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Towards the identification of metabolite markers of nipple pain and inflammation in human milk

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4 Discussion

Breast milk is the preferred source of nutrition for the developing infant, with breastfeeding linked to many health, nutritional, immunological, developmental, psychological, social, economic, and environmental benefits (Anatolitou, 2012). Nipple pain and mastitis during the first three weeks of breastfeeding are important clinical factors associated with early cessation of breastfeeding (Schwartz et al., 2002). The intensity and duration of maternal nipple pain during breastfeeding have been correlated to interference with breastfeeding and general quality of life (McClellan et al., 2012). Women who experience pain in the first three weeks post-partum are also more likely to give up breastfeeding than women who experience pain after the first three weeks (Schwartz et al., 2002), thus jeopardising both their own and their infants short and long term health. Rapid identification and treatment of these conditions would potentially reduce premature weaning and extend breastfeeding duration. Despite initiation of breastfeeding increasing in Perth (90-96% on discharge from hospital), the prevalence of breastfeeding at 6 (50-60%) and 12 months (22-28%) are still below the national targets of 80% and 40%, respectively (Scott et al, 2006; NHRMC, 2010).

A recent Australian study recorded the incidence of nipple pain in 360 breastfeeding women who intended to breastfeed beyond 8 weeks postpartum (Buck, Amir, Cullinane & Donath, 2014). They found that 79% of women reported nipple pain in the first week post partum and 58% experienced nipple damage, including cracked or grazed nipples (Buck et al., 2014). The prevalence of nipple pain and nipple trauma had at best reduced by half at 4 weeks post partum (43% and 24%, respectively) with

a further decline identified at 8 weeks post partum where 20% reported nipple pain and 8% reported nipple damage (Buck et al., 2014). In this study 20 women ceased breastfeeding before the end of the study (>8 weeks) as a result of developing nipple pain and trauma (Buck et al., 2014).

Despite the highest incidence of nipple pain identified in early lactation, women in the first four weeks of lactation were deemed not acceptable for this study. This study aimed to identify compositional changes in breast milk associated with nipple pain and trauma. However, the composition of milk undergoes many changes over the first three weeks post parturition until it reaches the mature phase which is maintained for the remainder of lactation (Hassiotou & Geddes, 2013). Therefore, the composition of milk is too variable in the initial stage of lactation to enable identification of changes resulting from breast pathology compared to transitional changes of the milk to mature milk. For this reason participants in this study were recruited between 1-6 months post parturition to eliminate compositional changes in the milk due to the establishment of lactation, thereby identifying only those changes associated with breast pathology.

The primary aim of this study was to determine compositional changes to human milk, consequential to mothers experiencing persistent nipple pain with and without evidence of trauma. It was hypothesized that the presence of nipple pain in lactating women would result in changes in the human milk metabolite profile possibly due to infection and/or inflammation of the breast, compared to asymptomatic women. A series of bovine samples from asymptomatic cows and cows presenting with mastitis served as comparative controls.

The treatment for inflammatory symptoms of the breast, including nipple pain and mastitis, has been under discussion for some time. This is largely due to the wide spectrum of symptoms under the umbrella of 'mastitis', the myriad of potential causes, and lack of international scale for the measurement of symptoms (Kvist, Larsson, Hall-Lord, Steen & Schalen, 2008).

McClellan et al. (2012) investigated a selection of pain scales as objective measures to compare the pain experienced by breastfeeding women. Differences in pain scale were reported for women with nipple pain with and without evidence of trauma. Pain scores reported for the mothers in the nipple pain with trauma group were significantly higher than the nipple pain without trauma group ($p<0.001$). Mothers experiencing nipple pain with trauma described their pain as 'piercing' 'tight' and 'tearing', whereas mothers in the nipple pain without trauma group most commonly referred to their pain as 'radiating'. In this study participants' pain was converted to a numerical value between 0-10; zero being no pain and 10 being the worst perceived pain possible. Participants in the control group all reported their pain to be 0, participants in the nipple pain group reported perceived pain levels between 4 to 10. The mean pain level was reported for the nipple pain without evidence of trauma was 5.83 with one participant reporting pain as high as 8; however this was not different to the TG subgroup with a mean pain score of 5.2. Compared to McClellan et al. (2012) the trauma group was not reporting as much pain and therefore may not have been as advanced in the mastitis continuum.

