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

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1 Literature Review

1.1 Introduction

1.1.1 Benefits of breastfeeding for mother and infant

Human milk is considered the 'gold standard' nutrition for infants as it provides optimal nutrition, promotes normal growth and development and reduces the risk of developing illness or disease (Heikkila & Saris, 2003). The WHO (2003) recommends exclusive breastfeeding for the first six months of age, with complementary breastfeeding up to two years and beyond. In Australia the National Health and Medical Research Council (NHMRC) Guidelines recommend exclusive breastfeeding for the first six months of age and the continuation of breastfeeding for up to at least one year (NHMRC, 2012). Where breastfeeding is not possible human donor milk is the preferred substitution ahead of infant formula (Hartmann, Pang, Keil, Hartmann & Simmer, 2007).

Breast milk is readily available, financially affordable and an environmentally sustainable source of sustenance for developing infants and contributes to numerous positive health outcomes for both the mother and infant. An American based study by Bartick and Reinhold (2010) found that if 90% of US families could comply with the medical recommendation to breastfeed exclusively for 6 months that the United States would save almost $13 billion a year and prevent an excess of 911 deaths annually, nearly all of whom would be infants (95%) (Table 1.1).

Table 1.1 Excess costs and deaths at current breastfeeding rates compared with projected costs if 90% or 80% of US parents complied with the medical recommendation to exclusively breastfeed for 6 months.

	Excess Costs Compared with 90% Compliance (Excess Deaths), 2007 $US	Excess Costs Compared with 80% Compliance (Excess Deaths), 2007 $US
Total	12 974 676 651 (911)	10 491 841 489 (741)
SIDS	4 725 328 464 (447)	3 722 074 013 (352)
NEC deaths	2 631 165 267 (249)	2 218 109 495 (210)
LRTI deaths	1 820 102 146 (172)	1 537 915 767 (146)
Otitis media	908 793 396	765 766 295
Atopic dermatitis	601 358 918	497 497 274
Childhood Obesity	592 100 121	404 195 504
LTRI hospitalisation	451 592 572	381 578 219
Childhood asthma	335 796 234	229 194 255
NEC	266 536 884	219 843 084
Childhood asthma deaths	216 869 872 (21)	148 022 294 (14)
Gastroenteritis	186 016 678	162 076 307
Childhood leukaemia deaths	133 422 239 (13)	133 422 239 (13)
Childhood T1D deaths	95 231 472 (9)	64 999 258 (6)
T1D	8 376 168	5 717 067
Childhood leukaemia	1 986 220	1 430 416

This table was reproduced from Bartick & Reinhold (2010)
LRTI- Lower respiratory track infection
NEC- Necrotizing enterocolitis
T1D- Type 1 diabetes

Breastfeeding has been linked to many immediate and lifelong benefits for the developing infant. Research has shown that an exclusively breastfed infant between the ages of 1-6 months consumes an average of 750-800ml over a 24 hour period (range 500-100 ml) (ABS, 2015). A study of nine year old children by McCrory and Layte (2012) found that children who were breastfed (>13 weeks) had a 38% reduced

risk of obesity and those who were breastfed for 26 weeks and beyond had a 51% risk reduction for obesity. Additional studies have found a dose-response effect on cognitive and neural development, where the duration of breastfeeding correlated positively with an increase in IQ (Michaelsen, Lauritzen, Mortensen & Jorgensen, 2003).

Functional nutrients such as human milk oligosaccharide (HMO) in human milk provide the microenvironment for gut protection and maturation (Newburg, 2005). Due to the immature nature of the infants gut at birth they are more susceptible to intestinal and systemic infections. The ingestion of breast milk, in particular colostrum, results in differentiation of immature to mature epithelia (with subclasses of enterocytes and lymphoid tissue) and active maturation of the infants own mucosal immune system for protection against infection and immune mediated disease (Walker, 2010).

Furthermore, research has found that breastfed infants are less susceptible to a range of serious illnesses and conditions such gastroenteritis, respiratory illness and otitis media (AIHW, 2009). Conversely, exclusive formula feeding presents numerous health risks to the developing infant including increased risk of allergies including eczema and atopic dermatitis, and (potential) ingestion of contaminants (Tait, 2000).

Breastfeeding has been found to provide many maternal benefits and research suggests a dose-response effect with breastfeeding and health risk (Godfrey & Lawrence, 2010). A history of lactation has been associated with a reduced risk of cancer including breast and ovarian and decreased incidence of type II diabetes (Ip,

Chung, Ramam, Trikalinos & Lau, 2009). The practice of breastfeeding enhances the mother-child bond and therefore positively correlated with a decrease in post-partum depression and associated with a decreased rate of neglect and abuse (Strathearn, Mamun, Najman & O'Callaghan 2009).

Numerous maternal benefits are associated with breastfeeding such as a decrease in maternal post-partum blood loss, more rapid involution of the uterus and a quicker return to pre-pregnancy body weight than mothers who don't breastfeed (American Academy of Pediatrics, 2012). In mothers with no history of gestational diabetes breastfeeding duration was found to decrease the risk of developing type II diabetes by 4-12% (Schwarz et al., 2010). Furthermore mothers who breastfeed are less likely to develop rheumatoid arthritis (Karlson, Mandl, Hankinson, & Grodstein, 2004), hypertension, hyperlipidaemia and cardiovascular disease including coronary heart disease (Godfrey & Lawrence, 2010).

Given the multitude of short and long term benefits of breastfeeding to the mother and infant, promotion of breastfeeding is of global importance given the increase in incidences of the diseases aforementioned, particularly in developing countries where breastfeeding rates have been falling.

1.1.2 Breastfeeding incidence and duration in Australia

In Australia and internationally breastfeeding has received increased attention as a means for improving public health, contributing to health, nutrition and wellbeing of infants and mothers. Benefits of breastfeeding are largely dose-dependent, therefore extending duration is highly desirable in terms of facilitating infant growth and

development and ensuring maximal maternal benefit. A national survey in 2001 found 87 % of infants aged up to three years of age had obtained nutrition from breast milk at some stage, in the form of exclusive breastfeeding or complementary breastfeeding with the addition of solids or substitutes (ABS, 2003). The incidence of breastfeeding post hospital discharge has increased from 40-45% in the 1970's to 82% and 83% in 1995 and 2001 respectively (ABS, 2003). Despite rising rates in initiation of breastfeeding, duration rates still dramatically decline despite recommended duration of one year by the NHRMC (2010) or up to two years by the WHO (2001).

