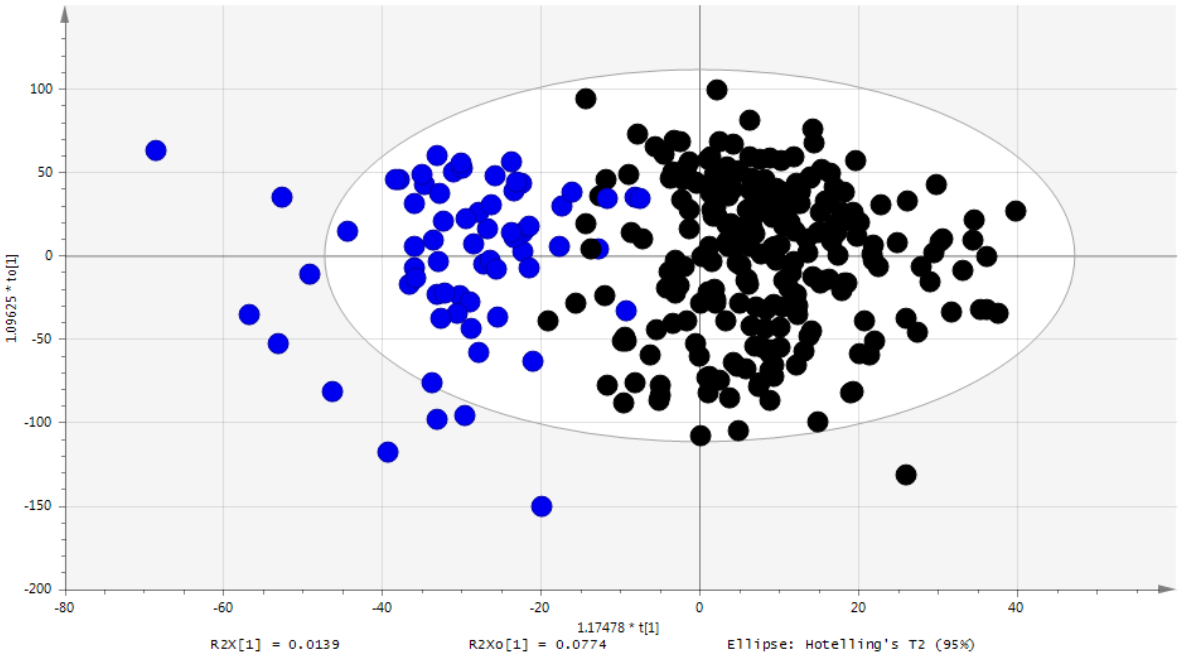


Figure 75: OPLS-DA scores plot of maternal urine samples collected at all time points, classified as either secretors (black circles) or non-secretors (blue circles), based on breast milk samples. R^2X=0.09, R^2Y=0.674, Q^2=0.383.

As seen in Figure 75, two groups are observed in the maternal urine samples, in high agreement with maternal secretor status, the classification of which was based on the mothers' breast milk samples. This model shows a good predictivity score, as displayed by the Q^2Y value.

In order to identify the metabolites which altered in abundance between these two groups, an OPLS-DA loadings correlation coefficients plot was created to highlight the regions which differed between these two groups, as shown in Figure 76.

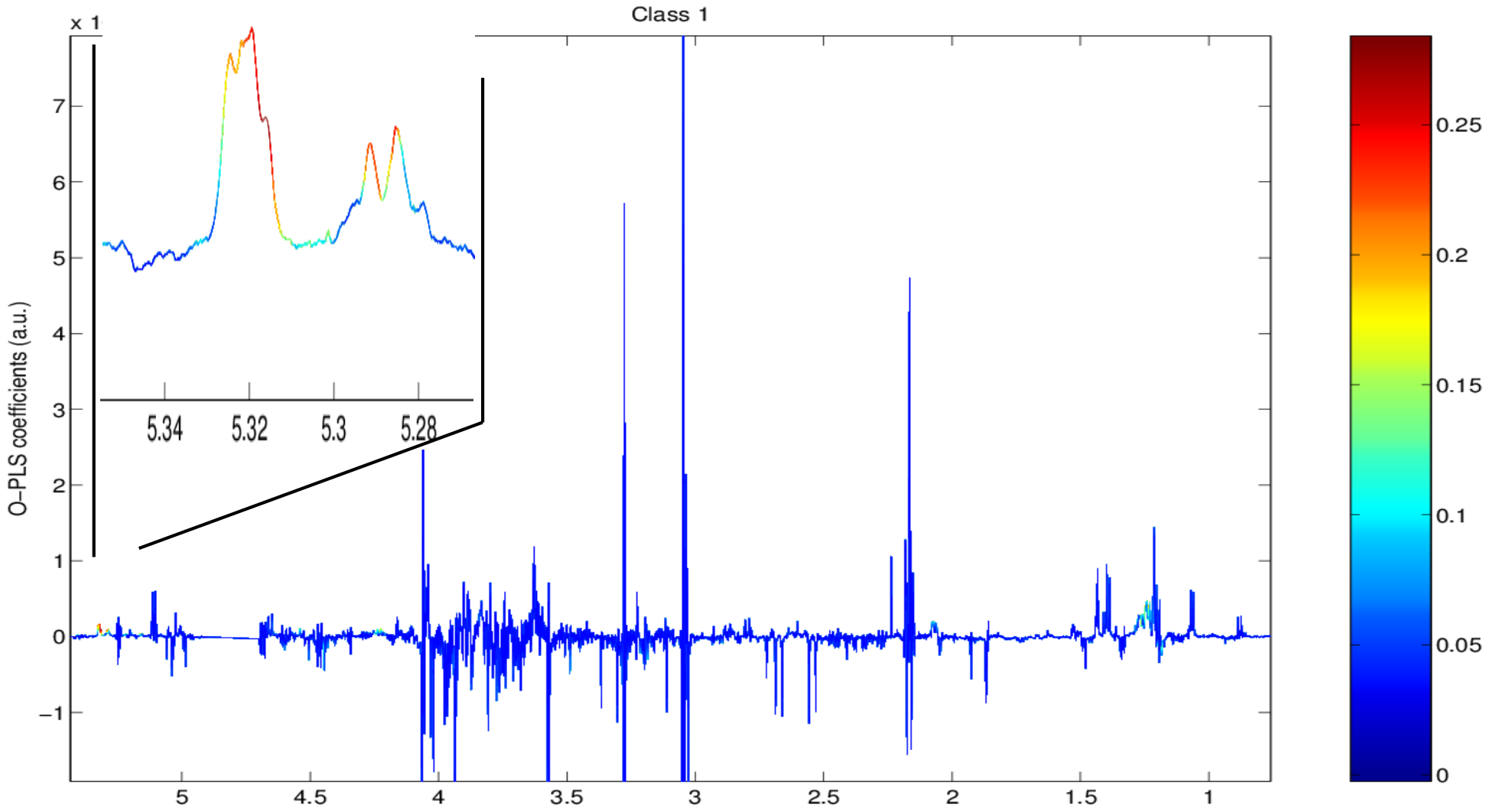


Figure 76: OPLS-DA loadings coefficients plot comparing all maternal urine samples classified as secretor (up) or non-secretor (down) based on breast milk samples $R^2X = 0.09$ $R^2Y = 0.80$ $Q^2Y = 0.24$. Model calculated with one orthogonal component.

As Figure 76 displays, the most discriminatory peaks in the secretor mothers' urine included a doublet at δ 5.29, and a multiplet at δ 5.32. In order to identify this metabolite, STOCSY was used, displayed in Figure 77.

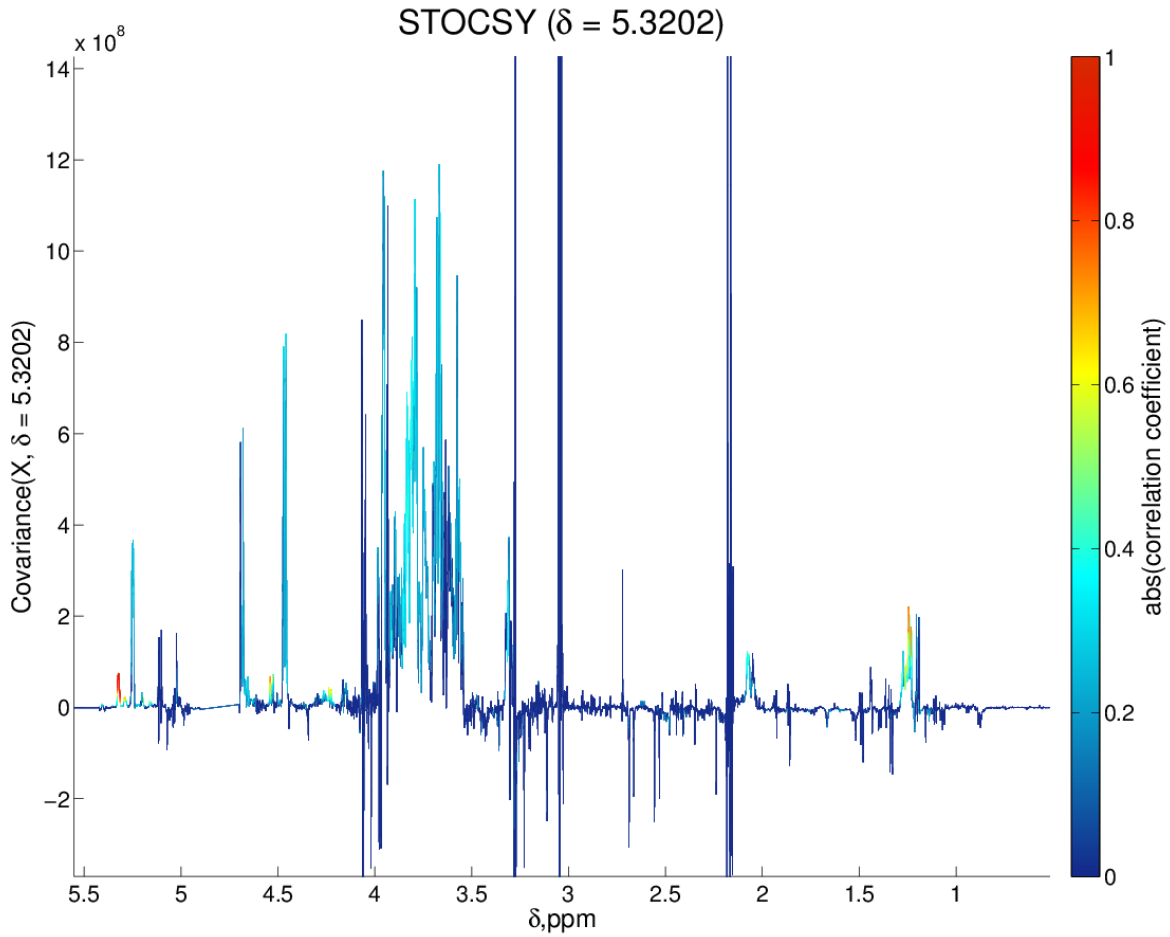


Figure 77: STOCSY of discriminatory peak between secretor and non-secretor mothers at δ 5.32. Highly correlated peaks included those at δ 1.24, 4.25, 4.53 and 5.29.

One potential identification of the metabolite highlighted in Figure 77 is 2'-Fucosylactose, as it contained many of the same peaks as a standard analysed for comparison (kindly donated by Jennewein Biotechnologie GmbH, Rheinbreitbach, Germany), displayed in Figure 78.

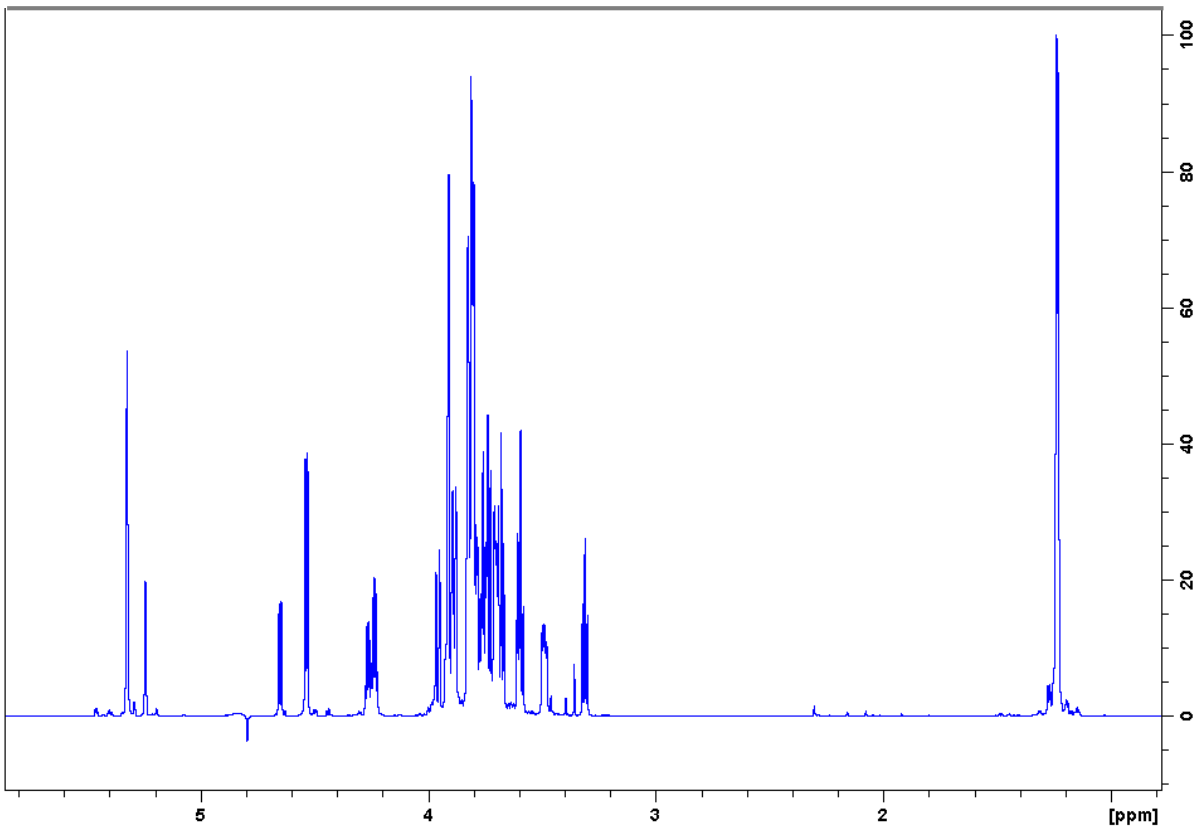


Figure 78: Standard ^{1}H NMR spectrum of 2'-fucosylactose.

5.4.9 Comparison of infant urine of infants born to secretor mothers against non-secretor mothers

Similarly to the maternal urinary samples, infant urine samples were classified by whether their mother was a secretor or non-secretor, again based on the HMO profile of the mother's breast milk. The maternal secretor status was therefore used as the class variable, and an OPLS-DA model produced, displayed in Figure 79.

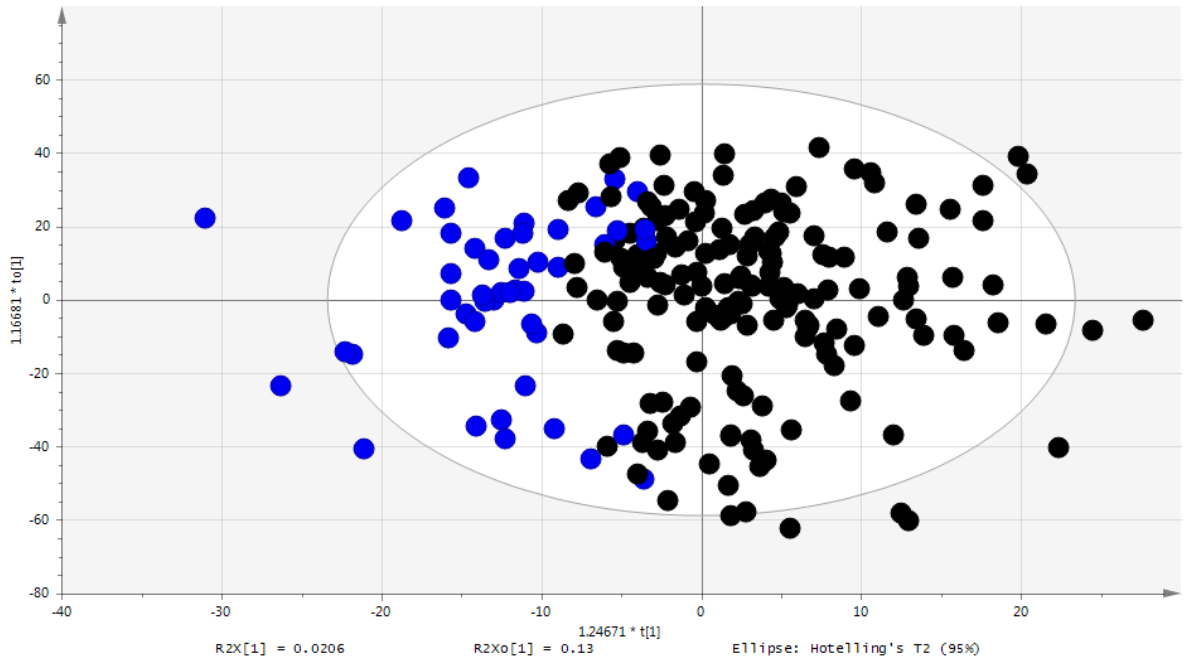


Figure 79: OPLS-DA scores plot of infant urine samples collected at all time points months post-partum, classified as either secretors (black circles) vs non-secretors (blue circles), based on maternal breast milk samples. R^2X=0.151, R^2Y=0.456, Q^2Y=0.214.

This figure demonstrates that there are systematic changes in the urinary metabolomes of infants of secretor/non-secretor mothers, as the Q^2Y score has a positive predictive value. To identify the metabolites responsible for this separation the OPLS-DA loadings coefficients were plotted, displayed in Figure 80.