The influence of bacteria in the development of nipple pain and nipple trauma is still not clear. Within the literature there are several studies surrounding the identification and quantification of pathogenic bacterial species in an attempt to determine the

causative agents of nipple pain and mastitis. Kvist et al. (2008) conducted a bacterial study (using PCR) to determine the role of bacteria in nipple pain and mastitis and found five main bacterial species to be the most prevalent between control and case samples; coagulase negative Staphylococci (CNS), viridans streptococci, *Staphylococcus aureus* (*S. aureus*), Group B streptococci (GBS) and *Enterococcus faecalis*. Despite this, viridans streptococci (OR: 1.43; p=0.04), *S. aureus* (OR: 1.81; p=0.001) and GBS (OR: 2.40; p<0.001) were found significantly more often in case samples than control. Additionally, CNS were detected significantly more often in the milk of healthy control mothers (90%) than in those suffering from mastitis (83%) (OR: 0.60; p=0.02) (Kvist et al., 2008). In this thesis study, CNS were found in 94% of human milk samples and 100% of human control samples. CNS was found in highest abundance in the human control samples (1,231 CFU/ml) compared to the nipple pain samples (370 CFU/ml). Kvist et al. (2008) concluded that CNS was an important bacterium in the protection against pathogens, and increasing bacterial counts did not influence the clinical manifestation of mastitis. Furthermore, a *S. aureus* study by Heikkila and Saris (2003) found that the commensal bacteria in breast milk might inhibit the growth of *S. aureus* and prevent maternal breast infections, which could explain the higher abundances in control milk samples.

The study of 346 mothers by Amir et al. (2013) using PCR found that *S. aureus* was found in 82% of women presenting with nipple pain and in 79% of women without symptoms of nipple pain or mastitis. Conversely, a microbial culture study by Rowan et al. (2008) looking at the potential of *S. aureus* as a causative agent in nipple pain and trauma found no *S. aureus* in their control group. Furthermore, Rowan et al. (2008) identified *S. aureus* in 38% of mothers with nipple pain with minimal trauma and no obvious infection and in 100% of the mothers presenting with nipple pain with

obvious nipple trauma and infection. The data presented in this thesis identified no *S. aureus* in the control group samples. *S. aureus* was positively identified in only one nipple pain sample out of a total eleven nipple pain samples. This sample was collected from a mother suffering from unilateral nipple pain (PG) and had 82% *S. aureus* growth (620 CFU/ml) in milk from her affected breast, with no *S. aureus* identified in the milk collected from her unaffected (control) breast. However, this was the sample from a mother with nipple pain where *S. aureus* growth was identified, suggesting that in this study nipple pain was not always the result of *S. aureus* infection.

It should be noted that traditional microbial culture is not always a clear representation of the microbial flora of a sample, as culture dominant species can over grow or inhibit the growth of other species, not all species are amenable to any one method of culture, or at all. Microorganisms isolated using standard culture techniques are rarely the dominant and/or functionally significant species in the communities from which they were obtained (Hugenholtz, 2002).

The study by Amir et al. (2013) found a statistically significant association between the presence of *Candida ssp.* and nipple pain symptoms presenting 2-8 weeks post partum ($p=0.014$). A PCR analysis of the milk of 346 lactating women found that 33% of women were positive for *Candida ssp.*, distinctly different from the 5% of milk samples from the same sample set that cultured positive for *Candida ssp.* (Amir et al., 2013). Furthermore, control samples that tested positive for *Candida ssp*. at any point were 87% more likely to develop nipple pain symptoms than mothers who tested negative for *Candida ssp*. (Amir et al., 2013).