Rates of duration of breastfeeding between 1995 and 2001 showed that 48% of mothers were still breastfeeding at 6 months, however this decreased to 23% at 1 year and only 1% of mothers were still breastfeeding 2 years after parturition (Figure 1.1) (NHRMC, 2003).

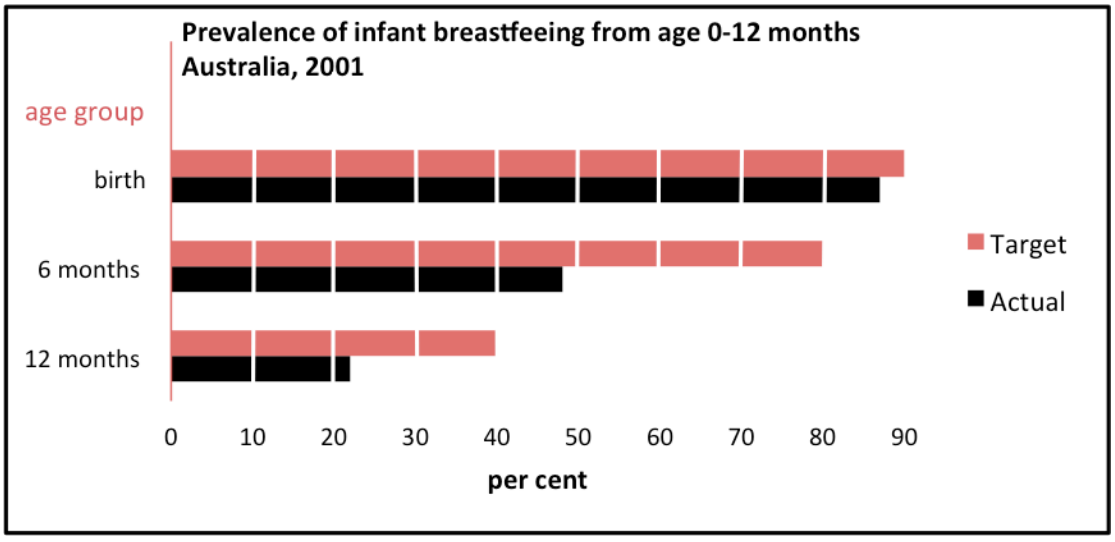

Figure 1.1 Prevalence of breastfeeding in Australia in 2001.
Reproduced from The National Health and Medical Research Council (2003).

More recent research suggests that the incidence and duration of breastfeeding in Perth, Western Australia, has increased significantly, in particular between 1992/3-

2002/3 where prevalence reached national targets for breastfeeding (>90% of mothers were breastfeeding at the time of discharge) (Graham, Scott, Binns & Oddy, 2005). In Perth 93% of mothers were breastfeeding at discharge from the hospital between 2002-2004 (Win, Binns, Zhao, Scott and Oddy, 2006). Additionally, the 2010 Australian National Infant Feeding Survey (NHMRC, 2010) found breastfeeding initiation rates have increase to 90-96% on discharge from hospital, with 50-60% and 22-28% still breastfeeding at 6 and 12 months respectively. Despite these initiation rates being higher than the national average in 2001, previously mentioned as 83%, and approaching those reported for Nordic countries (Lande et al., 2003; Ekstroem, Widstroem & Nissen, 2003), the increase in initiation doesn't appear to be accompanied with an increase in breastfeeding duration (Scott, Binns, Oddy& Graham, 2006). Breastfeeding rates dropped to 45.9 % (of whom 12 % were exclusively breastfeeding) by 6 months post partum and by one year only 19.2 % were reported to be breastfeeding (Scott et al., 2006).

Whilst hospital policy, promotion and support have increased breastfeeding initiation rates, breastfeeding duration has not substantially improved. This is likely due to the need to return to work and difficulties experienced by breastfeeding women such as perceived inadequate milk supply, nipple pain and mastitis which lead to premature weaning (Abou-Dakn, Richardt, Schaefer-Graf & Wockel, 2010).

1.2 Anatomy of the lactating breast

An understanding of the macroscopic and microscopic anatomy of the lactating breast can help us better understand the development of pathologies and their observed effects on the mammary tissue and its' secretions. Breast pathologies, for example

mastitis, can compromise the integrity of the breast, therefore changes in milk composition are considered to be an important indicator of the physiological state of the mammary tissue (McManaman & Neville, 2003). A fundamental knowledge of the anatomy of the breast can aid in diagnosis and treatment development.

1.2.1 Macroscopic anatomy

The human breast is comprised of two main tissues, adipose and glandular (secretory), held loosely together by a network of fibrous connective tissue called coopers ligaments (Ramsay, Kent, Hartmann, R. & Hartmann, P., 2005) (Figure 1.2*)*. It is important to note that there is large variation between women. Not only do the breasts of some women contain more adipose tissue, the amount of adipose tissue situated between glandular tissue is also highly variable (Geddes, 2007). In some cases the amount of glandular tissue was found to exceed adipose tissue by double (Geddes, 2007). A study by Ramsay et al. (2005) found no correlation with milk production or storage capacity and the estimated volume of secretory or glandular tissue (including the number of ducts and the mean diameter of the milk ducts) (Ramsay, et al. 2005).