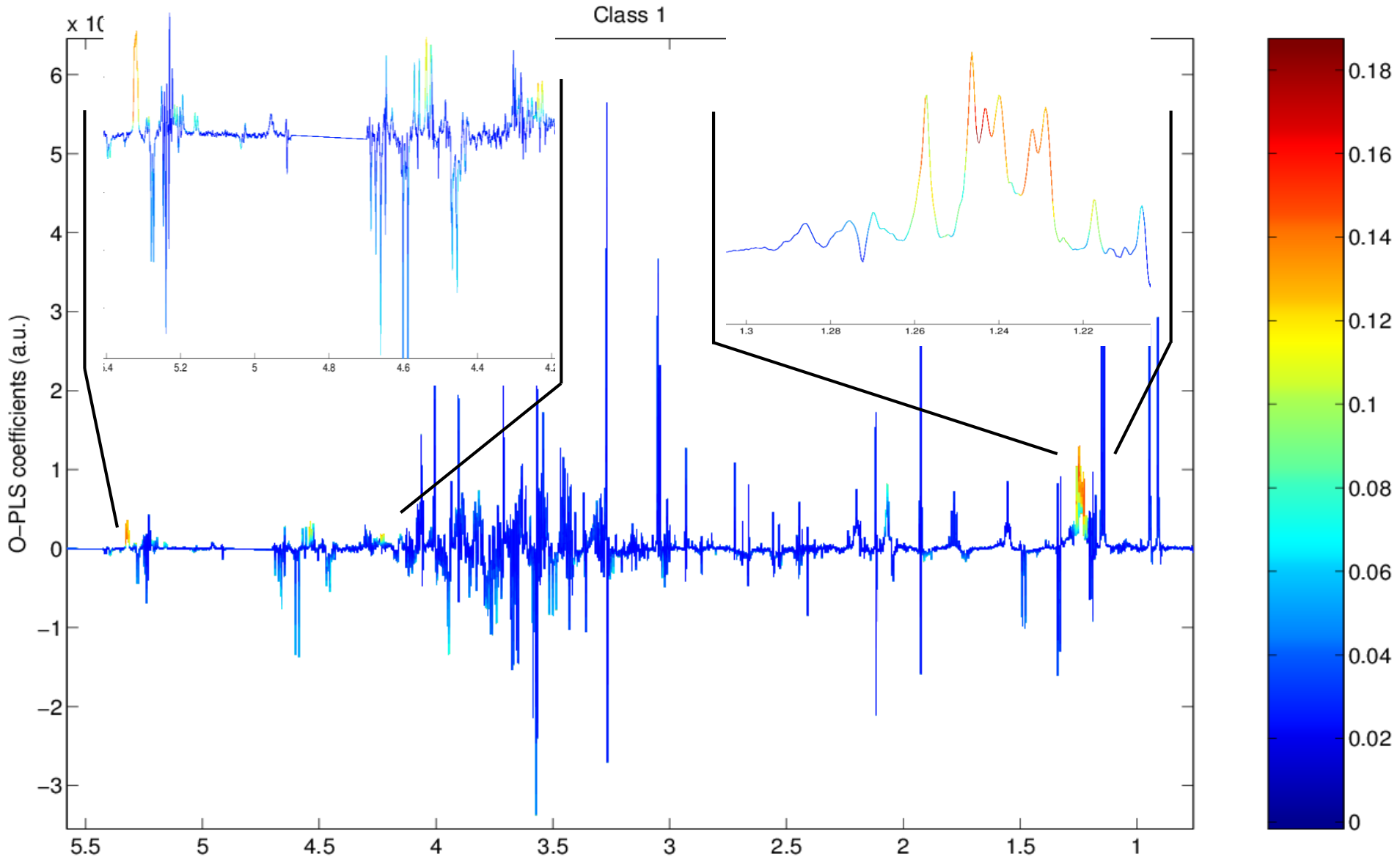


Figure 80: OPLS-DA loadings coefficients plot comparing all infant urine samples classified as infant of secretor (up) or non-secretor mothers (down) from breast milk samples. R^2X=0.152, R^2Y=0.576, Q^2Y=0.261. Model calculated with one orthogonal component.

In order to aid identification of these metabolites, STOCSY was used to detect correlated peaks. A STOCSY of the peak at δ 1.24 is shown in Figure 81. Peaks which were highlighted correlated were input to HMDB in order to identify metabolites possessing similar chemical shifts; with realistic hits investigated further.

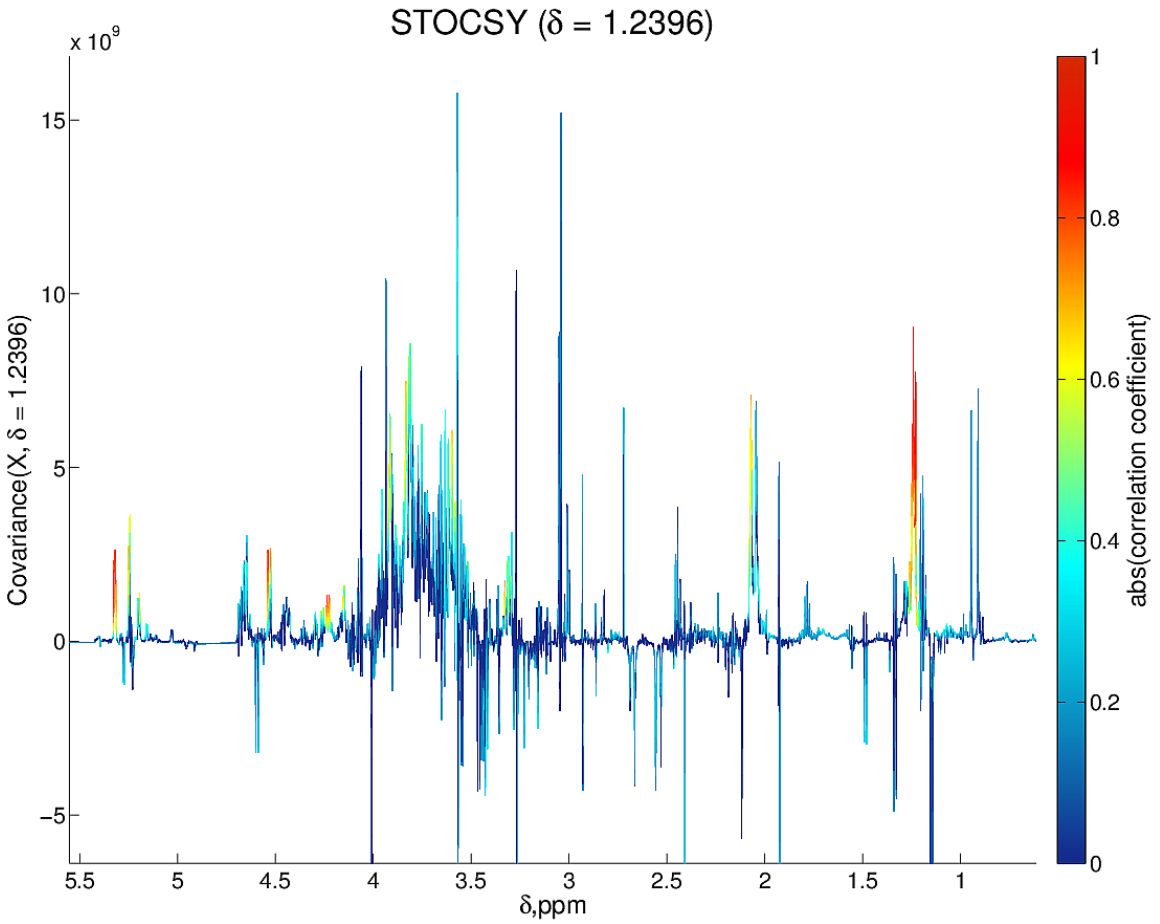


Figure 81: STOCSY correlation and covariance plot displaying peaks associated with the doublet at 1.24 ppm.

Again it appeared as if 2'-fucosyllactose was the discriminatory metabolite between the infants of secretor mothers and non-secretor mothers, displaying many of the same peaks as a standard analysed for comparison, displayed in Figure 78. Shared peaks included those at δ 1.23, 3.31, 3.59, 3.83, 3.91, 4.25, 4.53, 5.24 and 5.32.

5.5 Discussion

The principal aim of this chapter was to investigate whether maternal BMI influenced either the maternal or infant urinary metabolome, assessed by ^{1}H NMR spectroscopic analysis.

In regard to the influence of maternal BMI on maternal urinary metabolome, the only model possessing a Q^2Y value high enough to warrant further investigation was an OPLS model of samples collected at three months post-partum, using maternal booking BMI values. A potential reason for no strong correlation being identified between maternal BMI and urinary metabolome at the recruitment and week time points may be that effects due to maternal BMI were masked by the confounding effects of large metabolic changes immediately post-pregnancy, masking the effects of BMI on the urinary metabolome.

The corresponding loadings plot of this model indicated that the abundance of creatinine and TMAO was increased in the mothers possessing a higher BMI, whilst hippuric acid was increased in mothers possessing a lower BMI. However, no such observation was seen in samples collected at either the recruitment or one week time point.

The observation of a decreased abundance of hippuric acid in individuals of a higher BMI, has been recorded before, (Calvani et al., 2010). Hippuric acid is produced as a result of the co-metabolism of humans with their intestinal microbiota. Benzoic acid is firstly produced by several different strains of microbe, from various aromatic compounds in the diet; and then conjugated with glycine in the mammalian mitochondria (Nicholson et al., 2005).

Animal studies have identified hippuric acid as a metabolite which alters between calorie restricted rats, and those fed a normal diet. Calorie restriction is associated with multiple positive metabolic regulating effects and increased lifespan (Zhang et al., 2012). Further animal studies of obesity have identified hippuric acid as differing between obese and non-obese rodents (Waldram et al., 2009). This study also reports a difference in the microbial communities' between the obese and lean rats studied, with obese animals containing significantly lower numbers of total bacteria, as well as an altered microbial profile. This finding again highlights the importance of the microbiota in relation to the obese phenotype.

Hippuric acid has also been identified as a biomarker distinguishing north and south Chinese populations, who possess differing risks of cardiovascular disease (Yap et al., 2010). Furthermore, an inverse relationship between hippuric acid excretion in urine and individuals blood pressure has previously been reported, potentially identifying the protective mechanism (Holmes et al., 2008).

Higher concentrations of TMAO were associated with maternal BMI. Urinary TMAO occurs as a result of the interaction of both the individuals' diet, and microbial communities, particularly microbial metabolism of choline (Waldram et al., 2009).

Also increased in mothers of a higher BMI was creatinine. Previous research has indicated, in rats, that this metabolite is decreased in obese individuals (Waldram et al., 2009), not replicated in this work. Similarly, the effects of dietary restriction on the urinary metabolome of control, and diet restricted dogs have been evaluated using ^{1}H NMR spectroscopy. Here, it was found that diet restricted dogs had consistently elevated urinary excretion of creatinine, again not observed in this work (Wang et al., 2007).

However, in agreement with this work, obese individuals have been observed to have an increased amount of muscle mass in comparison to lean individuals (Mueller, 1988), and as creatinine positively correlates with muscle mass (Rikimaru et al., 1989), this finding is compatible with this research.

In regard to the results obtained from the infant urinary metabolome analysis, and the influence of maternal BMI on this, OPLS models produced comparing infants born to mothers of a high and normal BMI all possessed negative Q^2Y values. As these models have a negative predictive value, this suggests that there are no systematic differences between infants urinary metabolome born to mothers of differing BMI.

A possible explanation of why maternal BMI was not seen to influence infant urinary metabolome is by incorporating the results obtained in Chapter 4. Here, no differences were detected in the breast milk composition of overweight/obese compared to non-overweight/obese mothers. Therefore, as the composition of breast milk infants received from mothers with different BMI was similar, it may not be expected for differences to be detected in the infants' urinary metabolome.

One hypothetical reason to expect differences in the urinary metabolomes of infants born to mothers of a high, verses normal BMI may be detected, was the exposure of infants in utero to maternal obesity. However, this obviously did not have a detectable effect on the infants' urinary metabolome measured up to three months post-partum, as the models possessed negative Q^2Y values.

One limitation of this study was due to participants withdrawing from the study, particularly overweight and obese individuals. This resulted in a reduced power to detect differences in the urinary metabolomes of this group of mothers, as well as their infants, based on maternal BMI. This may be able to be determined with a larger cohort. Future studies should be designed to recruit greater number of overweight/obese mothers due to these mothers being more likely to stop breastfeeding, and therefore withdraw from study. However, overweight/obese mothers who are able to continue

breastfeeding may be different from mothers who stop breastfeeding, but this speculation would be difficult to test.

To investigate whether there were any run time effects, due to the analysis taking place over several days, an OPLS model was produced. This indicated that the analysis was reproducible and reliable over the course of the run, giving confidence in the results obtained, agreeing with previous literature indicating that urine is a relatively stable biofluid.

Although no identifiable differences in the infant urinary metabolomes were able to be detected in regard to maternal BMI, a number of other interesting influences on the urinary metabolomes of these individuals were able to be investigated.

One such influence, on the infant urinary metabolome, was whether the infant was mixed formula fed, or exclusively breast fed. On investigation of the urinary metabolomes, PAG was revealed to be increased in abundance in exclusively breast fed infants, in comparison to mixed formula fed infants.

The PAG metabolite occurs in urine as a consequence of microbial-mammalian co-metabolism (Yap et al., 2010). The intestinal microbiota putrefy dietary phenylalanine in the colon, converting it to phenylacetate, this is then transported to the liver where it is conjugated with glutamine (Swann et al., 2013). This demonstrates the early relationship between the intestinal microbiota and human metabolism. PAG has been associated with beneficial effects on cardiovascular health in later life (Yap et al., 2010). This finding suggests there are differences in the species or function, or both, of the microbiota in mixed formula fed infants in comparison to breast fed infants. It is well established that formula fed infants harbour a different microbiota compared to breast fed infants, (Harmsen et al., 2000).

PAG has also been described to be associated with age in American and Taiwanese individuals. In this study PAG was seen to positively associate with age, which the authors attributed to the influence of age on the host-microbiome metabolism (Swann et al., 2013).

Another interesting observation was the influence of gestational age on the infant urinary metabolome. Here, creatinine was found to be increased in infants born late term, whilst metabolites increased in abundance in infants born early term were acetate, TMAO and glycine. Previous research investigating the urinary metabolome of preterm infants against term born infants also observed an increase in TMAO in preterm infants urine and an increase of creatinine in term born infants, similar to the results presented here (Foxall et al., 1995). Creatinine has been shown to correlate to muscle mass, therefore this association of an increased concentration of creatinine in the infants of a higher gestational age, and therefore larger, makes sense physiologically (Rikimaru et al., 1989).

The authors of this study hypothesised the increased excretion of creatinine and lower excretion rates of amino, and organic acids could be ascribed to the maturation of the infants glomerular filtration and tubular function (Foxall et al., 1995).

Furthermore, analysis of the maternal urinary metabolome revealed that there were systematic alterations in the metabolomes of secretor verses non-secretor mothers (of 2'-FL and related HMO) This ability to distinguish maternal secretor status using maternal urine samples has been observed previously. The authors of one such study attributed this discriminatory potential to retrograde 'leakage' of HMO into maternal circulation, and its subsequent elimination in urine (Hallgren and Lundblad, 1977a) (Hallgren and Lundblad, 1977b). Whether HMO exerts any positive effects on maternal health is yet to be elucidated. It has however been hypothesised that, similarly to the protective effects HMO confer to neonates, HMO may protect the mammary gland from mastitis (Bode, 2012). For example, *Staphylococcus* has been shown to bind to 2'-FL in vitro, which may prevent it from binding to the mammary epithelium, initiating infection (Lane et al., 2011).

Likewise, investigation of the infant urinary metabolome revealed there were also systematic alterations in infants' urine born to secretor mothers, in comparison to non-secretor mothers. A potential discriminatory metabolite, seen to be increased in infants of secretor mothers, was 2'-FL. This is reasonable to expect as 2'-FL is one of the HMO present in the highest abundance and discriminate between secretor and non-secretor mothers.

The increased abundance of 2'-FL in the urine of infants of secretor mothers must be due to infant absorption of this HMO from the gastrointestinal tract. Previously, it has been thought that HMO are completely indigestible, but it has been demonstrated that certain HMO are able to be absorbed, and may be found in infant circulation (Goehring et al., 2014).

Excretion of HMO into infant urine was first described by Rudloff et al, who showed that intact HMO were present in the urine of breast fed, but not formula fed, infants (Rudloff et al., 1996). Rudloff et al further investigated their results using ^{13}C-labeling studies, administering labelled galactose and studying its metabolic fate. This research demonstrated approximately 1% of the HMO was excreted in the infant urine. The researchers hypothesise that these HMO, as well as having multiple important functions in the gastrointestinal tract, may also protect infants against urinary tract infections by acting as analogous receptors for pathogenic adhesion molecules (Obermeier et al., 1999) (Rudloff et al., 2006) (Rudloff et al., 2012).

5.5.1 Conclusions

There is some evidence to suggest there are alterations in the maternal urinary metabolome of mothers with a high BMI compared to a normal BMI, at three months post-partum, but not at delivery or one week post-partum.

No discernable differences were detected in the urinary metabolome of infants born to mothers with a high or normal BMI, at any of the three time points measured.

The data presented here suggest formula fed infants show a significantly different urinary metabolome compared to breast fed infants, likely driven by the differences in diet between these two groups of infant, at three months post-partum. One identified metabolite seen to be altered between these two groups of infant was phenylacetylglutamine, which had an increased abundance in breast fed infants. This metabolite is a product of bacterial co-metabolism, highlighting that differences in diet have the potential to impact infant intestinal microbiota.

One novel finding, to my knowledge, was the effect of gestational age on the infant urinary metabolome, in a group of term infants. Previously differences have been displayed in the urinary metabolomes of preterm vs term infants (Atzori et al., 2011).

A further finding was the impact of maternal secretor status on both the maternal and infant urinary metabolome.

Chapter 6
Discussion

Key conclusions

- A positive correlation between maternal BMI and breast milk leptin was identified using systematic review and an in vitro multiplex assay.
- No correlation between breast milk ghrelin, insulin, adiponectin, resistin, Peptide YY or GLP-1 and maternal BMI was detected, by either systematic review or *in vitro* analysis.
- Breast milk composition is conserved in relation to maternal BMI, identified using ^{1}H NMR spectroscopy, UPLC-MS and mid IR spectroscopy, investigating the aqueous, lipid and macronutrient content of breast milk.
- Changes in breast milk composition which were observed related to the well described differences in time of collection post-partum which samples are taken, as well as the differences between fore and hind milk.
- Maternal Secretor status can be readily identified using breast milk samples.
- Maternal BMI did not influence the infant urinary metabolome, at any time point measured.
- Maternal Secretor status influences both the maternal and infant urinary metabolome.
- Formula feeding was observed to influence the infant urinary metabolome.
- Maternal BMI influences the maternal urinary metabolome, with microbial co-metabolites implicated as differing between high and low BMI individuals.

6.1 Introduction

The objective of this thesis was to evaluate the hypothesis that maternal phenotype, specifically maternal BMI, has a discernable impact on breast milk composition. In order to investigate this, analytical techniques including metabonomics (^{1}H NMR spectroscopy and UPLC-MS), mid IR spectroscopy (in the form of a clinically utilised breast milk analyser), and a multiplex assay were employed to explore the metabolomic, nutrient and endocrine profile of breast milk.

Additionally, the sample set collected allowed for the analysis of multiple factors associated with alterations in breast milk composition, as well as differences in the urinary metabolomes, to be explored.

Evaluation of appropriate techniques for analysing breast milk composition, as well as the optimization of sample preparation methods prior to employing these analytical platforms are described in detail in the method development in Section 2.10, Chapter 2. Two peer reviewed publications have been produced from this method development work, together detailing the most comprehensive characterisation of breast milk to date (Villasenor et al., 2014), and (Andreas et al., 2015).

The impact of maternal BMI on various appetite regulating hormones present in breast milk was also determined, by both systematically reviewing the published literature, and performing an *in vitro* multiplex assay. The systematic review and *in vitro* studies both confirmed that breast milk leptin consistently correlated with maternal BMI. However, evidence for the other hormones analysed showed no clear association with maternal BMI. This systematic review was published in PLOS ONE (Andreas et al., 2014) and the in vitro analysis is currently under review, also at PLOS ONE.

Previous studies have reported a positive correlation between maternal BMI and breast fed infants' serum leptin from birth to 12 months. The authors of one such study propose that ingested leptin in breast milk is absorbed into the infants circulation, but this is yet to be confirmed (Savino et al., 2006). An alternative explanation could be that the leptin in infant circulation is synthesized by the infant, and correlates with maternal BMI due to the positive relationship between infant and maternal adiposity (Modi et al., 2011).

The majority of studies agree that with increasing maternal BMI there is a simultaneous increase in maternal plasma leptin, and an increase in breast milk leptin (Andreas et al., 2014). It is suggested that individuals who have a greater BMI become desensitised to leptins actions, resulting in its overproduction, i.e. leptin resistance. The quantity of leptin supplied to an infant through breast milk appears to be partially dependent on maternal adiposity. The leptin present in breast milk may reduce the appetite of infants, causing less weight gain in infants receiving higher concentrations of leptin. This has been suggested to be a mechanism to protect the infant against the impact of maternal obesity, protecting the infant against subsequent obesity and other metabolic disorders (Savino et al., 2009).

Doneray et al. found that the increase in breast milk leptin concentration over the first month of lactation is negatively correlated with the change in infant BMI over the same time period (Doneray et al., 2009). Likewise, Miralles et al. found a negative correlation between infant BMI at 18 and 24 months and breast milk leptin concentrations (Miralles et al., 2006). These studies suggest that milk

leptin concentrations are negatively correlated with infant BMI; however, results from these studies are correlations, and therefore not indicative of causality.