A study by Hale et al. (2009) using microbial culture and a β-glucan assay (where β-glucan concentration 80 pg/mL is considered a presumptive measure of *Candida* infection) found that β-glucan was more commonly found in higher concentrations in control samples than in symptomatic patients, although no control samples and one symptomatic sample cultured positive for *Candida* (1 CFU/15 μL of milk). Hale et al (2009) suggested that their results were the product of the extreme clean-catch technique used for milk collection (which formed the basis for the collection method used in this study) and that studies that draw conclusions on *Candida* mastitis or the role of *Candida* in nipple pain may be the result of insufficient aseptic techniques and evidence of microbial contamination through the infant's saliva on unsterilised nipples. Amir et al. (2013) suggested that the Hale et al. study (2009) may indicate alternative causes of breast and nipple pain, including mechanical causes such as infant tongue tie or bacterial infection.

A protocol similar to that of Hale et al. (2009) was used for milk collection in this study to ensure microbial contamination of the milk, from skin flora or environmental contaminants, did not influence the microbial composition of the milk. No fungal species were isolated from the human samples, with nipple pain or otherwise, in this study. Yeast species were isolated from bovine milk from both pooled vat samples collected from a single storage vat (BV01 and BV02), but not from individual bovine milk samples. The ability to culture yeast in pooled bovine samples showed that Sabouraud agar was a suitable medium for the isolation of fungal species, with evidence of only fungal species isolated by these means. Furthermore, these results indicate that there were no fungal species in the human samples. Molecular techniques (PCR or sequencing) would be required to confirm definitively that no

fungal species were present in the human milk samples, or identify species non-amenable to this culture technique.

Biochemically it is possible to detect changes in certain macronutrients and micronutrients in breast milk that are associated with closure of the tight junctions between the lactocytes at the onset of secretory activation (Pang and Hartmann, 2007). A study by Fetherston, Lai and Hartmann (2006) found that mastitis was associated with the reopening of the tight junctions and consequently paracellular pathways, accompanied by increased breast permeability, inflammation of the breast, and reduced milk synthesis. A significant increase in Na^+ concentration was observed in women with nipple trauma ($p<0.004$), and a further increase in mean Na^+ concentration was observed in women with clinical mastitis (21.8 mmol/L) (Fetherston et al., 2006). In this thesis, a significant difference in Na^+ concentration was observed between mothers in the control and TG groups ($p<0.001$). However, the mean Na^+ concentration for the TG subgroup (8.04 ± 2.40 mM) was much lower than that observed in women with mastitis in the Fetherston et al. study (2006), indicating that no participant in the study had mastitis, but possibly had the potential to develop into mastitis if left untreated.

The composition of milk is highly variable over the course of lactation, between feeds and between mothers (Chung, 2014) and can be influenced by maternal and infant health and medication use (McManaman & Neville, 2003). Paracellular pathways allow the direct exchange of interstitial or serum substances into the milk. This transport pathway closes during the initiation of lactation, due to the reduction of tight junctions between the epithelial cells (McManaman & Neville, 2003). This pathway is affected by the physiological state of the breast, and during an episode of

inflammation or mastitis, tight junctions between the epithelial cells can become compromised, allowing plasma components such as sodium and calcium to leak into the milk (Shennan & Peaker, 2000).

Assessment of the increased permeability of the paracellular pathway can be determined by measuring the solute content of the milk, in particular Na^+ and K^+ (Shennan & Peaker, 2000). Damage to the paracellular pathway can be identified by an increase in Na^+ concentration and decrease in K^+ concentration. However, measurement of the Na^+/K^+ ratio has been proposed as a more accurate indicator as it reduces the influence of individual differences due to the large variation in human milk composition between individuals. Areetey et al. (2008) proposed an elevated Na^+/K^+ ratio above 1.0 to be considered indicative of infection.