The glandular tissue contains a secretory system, which is drained by a ductal system that stores and transports milk to the nipple during lactation. Based on the 1840's Cooper's dissections of the lactating breast (Cooper, 1840), it was previously believed that the lactating breast contained 15-20 ducts (however, Cooper identified up to 22 ducts in one instance), with 7-12 generally found to be patent. However, more recent studies using 2D ultrasound (Ramsay et al. 2005) found the glandular tissue of each breast to contain approximately 9 milk ducts (range 4-18) with a mean diameter of 2 ± 0.8 mm (range 1.0-4.4 mm) (Ramsay et al. 2005). Another study by Love and Barsky

(2004), using a combination of in vivo and in vitro techniques, resolved that 90 % of nipples contained 5–9 milk ducts (with a nipple orifice), arranged in a central and peripheral orientation.

Furthermore Cooper (1840) described the proximal ducts to be large sac like structures that converged into one main milk duct, known as a lactiferous sinus. However, recent studies using 2D ultrasound imaging (Ramsay al. 2005) and 3D ultrasound imaging (Gooding, Finlay, Shipley, Duck & Halliwell, 2010) found an absence of lactiferous sinuses and rather the main milk ducts to be relatively small with expanding areas coinciding with merging ducts. Furthermore the ducts have been found to dilate during milk ejection in order to transport milk towards the nipple for the suckling infant (Ramsay et al. 2005).

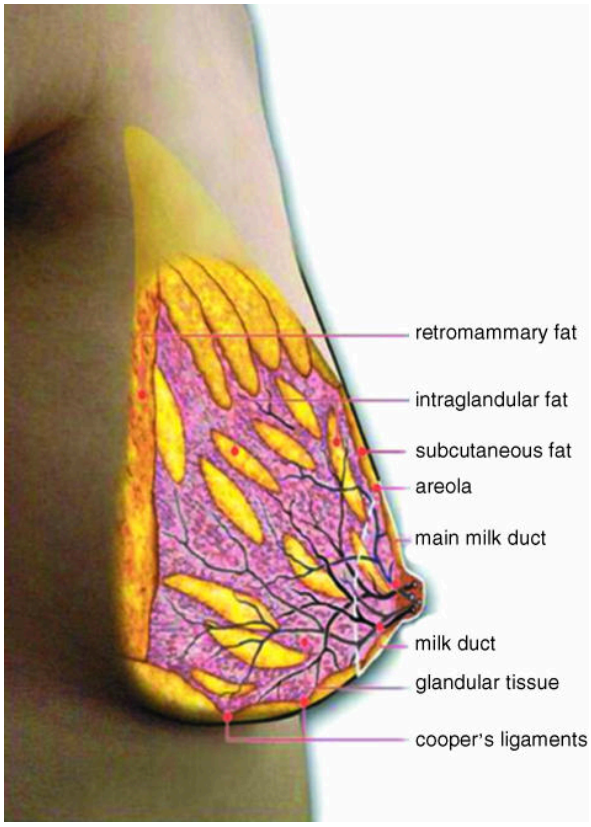

Figure 1.2 Macroscopic anatomy of the normal lactating mammary gland. *Reproduced from Ramsay et al. (2005).*

1.2.2 Microscopic anatomy

The mammary gland is comprised of lactocytes, ductal epithelial cells, myoepithelial cells and mammary stem cells (Berry, 2009). The lactocytes, secretory epithelial cells, line the alveoli of the breast and are responsible for producing and secreting milk into the luminal space of the alveoli (Berry, 2009). Lactocytes are cuboidal/columnar in shape and link with several specialised junctions, for example tight junctions, which prevent the passage of substances external to the alveolus during established lactation (Linzell & Peaker, 1971). The alveoli are surrounded by myoepithelial cells and a vascular connective tissue stroma that contains adipocytes and fibroblasts (McManaman & Neville, 2003). Myoepithelial cells function during milk ejection when suckling stimulus causes the release of oxytocin into the maternal circulation. Oxytocin binds to myoepithelial cells and causes them to contract (neuroendocrine reflex) thereby ejecting milk from the alveoli into the ducts towards the nipple to be removed by the infant or a breast pump (McManaman & Neville, 2003).

The cytoplasm of lactating alveolar cells is dense in mitochondria and there is an extensive rough endoplasmic reticulum network, as would be expected in a highly active secretory cell (McManaman & Neville, 2003). Additionally the cells contain a Golgi apparatus and secretory vesicles containing casein micelles located in the apical region of the cell (McManaman & Neville, 2003). The alveolar epithelial cells are connected through an apical junctional complex and the epithelial cells on the basal side of the alveolar contact the myoepithelial cells and basement membrane. This forms a separation between the epithelial cells and the stroma and vascular system, creating a barrier for the transfer of substances from blood or stromal cells to the milk.

1.3 Physiology of lactation

The prepubescent breast consists of a basic network of immature ducts, formed by epithelial cells, submerged in the mammary fat pad (Thomas, Williams and Hartmann, 2010). At puberty an increase in ovarian hormones stimulates the ducts to branch out and extend from the nipple and pervade the surrounding fat pad creating a complex ductal network (Thomas et al., 2010). However, the human breast does not reach maximal growth and functional development until pregnancy and parturition (Hale & Hartmann, 2007). The ability to secrete milk develops during pregnancy and is regulated by changes in multiple hormones.

Lactation is defined as the stage of sustained milk production (Pang & Hartmann, 2007). The initiation of lactation occurs in two stages, secretory differentiation and secretory activation (Pang & Hartmann, 2007). Secretory differentiation describes the stage of pregnancy where buds on the end of each duct proliferate and then differentiate to form alveoli lined with functional lactocytes capable of milk synthesis (Hale and Hartmann, 2007). Secretory activation describes the onset of copious milk secretion in association with changes in milk composition as a result of progesterone decline and increase in prolactin secretion at parturition (Pang & Hartmann, 2007). It is essential that secretory activation closely precede parturition to ensure the newborn has a continuous source of nourishment.

1.3.1 Secretory differentiation

Secretory differentiation usually occurs in the later stages of pregnancy at around 24 weeks gestation and is characterised by rapid growth of both the ductal and alveolar structures accompanied by accumulation of the first secretion (colostrum) within the

alveoli and ducts (Hassiotou & Geddes, 2013). Secretory mammary epithelial cells differentiate into lactocytes, functional mammary epithelial cells, with the ability to synthesize unique milk metabolites such as lactose, casein, alpha-lactalbumin and fatty acids (Pang & Hartmann, 2007). Milk synthesis describes the anabolic process leading to the accumulation of milk components in the lactocytes (Pang & Hartmann, 2007).

