

LACTATION

*Natural Processes,
Physiological Responses and
Role in Maternity*

*Lisa M. Reyes Cruz
Douglas C. Ortiz Gutierrez*
Editors

OBSTETRICS AND GYNECOLOGY ADVANCES

LACTATION

**NATURAL PROCESSES,
PHYSIOLOGICAL RESPONSES
AND ROLE IN MATERNITY**

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LISA M. REYES CRUZ
AND
DOUGLAS C. ORTIZ GUTIERREZ
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Preface

The primary function of the mammary gland is to provide nutrition for the young in the form of milk protein and fat. However, there are other benefits that are provided by lactation, such as the provision of immune factors that are secreted into the milk, which provide protection from infection. In this book, the authors discuss the natural processes, physiological response and role in maternity of lactation. Topics include gene expression in the mammary gland; human milk oligosaccharides and their health effects; the role of micro-RNAs in the lactation process; hormonal control of lactation, the role of breastfeeding in lactation; and Wnt signaling and ultrasound in lactation.

Chapter I - Nutrigenomics is the study of the interaction of dietary factors with genes, examining the influence that certain nutrients have on their expression and therefore may either contribute to health (along with other environmental factors), for example through a healthy balanced diet, or determine the onset of certain diseases (eg obesity). Nutrients modulate molecular processes involving DNA structure, gene expression and metabolism, and these in turn may affect the organism's development. In this context, breast milk, which constitutes the first food, has an essential role not only in promoting children's healthy growth, but also in preventing adult diseases, such as obesity.

This review focuses on the potential modulation of genes by dietary food compounds, devoting particular attention to gene expression in the mammary gland. In addition the knowledge on milk composition from a nutrigenomics perspective and the likely health-outcomes will be analysed, in particular those associated with energy metabolism and body weight control that could be related to changes in milk composition. Finally, the role of milk leptin as a key factor in epigenetics and developmental plasticity is evaluated. Leptin would represent an essential nutrient during lactation in the protection against obesity and its metabolic-related disorders in later life.

Chapter II - Human milk contains high concentrations of all the nutrients required for infant growth and development. The essential proteins, fats, lactose, vitamins and minerals are present in milk in amounts optimal for the growth and development of the neonate. Additionally, human milk contains a broad range of nonessential components with clear health benefits for the newborn. The most remarkable beneficial components in human milk are free oligosaccharides, which comprise the third most abundant component. This abundance is extraordinary because these molecules are not digestible by the newborn's gastrointestinal system, yet they have been conserved and amplified in human milk during evolution. Oligosaccharides in milk actively influence the bacterial species that colonize the

infant's gut. These molecules support the growth of selected bifidobacteria within the intestine, thus achieving a protective microbiota dominated by beneficial bacteria (*prebiotic activity*). Milk oligosaccharides also possess anti-infective and anti-toxic activities as they inhibit pathogens from binding to intestinal cells. This inhibition occurs because some human milk oligosaccharides have functional residues in common with the glycan structures found on the gut mucosa. These glycan structures regulate cell-to-cell communication, and they are also used by enteric pathogens to bind to the mucosa and cause infection. Another therapeutic activity of sialylated oligosaccharides is their contribution to the cognitive development of the infant, as sialic acid is an essential nutrient for brain development and is used for sialylation of brain gangliosides. Since human milk oligosaccharides show extreme diversity in structure and may contain various functional groups, they also exert different bioactivities. The biotherapeutic properties of human milk oligosaccharides, in particular those of fucosylated and sialylated oligosaccharides, are discussed as well as the analytical methods used to determine their composition and identify the potential for their commercial translation.

Chapter III - Micro-RNAs (miRNAs) are small RNA molecules known to participate in important regulatory mechanisms through the targeting of mRNAs by sequence specific interactions, leading to specific inhibition of gene expression. Ongoing studies have revealed the role of miRNAs in the regulation of mammary gland development but a role in lactation is not yet completely clear. Recently, the identification of significant quantities of selective miRNAs in the milk of a number of mammals, together with the recent characterisation of plant food miRNAs in the blood of people, have precipitated an investigation of the potential role of miRNAs in the regulation of the lactation process. This investigation should include both the process of milk production by the mother and the post-partum development of the young. In order to examine the role of milk miRNAs in the lactation process, the Authors propose a comparative framework for the analysis of lactation. The authors review mammalian lactation diversity and animal models of lactation and recent literature on milk miRNA. They also perform comparative and functional analysis of milk miRNAs and, discuss the function of milk miRNAs as informative markers of both lactation status and maternal physiology, as well as information carrying signals facilitating the timely delivery of maternal development signals to the young.