No individual in this study recorded a Na^+/K^+ ratio above one. The highest ratio was 0.811 recorded from the milk of a mother experiencing persistent nipple pain with trauma. The TG subgroup had a significantly higher ratio than the control group ($p<0.001$) and a mean ratio difference of 0.13 between the TG and PG nipple pain subgroups indicating, alike the pain scores, little difference between the groups.

Sodium and potassium analysis has been found to be a useful tool in relation to mastitis, however it appears less conclusive in the analysis of nipple pain. Mastitis can cause a significant amount of damage to mammary tissue, resulting in considerable alteration in milk composition. As nipple pain without incidence of mastitis is often less invasive, it is possible that the damage to the mammary tissue was not extensive enough to result in a significant increase in Na^+/K^+ concentration or ratio in this study.

All analyses of sodium and potassium concentrations presented here should be treated with caution, as the sample size can be considered too small to draw definitive conclusions. This is due to the pre-expected marginal differences between the groups due to the subtleties of the pain condition. To detect a true difference in Na^+/K^+ ratio between nipple pain samples in TG and PG subgroups, a larger sample size would be required. Using the independent nipple pain samples from this study, a t-test power calculator was used to determine the sample size required to improve the power of the study to 80 and 90% (Lenth, 2009). The two-sample group t-test found that an increase in sample size to 20 participants per group would be required to increase the power of the study to 80% and 26 participants per group would increase the power of the study to 90% (Lenth, 2009). It is assumed that each participant would be included in only one group, and that only one sample would be taken from each. If more samples were taken, or another study design was used, then smaller numbers might be appropriate.

The burgeoning area of metabolomics as a diagnostic medium has begun to be explored in the dairy industry. A metabolomics study by Sundekilde et al. (2013) identified a series of metabolite biomarkers including isoleucine, lactate, butyrate and acetate to be linked with an elevated somatic cell count in bovine milk, which is indicative of mastitis. Sundekilde et al. (2013) proposed that the detection of these biomarkers could be used to determine milk quality, diagnose mastitis and to determine the level of infection. The same concept could be applied to human milk: determining changes to metabolited as a means of diagnosis for breast complications. Metabolite profiling has the potential to illuminate how the presence of infection and inflammation affects breast milk composition, leading to a greater understanding of the effect of nipple pain and mastitis on the lactating breast and providing a means of

monitoring treatment. As nipple pain is often a precursor to mastitis, it is important to understand if underlying breast pathology exists. Consequently, if the severity of nipple trauma is associated with underlying infection, then profiling the metabolome of expressed milk will identify any differences in metabolite composition between mothers experiencing varying degrees of pain and discomfort.

Although there have been some studies that associate certain species of bacteria with incidences of nipple pain and mastitis, there is still no reliable diagnostic biomarker of pain or infection to enable rapid effective treatment. Metabolomics is widely used as a functional tool in biomarker detection and systems biology. Metabolomics involves the unbiased quantitative and/or qualitative analysis of the complete set of metabolites present in a biological system. Biostatistics plays an essential role in analysing differences in metabolomes and enabling the identification of metabolites pertinent to a particular phenotypic characteristic (Koek, Jellema, van der Greef, Tas & Hankemeier, 2011).

Generally a non-targeted metabolomics approach is used to gain new insights and a better understanding of the biological functioning of a cell or organism in an attempt to interpret biological outcomes (Koek et al., 2011). It is crucial that all steps of the analytical method, namely sample preparation, data acquisition and data processing, are addressed when determining a suitable experimental design. Optimisation, validation and quality control of analytical methods are of the highest importance (Koek et al., 2011).