Alveolar development occurs during the early stages of pregnancy and can be described as the proliferative activity that leads to the development of the mature milk secreting unit (Pang & Hartmann, 2007). Reproductive hormones; oestrogen, progesterone and prolactin together with metabolic hormone's growth hormone, glucocorticoids and insulin must be present for secretory differentiation to occur (Pang & Hartmann, 2007).

1.3.2 Secretory activation

Secretory activation occurs shortly after parturition in women, and it is defined as the onset of copious milk production and marks the commencement of milk secretion (Jensen, 1995). Clinical signs of secretory activation are an abrupt sensation of breast engorgement occurring between 24-72 hours post parturition (Arthur, Smith & Hartmann, 1989). Progesterone withdrawal at parturition, due to the expulsion of the placenta, initiates secretory activation, however the hormones prolactin, insulin and cortisol must also be present (Pang & Hartmann, 2007). Blood prolactin levels are high during early lactation, shown to stimulate milk synthesis and proliferation (Neville et al., 2002). This results in accumulation of milk in the alveoli followed by copious milk secretion.

Post parturition the basement membrane (separating the mammary stroma from the epithelium) experiences a change in integrity characterised by tightening and reduced permeability, resulting in the control of systemic and stromal signalling to the mammary epithelium (Hassiotou & Geddes, 2013). This serves to control the movement of milk components or their precursors via paracellular pathways between the systemic circulation lactocytes and alveolar lumen or the lactocytes (Hassiotou & Geddes, 2013).

1.4 Secretory pathways

Secretory alveoli are enclosed by a basement membrane separating them from the surrounding stroma (Thomas et al., 2010). The basement membrane is important in regulating the activity of the alveolar cells and the components that can pass from the mother's bloodstream or interstitial fluid into the milk (Thomas et al., 2010). There are a number of potential barriers that control the transfer of exogenous substances from the blood or stromal cells to the milk. Metabolites both endogenous and exogenous to the mother can enter the milk via means of transcellular and paracellular pathways.

1.4.1 Transcellular pathways

The transcellular pathway allows movement across the lactocytes and is the mode of movement employed by fat globules, ions (e.g. calcium), glucose, protein hormones, immunoglobulins, and water (Pang and Hartmann 2007).

The transcellular pathway can be divided into four general mechanisms of movement. Two exist for the secretion of endogenously generated molecules, aqueous solutes

including proteins and oligosaccharides and nutrients such as lactose, citrate, phosphate and calcium, and two exist for the transport of exogenous molecules, including numerous macromolecules derived from serum and stromal cells and many ions and small metabolites (McManaman & Neville, 2003).

1.4.2 Paracellular pathway

The paracellular pathway allows direct, bi-directional and extracellular movement of low molecular weight substances and macromolecular solutes from the serum or interstitial space into the milk (McManaman & Neville, 2003). The paracellular pathway becomes closed during lactation as a result of tight junctions between epithelial cells, at which point transcellular pathways act as the only route for transfer of solutes to milk (McManaman & Neville, 2003). The transport of metabolites through this pathway is largely affected by the functional capacity of the mammary gland and can be a direct indication of the physiological state of the lactating breast. Inflammation resulting from mastitis can cause the paracellular pathway to reopen, allowing small molecules including sodium, chloride and glucose to pass freely into the milk space, while molecules such as lactose, potassium and calcium pass from the milk space into the plasma (Jensen, 1995).

1.5 Pathology of the human breast

Breastfeeding is the preferred source of nutrition for all newborns however it is not always an option for all women, as some women experience physiological, psychological or clinical difficulties that prevent them breastfeeding either temporarily or for an extend period of time. A West Australian study of 306

breastfeeding women by Fetherston (1997) found that mastitis is the third most common reason for weaning, with one in four women citing mastitis as their reason for ceasing breastfeeding (Michie, Lockie & Lynn, 2003). More recently a study by Abou-Dakn et al. (2010) found that the most common reason for premature cessation of breastfeeding in early lactation, affecting up to 50% of mothers, is nipple pain and mastitis. In weeks 1-3 insufficient milk supply (37.3%), commonly due to mastitis (Abou-Dakn et al., 2010), followed by breast pain or mastitis (32.9%) was the most common reasons for cessation of breastfeeding (Schwatrz, 2002). Women who reported pain in the first three weeks of breastfeeding were more likely to cease breastfeeding than mothers who reported pain beyond three weeks (Schwartz et al., 2002).

The study by Schwartz et al. (2002) also found that women who developed mastitis in the first three weeks post partum were nearly six times more likely to cease breastfeeding than women not suffering from mastitis. Furthermore with every day of pain in the first three weeks there was an increased risk of 10-25% for termination of breastfeeding (Schwartz et al. 2002). Thus mastitis, pain and days with pain in the first three weeks post partum are important clinical factors associated with breastfeeding termination in mothers who prenatally identified themselves as mothers who intended to breastfeed (Schwartz et al., 2002).

Mastitis or, inflammation of the mammary tissue, is a debilitating disease that largely contributes significantly to weaning in the first three weeks post partum. Factors associated with mastitis include pain and discomfort when breastfeeding, ineffective

milk removal, reduced milk flow and the inability to provide sufficient nutrition for the growing infant (Foxman, D'Arcy, Gillespsie, Bobo & Schwartz, 2002).