As described in Chapter 4, the composition of breast milk appears to be extremely well conserved in relation to maternal BMI. The analytical platforms used (^{1}H NMR spectroscopy, UPLC-MS and mid IR spectroscopy), analysing the aqueous, lipid and macronutrient content of breast milk respectively, found no association between maternal BMI and alterations in breast milk composition, in the sample set collected during this study.

It might be expected that breast milk composition is tightly regulated. Demonstrating this tight regulation, one study found the concentration of iron, zinc and copper in breast milk was independent of the maternal mineral status. (Domellof et al., 2004). Furthermore, another study investigating breast milk production from well-nourished and under nourished women found that, even in women possessing a low BMI, milk volume was comparable to that of the well-nourished mothers (Nazlee et al., 2011). Another study, investigating the composition of breast milk from under-nourished and well-nourished mothers, demonstrated that in under-nourished women, the milk composition remained comparable to that of well-nourished mothers (Soliman et al., 2014). Similarly, milk quality and quantity are found to be maintained in women with common illnesses, such as infection, when compared to healthy women (Zavaleta et al., 1995) (Lonnerdal et al., 1996).

From an evolutionary point of view, this conservation of milk composition and volume may also be expected. In terms of natural selection, the principle factor in selection is the successful rearing of infants to reproductive maturity. Advantageous traits, promoting the survival of infants through lactation, are therefore likely to be highly conserved. New-born mammalian infants, especially humans, are particularly dependant on their mothers for nourishment. The lactation period is a critical time in determining the survival of neonates, meaning milk composition is likely to be optimised for this purpose, in order to ensure the survival of the neonate.

Of the differences which were observed in the composition of breast milk, these could be ascribed to previously well documented factors affecting breast milk composition, such as time of collection post-partum, as described in Section 2.10, Chapter 2. Also exerting a large influence on the concentration of macronutrients present in breast milk was whether fore or hind milk was analysed, outlined in Section 4.4.4, Chapter 4. This demonstrates the importance of standardised collection of fore and hind milk, or of an entire feed, when conducting studies of milk composition.

In the aqueous fraction of breast milk, assessed using ^{1}H NMR spectroscopy, the largest variation observed was accounted for by the different profiles of HMO individual mothers produced, known to

be genetically determined (Thurl et al., 1997). HMO are postulated to have multiple protective and beneficial effects for the infants ingesting them. HMO act as prebiotics, selectively encouraging the growth of beneficial bacteria, especially *Bifidobacterium longum* subsp. *longum*, *B. longum* subsp. *infantis*, and *Bifidobacterium breve*, (Pacheco et al., 2014). As well as this, they act as anti-adhesive antimicrobials (Simon et al., 1997), exhibit immunomodulatory effects (Eiwegger et al., 2004), and have been proposed to play a role in brain development (Bode, 2012).

The intestinal microbiota outnumber mammalian cells ten to one, additionally, the gene set of the microbiota is approximately 150 times larger than that of the human genome (Qin et al., 2010). This microbiome is crucial for both the immune and metabolic development of the infant, executing many important functions (German et al., 2002). Therefore, due to the importance of the intestinal microbiome to human health, one would expect factors regulating the growth of the microbiota to be highly conserved, as is observed with the very high concentrations of HMO found in colostrum, produced during the period when the infant is colonised with microbiota.

Also investigated was the impact of maternal factors on the maternal and infant urinary metabolome, explored using ^{1}H NMR spectroscopy. Here, it was established that maternal BMI did not influence the infant urinary metabolome, at any of the time points measured.

This could be expected, because, as demonstrated in Chapter 4, maternal BMI was not seen to influence breast milk composition, meaning maternal BMI would be unlikely to be able to influence the infant urinary metabolome, unless this occurred through some other mechanism.

A possible mechanism hypothesised to suggest why there may be a difference in the urinary metabolome of infants born to obese mothers include the fact that obese and overweight mothers are more likely to formula feed, an observation noted in this cohort. Formula feeding was observed to influence the urinary metabolome, as outlined in Chapter 4. However, when models were reanalysed using only exclusively breast fed infants against maternal BMI, the Q^2 values were still negative, suggesting maternal BMI exerted no influence on infant urinary metabolome.

Another possible factor which could have led to differences in the infant urinary metabolomes from infants born to obese mothers could be due to alterations in the microbiota profile. Infants are known to be colonised by their exposure to the maternal microbiota (in vaginally delivered infants) (Biasucci et al., 2008), and as obese individuals have an altered microbiota, this could be expected to be passed on to the infants. However, no alterations in infant urinary metabolome were observed, potentially due to the early period in which samples were collected, as the microbiota are unlikely to have been established at this point.

Maternal BMI did appear to influence the maternal urinary metabolome. This effect has been observed in previous studies, where changes in metabolite abundances related to the gut microbiome were particularly pronounced, also highlighted in this study (Calvani et al., 2010).

6.2 Implications of findings for clinical practice

From this research it appears that maternal BMI exerts little influence on breast milk composition, other than leptin concentration. Due to the well documented, broad benefits of receiving breast milk, the current advice of 'breast is best' is a good recommendation to all mothers who are able to breast feed. These benefits are not just isolated to the neonate, but also extend to the mother, as breast feeding is associated with a reduced risk of type 2 diabetes, breast and ovarian cancer and maternal post-partum depression (Ip et al., 2007). As maternal BMI has such a small effect on the composition of milk, obese women should be encouraged to breast feed wherever possible, in order to provide their infants with all the nutritional and protective benefits breast milk contains.

However, in relation to the effects of maternal overweight/obesity on breastfeeding itself, obese mothers have been shown to have difficulty in initiating, and maintaining, breastfeeding (Donath and Amir, 2008, Oddy et al., 2006). A meta-analysis indicated that obese women have significantly reduced ability to initiate lactation, but once initiated, there is no difference from women of normal weight in lactation duration, meaning supporting of lactation initiation is key (Prior et al., 2012). It has also been reported that mothers who are overweight produce a smaller volume of breast milk (Barbosa et al., 1997). The authors of this study proposed that the reduced intake of breast milk of infants of obese mothers is balanced by an increase in the calorific density of breast milk from obese mothers (Barbosa et al., 1997). However, this increase in calorific density was not seen in the cohort of mothers recruited for this study.

In agreement with this observation, previous studies have also demonstrated infants of overweight/obese mothers have decelerated early growth, gaining less weight and less fat mass, with these results remaining unaltered after adjustment for breastfeeding status (Ode et al., 2012). However, studies on the effect of maternal BMI on early infant growth have produced varied results, with some studies concluding that maternal BMI has no effect on early infant growth (Regnault et al., 2010), whilst others showing increased maternal BMI increases early infant growth (Ong et al., 2008), and others showing reduced infant growth for infants of overweight obese mothers (Ode et al., 2012). This discrepancy in findings may be accounted for by studies solely measuring infant weight rather than body composition. Further reasons for this discrepancy may be due to bottle feeding versus self-regulated suckling, or the infant either being formula or breast fed.

The results from my study may help to explain the effect of reduced growth rates in infants of obese mothers. shown that milk from obese mothers appears to be of the same nutritional quality as that of lean mothers; if obese mother's infants have a lower milk intake, they may receive less energy than infants of lean mothers, explaining the poorer growth rates seen in obese women's infants.

Alternatively the slower growth rate of infants born to obese mothers may reflect physiological “catch-down” growth following a period of intrauterine over-nutrition.

The data presented here should encourage doctors, lactation consultants and overweight/obese mothers to support breast feeding, given that the composition of overweight/obese mother’s milk is comparable to that of normal weight mothers.

In this work, a mid-IR human milk analyser was used to investigate the macronutrient content of breast milk. This instrument is intended for use in neonatal units to aid clinical decision making, to assess whether breast milk intended for premature infant requires nutritional fortifying. As results obtained from this instrument will be heavily influenced by the time of sample collection post-partum, and whether the sample was collected at the beginning or end of a feed, potentially limiting its clinical usefulness. A clinical research group in the Netherlands has employed mid IR analysis in order to combine milk samples with a high protein content to feed premature infants (Michaelsen et al., 1990). However, the utility of this approach has not yet been demonstrated, and furthermore the optimal growth velocity from preterm babies is not known.

In conclusion, breast milk composition is well conserved across the maternal BMI range, with the exception of leptin concentration. The functional relevance of increased breast milk leptin content to the offspring is unclear. Overweight and obese women have difficulty in initiating lactation and hence added efforts are recommended to support them.

6.3 Strengths and limitations

My thesis has a number of strengths and limitations. One limitation was the use of BMI as a proxy marker, as previously this has been shown to have a poor correlation with body composition, highlighted by methods such as whole body MRI (Thomas et al., 2012). Thomas et al describe a number of metabolic phenotypes, where an individual may have a healthy BMI, but possesses excess intra-abdominal adiposity (associated with an adverse metabolic profile). Also described are individuals with a high BMI, but low quantities of intra-abdominal fat, who are metabolically normal.

Furthermore, the time at which these mothers were investigated meant their BMI values were particularly unstable, displayed by the increase and subsequent decrease in the average maternal BMI over the study period. These differences in BMI values are unlikely to be wholly accounted for by increased maternal adipose tissue, but influenced by other factors such as maternal fluid balance. Alterations in adipose tissue quantity and distribution may have been better classified by magnetic resonance imaging (MRI), as this technique is able to delineate individual adipose tissue depots. MRI was not carried out due to the associated increased costs and time required, as well as difficulty in recruiting patients when requesting multiple MRI scans. In order to account for this the mothers booking BMI values were used.

Another limitation faced was the relatively small number of samples originating from mothers with a higher BMI included in some of the analyses. This was due to the rapid post-partum weight loss many mothers experienced, meaning they were classified as having a BMI >25-30kg/m^2 at recruitment, but not at the subsequent study visits. Analyses were carried out using maternal booking BMI values in order to account for this; however the same issue was apparent here as there were not equal numbers of individuals in the overweight/obese group against the lean group at the booking time point. This meant there were significantly fewer participants with a BMI over 25-30kg/m^2 than expected at the time one week and three month time points.

Additionally, another point to highlight is the specific population investigated throughout this thesis. All the mothers were recruited from a single hospital in west London and can be classified as 'WEIRD' (Western, Educated, Industrialized, Rich, and Democratic). This raises the question of how generalizable the results are to other cohorts of women, as the recruitment procedure used may have resulted in a non-representative population being sampled. However, if we make the assumption that the techniques used here are investigating the basic biology and metabolism of the mothers and infants, then this presumably means the results can be extrapolated to different groups of mother. However, in order to confirm this, further studies must be undertaken, recruiting mothers in different environments to the group here. An important consideration in these studies would be the different

diets of the mothers, which is known to have a particularly large influence on the fatty acid profiles of breast milk.

A major strength of this work was the standardisation of breast milk sample collection. All samples were collected mid-morning to control for variation in breast milk composition over the course of the day, as fat concentrations in breast milk are known to peak mid-morning. Furthermore, the vast majority of samples were collected on day seven for the one week sample collection, reducing the variability in breast milk composition with regard to time of collection post-partum. Additionally, a distinction between fore and hind milk was made, allowing further influencing factors to be investigated, and introducing further control of sample composition.

Strengths of this work include the assessment of each breast milk sample by both ^{1}H NMR spectroscopy, in order to characterise the aqueous phase, and UPLC-MS, for the characterisation of the lipid phase, allowing greater coverage of the breast milk metabolome.

Furthermore, like any piece of work requiring an analytical platform, there are various limitations imposed by the limitations of the instruments themselves. For example, NMR spectroscopy has a relatively high limit of detection in terms of the concentration of metabolites present, in relation to MS, meaning, in order to characterise metabolites present in lower concentrations, MS must be used.

Likewise, the sample preparation methods employed are also able to introduce analytical artefacts, for example, contaminants present in solvents and collection tubes used. One problem encountered, introduced by the sample preparation method, was the binding of TSP to the remnants of protein which remained in the sample. This resulted in the TSP precipitating out of solution and therefore not being measured, meaning for the breast milk samples analysed by NMR, quantification of metabolite concentrations across samples was not possible, as there was no valid comparator. These factors limit the ability to compare across different studies, if different sample preparation protocols were employed.

It was expected that the solvent extraction process would remove the protein from samples, however a proportion must have remained, which was unfortunately missed during the method development period. A possible approach to address this issue would be to use a greater volume of solvent, if the solution was saturated with protein. However, using a greater volume of solvents accrues greater costs, as well as increasing issues of disposal and the health implications of handling these solvents.

Solely investigating breast milk composition reduces the complexity of the relationship between the mother and infant communicated via breast milk. For example, factors present in breast milk are

altered during digestion, and can take on new properties, such as peptides produced from the digestion of proteins capable of acting as antimicrobial agents.

Additionally, breast milk contains a number of enzymes, which not only aid the digestion of milk, but also play important roles in gut development. The gut of the human infant has little lipase activity, relying on the presence of maternally derived lipoprotein lipase and bile salt stimulated lipase for digestion of the tri- and diacylglycerols present in breast milk (Blackberg and Hernell, 1981). The relative changes in lipase concentrations in the breast milk over the course of lactation, is a possible cause of altered lipid profile. This means, depending on the lipase activity, nutrition may be provided to the infant, but remain unavailable were it to go undigested.

Furthermore, breast milk composition also influences the intestinal microbiota, meaning the quantity and profile of HMO produced will influence the abundance and classes of commensal bacteria present in the gut. As different microbes are able to carry out different functions, it is likely that this will affect infant physiology, which was not investigated in the present study.

6.4 Future Directions

In relation to the investigation of how maternal overweight/obesity may influence breast milk composition I was unable to reject my null hypothesis, suggesting breast milk composition is extremely tightly regulated, even in conditions such as obesity. Furthermore, where breast milk composition does vary, such as in relation to fore or hind milk, or over lactation, this occurs in a predictable manner.

If infants fail to grow properly, as milk composition is so tightly regulated, this suggests that it may not be the quality of milk, but the supply which determines failure to grow adequately. This finding should encourage further investment into the provision of lactation consultants for new mothers, who are able to support breast feeding. This will ensure the proper growth of infants, and that both infants and their mothers receive all the health benefits of breast feeding.

As breast milk composition is so well conserved, determining its composition would have little value in a healthy term pregnancy. One situation in which screening of breast milk may be worthwhile is as a stratifying variable in neonatal clinical trials. Trials investigating the efficacy of antibiotics may want to assess the HMO profile of mothers, as HMO have been shown to influence the incidence of various infections, they may act as a confounding factor, obscuring the true efficacy of investigational treatments.

Furthermore, for mothers of premature infants, and those at risk of necrotising enterocolitis (NEC), there may, in the future, be a benefit of screening mother's breast milk for the presence of specific protective factors.

For example, previous research has demonstrated that, in neonatal rats, the HMO disialyllacto-N-tetraose can prevent NEC (Jantscher-Krenn et al., 2012). This protective effect has also been observed for human infants (Van Niekerk et al., 2014), meaning there may be a case for screening mothers breast milk for the presence of this oligosaccharide, and supplementing breast milk with this HMO in infant populations who experience a high incidence of NEC.

Likewise, HIV is less likely to be transmitted to breastfeeding infants if the mother's breast milk contains a higher HMO concentration, specifically lacto-N-neotetraose (Bode et al., 2012). Again, this poses the possibility of supplementing HIV infected mothers breast milk with this HMO to prevent the transmission of HIV to her infant.

There are multiple instances where it may be feasible to screen breast milk for different protective factors, and supplement with appropriate factors in order to reduce the burden of disease resulting from vertical transmission of infections from mother to infant.

This work has highlighted the importance of sample preparation methods prior to metabolomic analysis, and attempted to optimise these, resulting in a publications in Analytical Chemistry (Villasenor et al., 2014), and Electrophoresis (Andreas et al., 2015).

Future studies must ensure that sample collection is, as far as possible, standardised, especially in regard to time of collection post-partum, as there is a large amount of variation in breast milk composition in regard to this. In addition, this work provides data to ensure future experiments investigating these factors are adequately powered. One factor which potentially has an impact on breast milk composition is maternal diet. This was not explored in this work, but is an interesting avenue of future research.

Likewise, this work has drawn attention to the influence of diet on infant metabolism, this was clearly demonstrated by the separation in the urinary metabolomes of the formula fed, and breast fed infants, and the subsequent identification of phenylacetylglutamine (PAG) as a metabolite discriminating between these groups, being increased in the breast fed infants. This indicates a clear relationship between infant diet and urinary metabolome.

This work may also aid in the development of better formula compositions, improving the health and development of formula fed infants, as there are clearly many improvements to be made. I believe the formula industry must prioritise research into the manufacturing of HMO in large quantities, and the addition of these to formula milk, due to the clear benefits these compounds have for infants.

However, even if the formula industry is capable of more closely reflecting the composition of breast milk, there are still advantages for both mother and infant from the act of breastfeeding itself. For example, not breastfeeding is associated with an increased risk of post-partum depression (Warner et al., 1996). In terms of benefits for the infant, previous research has indicated that regardless of whether fed breast or formula milk, bottle feeding is associated with increased infant weight gain, as infants are less able to regulate their own intake (Li et al., 2012).

This work highlights the importance of breast milk not only as a source of nutrition, but for the multitude of other factors present, essential for the proper development of the infant, of which we are just starting to learn. Future studies could also investigate the alterations of various metabolites present in partially digested/digested breast milk, and attempt to ascribe functions to them, as they may take on new properties.

For example, greater understanding of multifunctional metabolites, such as HMO, will mean more importance will be given to breast feeding as the optimal way to feed infants. Future work should focus on the specific interactions of HMO with the microbiota, as well as the functions of the

microbiota themselves. It would be of interest to investigate how different profiles of HMO may selectively stimulate specific species of microbiota.

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Appendices

Appendix 1 - Background

Appendix 1A: Table outlining the metabolites present in breast milk.