Optimisation of sample preparation, namely sample volume, was important to identify the dynamic range in metabolite concentrations that would allow the unbiased

measurement of sample components for any given instrumental analysis. The optimisation of the sample loading on the GC was required to determine an amount/mass of metabolite extract that would not overload the instrument while still being large enough to identify metabolites present at low abundance. Milk sample volumes of 25 µl, 50 µl, 75 µl and 100 µl were analysed with the addition of an internal standard ($^{13}C_6$ labelled D-sorbitol) to determine reproducibility and to assess the capacity for this internal standard to reliably correct for changes in signal intensity across data from GC-MS acquisition.

Extraction and derivatisation efficiency can be estimated using labeled n-alkanes as reference compounds and comparing their response to the response of derivatised metabolites (Koek, Muilwijk, van der Werf, & Hankemeier, 2006). In this instance we sought to assess the reproducibility of the peak intensity of a series of n-alkane analytical standards (C_{12}, C_{15}, C_{19}, C_{22}, C_{28}, C_{32}, C_{36}) added to derivatised milk extracts of differing concentration and the capacity for the corresponding peak area of the internal standard, $^{13}C_6$ labelled D-sorbitol, to correct for differences in analytical reproducibility among a sample set. The %RSD of the alkanes was used to determine the reproducibility of the four test volumes. Food and Drug Administration guidelines (FDA) for biomarker studies specify a RSD of < 20% to be an acceptable level of precision (Kirwan et al., 2013). The dried 50 µl milk volume metabolite extract was the largest sample volume that could be dissolved in a single derivatising volume while maintaining reproducibility of the added compounds (alkanes) below a %RSD of 30. Larger sample volumes may be compromised due to inadequate amounts of TMS reagent for complete derivatisation, a factor of milk being a complex biological matrix, as well as potential interference with deconvolution and therefore accuracy in measurement of peak areas. In order to not compromise the measurement of the

lower abundant metabolites, the largest reproducible volume, 50 µl, was used for subsequent metabolomics analyses.

Many metabolites contain polar functional groups, and are thermally unstable at the temperatures required for injection into a GC or are simply not volatile. Derivatisation prior to GC analysis is often needed to extend the application range of GC based methods (Koek et al., 2011). The majority of GC-based metabolomics methods are based on derivatisation using an oximation reagent followed by silylation (often by MSTFA), or solely silylation (Gullberg et al., 2004; Jonnson et al., 2006; Zhang, Wang, Du, Zhu & A, 2007).

Koek et al. (2006) tested several derivatisation reagents including BSTFA, MSTFA, MSTFA with 1% TMCS, BSA, TMSI and TMSI/BSA/TMCS 3:3:2, and found the highest recoveries and smallest RSDs were found using MSTFA. Within the literature a range of derivatisation temperatures has been tested. Gullberg et al. (2004) trialled MSTFA derivatisation at 20°C, 40°C and 60°C and found that 60°C was the optimal temperature. Dunn et al. (2011) used rapid derivatisation at 80°C. Gummer et al. (2013) used 37 °C for MSTFA derivatisation. Bressanello et al. (2014) incubated at 100°C following the addition of MSTFA.

For this study a range of derivatisation temperatures were assessed to determine the optimal derivatisation temperature. Samples were prepared using methoxymation followed by MSTFA derivatisation incubated at 37°C, 45°C, 60°C, 75°C and 90°C. Depending on the target metabolites, different analytical approaches are required and different requirements are posed on analytical performance, including detection limits, accuracy of compound identification and reproducibility (Koek et al., 2011). In

this instance, reproducibility is of the upmost importance due the already highly variability of human milk composition between individuals.

Analysis of the tested derivatisation temperatures found that incubation at 75 °C resulted in high signal intensity, but more importantly the highest reproducibility (determined by comparison of standard deviation across the three replicates for each of the test temperatures). The reliability and suitability of sample preparation, data acquisition, data preprocessing and data analysis are imperative for accurate biological interpretation (Koek at al., 2011).