1.5.1 Nipple pain

Nipple pain, with or without trauma, is a complication associated with breastfeeding found to have a significant impact on breastfeeding in the first few weeks post partum. The incidence is reported to range from 34% up to 96%, with the highest prevalence on day 3 and decreasing by day 7 (Page, Lockwood & Guest, 2009). Incorrect positioning and attachment has been implicated as the major cause of nipple pain, with speculation that increases in suction pressure applied by the infant may be a cause of pain in some women (McClellan et al., 2008); vasospasm, tongue tie and eczema are less common causes of nipple pain. Nipple infection accounts for a proportion of the cases of nipple pain and is thought to be a consequence of nipple trauma. Determining the cause of nipple pain is often difficult, for example severe pain combined with whitish changes of the nipple is often misdiagnosed as *Candida spp*., resulting in many breastfeeding women receiving incorrect treatment (Holmen & Bache, 2009). The involvement of bacteria in nipple pain is still largely unknown, however a study by Eglash, Plane & Mundt (2006) stated that women with nipple pain without symptoms of acute mastitis were 3 times more likely to culture pathogenic bacteria, most commonly *Staphylococcus aureus*, than candidiasis.

Most lactation consultants agree that nipple soreness in the first week post partum is quite normal, however nipple pain that exceeds the first week is normally a sign of a greater problem that requires skilled assessment and observation (Tait, 2000). Associated with both frictional and suction lesions, pain can range from an

uncomfortable feeling to severe pain possibly preventing the continuation of breastfeeding (Page et al., 2009). As a result, as many as one third of mothers who experience these complications may change to alternate methods of infant nutrition in the first six weeks after birth (Page et al., 2009).

1.5.2 Mastitis

Mastitis and breast abscess occurs in all populations and at any stage of lactation. WHO (2000) reported the incidence of mastitis to affect ~20% of all lactating women, with 74-95% of cases occurring in early lactation (first 12 weeks after birth). Mastitis can be defined as cellulitis of the interlobular connective tissue within the mammary gland of the breast (Foxman et al., 2002). Clinical symptoms range from focal inflammation with minimal systemic symptoms to abscess and septicaemia in more severe cases (Foxman et al., 2002). Systemic symptoms such as pyrexia and flu like symptoms are often sudden in their onset and vary in severity, with women reporting duration of symptoms ranging from one to 12 days (Fetherston, 2001). The affected breast may appear red, hot and swollen. Factors associated with mastitis also include pain, discomfort when breast feeding and poor drainage (reduced milk removal) which may cause some women to cease breastfeeding (Foxman et al., 2002).

Past research has determined that mastitis is most frequently the result of stasis of milk, without significant deviation in 'normal/healthy' bacterial counts and species. On occasions where milk stasis is not the cause of mastitis, milk infection is often the cause of bacterial colonisation of the breast and can be detected through increased colony counts and predominance of a small number of bacterial clones (Michie et al., 2003). Consequently, mastitis is frequently defined as infectious or non-infectious. The most common type of mastitis is non-infectious mastitis, where inflammation of

the breast tissue results from milk stasis, blocked ducts, engorgement or physical injury (Crepinsek, Crowe, Michener & Smart, 2012).

Infectious mastitis may result from trauma to the skin of the nipple, damaging the integrity of the breast and consequently providing a route for microbial infection (Crepinsek et al., 2012). The most common portal of entry for bacterial infection in women with mastitis is assumed to be through nipple pores into lactiferous ducts (Fetherston, 2001). Infectious mastitis is most often associated with *Staphylococcus aureus*, an organism that can cause an abscess to develop if left untreated (Amir, Forster, McLachlan & Lumley, 2004). A study by Delgado et al. (2009) found that *Staphylococcus epidermidis* was the most prevalent staphylococcus species isolated from mastitic milk and was prevalent in concentrations significantly higher than that normally present in the healthy mother. *S. epidermidis* has been increasingly recognised as an opportunistic pathogen and as a casual pathogen of mastitis, despite being a normal inhabitant of healthy human skin and mucosal microflora (Delgado et al., 2009). *Staphylococci* are known for their pronounced genetic variability and *S. epidermidis* has been found to have mechanisms for adhesion and biofilm formation. Its resistance to certain antibiotics has increased in recent years and it is consequently emerging as a common nosocomial pathogen (Ziebuhr et al., 2006).

Alternatively mastitis can be viewed as a continuum of a disease where the initial non-infectious mastitis develops into a secondary infectious mastitis resulting in the formation of an abscess (Crepinsek et al., 2012). Hence infection, when it occurs, is not primary, but the result of stagnant milk providing a medium for bacterial growth.

Although effective milk removal through feeding, pumping or both is the foundation for all treatment to remove stagnant milk, antibiotics are usually prescribed prophylactically to cover possible bacterial infection (Jahanfar, Ng & Tang, 2013). However, antibiotic prescription is not based on analysis of breast milk, therefore it is not known how many cases are unnecessarily adding to the increase in antibiotic resistant strains of bacteria. To reduce the number of bacteria becoming resistant to antibiotics it is important to correctly diagnose each case of mastitis to reduce their inappropriate use. Thomsen, Hansen & Moller (1983) proposed that levels greater than 10^3 CFU/ml of pathogenic bacteria in breast milk was an indication that antibiotic treatment is required. They concluded that a high bacterial count together with leukocytosis was indicative of infection. Note that the colony forming unit (CFU) count does not take into account the normal bacterial content in milk.

1.5.3 Breast thrush

Some breastfeeding mothers also experience a burning pain in the nipple/breast known as breast thrush, which occurs in 10% of women. Although the exact cause of breast thrush has not yet been confirmed, many researchers believe it is the result of *Candida albicans* infection (Amir et al., 2011). However, due to the presence of other microorganisms it is difficult to identify *C. albicans* as the sole cause. Consequently, it is possible that breast thrush is the result of co-infection caused by the presence of multiple microorganisms such as *S. aureus* or *E. coli* as well as *C. albicans* or other *Candida spp*.

Australian milk banks currently examine the bacteriology of donor milk by culturing all donations on cysteine-lactose-electrolyte deficient (CLED) agar and 5% horse blood agar and quantifying colony growth (Hartmann et al., 2007). Critical limits have been defined for the level of contamination acceptable in raw milk and donations containing a confluent growth of microorganisms exceeding 10^5 CFU/ml are discarded (Hartmann et al., 2007). However, this is not routine practice for the diagnosis of mastitis or causative agents of nipple pain, nor is it conducted before the administration of antibiotics or alternative medications.