Metabolite	Concentration	Notes	References
	g/litre		
Lactose	72.0±2.5		(1985)
Oligosaccharides	20.9±5, 12.9±3.30	Day 4, Day 120	Coppa et al
Protein	10.5±2.0		(1985)
Fat	39.0±4.0		(1985)
	mg/litre		
Calcium	280±26		(1985)
Phosphorous	140±22		(1985)
Magnesium	35±2		(1985)
Sodium	180±40		(1985)
Potassium	525±35		(1985)
Chloride	420±60		(1985)
Iron	0.3±0.1		(1985)
Zinc	1.2±0.2		(1985)
Copper	0.25±0.03		(1985)
Vitamin E	2.3±1.0		(1985)
Vitamin C	40±10		(1985)
Thiamine	0.210±0.035		(1985)
Riboflavin	0.350±0.025		(1985)
Niacin	1.5±0.2		(1985)
Vitamin B_6	93±8		(Styslinger and Kirksey, 1985)
Pantothenic acid	1.8±0.2		(1985)
	µgrams/litre		
Vitamin A	670±200		(1985)
Vitamin D	0.55±0.10		(1985)
Vitamin K	2.1±0.1		(1985)
Folate	85±37		(Brown et al., 1986)
Vitamin B12	0.97		(Sandberg et al., 1981)
Biotin	4±1		(1985)
Iodine	110±40		(1985)
Selenium	20±5		(1985)
Manganese	6±2		(1985)
Fluoride	16±5		(1985)
Chromium	50±5		(1985)
Molybdenum	Not reported		(1985)
Amino acids	**µmol/dl Colostrum, Transitional, Mature**		(Carlson, 1985)

Glutamic acid	36-68, 88-127, 101-180		
Glutamine	2-9, 9-20, 13-58		
Taurine	41-45, 34-50, 27-67		
Alanine	9-11, 13-20, 17-26		
Threonine	5-12, 7-8, 6-13		
Serine	12, 6-11, 6-14		
Glycine	5-8, 5-10, 3-13		
Aspartic acid	5-6, 3-4, 3-5		
Leucine	3-5, 2-6, 2-4		
Cysteine	1-3, 2-5, 3-6		
Valine	3-4, 3-6, 4-6		
Lysine	5, 1-11, 2-5		
Histidine	2, 2-3, 0.4-3		
Phenylalanine	1-2, 1, 0.6-2		
Tyrosine	2, 1-2, 1-2		
Arginine	3-7, 1-5, 1-2		
Isoleucine	2, 1-2, 1		
Ornithine	1-4, 1, 0.5-0.9		
Methionine	0.8, 0.3-3, 0.3-0.8		
Phosphoserine	8, 5, 4		
Phosphoethanoamine	4, 8, 10		
α-aminobutyrate	1, 0.4-1.4, 0.4-1		
Tryptophan	5, 1, 1		
Proline	-, 6, 2-3		
Citrulline			
Protein nitrogen	**g/l**		(Forsum and Lonnerdal, 1980)
Casein nitrogen	2.5		
Whey protein nitrogen	6.4		
α-Lactalbumin	2.6		
Lactoferrin	1.7		
B-Lactoglobulin	-		
Lysozyme	0.5		
Serum albumin	0.5		
IgA	1		
IgG	0.03		
IgM	0.02		
Proteins	415 identified		
Non-protein nitrogen	**g/l**		
Urea nitrogen	0.25		(Forsum and Lonnerdal, 1980)
Creatine nitrogen	0.037		
Creatinine nitrogen	0.035		

Uric acid nitrogen	0.005		
Glucosamine nitrogen	0.047		
α-Amino nitrogen	0.13		
Ammonia nitrogen	0.002		
Amino sugars			
N-Acetylglucosamine	230mg N/L	N/L=nitrogen/litre	
N-Acetylneuraminic acid	63 mg N/L		
Peptides			
Epidermal growth factor	88 ng/ml		
Somatomedin-C/insulin-like growth factor	18 ng/ml		
Delta sleep-inducing peptide	30ng/ml		
Carnitine	62.9 nmol/ml	Mean over 50 days	(Novak et al., 1981)
Choline	0.21±0.05 mmol/l		(Holmes et al., 2000)
Phosphocholine	0.48±0.07 mmol/l		(Holmes et al., 2000)
Glycerophosphocholine	0.41±0.08 mmol/l		(Holmes et al., 2000)
Phosphatidylcholine	0.10±0.01 mmol/l		(Holmes et al., 2000)
Sphingomyelin	0.10±0.01 mmol/l		(Holmes et al., 2000)
Citrate mg/dL	41.9 ± 5.9, 116.7 ± 5.7, 64.8 ± 8.8	Days 2-3, 14-12, 120-180.	
Prostaglandins	**pg/ml**		
Prostaglandin E	149±37, 224±64	5-7 days, fore/ hind milk	(Lucas and Mitchell, 1980)
Prostaglandin F	425±152, 671±322	5-7 days, fore/hind milk	(Lucas and Mitchell, 1980)
Prostaglandin E2	162 ± 135, 198±148	3 days post-partum Foremilk, hind milk	(Alzina et al., 1986)
6-ketoprostaglandin F1 **α**	818 ± 386, 795 ± 487	3 days post-partum Fore/hind milk	(Alzina et al., 1986)
Thromboxane B2	3557 ± 2770, 4476 ± 4053	3 days post-partum Fore/hind milk	(Alzina et al., 1986)
$PGF_{2\alpha}$	221 ± 70, 534 ± 350	Fore/hind milk	(Reid et al., 1980)

Nucleotides	**mg/dl**		(Janas and Picciano, 1982)
CMP	461±17.9		
UMP	179±19.8		
AMP	175±12.8		
IMP	228±14.5		
GMP	138±8.5		
UDP	174±12.8		
CDP	474±41.5		
ADP	69±17.9		
GDP	96±8.9		
Polyamines	**nmol/L**		
Putrescine	0-615		(Pollack et al., 1992)
Spermidine	73-3512		(Pollack et al., 1992)
Spermine	722-4458		(Pollack et al., 1992)
Cadavenne	Present		(Sanguanser msri et al., 1974)
Enzymes			
Lysozyme	0.2mg/ml		
Secretory leukocyte protease inhibitor	19 µg/l		(Semba et al., 1999)
Monosaccharides	**g/dl**		
Glucose	1.4		
Galactose	1.2		
Fucose			
Hormones			
Oxytocin	3.3-4.7mg/ml	Increases with nursing	(Takeda et al., 1986)
Leptin	1.5 ± 0.87ng/ml		(Houseknec ht et al., 1997)
Ghrelin	571 ± 128pg/mL		(Aydin et al., 2007)
Obestatin	528.5 pg/mL		(Aydin et al., 2007)
Resistin	375±29 pg/mL		(Ilcol et al., 2008)
Adiponectin	29.5 ± 6.4 ng/mL		(Dundar et al., 2010)

Progesterone	10-40ng/ml		(Koldovsky, 1995)
Pregnanediol	0-450ng/ml		(Koldovsky, 1995)
Prolactin	0.5 ng/mL		(Hines et al., 2007)
Estradiol	0.6 pg/mL		(Dawa et al., 2009)
Oestrogens	15-840ng/ml		(Koldovsky, 1995)
Transforming growth factor alpha	0-8.4 ng/ml		(Koldovsky, 1995)
Gastrin	10-30pg/ml		(Koldovsky, 1995)
Gastric inhibitory peptide	33-59ng/ml		
Gastric regulatory peptide	31-55pg/ml		
Neurotensin	7-15pg/ml		
Peptide histidine methionine	3-32pg/ml		
Peptide YY	15-30 pg/ml		
Insulin	21 ng/ml		
Lipids	**% weight**		(Specker et al., 1987)
Saturates			
8:0	0.22±0.01		
10:0	1.57±0.09		
12:0	5.47±0.66		
14:0	6.54±0.73		
16:0	20.48±0.64		
18:0	8.14±0.55		
20:0	0.57±0.03		
Total % weight	42.99%		
Monounsaturates			
16:1	3.35±0.28		
18:1	34.7±0.86		
Total	38.08%		
Polyunsaturates			
18:2	14.47±1.98		
20:2	0.50±0.03		
20:3	0.56±0.03		
20:4	0.68±0.03		
18:3	1.85±0.16		
22:6	0.27±0.08		
Total	18.33%		
	% total of lipid		(Koldovsky, 1995)

Triacylglycerol	58		
Diacylglycerol	8		
Monoacylglycerol	0.6		
Sterols	0.7		
Unesterified fatty acids	7.3		
Phospholipids	23		
	% phospholipid		
Sphingomyelin	26		
Phosphatidyl choline	30		
Phosphatidyl ethanolamine	37		
Phosphatidyl inositol	5		
Phosphatidyl serine	1		
Lysophosphatidyl choline	2		
Glycosylceramides	1–20 mg/L		(Jensen, 1999)
Type of triglyceride	**% of total (mol/100 mol TG)**		(Jensen, 1999)
16:0-18:1-18:1	11.8		
16:0-18:1-18:2	10.0		
16:0-16:0-18:1	4.4		
18:1-18:1-18:2	3.3		
14:0-16:0-18:1	3.3		
16:0-18:2-18:2	3.2		
12:0-16:0-18:1	3.1		
16:0-18:0-18:1	3.1		
14:0-18:1-18:1	2.8		
16:0-16:0-18:2	2.4		
14:0-18:1-18:2	2.3		
18:1-18:1-18:1	2.1		
16:0-18:1*t*-18:1	2.1		
18:1-18:2-18:2	1.9		
16:0-16:1-18:1	1.9		
16:0-18:0-18:2	1.7		
14:0-16:0-18:2	1.5		
12:0-18:1-18:1	1.4		
12:0-16:0-18:2	1.3		
12:0-18:1-18:2	1.2		
12:0-14:0-18:1	1.1		
18:0-18:1-18:2	1.0		
Oligosaccharides	**g/l**		(Kunz et al., 2000)
Lacto-n-tetraose	0.5-1.5		
Lacto-n-fucopenatose I	1.2-1.7		
Lacto-n- fucopenatose I	0.3-1.0		
Lacto-n- fucopenatose I	0.01-0.2		

Lacto-n- difucohexose I	0.1-0.2		
NeuAc(**α**2-6)lactose	0.3-0.5		
NeuAc(**α**2-3)lactose	0.1-0.3		
NeuAc-lacto-N-tetraose a	0.03-0.2		
NeuAc-lacto-N-tetraose c	0.1-0.6		
$NeuAc_2$-lacto-N-tetraose	0.2-0.6		
Cytokines	**pg/mL**		(Goldman et al., 1996)
IL-1β	1130±478		
IL-6	151±89		
IL-8	3684±2910		
IL-10	3400±3800		
TNF-**α**	620±183		
G-CSF	~358		
M-CSF	17120 U/mL		
EGF	~200,000		
TGF-**α**	~2200-7200		
TGF-β_2	130±108		
sCD-14	5.08 µg/mL		(Collado et al., 2012)
INF-y	196.57 pg/mL		(Collado et al., 2012)
IL-4	20.6		(Collado et al., 2012)
IL-2	22.73		(Collado et al., 2012)
Cholesterol and precursors		2nd month of lactation	
Cholesterol	10-20mg/dl		(Jensen, 1999)
Squalene	386µg/dL		
Lanosterol	94µg/dL		
Dimethylsterol	45µg/dL		
Methostenol	48µg/dL		
Lathosterol	43µg/dL		
Desmosterol	1509µg/dL		
Bile Salts	µmol/L		(Forsyth et al., 1983)
Cholate	0.89±0.13		
Chenodeoxycholate	0.26±0.057		
Antioxidants			

Malondialdehyde	0.72±0.44 μM		(Silvestre et al., 2010)
Glutathione Peroxidase	18.19±9.45 mM/L·min		(Silvestre et al., 2010)
Cells			
Total cell count	593 ± 205 × 10^6/L 1.74 ± 0.97 × 10^6/L	Colostrum Mature	(Jin et al., 2011)

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Appendix 2 - Methods and Method Development

Appendix 2A

NRES Committee London - Fulham
Charing Cross Hospital
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Fulham Palace Road
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Telephone: 020 331 17282
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27 February 2012

Professor Neena Modi
Professor of Neonatal Medicine
Imperial College London
Neonatal Medicine
Chelsea and Westminster Hospital
369 Fulham Road, London
SW10 9NH

Dear Professor Modi

Study title: **The influence of maternal metabolic phenotype on breast milk composition, infant urinary metabolome and infant stool microbiome.**
REC reference: **12/LO/0203**

The Research Ethics Committee reviewed the above application at the meeting held on 20 February 2012.

Ethical opinion

Dr Matthew Hyde, Suzan Jefferies, and colleague attended the meeting.

They explained that the volume of feed the baby took from the breast depended the baby's age and weight. However it was estimated that on day 1 the baby would probably take 7mls and this might rise to 20 mls by day 7. The mother usually produces 750-1000mls every 24 hours. If the breast became dry then the hind milk would not be taken.

It was understood that a milk sample of 25mls was required, by the investigators, as the whole milk has fat content and the lab needed enough of the milk sample to cover that.

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

NHS Sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

Appendix 2A (continued)

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations

It is responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering Letter		24 January 2012
Evidence of insurance or indemnity	1	24 January 2012
Investigator CV	Neena Modi	30 January 2012
Investigator CV	Student - Nicholas James Andreas	30 January 2012
Letter from Sponsor	Letter from Funder - Westminster Medical School	26 April 2011
Other: 7-Day food record diary	1	23 January 2012
Other: CV- Dr Matthew James Hyde		30 January 2012
Other: CV - Professor Elaine Holmes		30 January 2012
Participant Consent Form	1	23 January 2012
Participant Information Sheet	1	23 January 2012
Protocol	1	24 January 2012
Questionnaire: Patient recruitment forms	1	30 January 2012
REC application	3.3	24 January 2012
Referees or other scientific critique report	Caroline King - Paediatric dietitian	30 January 2012

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Appendix 2A (continued)

Dr Colin Michie said he worked with Neena Modi. The Committee agreed that he could help with any technical issues during the discussion, but the final decision would be taken without his vote.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

12/LO/0203	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project

Yours sincerely

pp.
Dr Charles Mackworth Young
Chair

Email: lara.callaghan@imperial.nhs.uk

Enclosures: *List of names and professions of members who were present at the meeting and those who submitted written comments*
"After ethical review – guidance for researchers"

Copy to: *Ms Becky Ward*
Mrs Doris Daby, Chelsea and Westminster NHS Foundation Trust

Appendix 2A (continued)

NRES Committee London - Fulham

Attendance at Committee meeting on 20 February 2012

Committee Members:

Name	*Profession*	*Present*	*Notes*
Mrs Marian Cohen	Retired Lawyer	Yes	
Prof Alison Crombie	Anthropologist Nurse	Yes	
Dr David Evans	General Practitioner	No	
Dr Kanagasabai Ganeshaguru	Retired Scientist	Yes	
The Rev'd Nigel Griffin	Hospital Chaplain	Yes	
Dr Shaun Griffin	Director of Communications and Public Affairs	No	
Dr Akil Jackson	Physician	Yes	
Mr David Leonard	Pharmacist	Yes	
Mr David Leonard	Pharmacist	Yes	
Dr Colin Michie	Paediatrician	Yes	
Dr Frank Miskelly	Physician (Vice-Chairman)	Yes	
Dr Shirlony Morgan	Psychiatrist	Yes	
Professor Sandra Oliver	Retired Academic	Yes	
Mr Vassilios Papalois	Surgeon	Yes	
Lady Roche	Lay Member	Yes	
Mrs Gillian Sichau	Occupational Therapist	Yes	
Mrs Katie Wilkinson	Clinical Trials Centre Manager	No	
Dr Ruth Williamson	Radiologist	Yes	
Dr Charles Mackworth Young	Consultant in Rheumatology	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Ms Lara Callaghan	Assistant Coordinator
Rosalind Cooke	Committee Coordinator

Appendix 2B

Chelsea and Westminster Hospital NHS
NHS Foundation Trust

Information Sheet for Parents and Guardians

Title of Project:

Breast milk: A messenger between mother and baby?

We would like to invite you and your baby to participate in a research project.
Please read this information carefully. Ask us if there is anything that is not clear. Take time to decide whether or not you wish to take part.

What is the purpose of this study?
Human breast milk is complex. In addition to providing your baby with nutrients, breast milk contains hormones, such as insulin, immune factors and complex sugars which help protect your baby against infection. Little is known about the purpose of many of these components, but we do know that breast milk composition is unique to each mother. Breast milk has positive benefits for infant health and we wish to understand how different components influence this.

In order to study this, we wish to collect small samples of breast milk and urine from mums, and small samples of urine and stool from their babies. We will use new techniques to identify a large number of metabolites in the samples we collect. This will enable us to examine the relationship between a mother's metabolism, her breast milk and her baby's metabolism.

Why have we been chosen to take part in this study?
We are approaching parents of full-term healthy babies who are being breastfed.

Do I have to take part?
It is up to you to decide whether you would like to take part. If you agree you will be given this information sheet to keep and asked to sign a consent form a copy of which will be given to you. If you decide to take part you are still free to withdraw at any time and without giving a reason. A decision not to take part will not affect the standard of care you and your baby receives.

What does the study involve?
If you agree to take part in the study we would like to see you three times:

1) While you are still in hospital, if you are producing excess milk to what your baby requires, we will ask you for a small breast milk sample (5ml) and a small urine sample (15ml). From your baby we will collect a small (15ml) urine sample using cotton wool placed in your baby's nappy and a small stool sample, also from your baby's nappy. We will also collect some basic information about how your baby was born, your diet, and family history of allergy and weigh and measure you and your baby.
2) Between 7 and 14 days after your baby's birth, we will arrange to see you again. This can either be a home visit by our research nurse, or you can come into the hospital if you prefer. We will ask you not to suckle your baby from one breast for 1 hour before collection. At the time of collection we would ask you for a sample of up to 20ml of breast milk. Your baby can then have a feed and at the end we will ask you to collect another small sample (5ml). We will also collect another small (15ml) urine sample from your baby and you; and a small stool sample from your baby; and weigh and measure you and your baby.
3) Around 3 months after you have had your baby, we will arrange a final visit, again either at home, or in the hospital-if you are still breast feeding. We will ask you to collect a breast milk sample in the same way as before, and will collect another small (15ml) urine sample from your baby and you; plus a small stool sample from your baby; and weigh and measure you and your baby.

Appendix 2B (continued)

How will the samples be analysed?
The samples we collect in this study will be analysed at Imperial College London. A small part of the analysis will be carried out by Imperial College PhD student, Nicholas Andreas, in the laboratories of Danone International (based in The Netherlands and Germany). This is because Danone has access to research facilities which are not available at Imperial College. No personal information will be sent to Danone. All data arising from your samples will belong to Imperial College London.

The tests used to analyse the samples are not diagnostic tests and will not tell us if your baby is ill. Your baby's involvement in the study will cease following collection of the samples.

After analysis samples will remain in secure storage at Imperial College London for up to 10 years for possible use in future research studies, but this would only be if additional research ethics approval is obtained. If no further studies are approved the samples will be destroyed.

What are the possible disadvantages and risks of taking part?
There are no known risks involved with participation.

What are the possible benefits of taking part?
There are no direct benefits to you or your baby. The information from this study will help us understand human milk as a potential biological determinant of infant health and wellbeing.

What happens if something goes wrong?
It is extremely unlikely that anything will go wrong. However we are required to inform you that in the unlikely event of your suffering any serious adverse effects directly related to your participation in this study, you are eligible to claim for compensation through the Imperial College School of Medicine's "No Fault" Compensation Scheme.

Will my taking part in this study be kept confidential?
All information collected in the course of the research will be kept strictly confidential.

What will happen to the results of the research study?
This project is being undertaken as part of a PhD at Imperial College London. We will publish the results in a scientific journal. No participant will be identified in any publication. If you wish we will send you a copy of the results.

Who has reviewed this study?
The Fulham Research Ethics Committee have reviewed and approved this study.

Thank you for reading this. Please do not hesitate to ask us if you have any questions.

Prof. Neena Modi Professor of Neonatal Medicine Imperial College London Tel 020 33155102	Dr Matthew Hyde Research Associate Imperial College London Tel 020 33158241	Suzan Jeffries Research Nurse Imperial College London Tel 020 33153157	Nicholas Andreas PhD Student Imperial College London Tel 020 33158241

Appendix 2B (continued)

Chelsea and Westminster Hospital NHS
NHS Foundation Trust

Consent Form

Title of project: Breast milk: A messenger between mother and baby?

Patient's Name (Mother):

Patient's Name (Baby):

The mother should complete the whole of this sheet by herself.