Metabolomics revolves around the central concept that an individual's metabolic state is a close representation of his/her current physiological state (Fanos et al., 2012). Analysis of an individual's metabolome is indicative of their current heath and disease status (Fanos et al., 2012). Here, we used a metabolomics analysis of human milk from control mothers, and mothers experiencing persistent nipple pain with or without evidence of trauma, to identify metabolite difference reflective of underlying physiological breast health.

Due to the large variation in breast milk composition between individuals, irrespective of their health status, it was important to ensure appropriate quality controls and internal standards were used in the metabolomics analysis to account for noise within the data and identify true biological variation between the control and nipple pain subgroups. Using only a single internal standard for purpose of normalisation can also be presumptuous in that it assumes that all metabolites in a sample are subject to the same amount of unwanted or external variation, often failing to remove unwanted factors (De Livera et al., 2012). In this case a labelled internal

standard, $^{13}C_6$ labelled D-sorbitol, with the addition of pooled QCs, a mixture of the entire sample set under study, were used to normalise and interrogate the data. A labelled or synthetic internal standard prevents variation resulting from chemical properties and confusion of the internal standard with metabolites of interest that may co-elute or appear similar in structure (De Livera et al., 2012). The use of multiple internal standards or similar, the use of pooled QCs for example, has been found to be more effective at removing unwanted instrumental interference and leads to lower variability of the normalised metabolite abundances (De Livera et al., 2012). Normalisation allows the reduction of variation or interference between sample extraction efficiency and instrumentation. Resulting values can then be scaled to the median intensity (as opposed to mean; which has been found to be less effective) for each metabolite across the data set (Veselkov et al., 2011). The data manipulation employed in this thesis was to reduce the influence of the highest abundant metabolites within the model by reducing the range of dynamic abundance and therefore the bias within the raw data. A log scaling approach was also used as an additional level of scaling for data modelling (Veselkov et al., 2011). Additionally within this thesis, PCA was used for quality control purposes and initially focused on the pooled milk (quality control) samples, which were run at regular intervals between samples throughout data acquisition.

It is known that the first few injections of samples on the instrument show variability and give unrepresentative results within metabolomics analyses due to small changes in signal intensity and retention time (Veselkov et al., 2011). For this reason, the column was ‘conditioned’ using five injections of the same biological matrix as the sample set, in this case pooled QC samples, to reduce this initial variability between sample replicates.

The ion source of the MS can become contaminated, especially when using metabolite dense biological matrices such as human and bovine milk, resulting in a gradual decline in instrument sensitivity over time (Veselkov et al., 2011). A slow degradation of signal intensity was observed over the duration of analysis within this study despite preventive cleaning of the ion source and regular monitoring and maintenance of GC consumables. Internal standards and QCs were unable to correct for instrumental error and decline in signal intensity. This indicated that the observed decline in signal intensity was not uniform across the chromatogram and could unlikely be corrected. As a consequence any samples analysed within the analytical sequence amongst the QC samples that failed to group by PCA were determined as not meeting the requirements of the quality control and were excluded from the final interpretation.

Within the sample data, the PCA showed that the first three principal components exhibited little significant grouping of the human nipple pain groups. PC-4, associated with 8% of the variance between the human milk and samples, best showed difference in metabolite composition between the control and trauma samples. Whilst ideally the first components of a PCA would distinguish sample groupings consistent with the study design, the result of finding PC4 of most relevance was not entirely unexpected due to the subtlety of the condition under study (nipple pain) as demonstrated throughout this thesis, such as in the Na^+/K^+ results, and the inherent variation in milk composition between individuals and within individuals over time (Grote et al., 2015).