1.6 Composition of human milk

Milk is a complex biological matrix made up of thousands of compounds. The complexity of milk reflects the activities of the mammary secretion and transport processes, the physiological condition of the breast and the unique nutritional requirements of the developing newborn (McManaman & Neville, 2003). The constituents in milk provide nutrition, structural components for cellular membranes and non-nutritive functional components e.g. immunological factors (Jensen, 1995).

The composition of human milk is dynamic and highly variable. Variation occurs over the course of lactation, between and within feeds, diurnally, between mothers and with treatment of expressed milk including storage and pasteurisation (Chung, 2014). The mother's nutrition, body mass index (BMI) and parity have also been found to influence milk composition (Hsu et al., 2014). A study by Eilers et al. (2011) found a positive correlation with milk leptin concentration and BMI, suggesting that mothers' adiposity may increase the leptin levels in milk.

Milk composition changes over the course of lactation, which can be divided into the known milk stages colostrum, transitional milk and mature milk. Colostrum marks the first phase of lactation spanning the first 3-5 days after parturition. Colostrum has a distinct biochemical and cellular composition, characterised by high concentrations of protein, fat-soluble vitamins, minerals, and immunoglobulin, designed to provide enhanced immunological protection and nutritional and developmental support to the infant (Hassiotou & Geddes, 2013).

Transitional milk proceeds the colostrum stage lasting up until 2-3 weeks postpartum and has higher levels of fat, lactose and water soluble vitamins and contains more calories than colostrum (Jensen, 1995) (Table 1.2). Thereafter, breast milk is said to have reached the mature phase, the final stage of milk transition, which is maintained for the remainder of lactation (Hassiotou & Geddes, 2013). Mature milk is comprised of 90% water and 10 % carbohydrates, proteins and fats.

Table 1. 2 Human milk composition between 1 and 28 days post partum.

Component	Days post partum						
	1	**2**	**3**	**4**	**5**	**14**	**28**
Yield g/24 hr	50	190	400	625	700	1100	1250
Lactose (g/L)	20	25	31	32	33	35	35
Fat (g/L)	12	15	20	25	24	23	29
Protein (g/L)	32	17	12	11	11	8	9

Recreated from Jensen (1995). Handbook of Milk Composition.
The volume of milk, lactose and fat increase and protein decreases as lactation progresses days post partum.

1.6.1 Macronutrients and micronutrients

Milk is a highly complex suspension of lipids, proteins, carbohydrates, secretory immunoglobulins, calcium and various other macro and micro molecules, ions and bioactive factors (Thomas et al., 2010). Table 1.3 is a summary of macronutrient composition of human milk findings from past studies. Fat content (grams per 100ml) can be identified as the most variable nutrient across populations, however individuals within a population showed equal if not greater variations (Prentice, 1995; Wojcik, Rechtman, Lee, Montoya & Medo, 2009).

Table 1.3 Summary of macronutrient composition from past studies.

Population	Fat	Lactose	Protein	Reference
Philippines (Manila)	3.93	7.31	0.85	WHO, 1985
The Gambia	3.78	7.74	1.09	Prentice et al., 1981a
Australia	3.74	6.14	0.92	Mitoulas et al., 2002
Bangladesh	2.66	8.08	1.00	Brown et al., 1986
Sweden	5.69	6.70	0.83	WHO, 1985
Guatemala	2.40	8.00	0.94	WHO, 1985
Zaire	3.30	6.30	1.30	WHO, 1985
USA (DARLING)	3.80	7.40	1.10	Nommsen et al., 1991
Mean	**3.66**	**7.21**	**1.00**	

Reproduced from 'Predictors of breast milk macronutrient composition in Filipino mothers', Quinn, Largado, Power & Kuzawa (2012).
Contents recorded in grams per 100ml and calculated where necessary using a nitrogen to protein conversion factor of 6.38.
The summary of milk macronutrients from past studies found that fat content of human milk varied between 2.4 to 5.69 g/100ml across populations, however women within the same population showed equal if not greater variation than different populations. Carbohydrate and protein content of milk showed less variance, with carbohydrate ranging from 6.14 to 8.08 g/100ml and protein ranged from 0.83 to 1.03 g/100ml.

During the initiation of secretory activation the paracellular pathway closes, preventing movement of small molecules from serum or interstitial space into the milk and vice versa. The closure of tight junctions blocks the paracellular pathway preventing lactose (made by the epithelial cells) from passing from the alveolus to the plasma and sodium and chloride from entering the alveolar lumen from the interstitial space (McManaman & Neville, 2003). This resulted in a fall in sodium and chloride concentration and an increase in lactose concentrations in milk (Table 1.4), which occurs immediately after birth and is complete by 72 hours post delivery (McManaman & Neville, 2003).

Table 1.4 Changes in selected milk components in early lactation.

Component (mmol/L)	Hours post partum				
	21	**48**	**60**	**96**	**120**
Volume (ml/day)	-	180	350	560	540
Lactose	100	140	160	160	160
Potassium	13.8	15	18	18	18
Sodium	34	25	16	14	14
Chloride	44	35	25	20	20
Calcium	4.0	6.0	6.6	7.6	8

Recreated from Jensen (1995). Handbook of Milk Composition.
The volume of milk and amount of lactose, potassium and calcium increase and sodium and chloride decrease as lactation progresses hours post partum.

Epithelial cells are connected via an apical junctional complex composed of adherens and tight junctional elements that act to prevent direct paracellular exchange of interstitial and milk components (McManaman & Neville, 2003). However, during an episode of mastitis or inflammation the tight epithelial junctions dividing milk and plasma become compromised and the paracellular route reopens, causing plasma components such as sodium and chloride to leak into the milk (McManaman &

Neville, 2003) and components such as lactose and potassium to pass from the milk into the plasma (Jensen, 1995). An elevated milk sodium concentration above the norm (5-6mmol/L) has previously been considered indicative of infection. However, as milk composition varies largely between individuals, it is difficult to determine if sodium concentration levels are an accurate measure of infection or inflammation and consequent damage to the mammary tissue. For this reason, the sodium to potassium ratio has recently been suggested as a possible indication of infection or inflammation as it accounts for individual differences, with a elevated sodium to potassium ratio above 1.0 being considered indicative of mastitis (Aryeetey, Marquis, Timms, Lartey & Brakohipa, 2008).