		Please initial
1)	I have been fully informed of what the study involves for me and my baby.	☐
2)	I have read the Information Sheet for Parents (**Breast milk: A messenger between mother and baby?/Version 1/230112**) and have been given the opportunity to ask questions and discuss this study.	☐
3)	I understand that I and my baby are free to withdraw from the study at any time, without having to give a reason and without affecting our medical care or legal rights.	☐
4)	I understand that the medical notes belonging to me and my baby may be looked at by researchers, sponsor or regulatory bodies, involved in this study.	☐
5)	I agree that samples collected during this study may be stored for future ethically approved projects.	☐
6)	I agree to take part in this study.	☐
7)	I agree to my baby taking part in this study.	☐
8)	I would like to have a copy of the results sent to me	☐

Name of Mother	**Date**	**Signature**
Name of Person taking consent (if different from researcher)	**Date**	**Signature**
Researcher	**Date**	**Signature**

Appendix 2C

Imperial College London

Chelsea and Westminster Hospital NHS
NHS Foundation Trust

Breast milk: A messenger between mother and baby? 12/LO/0203 version 1/230112

PATIENT RECRUITMENT FORM

FORM COMPLETED *(initials)* NA / SJ on *(date)* __ / __ / 20 __	RECRUITED BY *(initials)* NA / SJ

STUDY IDENTIFIER:

THIS SHOULD BE ENTERED ON ALL PARTS OF THIS FORM:

__/ _ _ _ _ _/ _ _ _ _

INFANT INFORMATION:

INFANT'S SURNAME: INFANT'S FIRST NAME:

INFANT'S HOSPITAL NO.: INFANT DATE OF BIRTH (DD/MM/YYYY): __/ __ / 20 __

INFANT GENDER: MALE ☐ FEMALE ☐

PARENTAL INFORMATION:

MOTHER'S FULL NAME:

MOTHER'S HOSPITAL NUMBER: MOTHER'S DATE OF BIRTH (DD/MM/YYYY): __/ __ / 19 __

FATHER'S FULL NAME:

Do the parents wish to be contacted regarding study outcome?	Yes ☐	No ☐
Are the subjects willing to be re-contacted to participate in future studies?	Yes ☐	No ☐

If *YES* to either of the above options, please complete:

EMAIL ADDRESS:

HOME ADDRESS:

MOBILE:

HOME:

GP DETAILS:

NAME OF GP:

ADDRESS OF GP:

Appendix 2C (continued)

Imperial College London

Chelsea and Westminster Hospital NHS
NHS Foundation Trust

Breast milk: A messenger between mother and baby? 12/LO/0203 version 1/230112

Recruitment Data Collection Form

FORM COMPLETED *(initials)*: NA / SJ **on** *(date)*: __ / __ / 20 __	**RECRUITED BY** *(initials)*: NA / SJ
TRANSFERRED TO DATABASE *(initials)* on *(date)*	QC *(initials)* on *(date)*

Characteristics At Birth

Study identifier *(from patient recruitment form)*: _ / _ _ _ _ / _ _ _ _		
Male (1) ☐	**Female** (2) ☐	**Date of birth** (DD/MM/YYYY): __ / __ / 20 __
GA at birth (weeks+days):	**Was this GA calculated from an early scan?** No (0) ☐	Yes (1) ☐
Birth weight (kg):	**Birth HC** (cm):	**Birth length** (m):
List all medications received since birth:		

About This Pregnancy

Number of pregnancies *(including this one)*:	**Number of births** *(including this one)*:
How many fetuses survived the 1st trimester of this pregnancy? *Please state:*	

Labour and Delivery

List any drugs given in the week before delivery:
List any drugs given during delivery:
List any drugs given in the week after delivery:
List any vitamin, mineral or herbal supplements taken by the mother during pregnancy:

Mode of delivery *(Please tick one mode of delivery)*	Vaginal – Unassisted (1) ☐
Vaginal – Assisted (2) ☐	C-section in labour (3) ☐
Pre-labour C-section (4) ☐	***Please specify reason*** *(if known)*:

Maternal History

Year of birth:	**Date of booking** (DD/MM/YYYY): __ / __ / 20 __		**Blood group:**
Height (m):	**Weight pre-pregnancy** (kg):	**Weight at booking** (kg):	**Current weight** (kg):

Mothers occupation:	**Years of maternal education post age 11:**
Highest qualification achieved: Pre GCSE (0) ☐ GCSE level (1) ☐ A-level/HND/GNVQ/foundation level (2) ☐ Undergraduate degree (3) ☐ Postgraduate degree (4) ☐	

What was the mini OGGT result?	**Is the mother diabetic?** No (0) ☐ Yes (1) ☐
If *YES*, is the diabetes: Pre-pregnancy Type 1 (1) ☐ Pre-pregnancy Type 2 (2) ☐ Gestational (3) ☐	
Please specify treatment:	None (0) ☐ Insulin (1) ☐ Diet (2) ☐
What was the full OGTT value? 0hr___ 1hr___ 2hr___	**HbA1C values:** Date_____ Value___ Date_____ Value___

Is the mother dyslipidaemic? No (0) ☐ Yes (1) ☐

Is the mother being treated for hypertension? (>140/90 mmHg) No (0) ☐ Yes (1) ☐

Appendix 2C (continued)

If *YES*, is the hypertension: Essential (1) ☐	Secondary (2) ☐	Pregnancy related (3) ☐

MATERNAL DIET/LIFE STYLE DURING PREGNANCY

During the average week in pregnancy, how many meals contained: Red meat __ Fish __
Soft drinks (portions per week, 1 portion ≈ 325 ml or 1 can/1 glass) *(any sugary drink inc non-fizzy e.g. fruit juice)*
What is your average weekly alcohol consumption? (units):
Please list any dietary exclusions:

What statement best describes the mother's smoking status:		
Never smoked (0) ☐	Have smoked, but not currently smoking (1) ☐	Currently smoking (2) ☐
If the mother *HAS* smoked in the past, but is *NOT* currently smoking, when did she stop smoking?		
Before pregnancy (0) ☐	1st Trimester (1) ☐	2nd Trimester (2) ☐ 3rd Trimester (3) ☐
If the mother is currently smoking, on average how many cigarettes does she smoke each day?		

ATOPY AND ALLERGY *(Please tick all that apply)*

	Asthma	Eczema	Hay fever	Food allergy	Other *State:*	None
Maternal	☐ (1)	☐ (1)	☐ (1)	☐ (1)	☐ (1)	☐ (0)

FAMILY ETHNICITY

	What is the family's ethnic origin? *	What was the country of birth?
Maternal		
Paternal		

*Ethnic origin was classified as; White British, White Other, Chinese, Japanese, Hispanic, Indian, Middle Eastern, Other Asian, Other mixed.

Appendix 2C (continued)

Imperial College London

Chelsea and Westminster Hospital NHS
NHS Foundation Trust

DATA COLLECTION FORM AT 7 DAYS AND 3 MONTHS

FORM COMPLETED *(initials)*: NA / SJ **on** *(date)*: _ _ / _ _ / 20 _ _	**RECRUITED BY** *(initials)*: NA / SJ
TRANSFERRED TO DATABASE *(initials)* on *(date)*	QC *(initials)* on *(date)*

Study identifier *(from patient recruitment form)*: _ / _ _ _ _ / _ _ _ _

VISIT 1 CHARACTERISTICS (7 DAYS)

Completed by *(Initials)*:	**Visit 1 Date** (DD/MM/YYYY): _ _ / _ _ / 20 _ _
Infant head circumference (cm):	**Maternal Length** (m):
Infant length (m):	**Maternal Weight** (kg):
Infant weight (kg):	**Maternal urine sample collected** ☐
Infant urine sample collected ☐	**Maternal breast milk sample collected** ☐
Infant stool sample collected ☐	
Method of feeding *(circle)*: *1 = breast, 2= predominantly breast, 3 = 50:50, 4 = predominantly formula, 5 = formula* **1 2 3 4 5**	Additional comments regarding feeding:
Type of formula *(list if >1, marking predominant formula)*:	
Is the mother is currently taking any medication?	
Is the infant currently taking any medication?	
If the mother is currently smoking, on average how many cigarettes does she smoke each day?	

VISIT 2 CHARACTERISTICS (3 MONTHS)

Completed by *(Initials)*:	**Visit 2 Date** (DD/MM/YYYY): _ _ / _ _ / 20 _ _
Infant head circumference (cm):	**Maternal Length** (m):
Infant length (m):	**Maternal Weight** (kg):
Infant weight (kg):	**Maternal urine sample collected** ☐
Infant urine sample collected ☐	**Maternal breast milk sample collected** ☐
Infant stool sample collected ☐	
Method of feeding *(circle)*: *1 = breast, 2= predominantly breast, 3 = 50:50, 4 = predominantly formula, 5 = formula* **1 2 3 4 5**	Additional comments regarding feeding:
Type of formula *(list if >1, marking predominant formula)*:	
If the mother is currently smoking, on average how many cigarettes does she smoke each day?	

FORMULA FEEDING

Age when first formula fed (DD/MM/YYYY): _ _ / _ _ / 20 _ _
Age when first predominantly formula fed (DD/MM/YYYY): _ _ / _ _ / 20 _ _

Appendix 2D

Protease inhibitor cocktail preparation

Lyophilized powder from Sigma-Aldrich containing; AEBSF at 2mM, Aprotinin at 0.3µM, Bestatin at 130µM, E-64 at 14mM, Leupeptin at 1mM and EDTA at 1mM.

Dilute in 100ml distilled H_2O;

Aliquots 20µl into Eppendorf tubes and allow to dry;

Freeze;

Add 1 ml of breast milk once collected.

Appendix 2E

Buffer Preparation for urine for NMR spectroscopy (500ml)
Reagents must be of analytical grade.

Reagents:
a) 1.5M KH_2PO_4/D_2O, pH 7.4 (with KOD/D_2O)
b) 2mM NaN_3
c) 0.1% TSP (3-Trimethylsilyl propionic acid)

Preparation:
1) Dissolve 102g KH_2PO_4 in 400ml D_2O
2) Dissolve 500mg TSP and 65mg of NaN_3 in 30-50ml D_2O
3) Mix both solutions very well under ultrasonication
4) Adjust pH to 7.4 with KOH-tablets or strong KOD-solution (KOH-tablets in D_2O)
5) Fill to 500ml and mix well

Appendix 2F – CE-MS system parameters

The software used to operate the CE unit was 3D-CE ChemStation Rev B.04.03 (Agilent Technologies, Wilmington, USA). Separations were carried out in normal polarity in a fused-silica capillary (Agilent) with an internal diameter of 50µm, a total length of 60cm and with a background electrolyte containing 0.1mol/l formic acid solution. The capillary was conditioned with a first flush of 300 seconds of 2M ammonium hydroxide (Sigma-Aldrich, UK), a second flush of 240 seconds of deionised water and a third flush of 300 seconds of run buffer. The temperature of the capillary was set at 25˚C. Samples were injected into the instrument hydrodynamically, at 50mBar for 3 seconds, in a stacking manner. A voltage of 25kV was applied for 20 minutes. The sheath liquid was delivered at a flow rate of 0.6ml/min with split 1:100, and comprised of methanol/water (70% v/v) containing 1.0mmol/l formic acid. Auxiliary liquid flow was set to 6µl/min.

The software MassHunter WorkStation Acquisition B.02.01 was used to acquire the MS data (Agilent Technologies, Wilmington, USA). Data were collected in centroid mode with an acquisition rate of 5 spectra per second. The nitrogen gas flow rate was 10l/min, set to 200°C. The nebulizer was set to 10psi 30 seconds after injection. The fragmentor and octupole voltage were set to 150 and 750V respectively. A solution containing 10ml water, 90ml methanol, 100µl formic acid, with two reference masses; 600µl of 5mmol/l purine, and 600µl of 2.5mmol/l HP 0921 was infused directly into the ion source. The reference mass spectra were collected simultaneously with the analytical data and used for accurate mass correction and highly accurate mass measurement in the MS system. A mass acquisition range of 50-1700 *m/z* was used.

CE-MS data processing

Background noise and unrelated ions were cleaned from the CE-MS data by the Molecular Feature Extraction (MFE) tool in MassHunter (B.04.00, Agilent). The MFE creates a list of all possible components extracted from the full TOF mass spectral data and migration times. The following parameters were used to obtain these: target data type of small molecules (chromatographic); peaks with height ≥200 counts; Peak spacing tolerance=0.0025; *m/z*, ±7.0 ppm; isotope model =common organic molecular; limited assigned change=2.

Alignment was performed first on all samples with QC samples, and later only for the samples under investigation. Corrections applied for the alignment were 2% migration time correction, and ±10 ppm mass correction. Data were normalised using total area normalisation. The filtering and alignment of the data were performed with GeneSpring version 12.6 (Agilent) selecting features in the range of 0.5 minutes to 20 minutes. Filtering was performed by eliminating features present in the QC samples

with a coefficient of variation above 30%. SIMCA P+ 13.0 software (Umetrics, Umeå, Sweden) was used for multivariate analysis. Variables with a |t-statistic| ≥ 1.96 (z-score, corresponding to the 97.5 percentile) [17] were considered significant.

Appendix 2G

Experimental design of breast milk hormone analysis, adapted from;

http://www.bio-rad.com/webroot/web/pdf/lsr/literature/10010747.pdf

Reconstitute standards;

1) Add 500µl of standard diluent and vortex for 5 seconds
2) Incubate on ice for 30 minutes
3) From each of the reconstituted vials add 128µl of standard to S1 and 72 µl standard diluent, then vortex for 5 seconds.
4) Place 150µl of standard diluent into the remaining tubes S2-blank
5) Starting at S1 transfer 50µl of Standards into tubes S2-S8. Vortex for 5 seconds after each volume transfer.

Prepare coupled beads

1) Vortex stock coupled beads at medium speed for 30 seconds
2) Add required volume of assay buffer to each falcon as below and cover with foil
3) Dilute beads according to table below combine with assay buffer

	Plate 1 ~105 wells Volume µl
Leptin	262
Resistin x20	262
Ghrelin	262
Insulin	262
Assay buffer	4200
Total volume	5250

Prepare samples

1) Take samples from -80 freezer and allow to thaw keep on ice
2) Centrifuge at 4 degrees for 10 minutes at 10,000RPM

Combine components

1) Vortex beads at medium speed for 30 seconds
2) Add 50µl of beads to each well
3) Wash plate twice; add 100µl of wash buffer to each well, place plate on magnetic bead holder, wait 1 minute. Throw out solution, and dab on absorbent towels. Remove from magnetic bead bolder and wash again
4) Vortex standards and samples for plates gently for 5 seconds
5) Transfer 50µl to each well, different pipette tip after each transfer, making sure you adhere to plate map carefully
6) Cover whole plate with sealing tape and foil.
7) Incubate on plate shaker at 850RPM at room temperature for 1 hour

Appendix 2G (continued)

Prepare detection antibodies

1) Vortex stock antibodies before dilution for 15-20 seconds at medium speed
2) Add Bioplex Detection Antibody diluent to the a falcon tube as detailed in table below:

	Plate 1 (~105 wells) Volume µl
Leptin antibody	132
Insulin antibody	132
Ghrelin antibody	132
Resistin antibody	132
Antibody diluent	2097
Total volume	2625

3) Add detection antibody to a falcon tube as above.
4) Wash plate 3 times with 100µl wash buffer.
5) Vortex diluted detection antibodies gently for 5 seconds and transfer 25µl of diluted antibody to each well
6) Cover the plate with new sealing tape and foil.
7) Incubate on shaker at 850+/- 50 rpm for 30minutes at room temperature

Prepare and add Streptavidin-PE

1) Vortex the 100xSA-PE for 5 seconds at medium speed
2) Add assay buffer as below table into each labelled falcon tube:

No. of Wells	**100xSA-PE, µl**	**Assay buffer, µl**	**Total volume**
Plate 1 (105)	53	5197	5250
Trial (50)	25	2475	2500

3) Pipette the required SA-PE into each labelled falcon, then cover with foil until use.
4) After incubation of antibody is completed, remove and discard sealing tape.
5) Wash the plate 3 times Vortex diluted SA-PE medium at medium speed for 5 seconds. Pour into reagent reservoir and transfer 50 µl to each well using a repeat pipette.
6) Cover with sealing plate and with foil.
7) Incubate plate on shaker at 850 +/- 50rpm for 10 minutes at room temperature.
8) After incubation step, wash plate three times
9) Add 125 µl of assay buffer to each well, cover with foil.
10) Take to Bioplex machine and read plate.

Appendix 2H

	PCA Models (Figure 23)	R^2	Components	Outliers	t[1]	t[2]	Scaling
A	NMR aqueous	0.406	2	3	0.27	0.128	UV
B	CE-MS; Positive mode	0.333	1	0	0.23		Par
C	NMR Lipid	0.484	2	3	0.35	0.12	UV
D	UPLC-MS; Positive mode lipid	0.567	2	0	0.39	0.17	Par
E	UPLC-MS; Negative mode lipid	0.331	2	0	0.23	0.1	UV
	PLS-DA Models (Figure 24)	R^2	Q^2	**Components**	**Outliers**	**t[1]**	**t[2]**	**Scaling**
A	NMR aqueous	0.429	0.304	2	1	0.20	0.19	UV
B	CE-MS; Positive mode	0.833	0.458	2	0	0.23	0.06	Par
C	NMR Lipid	0.295	0.104	2	1	0.30	0.12	UV
D	Lipid UPLC-MS; Positive mode	0.589	0.04	2	0	0.23	0.22	Par
E	Lipid UPLC-MS; Negative mode	0.659	0.09	2	0	0.2	0.08	UV
	OPLS-DA Models (Figure 25)	R^2X	R^2Y	Q^2	**Components**	**Scaling**		
A	NMR aqueous	0.381	0.582	0.249	2	UV		
B	CE-MS; Positive mode	0.331	0.984	0.766	2	Par		
C	NMR Lipid	0.462	0.567	-0.327	2	UV		
D	Lipid UPLC-MS; Positive mode	0.246	0.959	0.489	2	Par		
E	Lipid UPLC-MS; Negative mode	0.294	0.932	0.127	2	UV		
	OPLS-DA Models (Figure 26)	R^2X	R^2Y	Q^2	**Components**	**Scaling**		
A	NMR aqueous	0.412	0.927	0.866	2	UV		
B	CE-MS; Positive mode	0.34	0.967	0.735	2	Par		
C	NMR Lipid	0.416	0.572	-0.05	2	UV		
D	Lipid UPLC-MS; Positive mode	0.564	0.837	0.531	2	Par		
E	Lipid UPLC-MS; Negative mode	0.356	0.938	0.43	2	UV		
	OPLS-DA Models (Figure 27)	R^2X	R^2Y	Q^2	**Components**	**Scaling**		

A	NMR aqueous	0.41	0.813	0.713	2	UV
B	CE-MS; Positive mode	0.249	0.988	0.301	2	Par
C	NMR Lipid	0.443	0.455	0.167	2	UV
D	Lipid UPLC-MS; Positive mode	0.622	0.792	0.429	2	Par
E	Lipid UPLC-MS; Negative mode	0.346	0.962	0.165	2	UV

Appendix 3

The effect of maternal body mass index on the concentration of appetite regulating hormones present in breast milk: a systematic review and *in vitro* analysis

PLOS ONE

RESEARCH ARTICLE

Effect of Maternal Body Mass Index on Hormones in Breast Milk: A Systematic Review

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: NJA is funded by Westminster Medical School Research Trust, CG is supported by the Chelsea and Westminster Hospital Charity.

Competing Interests: NJA has received support from Medela to attend an educational conference, but declared no other conflict of interest. MJH has received support from Danone International to attend an educational conference, but declared no other conflict of interest. CG has received support from Pfizer Nutrition to attend an educational conference, but declared no other conflict of interest. In the last five years NM has received consultancy fees from Ferring Pharmaceuticals, speaker honorarium for an educational meeting funded by Nestle International in which they had no organisational involvement, and grants from the Medical Research Council, National Institute of Heath Research, Westminster Children's Trust

Abstract

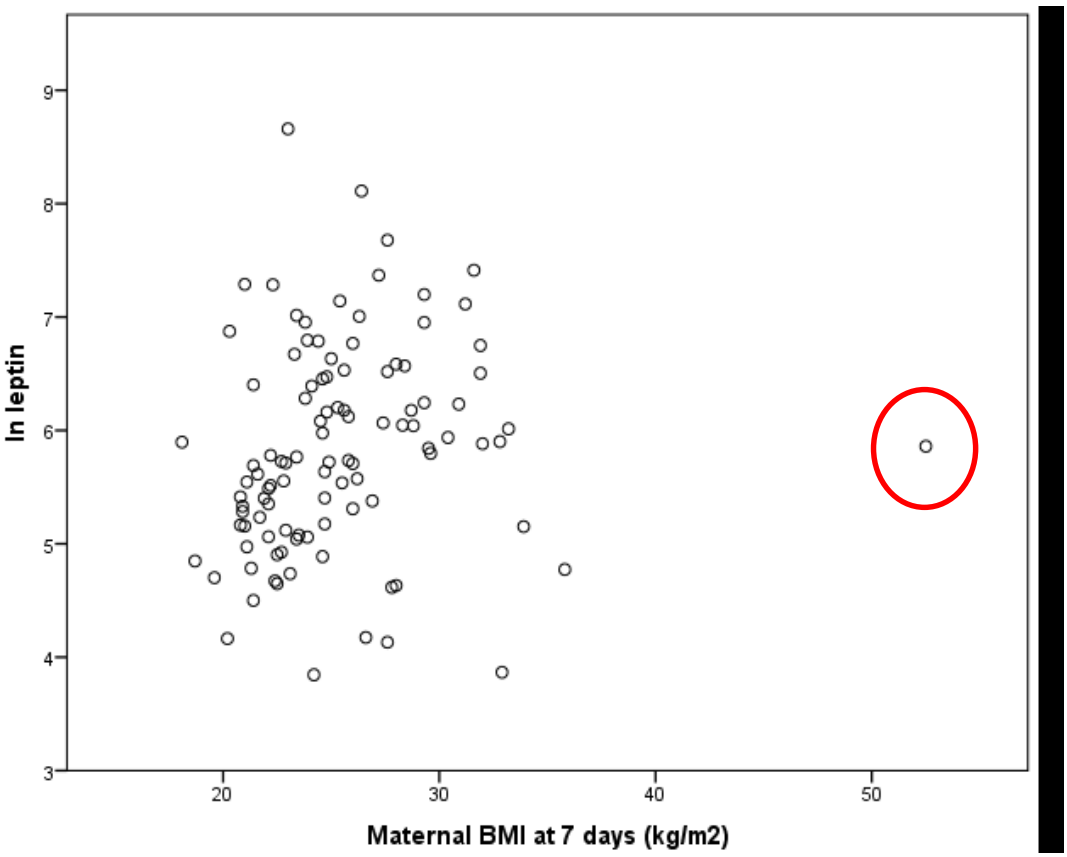
Background: Maternal Body Mass Index (BMI) is positively associated with infant obesity risk. Breast milk contains a number of hormones that may influence infant metabolism during the neonatal period; these may have additional downstream effects on infant appetite regulatory pathways, thereby influencing propensity towards obesity in later life.