The projected position of each of the samples on the scores plot (Figure 3.19) is a measure of their individual metabolite abundances relative to the metabolite abundances of the other samples on the plot. Samples found close to each other on the scores plot indicate high positive correlation. This can be seen with Hs_cont_6 (C02) and Hs_NT_09 (NP05), which are closer to each other on the scores plot than other samples, indicated by the black square in Figure 3.19. These samples were retrieved from a single mother (M03) who donated both a control and nipple pain sample. The mother was suffering from unilateral nipple pain with no evidence of trauma and had a Na^+/K^+ ratio of 0.54 (where a ratio above 1 is considered indicative of infection) with no evidence of pathogenic bacteria in her milk, it is therefore consistent that these samples are compositionally similar. In contrast, samples located in diagonally opposite quadrants have a tendency to be negatively correlated, indicating a compositional difference between nipple pain with trauma samples (Hs_T_13, Hs_T_18 and Hs_T_23) located at the bottom of the scores plot, with a negative PC-4 score coordinate, and the control samples present in the opposite quadrants, with a positive PC-4 score coordinate (Figure 7.7). Additionally, the trauma samples recorded the highest Na^+/K^+ ratio; of note was sample Hs_T_13 (NP07), which had the highest ratio of 0.81, a measurement used as an indication of breast trauma resulting in reopening of paracellular pathways in the mammary tissue (Aryeetey et al., 2008).

The correlation loadings plot (Figure 3.20) displays all of the measured metabolites from the human milk and was used to identify which metabolites were able to constitute to the variance between the human sample set as well as identify metabolites that are positively and negatively correlated with control and nipple pain samples. Metabolites found closer to the middle of the plot do not contain enough structured

variance and cannot be explained by the plot. These were determined inconsequential to a nipple trauma event.

Amino acids, in particular isoleucine and proline, were amongst the metabolites found most positively correlated with human trauma samples. Within the literature there are several studies that have identified a link between an increase in amino acid concentration in response to inflammatory diseases including osteoarthritis, Crohn's disease and inflammatory bowel conditions. Alteration in tissue concentration of amino acids is likely the result of gross tissue destruction or cellular apoptosis (Fitzpatrick & Young, 2013).

A study by Marchesi et al. (2007) of faecal matter from healthy participants and participants with Crohn's disease and ulcerative colitis found a significantly higher quantity of amino acids in participants with inflammatory bowel conditions. An increase in isoleucine concentration (+ 0.69) was observed in the faecal matter of patients with Crohn's when compared to control samples (Marchesi et al., 2007). Other amino acids showing increases included leucine, alanine, lysine and valine. A study by Zhai et al. (2010) of serum from patients with osteoarthritis found an increase in amino acids, in particular valine and leucine, in test patients compared to controls. Additionally Griffin et al. (2004) used an adenoviral vector to induce a focal inflammatory lesion in rats and identified an increase in urinary amino acids, including leucine, isoleucine and valine, in response to tissue inflammation.

Examples relating to bovine milk also exist in the literature. Sundekilde et al. (2013) profiled bovine milk from cows with mastitis to identify metabolites associated with increased somatic cell count. Again, isoleucine was positively correlated with an

increased somatic cell count and a significant difference was found between healthy cows and cows with mastitis ($p=0.014$). A preliminary bovine study by Ianni et al. (2015) profiled selective amino acids in milk from cows with sub-clinical and clinical mastitis to identify metabolite differences in milk from cows with varying levels of mastitis. This study found that amino acids, aspartic acid, isoleucine and valine were only detectable in the milk from cows with clinical mastitis and not in the sub clinical mastitis milk samples (Ianni et al., 2015). Consequently, it was proposed that these three potential biomarkers could be useful for the confident identification of clinical mastitis in cases that are difficult to diagnose (Ianni et al., 2015). Similarly the measured increase in amino acids in breast milk presented in this thesis may serve as an indication of inflammation and damage to mammary tissue including paracellular pathways.

Due to the small sample numbers resulting from technical problems and instrumental error, we are unable to conclude complete significance of these results. However, the conditions determined and the data provide promising direction for future metabolomics studies. Metabolomics offers opportunities for further refinement of these presented analyses and potential application for more complete human milk analysis if complementary techniques together with the GC-MS analyses presented here were to be combined for a more complete understanding of the milk metabolome.