1.7 New methods of milk analysis

There have been many studies in recent years on bovine mastitis, described as a production disease, as it is the most expensive disease effecting dairy farms world wide, causing enormous financial loss to the dairy industry (Hogeveen, Huijps & Lam, 2011). Metabolomics has been utilized increasingly within the food industry and has been proposed as a useful tool in the dairy industry to ensure proper milk composition and milk of the highest quality (Boudonck, Mitchell, Wulff & Ryals, 2009). Previously diagnosis of mastitis in domesticated animals focused predominantly on quantitative measures, most commonly monitoring milk somatic cell count, which is known to increase during an episode of mastitis (Michie et al., 2003). Estimates of the milk cell count are widely employed to assess milk quality, with lower cell counts attracting higher prices. More recently, a study by Sundekilde et al. (2013) found a series of metabolite biomarkers, including isoleucine, lactate, butyrate and acetate, that were associated with elevated somatic cell count in bovine

milk and suggested that detection of these could be a potential tool to determine milk quality, diagnose mastitis and consequently determine whether milk should be discarded. This knowledge and technology could also be applied to human milk, determining metabolome changes as a means for diagnosis of breast complications particularly mastitis. Furthermore, it can be reasoned that if bacterial and fungal infections are a causative agents of nipple trauma and mastitis, their presence and associated endogenous metabolites will contribute to the composition of expressed milk. Consequently, if severity of trauma is correlated to underlying infection, then profiling the metabolome of expressed milk may identify differences in metabolite composition between mothers experiencing varying degrees of pain and discomfort.

1.7.1 Metabolomics

Metabolomics revolves around the central concept that an individual's metabolic state is a close representation of their current physiological state indicating their health or disease status (Fanos, Barberini, Antonucci & Atzori, 2012). Our metabolome is not solely determined by our genes but also influenced by our environment and unique body flora and therefore consists of a mix of endogenous and exogenous metabolites, some of which may include food component or environmental chemicals. Metabolomics aims to improve understanding of physiology and metabolism by using analytical chemistry techniques to assess metabolic changes in biofluids, tissues and cell extracts to create a metabolic profile (Veselkov et al., 2011).

Metabolomics aims at a quantitative analysis of a large number of low molecular weight metabolites existing as substrates or products in metabolic pathways present in all living systems (Moco, Collino, Rezzi & Martin, 2013). The metabolomics

approach is based on highly sensitive analytical methods with data obtained by quantifying multiple metabolites or small molecules in test samples (Fanos et al., 2012). A typical metabolomic data matrix consists of metabolites and their relative abundances for a sample set including two or more conditions (control and study group/s). The direction of statistical analysis is to identify differences in presence and abundance of metabolites between control and study groups (De Livera et a., 2012). Current techniques commonly used in metabolomic analysis include mass spectrometry coupled with gas chromatography (GC-MS) or liquid chromatography (LC-MS) and nuclear magnetic resonance (NMR) (Fanos et al., 2012).

1.7.2 Gas chromatography mass spectrometry (GC-MS)

GC-MS is a synergistic combination of two techniques, firstly gas chromatography which separates the components of a mixture of molecules and the second, the mass spectrometer which provides structural information of each component measured (Kitson, Larsen & McEwen, 1996). In GC-MS-based metabolomics, complex mixtures of metabolites from a cell, tissue or biofluid are analysed.

Gas chromatography involves volatilization of the sample in a heated inlet, separation of the components of the mixture in a capillary column and detection of each component at the detector (Figure 1.3). A carrier gas (mobile phase) is used to transfer the volatilised sample from injector through the column where separation of each analyte is determined by the partition of each component between the mobile and stationary phase. Only materials that can be volatilised without decomposition are suitable for analysis by gas chromatography.

Within the mass spectrometer analytes are ionised and measured as a function of their mass to charge ratio and represented as a mass spectrum of ions each in relative abundance, which provides a quantitative measure of the abundance of each ionic species as it elutes from the column (Hubschamann, 2008). The measurements are calibrated against ions of known mass to charge ratio and compared to a database of known metabolites to determine presence and abundance of metabolites in a sample (Hubschamann, 2008).