Objective: To conduct a systematic review of studies examining the association between maternal BMI and the concentration of appetite-regulating hormones in breast milk.

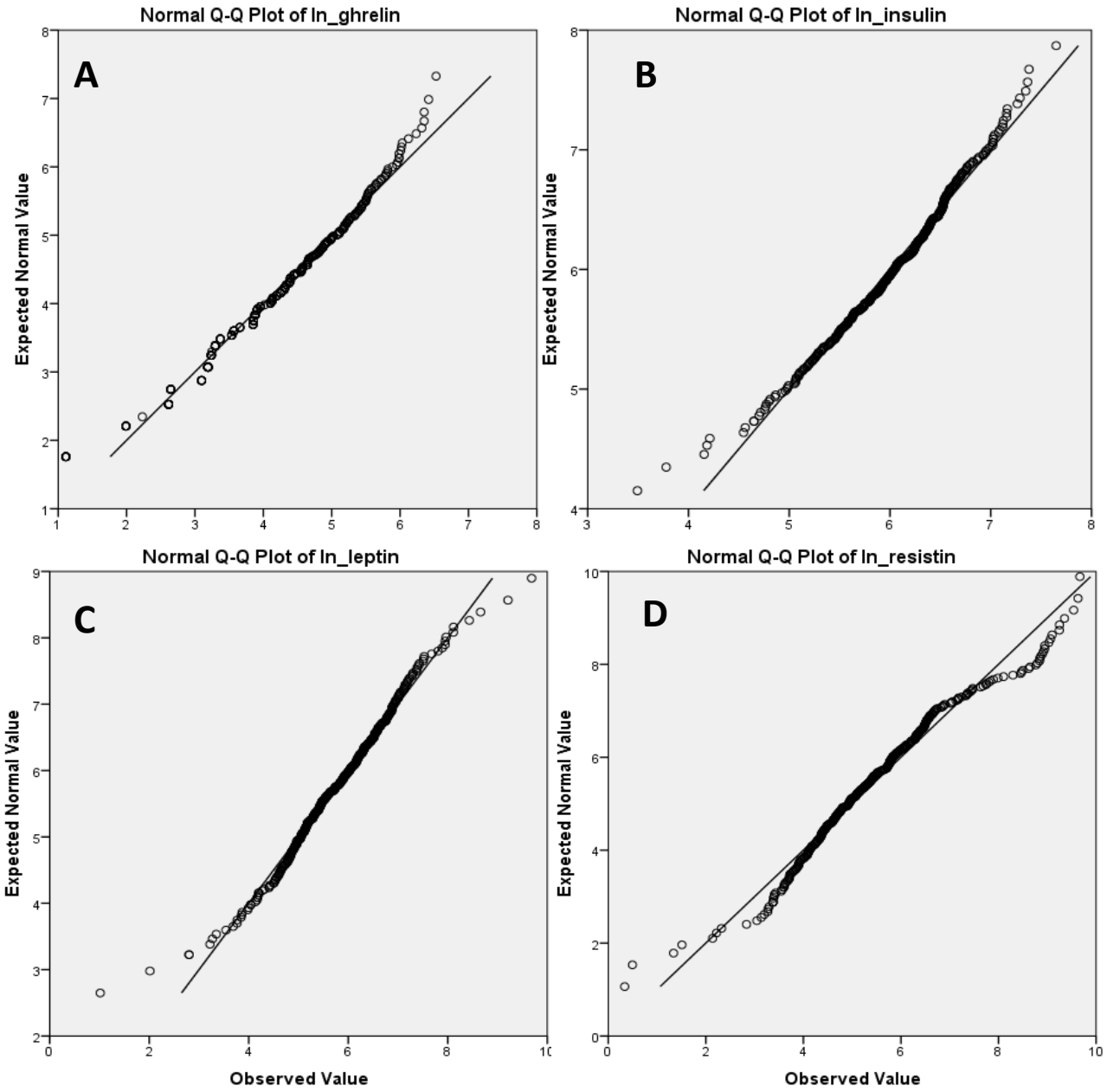
Method: Pubmed was searched for studies reporting the association between maternal BMI and leptin, adiponectin, insulin, ghrelin, resistin, obestatin, Peptide YY and Glucagon-Like Peptide 1 in breast milk.

Results: Twenty six studies were identified and included in the systematic review. There was a high degree of variability between studies with regard to collection, preparation and analysis of breast milk samples. Eleven of fifteen studies reporting breast milk leptin found a positive association between maternal BMI and milk leptin concentration. Two of nine studies investigating adiponectin found an association between maternal BMI and breast milk adiponectin concentration; however significance was lost in one study following adjustment for time post-partum. No association was seen between maternal BMI and milk adiponectin in the other seven studies identified. Evidence for an association between other appetite regulating hormones and maternal BMI was either inconclusive, or lacking.

Conclusions: A positive association between maternal BMI and breast milk leptin concentration is consistently found in most studies, despite variable methodology. Evidence for such an association with breast milk adiponectin concentration,

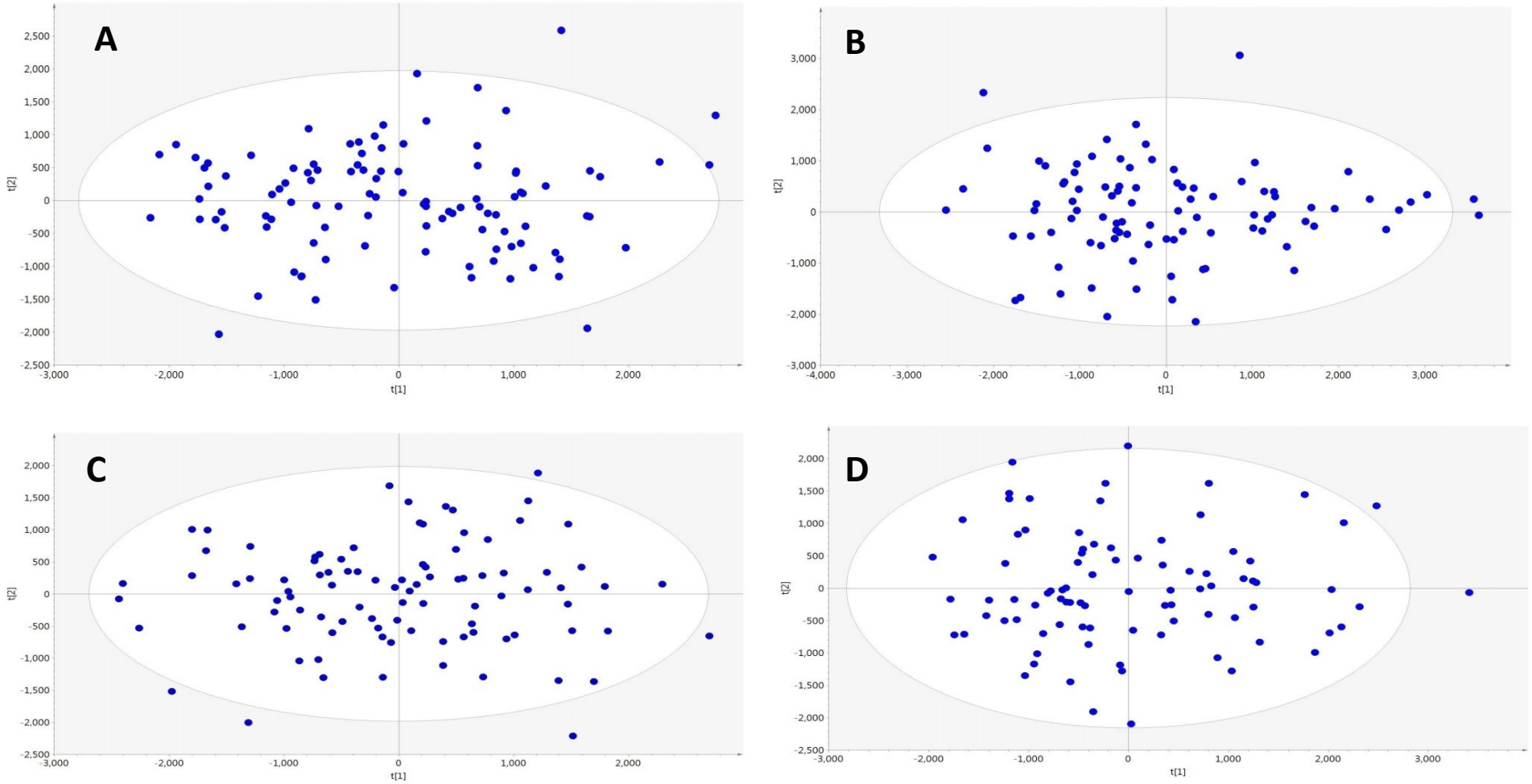


Appendix 3B: Scatter plot displaying the outlier mother at 50kg/m2, outlined in red. This point unduly influenced results obtained, and was therefore excluded from further analysis.

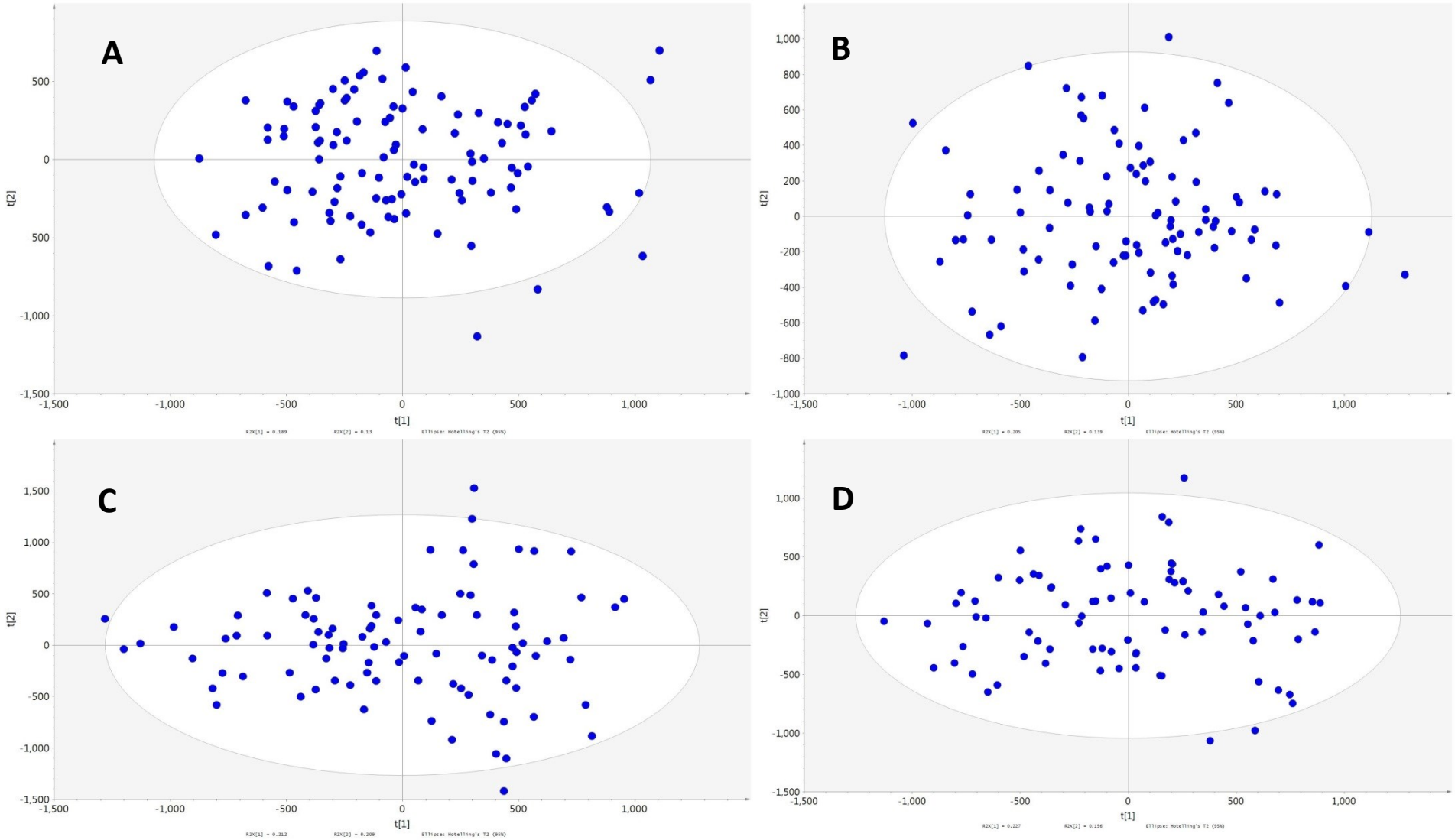


Appendix 3C: Normal Q-Q plot for breast milk (A) Ln ghrelin concentrations **(B)** Ln Insulin concentrations **(C)** Ln leptin concentrations and **(D)** Ln resistin concentrations.

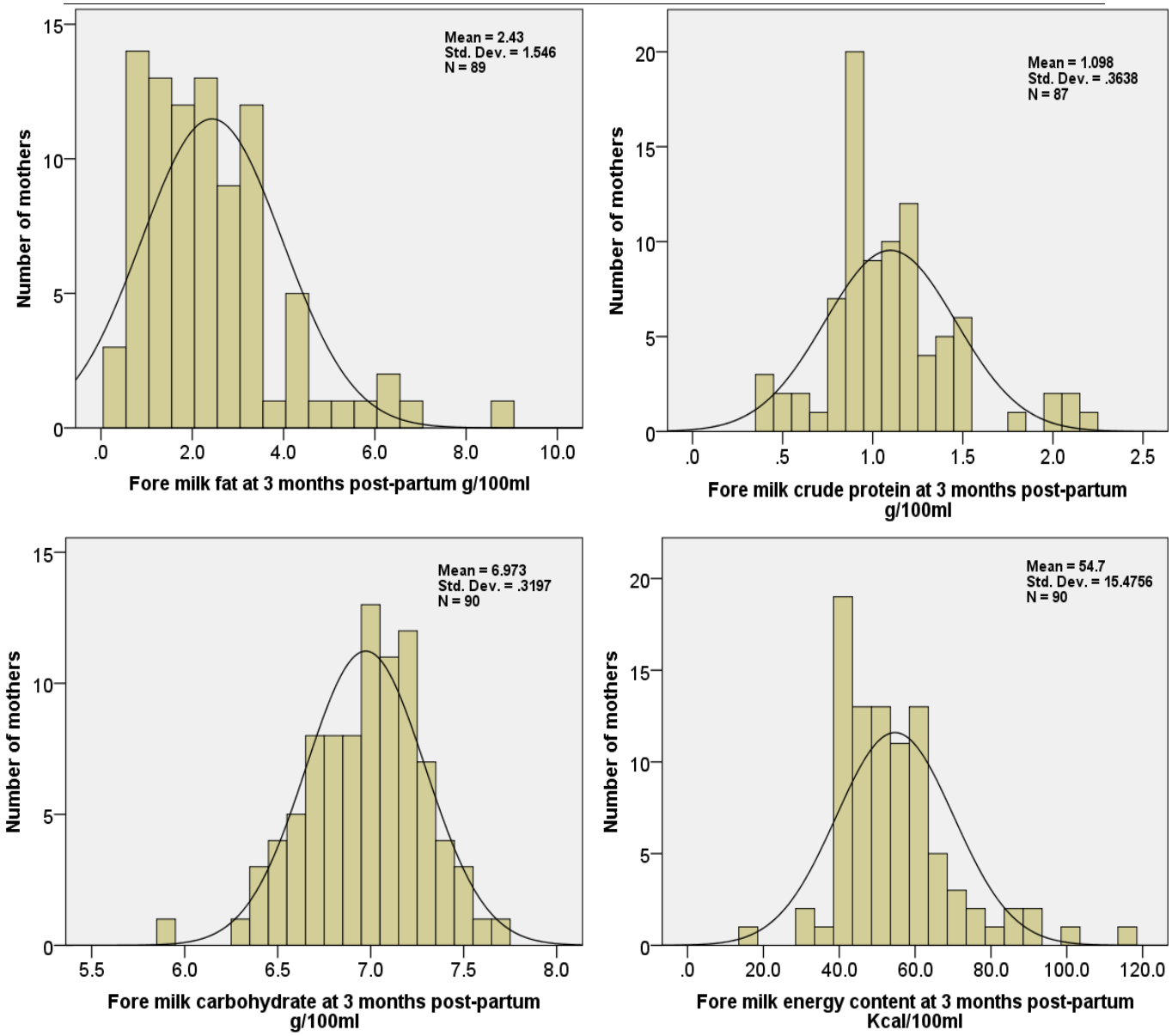
Appendix 4 - Breast milk metabonomic and nutritional analysis



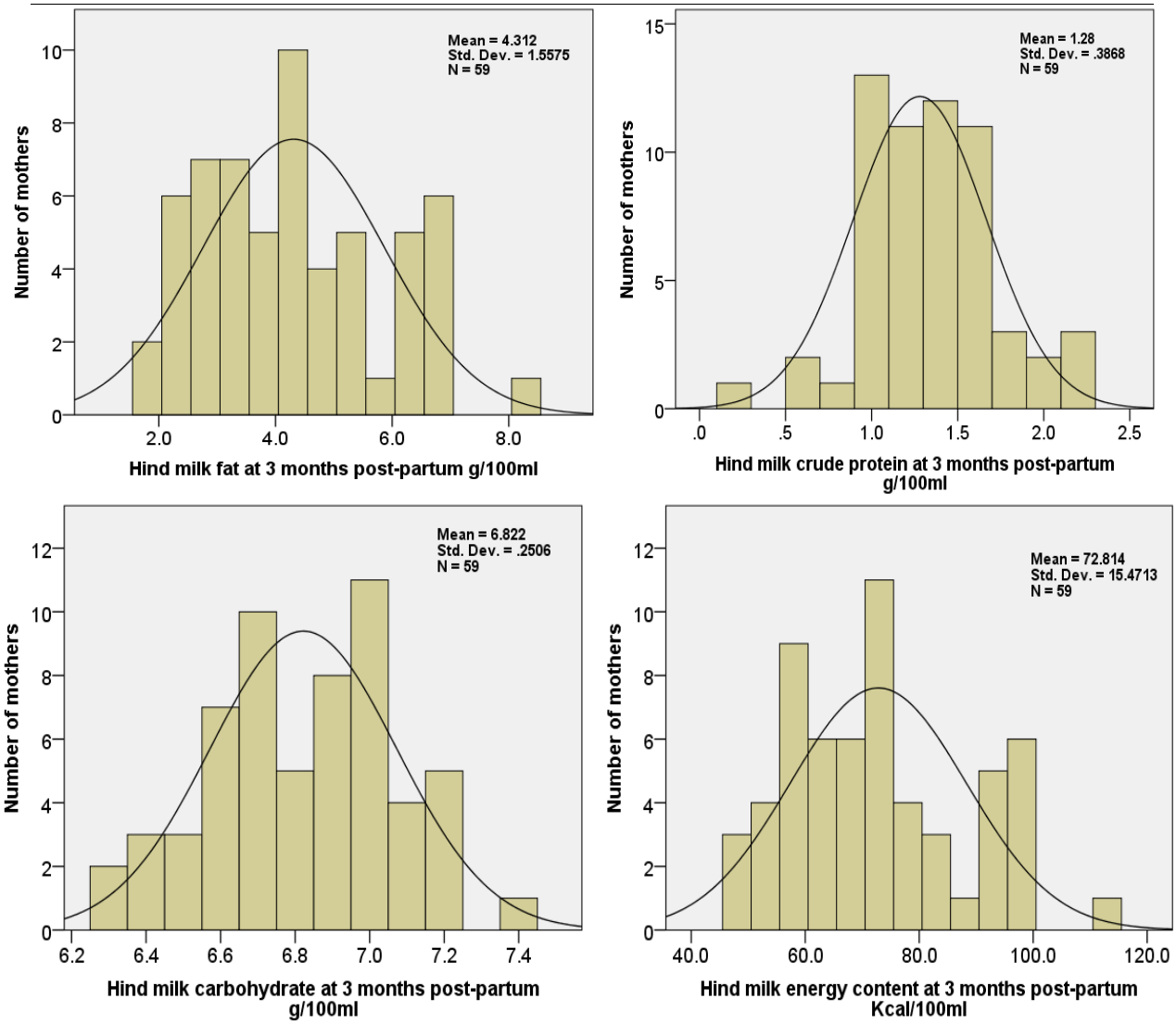
Appendix 4A: PCA scores plots of; A Fore milk samples collected at one week post-partum, R^2=0.301, Q^2=0.218. **B** Hind milk samples collected at one week post-partum, R^2=0.28, Q^2=0.197. **C** Fore milk samples collected at 3 months post-partum, R^2=0.384, Q^2=0.312. **D** Hind milk samples collected at 3 months post-partum, R^2=0.299, Q^2=0.201 in positive ionisation mode.