Chapter IV - Lactation is of paramount importance to the survival, development, and growth of mammalian species. The mammary gland development starts during fetal life. The ductal development is particularly associated with puberty, and the alveolar development is associated with proliferative activity in the luteal phase of the reproductive cycle and in the early stages of pregnancy that leads to formation of the milk secreting unit. Parturition and lactation are two processes that are closely coordinated. Profound changes in several key hormones, such as progesterone, estrogen, prolactin, cortisol, placental lactogen, and insulin, occur early in pregnancy and parturition. These prepare and assure milk production by the mammary gland after delivery. For milk ejection, a neuro-hormonal reflex leading to the contraction of the myoepithelial cells surrounding the alveoli is stimulated by the action of the oxytocin. After ceasing the stimulus for lactation, involution by apoptosis leads to regression of the mammary gland to the quiescent non-lactating state. Besides these physiological responses, the hormones can also influence cognitive functions and maternal behavior. Lactation is also important for establishing an affective linkage between mother and baby. This review will focus on natural processes of mammary gland development, on the hormonal control of lactation and on the role of breastfeeding in maternity.

Chapter V - The mammary gland is unique in that most of its development and dynamic morphogenesis occurs postnatally in response to changes in the hormonal milieu. Multiple components of the Wnt/ β -catenin signaling pathway have been implicated in mouse mammary gland development. It is clear that the Wnt proteins themselves are important regulators of numerous stages throughout postnatal mammary gland development. Mouse models have been developed to analyze whether β -catenin stabilization, expression of pathway components, or expression of Wnt/ β -catenin target genes is sufficient to disrupt mammary gland development and lactation. In addition to the role of β -catenin in the Wnt signaling pathway, it is also a major component of the adherens junctions and as such, has a role in maintaining epithelial integrity, which is essential for lactation. Multiple components of the Wnt pathway are known to have cross-regulation with other signaling pathways involved in the lactogenic phenotype. Furthermore, investigation into the regulation of Wnt pathway components has demonstrated that hormones, such as progesterone, can regulate components of the Wnt pathway. Through various mouse models of mammary gland development, it has become clear that the Wnt/ β -catenin signaling pathway is a critical regulator of normal mammary gland development. Interestingly, the specific roles of Wnt pathway regulators and components in lactation have given us insight to alterations that occur in breast tumor development.

Chapter VI - Breast milk is the 'gold standard' of infant nutrition providing not only nutrition for optimal growth but immune protection as well. Many women initiate breastfeeding however few continue to breastfeed for the recommended 6 months (WHO). Management of lactating women is predominately experience-based therefore lack of diagnostic tests and evidence-based treatment is likely to contribute to early weaning. Ultrasound imaging is not routinely used as a diagnostic tool during lactation however new research suggests that is a promising modality capable of identifying both breast and infant sucking pathologies. Imaging of the non-lactating breast is well established however little imaging is performed during lactation.

Ultrasound during lactation is relatively simple provided settings are optimized to accommodate the increased amount of glandular tissue. Furthermore an understanding of the growth of the breast during pregnancy and changes during lactation as well as lactation pathology enhance diagnoses. Ultrasound can also be utilized to confirm normal function of the lactating breast. While sufficient milk must be synthesised for the optimal growth of the infant it must also be released during breast feeding or breast expression by the milk ejection reflex. Increasing duct diameter and visualisation of milk flow at milk ejection confirms that the reflex is intact. A successful lactation depends upon the infant's ability to remove milk from the breast. Infant tongue action can be visualised during both breast and bottle feeding. Recently this technique has been employed to assess infants with oral anomalies such as ankyloglossia. It can also be applied to the infants of mother experiencing pain during breastfeeding to determine if compression of the nipple is a contributing factor. Ultrasound techniques have also been developed to image swallowing in both breast and bottle fed infants but have not yet been used extensively to identify swallowing pathology.

Nutrigenomics and Breast Milk, Perspectives in Obesity

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Abstract

Nutrigenomics is the study of the interaction of dietary factors with genes, examining the influence that certain nutrients have on their expression and therefore may either contribute to health (along with other environmental factors), for example through a healthy balanced diet, or determine the onset of certain diseases (eg obesity). Nutrients modulate molecular processes involving DNA structure, gene expression and metabolism, and these in turn may affect the organism's development. In this context, breast milk, which constitutes our first food, has an essential role not only in promoting children's healthy growth, but also in preventing adult diseases, such as obesity.

This review focuses on the potential modulation of genes by dietary food compounds, devoting particular attention to gene expression in the mammary gland. In addition the knowledge on milk composition from a nutrigenomics perspective and the likely health-outcomes will be analysed, in particular those associated with energy metabolism and body weight control that could be related to changes in milk composition. Finally, the role of milk leptin as a key factor in epigenetics and developmental plasticity is evaluated. Leptin would represent an essential nutrient during lactation in the protection against obesity and its metabolic-related disorders in later life.

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Mammary Gland

Lactation is a physiological process conditioning offspring growth. Breast milk is the most complete food for the growing infant; it contains all nutrients and substances needed for successful developmental progression of the newborn. As will be shown throughout the chapter, correct feeding during the first months of life has a relevant influence in the future health of the offspring.

Mammary gland has a fundamental role in production and secretion of milk for progeny. Mammary gland morphogenesis begins during embryonic development and continues during the postnatal period. During three major developmental stages—puberty, pregnancy and involution—the gland undergoes profound morphological and functional changes. These changes correspond to periods of cell proliferation, apoptosis and differentiation, in conjunction with changes in gene expression patterns [1-5]. Although mammary gland development enables lactation to occur after delivery, previous environmental inputs along life may condition its development and therefore, the so called epigenetic modulation, may affect the performance of the gland as a synthesizing organ [6, 7].