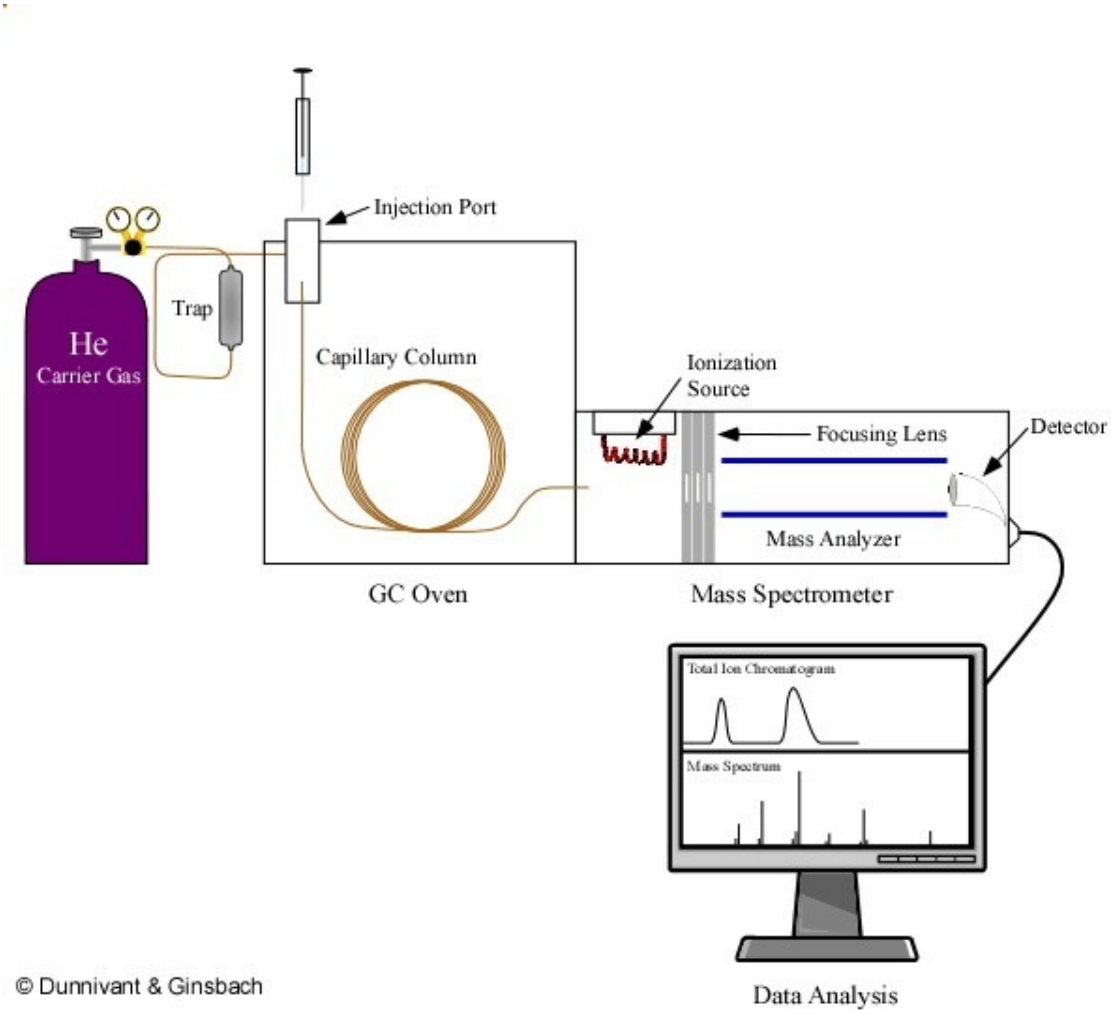

Figure 1.3 A simplified model of a gas chromatograph mass spectrometer.
Reproduced from Dunnivant and Ginsbach, 2008.

Interrogation of the GC-MS data requires deconvolution of the chromatogram to distinguish metabolites from non-biological analytes (e.g. artefacts from storage conditions) and to identify these from instrument noise and co-eluting analytes. GC retention times and calculated retention indices together with reproducible and predictable mass fragmentation (for comparison of the features with analysed metabolite standards) are used to identify metabolites by composition, against a library of metabolite mass spectra (Gummer et al., 2012). When used for metabolomics studies, the use of appropriate quality control measures, including internal standards to account for sample extraction and instrumental inter-sample efficiencies and system equilibration, is imperative (Gummer et al., 2012).

1.7.3 Untargeted metabolomics

Metabolomics is a reflection of genetic factors with the expressed metabolites defined as the end point. Mapping a person's metabolome against their phenotype has been proposed as a useful tool for clinical systems biology to detect metabolic changes even before disease symptoms appear (Smolinska, Blanchet, Buydens & Wijmenga, 2012). Blood and urine samples are frequently used to anlyse the human metabolome. Milk is an ideal bio-fluid for metabolomics studies since it can be obtained noninvasively, and the composition is directly reflective of genetic and environmental factors affecting breast health, more specifically mammary tissue and the milk secreting cells (lactocytes). With respect to lactation, nipple pain and mastitis have been found to produce biochemical changes in human milk including an increase in sodium and protein concentrations. As a result of cellular changes such as increased neutrophil count and activation of leukocytes causing them to extravasate into the milk at the site of inflammation (Michie et al., 2003; Hassiotou et al., 2013).

1.7.4 Targeted metabolomics

Bacterial and fungal metabolites can be used to detect and quantify bacteria and fungi in a solution. Furthermore metabolites only present in specific species of bacteria could allow more specific detection of target bacteria. GC-MS has successfully been used to identify biomarkers in complex matrixes (e.g. blood and urine) and provides high sensitivity and detection of markers even when present at nanogram levels (Sebastian & Larsson, 2003).

1.7.5 Summary

Research has already begun to identify the milk metabolome, however these studies lack focus on nipple pain and mastitis and their causative agents. There has been no untargeted analysis of metabolites linked to mastitis that may be present in the milk during a mastitis event. Identification of changes to the metabolome in the presence of nipple pain or mastitis, if established, could be a useful tool in clinical diagnosis and determining the underlying problem. Therefore, metabolite profiling has the potential to provide a diagnostic tool for the early identification of inflammatory processes contributing to nipple pain and mastitis. Targeted metabolomics could be used to identify the presence of specific metabolites, such as bacterial metabolites, to determine the influence of bacteria and identify the presence of bacteria as a possible cause of nipple pain and mastitis. This research provides an investigative model for the current work in the human metabolome and provides direction for more specific analysis following the optimisation of untargeted metabolite profiling.

1.8 Aims and Hypothesis

1.8.1 Project outline

This project aims to use current knowledge of human and bovine milk together with traditional microbial and biochemical methods to identify where new generation methods can be useful to gain further knowledge into breast milk composition and the effect of infection and inflammation. Metabolomics using gas chromatography mass spectrometry (GCMS) will allow metabolite profiling of human and bovine milk samples and the identification of possible biomarkers for nipple pain that could potentially develop into mastitis.

1.8.2 Hypothesis

The presence of persistent nipple pain, with and without evidence of trauma, in lactating women will result in changes in human milk metabolite profile due to infection and inflammation compared to asymptomatic women (controls).

1.8.3 Aims

Aim 1: To identify the presence of bacteria and fungi using conventional culture techniques, and to quantify bacteria and fungi detected.

Aim 2: To measure the effects of nipple pain and trauma on the paracellular pathway of the breast by measurement of the sodium and potassium concentration and ratio in human and bovine milk.

Aim 3: To optimise methodology for GC-MS (untargeted) measurement of metabolites in human milk and bovine milk.

Aim 4: To identify differences in the metabolic profile of human milk in mothers with nipple pain (with and without trauma) compared to healthy control mothers (asymptomatic), using untargeted analysis.