Appendix 4B: PCA scores plot of; A Fore milk samples collected at one week post-partum, R^2=0.319, Q^2=0.246. **B** Hind milk samples collected at one week post-partum, R^2=0.344, Q^2=0.289. **C** Fore milk samples collected at 3 months post-partum, R^2=0.421, Q^2=0.347. **D** Hind milk samples collected at 3 months post-partum, R^2=0.382, Q^2=0.319 in negative ionisation mode.

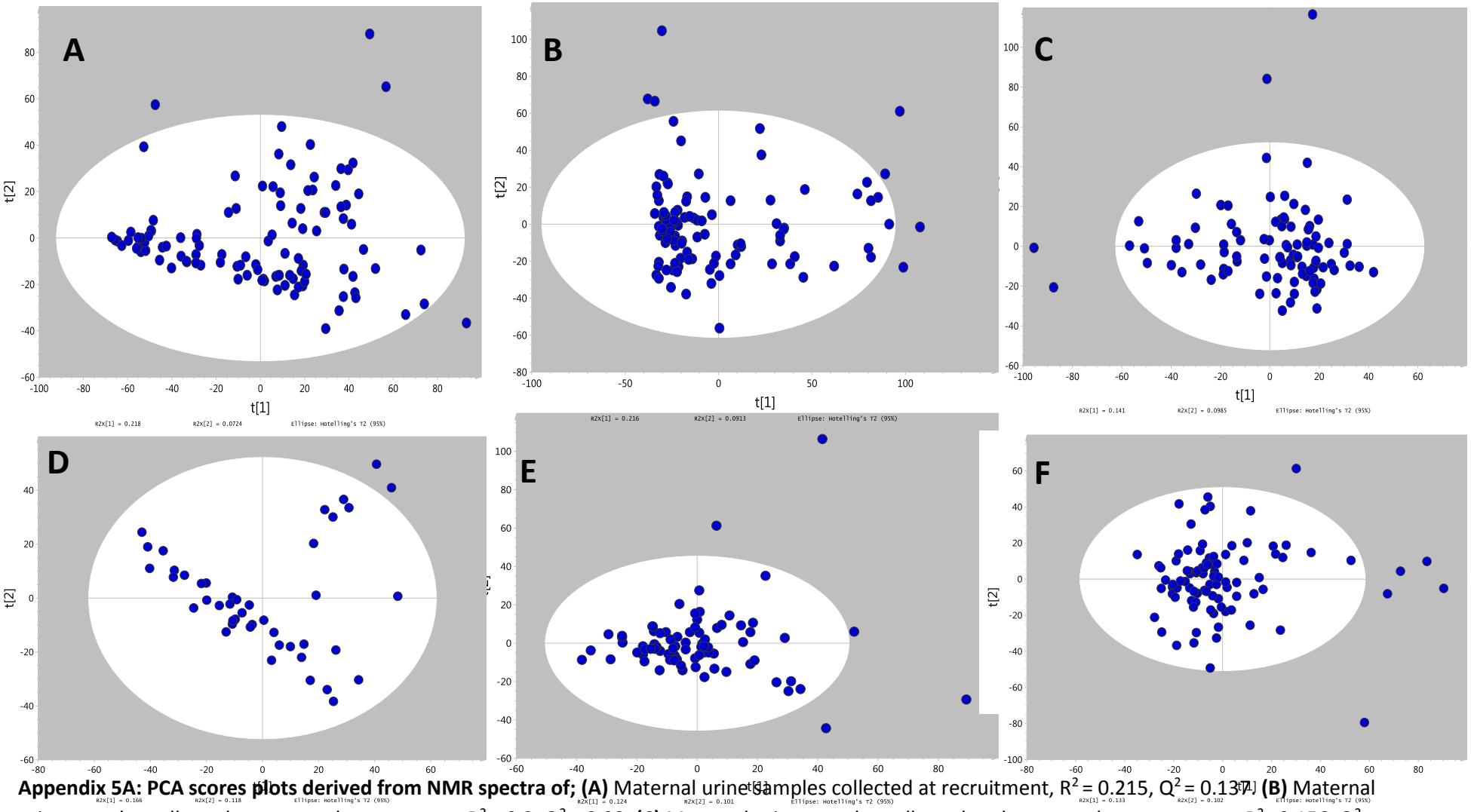


Appendix 4C: Histograms displaying the distribution of macronutrients in fore milk samples collected at 3 months post-partum, fat **(A)**, crude protein **(B)**, carbohydrate **(C)** in g/100ml and energy content **(D)** Kcal/100ml of breast milk analysed.

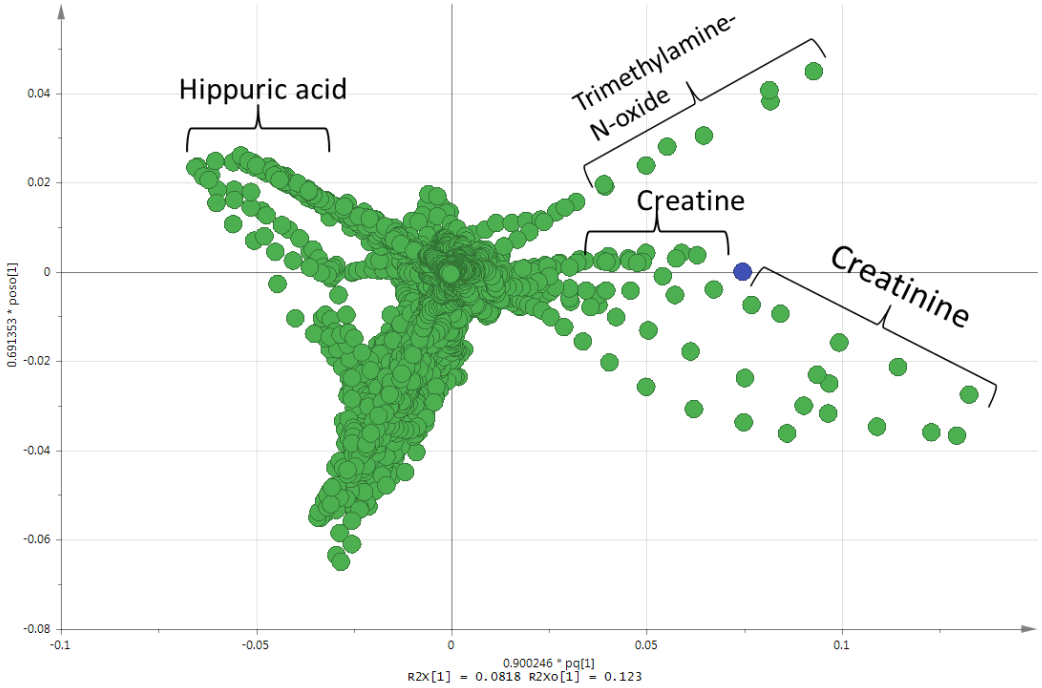


Appendix 4D. Histograms displaying the distribution of macronutrients in hind milk samples collected at 3 months post-partum, fat **(A)**, crude protein **(B)**, carbohydrate **(C)** in g/100ml and energy content **(D)** Kcal/100ml of breast milk analysed.

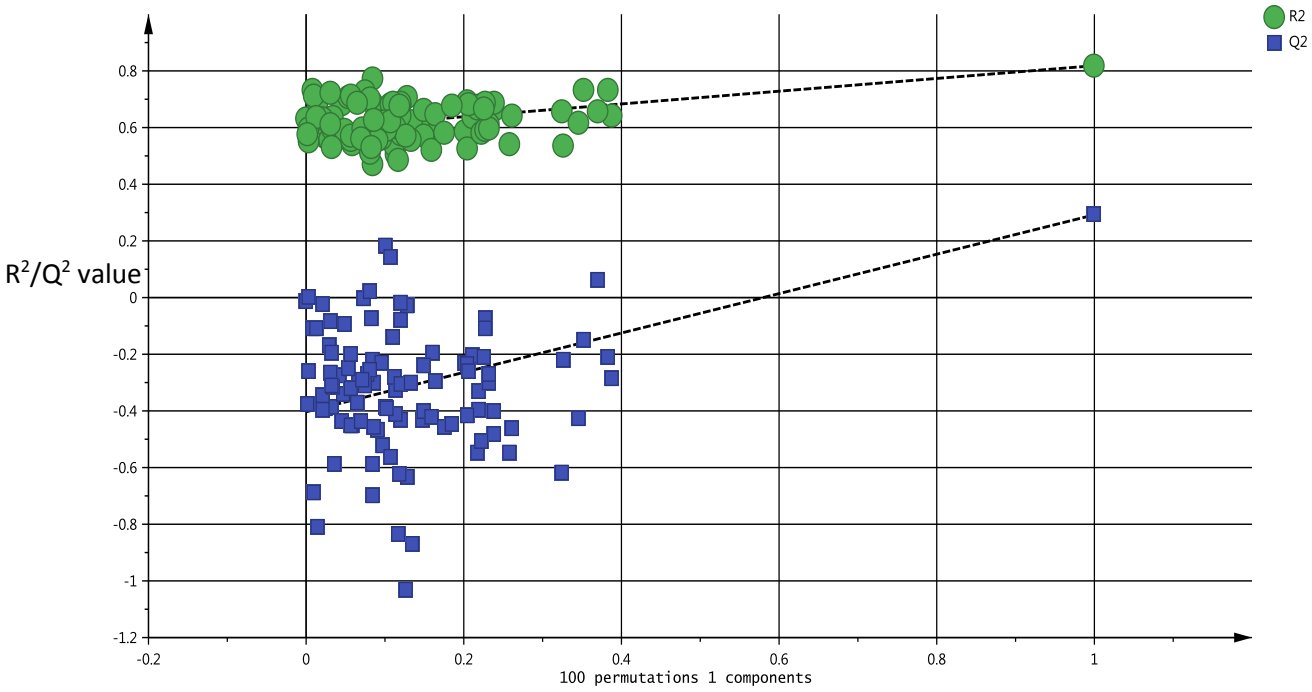
Appendix 5 - Infant and maternal urinary metabolome



Appendix 5A: PCA scores plots derived from NMR spectra of; (A) Maternal urine samples collected at recruitment, $R^2 = 0.215$, $Q^2 = 0.137$. **(B)** Maternal urine samples collected at one week postpartum, $R^2 = 0.2$, $Q^2 = 0.08$. **(C)** Maternal urine samples collected at three months post-partum, $R^2 = 0.156$, $Q^2 = 0.07$. **(D)** Infant urine samples collected at recruitment, $R^2 = 0.272$, $Q^2 = 0.152$. **(E)** Infant urine samples collected at one week post-partum, $R^2 = 0.192$, $Q^2 = 0.127$. **(F)** Infant urine samples collected at three months post-partum, $R^2 = 0.176$, $Q^2 = 0.09$.



Appendix 5B: Loadings plot corresponding to Figure 67, loadings to the right correspond to metabolites increased in the urine of mothers with a higher BMI whilst metabolites on the left of the plot correspond to metabolites increased in mothers with a lower BMI.



Appendix 5C: Permutation testing of the first model in Table 47. This figure strongly suggests the model is valid. Model comparing infants with an earlier gestational age, against infants born at a later gestational age.

Submitted Papers and Abstracts Related to this Thesis

Presented at the Neonatal Society 2013 spring meeting.

Title: The association between leptin concentrations in breast milk and maternal characteristics

Authors: Andreas NJ[1], Parkinson JRC[1], Hyde MJ[1], Jeffries S[1], Xiong R[1], Fitz-Simon N[1], Modi N[1]

Institution: [1]Section of Neonatal Medicine, Chelsea and Westminster campus, Imperial College London, UK

Background/Introduction: Leptin plays a fundamental role in the development and subsequent control of key metabolic pathways. The aim of this study was to determine if there was a relationship between leptin concentration in breast milk and key maternal characteristics.

Methods: This study was carried out under research ethics approval (REC: 12/LO/0203). Mothers were recruited to the study from the post-natal ward at Chelsea and Westminster Hospital. Breast milk samples (10ml) were collected seven days post-delivery from lactating mothers (n=37), with the aid of a breast pump. Timing of sample collection was standardised to the morning in order to reduce the influence of diurnal variations in breast milk composition. Samples were stored at -20°C. 1ml of sample was sonicated using an ultrasound probe prior to analysis. Leptin was measured using commercially available leptin ELISA kit (Millipore, UK). Concentrations of leptin in breast milk were correlated with maternal data (age, pre-pregnancy BMI, pre-pregnancy weight, duration of pregnancy, 28 week glucose tolerance test, weight and BMI at the time of sample collection). Linear regression was performed in SPSS 20.

Results: Correlation analysis revealed a significant association between leptin and both maternal weight at sample collection (r=0.4, R^2 = 0.15, p=0.015) (Figure 1) and maternal BMI at sample collection (r: 0.34, R^2=0.12, p=0.05). Linear regression analysis revealed the change in leptin per 1kg increase in maternal weight at sample collection was 0.003ng/ml (95%CI: 0.001, 0.005). No association was observed between leptin and other maternal characteristics (age, pre-pregnancy BMI, pre-pregnancy weight, duration of pregnancy, 28 week glucose tolerance test).

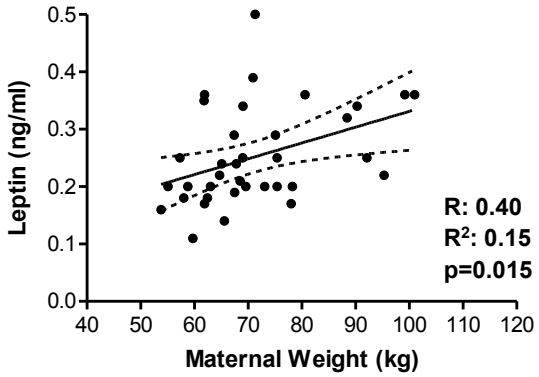


Figure 1 Plot of maternal weight at sample collection (kg) and leptin concentration (ng/ml). Line of best fit (solid) and 95% CI (hatched) are also shown.

Conclusions: These data indicate an association between foremilk leptin concentration with maternal weight and BMI, at the time of sample collection. Though the bioactivity of breast milk leptin is unknown, we speculate that this may be part of a feedback mechanism serving to regulate infant milk intake.

References:

(1) Konstantinos M (2012) The complex interaction between obesity, metabolic syndrome and reproductive axis: A narrative review. Metabolism

(2) Koo M et al. The effect of maternal BMI on the concentration of appetite regulating peptides in breast milk. Neonatal Society Autumn 2011.

Presented at the 21st European Neonatal Workshop, autumn 2013.

A NEWBORN IS NOT A SMALL ADULT: ANOTHER ILLUSTRATION OF THE NEED TO PERFORM PHARMACOKINETIC STUDIES

K. Allegaert

Neonatal Intensive Care Unit, University Hospitals Leuven, Belgium

Introduction: Propylene glycol (PG) is often used as an excipient. We aimed to characterize renal and hepatic PG elimination in neonates.

Methods: The pharmacokinetic analysis of PG was performed in NONMEM 6.2. on the basis of PG concentrations in plasma and/or urine samples for a total of 69 (pre)term neonates (birth weight 630–3980 g, gestational age 24–41 weeks, postnatal age 1–29 days) who received PG co-administered with paracetamol, phenobarbital or both. To capture the time dependent trend in renal excretion of PG, different models based on time after first dose, urine volume and urinary creatinine were tested.

Results: A one compartment model parameterized in terms of renal clearance, hepatic clearance and volume of distribution described the observations in both plasma and urine. Renal PG elimination was 15% of total clearance, which increased over time to 25% at 24 hours after the first dose of PG. This increase was best described using a hyperbolic function based on time after the first dose.

Conclusions: Renal PG elimination in neonates is low compared to hepatic clearance, particularly compared to the reported percentage of 45% in adults. This has implications related to co-morbidity (hepatic/renal failure) and co-excipient (ethanol) in neonates.

INSULIN CONCENTRATION IN BREAST MILK AND ITS ASSOCIATION WITH MATERNAL BMI

N.J. Andreas, M.J. Hyde, J.R.C. Parkinson, S. Jeffries, S. Santhakumaran, N. Modi

Department of Academic Neonatal Medicine, Faculty of Medicine, Imperial College London, UK

Introduction: Previous literature suggests that breast milk from diabetic mothers increases the offspring's likelihood of becoming overweight. As breast milk insulin is increased in these mothers, and plays a key role in many metabolic pathways, we aimed to investigate the relationship between maternal characteristics and insulin concentrations in breast milk.

Methods: Research ethics approval code; 12/LO/0203. Funding received from Westminster Medical School Research Trust. 37 non-diabetic mothers were recruited on the postnatal ward in Chelsea and Westminster Hospital. Mothers height and weight were measured in order to calculate BMI at booking and sample collection. Breast milk samples were collected 7 days post-partum using a breast pump and stored at –20°C. 1ml of sample was sonicated using an ultrasound probe prior to analysis. Insulin concentration was measured using commercially available ELISA kit (Millipore, UK). Concentrations of insulin in breast milk were correlated with maternal characteristics using Spearman's correlation. Linear regression was performed in SPSS 20, using log insulin concentrations.

Results: The median concentration of insulin in breast milk was 11.33 ng/ml (interquartile range, lower value = 7.1 ng/ml, upper value = 17.3 ng/ml). Spearman's correlation revealed there was a significant positive correlation between breast milk insulin concentration and maternal BMI at sample collection $r = 0.38$, $p = 0.02$. No association was observed between insulin and other maternal characteristics including age, duration of pregnancy and booking BMI.

Linear regression was used to quantify the association between maternal BMI and insulin content of the breast milk. The percentage change in insulin concentration per unit increase in maternal BMI at sample collection was 2.6% (95% CI: 0.2%, 5%).

Conclusions: The concentration of insulin found in breast milk appears to be related to maternal adiposity. This may be due to mothers with a higher BMI displaying increased insulin resistance, which is reflected by an increased concentration of insulin in the breast milk. The finding that there was no correlation between breast milk insulin concentrations with booking BMI suggests that due to maternal pregnancy weight gain and the concomitant increase in insulin resistance this measure no longer reflects the metabolic status of the mother.

Whether it is this increase in insulin content of breast milk of overweight mothers which influences the infants likelihood of becoming overweight is yet to be established, but may be a contributing factor towards this effect.

SYNERGISM OF CAFFEINE AND OCULAR KETOROLAC ON RETINAL ANGIOGENESIS IN RAT MODEL OF OXYGEN-INDUCED RETINOPATHY

J.V. Aranda, C. Cai, P. Sharma, J.H. Min, A. D'Souza, V. Bronshtein, Z. Ibrahim, G.B. Valencia, D.E. Lazzaro, K.D. Beharry

Neonatal Research Laboratories and NIH U54 Research Center, State University of New York Downstate Medical Center, Brooklyn, NY, USA

AUTHOR COPY

Presented at the Neonatal Society 2012 Summer Meeting.

Propylene glycol: a natural protection against cold stress at delivery?

Hyde MJ, Romero MG, Wijeyesekera AD, Perez IG, Jeffries S, Logan K, Andreas N, Gale C, Holmes E, Modi N

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Background: We have previously used ^{1}H NMR to study the hepatic metabolome in 7 day post-partum piglets, delivered vaginally (VD) or by pre-labour Caesarean section (CS) (1). In the present study we aimed to evaluate the urinary metabolome during the first 7 days post-partum in human babies born by VD or CS. The study was conducted with Research Ethics Committee approval (08/H07/11/43).

Methods: A urine sample was collected on cotton wool from 30 infants within 72 hours of birth, at Chelsea & Westminster Hospital, and stored at -80ºC until analysis. Samples were analysed using one-dimensional ^{1}H NMR spectroscopy at 300K on a Bruker Avance 600 MHz spectrometer, using standard parameters and pre-processing algorithms, as previously described (2). The spectra were automatically phased; baseline corrected, referenced using an in-house routine written in MATLAB, normalised using probabilistic quotient normalisation to the median profile and aligned; continuous spectral data were used for statistical analysis. Spectra were analysed on the basis of experimental group using principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA) to explore the variation between the groups.

Results: Urinary samples were analysed from 15 VD and 15 CS infants (Pre-labour CS = 7; Emergency CS = 8) born between 37-42 weeks gestation. There was a significant difference in the urinary metabolome of infants born by CS and VD when examined using PLS-DA (Fig 1a). One of the metabolites with greatest difference between CS and VD babies was propylene glycol (doublet at 1.14ppm; Fig 1b). As this was unexpected, we reviewed previously analysed data acquired from 15 term baby urine samples. We classified these according to mode of delivery (6 VD and 9 CS) and found a similar difference in propylene glycol between CS and VD babies.

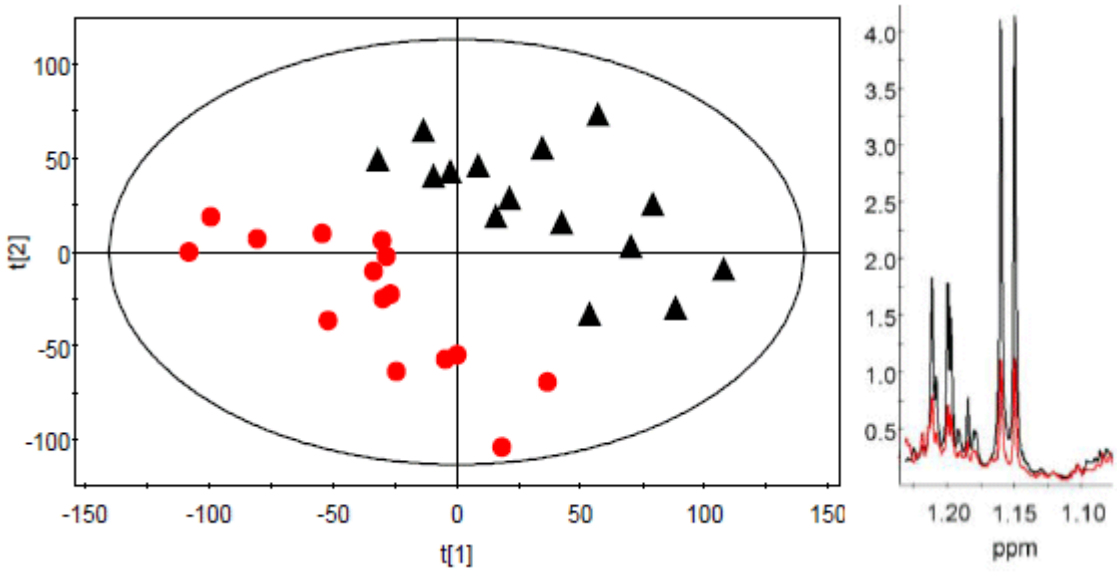


Figure 1: a) PLS-DA scores plot of UV scaled data from ^{1}H NMR spectra of urine collected from term infants born by CS (●; n = 15) vs. VD (▲; n = 15) infants. (R2 = 0.831; Q2 = -0.049). b) Representative NMR spectra (red = CS; black = VD) showing the doublet associated with propylene glycol.

Conclusion: In our previous work one of the metabolites which differed strongly between VD and CS delivered piglets was also propane-1,2-diol (propylene glycol), which was increased in the former. We previously attributed propylene glycol in the liver of VD animals to its use as a vehicle for sodium pentobarbital used for euthanasia, and hypothesised that differences between CS and VD piglets reflected altered underlying metabolism. We now show that propylene glycol is also increased in human infants born by VD compared to CS. Upon review of published literature we find reports of propylene glycol in cerebral in vivo ^{1}H NMR spectra in the human neonate following asphyxial injury and also in urine; these authors concluded that the source of the propylene glycol was phenobarbitone vehicle (3,4). The presence of propylene glycol in healthy VD infants suggest an alternative hypothesis, namely that propylene glycol, through its involvement in lactate/pyruvate metabolism, may be produced in VD infants in response to the transient hypoxaemia associated with VD. Propylene glycol is commonly used as antifreeze, and though postulating a role as a natural protection against cold stress at delivery may be implausible, its significance to the physiology of the newborn is intriguing. Our finding is novel and demands further study.

References

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2. Beckonert, O. (2007) Nature Protocols. 2, 2692-703.
3. Cady, E.B. (1994) MRM. 32, 764-7.
4. Ma, S. (1995) Appl Biochem and Biotech. 53, 37-51.

Presented at the Neonatal Society 2012 Summer Meeting.

Infants of mothers with diabetes have altered urinary metabolic profile at birth

Logan KM, Wijeyesekera AD, Perez IG, Hyde MJ, Romero MG, Jeffries S, Andreas N, Gale C, Holmes E, Modi N

Imperial College London, UK

Background: In addition to their genetic risk, babies exposed to diabetes in the womb have greater risks of childhood obesity (1) and a fourfold increased risk of T2D (2). Identification of pathways and markers of T2D risk in infants of mothers with diabetes (IDM) could bring targeted surveillance and early detection nearer. Using metabolomics, large numbers of metabolites may be analysed in small volumes of urine. Metabolic profile has not been studied in newborn IDM and we hypothesised that this differs between IDM and control infants in the newborn period. The study was conducted with Research Ethics Committee approval (08/H07/11/43).

Methods: Infants were recruited from the postnatal wards at Chelsea and Westminster Hospital between July 2010 and Jan 2012. A urine sample was collected within 72 hours using cotton wool and stored at -80ºC until analysis. Samples were analysed using one-dimensional ^{1}H NMR spectroscopy at 300K on a Bruker Avance 600 MHz spectrometer, using standard parameters and pre-processing algorithms, as previously described (3). The spectra were automatically phased; baseline corrected, referenced using an in-house MATLAB routine, normalised using probabilistic quotient normalisation to the median profile and aligned; continuous spectral data was used for statistical analysis. Spectra were analysed on the basis of experimental group using principal component analysis (PCA) and partial least square-discriminant analysis (PLS-DA).

Results: We analysed urine samples from 18 IDM and 12 healthy term control infants. The urinary metabolome of IDM and term neonates appears to differ (Fig 1). Differences appear to be seen in the following metabolites; glucose and formate, fumarate, succinate and citrate, which are involved in the TCA cycle.

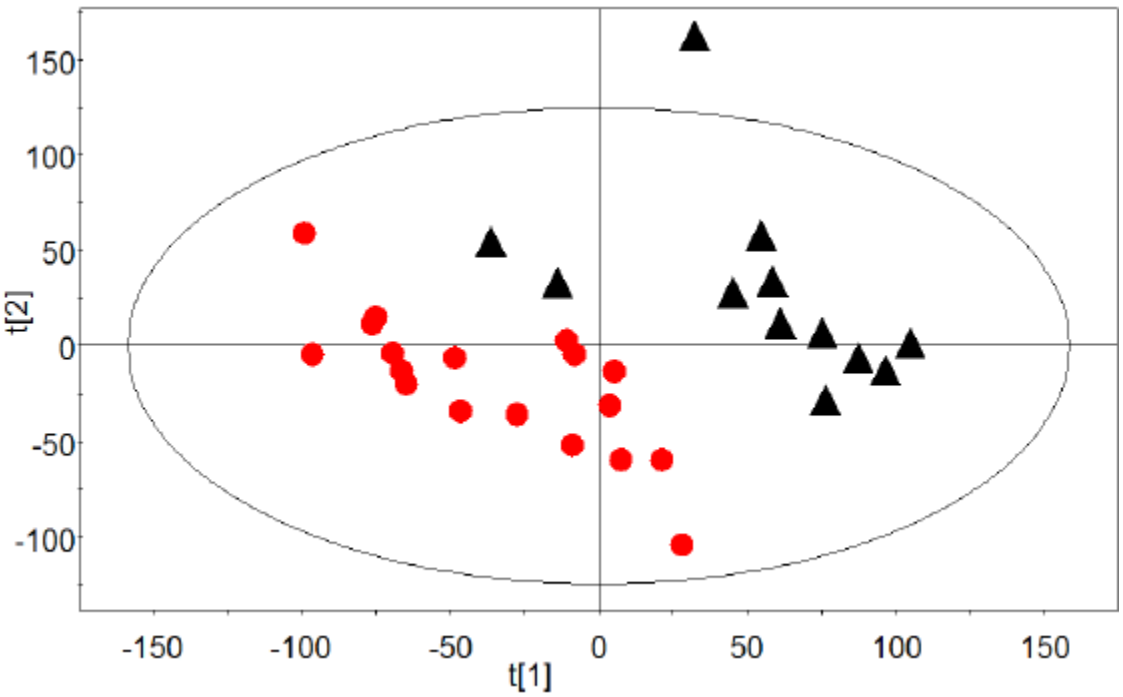


Figure 1: Partial least squares discriminant analysis (PLS-DA) scores plot of UV scaled, continuous data

from ^{1}H NMR spectra of urine collected within 72 hours of birth from infants of diabetic mothers(●; 37+3 - 39+5 weeks GA; n=18) compared with healthy term infants (▲; born 39+2 - 42 weeks GA; n=12). R^2=0.79, Q^2=0.09.

Conclusion: These preliminary data indicate differences in the metabolism of IDM and control infants in the newborn period. These findings require confirmation and reassessment in later infancy. The next step will be to study infant metabolite alterations in relation to the continuum of maternal glycaemia in pregnancy.

References

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Presented at the Neonatal Society 2014 Autumn Meeting.

Title (Upper case)

THE DIVERSE HUMAN MILK OLIGOSACCHARIDE CONTENT OF HUMAN BREAST MILK

Authors *(Presenting author underlined. If no author is a Society member please provide the name of the member introducing the author to the Society)*

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Introduction *(include hypothesis)*

Previous research has identified Human Milk Oligosaccharides (HMO) as a highly variable component of human breast milk. HMO are complex carbohydrates that serve multiple functions, including acting as an anti-infective agent for the infant. HMO biosynthesis is in large part determined by Lewis blood group and secretor status, which are genetically determined [1]. We hypothesised that the diverse profiles of HMO could be assessed using ^{1}H NMR spectroscopy, and that individual HMOs contributing to differences between profiles would be identifiable.

Methods (*include source of funding and ethical approval if required)*

Human milk samples were obtained from 108 mothers, at 7 days and 3 months post-partum. Samples were solvent extracted and ^{1}H NMR spectra acquired for the aqueous fraction. Data were analysed using Principal Component Analysis (PCA) to define clustering of data in relation to the component describing the largest proportion of variation. Data were modelled using Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) to compare different groups of mothers, defined by their spectral profiles. The study has received Research Ethics approval (12/LO/0203) and is funded by Westminster Medical School Research Trust.

Results

Large differences in the abundance of HMO were observed between the 7 day and 3 month time points. There was a substantial decrease in HMO quantity at 3 months. Furthermore, large differences in the specific types of HMO were identified. In the PCA plot, two broad groups of mothers were identified, each identified by distinct HMO profiles, known to be determined by maternal genotype. Based on the types of oligosaccharides produced one group, thought to be secretors (Se^{+}), produced a preponderance of HMO known to be products of the gene fucosyltransferase 2, including 2'-fucosyllactose, lactodifucotetraose and lacto-N-fucopentaose 1. The other group, thought to be non-secretors (Se^{-}), did not produce these HMO, but had an increased quantity of other HMO, including 3'-fucosylactose, present in their breast milk. Overlaying the NMR spectra, allows us to identify the types of HMO produced, associated with the Lewis blood groups. The interaction between the Lewis blood group (Le^{+}/Le^{-}) and secretor gene status produce four types of breast milk. All four types were present in our cohort, (Se^{+}/Le^{+}), (Se^{+}/Le^{-}), (Se^{-}/Le^{+}) and (Se^{-}/Le^{-}).

Conclusions

HMO are believed to have important prebiotic properties. Additionally, data show an association between maternal non-secretor status, and increased infant infection risk [2]. We have shown that human milk HMO profile may be characterised rapidly using ^{1}H NMR spectroscopy. This provides opportunity for a stratified medicine approach in future interventional studies involving manipulation of the infant microbiome to reduce

References *(include acknowledgement here if appropriate)*

[1] Kobata, A., Proc Jpn Acad Ser B Phys Biol Sci, 2010. 86(7): p. 731-47.
[2] Morrow AL, et al. J Pediatr, 2004, 145: 297-303.

Presented at the Neonatal Society 2015 Spring Meeting.

Title (Upper case)

THE ASSOCIATION BETWEEN BREAST MILK MACRONUTRIENT CONTENT AND MATERNAL BMI

Authors *(Presenting author underlined. If no author is a Society member please provide the name of the member introducing the author to the Society)*

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Institution(s)

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[2] Section of Computational and Systems Medicine, Faculty of Medicine, Imperial College London, UK

Introduction *(include hypothesis)*

Previous research on donor breast milk has suggested a positive correlation between maternal Body Mass Index (BMI) and breast milk concentration of protein, carbohydrate and fat[1]. We sought to further study the relationship between maternal BMI and fore and hind milk composition, over the first three months of lactation. We tested the hypothesis that we would observe differences in breast milk composition between high and normal BMI mothers.

Methods (*include source of funding and ethical approval if required*)

We obtained fore and hind milk samples from 95 mothers delivering at full-term, at seven days and three months post-partum. 52 mothers had a BMI <25, 43 a BMI >25. Samples were frozen at -80°C until analysis, when they were thawed and homogenised using sonication prior to analysis. Macronutrient content was determined using mid-infrared spectroscopy (Miris Human Milk Analyser loaned by MIRIS AB, Sweden), in accordance with manufacturer instructions. We used ANOVA (SPSS) to assess the relationship of breast milk macronutrient content in relation to maternal BMI. The study received Research Ethics approval (12/LO/0203) and was funded by Westminster Medical School Research Trust.

Results

We obtained a total of 284 samples (1 week, fore milk=95, hind milk=40; 3 months, fore milk=90, hind milk=59). We found no statistically significant or clinically relevant correlations between maternal BMI and breast milk protein, carbohydrate or fat content at either of the time points, nor did maternal pregnancy weight gain influence the concentration of macronutrients. In agreement with previous reports at three months only 24% of women with a BMI >25 were still breast feeding compared to 75% of mothers with a BMI <25. Also in keeping with other reports, we found that hind milk has significantly more fat and less carbohydrate than fore milk, regardless of stage of lactation. However, protein concentration in fore and hind milk differed with stage of lactation: at 7 days, there was no difference between fore and hind milk; but at 3 months there was more protein in hind milk ($p=0.009$). Between 7 days and 3 months of lactation, in fore milk, the concentration of fat remained stable, carbohydrate increased and protein decreased; in hind milk there were significant increases in fat and carbohydrate, but a significant decrease in protein.

Conclusions

In contrast to previous research, we did not observe any association between maternal BMI and breast milk macronutrient content at any of the time points studied. Our data suggest that breast milk composition is regulated independent of maternal BMI.

References *(include acknowledgement here if appropriate)*

[1] Michaelsen, K. F. J Pediatr Gastroenterol Nutr. 1990 11(2); p. 229-39

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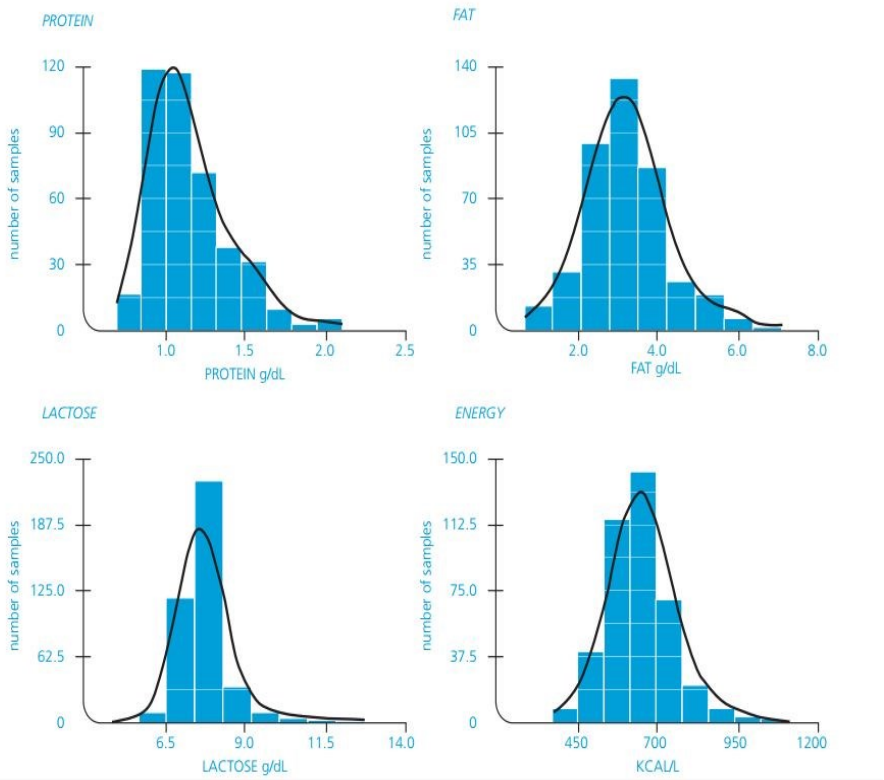
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Figure 5. Structures of 2 and 3'-fucosyllactose

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