Epigenetic Evidences in Mammary Gland

Epigenetics is a modulatory metabolic process, characterized by stable alterations in gene expression that arise during specific developmental stages (to be adapted to novel and changing environmental conditions) and may have phenotypical consequences, even in the descendants. Epigenetics is relevant in the context of this chapter because epigenetic dysregulation can cause obesity. Dietary components and other factors, including maternal obesity during pregnancy and lactation, might cause metabolic imprinting in the offspring, perpetuating, or even amplifying, obesity susceptibility across generations. Such epigenetic modifications can affect different organs, tissues and metabolic pathways associated with obesity (adipose metabolism, neural networks of energy balance regulation and so on) [8, 9].

In this section we are going to review the actual knowledge on the potential epigenetic imprinting that may affect mammary gland development and therefore milk composition. Following sections will cover specific knowledge on epigenetic mechanisms affecting milk composition and, if it is known, the impact on body weight regulation of the newborn.

At a biochemical level, epigenetic changes lead to alterations in chromatin conformation. These changes in chromatin are brought about by DNA methylation, histone modification, non-histone chromatin proteins and even non-coding RNAs (ncRNA) [10-14].

The most characterized epigenetic mechanism is methylation. Methylated cytosines serve as docking sites for proteins that prevent transcription factors from accessing their binding sites on the gene promoter. Histones are also susceptible to modification by a number of functional groups, including methylation at specific amino acids and, as a consequence, chromatin accessibility to regulating factors is amended and adapted to environmental stimulus. Extensive work in the agouti viable yellow (A^{vy}) mouse provided the first clear demonstration that supplementation of the mother's diet during the perinatal period, with nutrients affecting availability of compounds involved in the methylation pathway, can cause a permanent phenotypic change in the offspring via an epigenetic mechanism. Specifically,

genotypic obese mice show a lean phenotype associated with hypermethylation of the agouti locus. Recent studies provide compelling evidence that epigenetic regulation at specific genomic loci is susceptible to environmental influences during development in humans [8, 15].

Some studies have reported the inverse correlation between expression of major milk protein genes and methylation status, of either the promoter or the regulatory regions, in the lactating mammary gland, which occurs in pregnancy and puberty. This is partly modulated by lactogenic hormones, which act inducing an open chromatin conformation at regulatory regions. Evidences of other epigenetic mechanisms associated with transcription activation of mammary gland during lactation have also been documented [6].

Gene Expression in Lactating Mammary Gland

Physiology, gene expression and regulation in human mammary gland are not well known because of the ethical and practical problems associated to sampling. *In vitro* studies lack the complexity of the number of cells involved in breast: adipocytes, stromal and epithelial tissue, macrophages and lymphocytes. Some existing data on gene expression in human mammary epithelium have been obtained from milk fat globules (MFG). Mammary epithelium cells (MEC) RNA is isolated from MFG and the obtained transcriptome of human MFG is similar to that described in mammary glands of lactating rats [16]. A number of comparative studies have been addressed to analyze gene expression of mammary glands from different animal species in relationship with specific functions (e.g. immune function) [17] and in comparison with human milk composition [2]. Mechanisms that regulate proliferation, apoptosis, differentiation and tissue remodeling of mammary glands have been studied at different stages in different species and compared with other tissues, such as liver or adipose tissue by microarray [18], RT-PCR techniques or immunohistochemistry [19]. Recently, novel bioinformatic tools have been applied to data sets to get some insight on the potential regulatory gene networks in lactation [20]. However, molecular physiology of human lactation is almost unknown and most of the knowledge has been inferred from animal or cellular models [16].

The ability to secrete milk develops during pregnancy when the mammary gland is transformed from a simple ductal tree to a highly efficient exocrine organ with expansive lobular-alveolar structures. There is marked proliferation of ductal and alveolar epithelial cells during pregnancy, implying a progressive reorganization of the mammary gland, replacing adipose tissue by secretory epithelium. Secretory differentiation of the mammary gland begins around mid-pregnancy, but is at parturition when the secretory activation is fully functional and homogeneous expression of milk protein genes and biosynthetic enzymes by alveolar cells is reached [2, 21].

The mammary epithelium is composed by two layers of cells: luminal and basal cells. Luminal cells produce and secrete milk whereas basal or myoepithelial cells are responsible for the contraction that allows milk output. The correct development and function of the mammary gland during lactation depends on genes such as IRS-1 and -2 (Insulin receptor substrate 1 and 2) or IGF-1 (Insulin-like growth factor 1). These genes are sensitive to hormones that regulate postnatal development of the gland and are involved in either

apoptosis or stimulation of mammary epithelial cells. These protein genes, highly induced in mammary gland, are involved in lactogenesis and in milk synthesis [22, 23].

The Lactome

The lactome, comprising the subset of genes responsible for lactation, is spatially and temporally complex [24]. MEC are essentially biofactories of lipids, proteins and carbohydrates for milk during lactation. Milk production goes by hand with a broad suppression of functions to effectively push all of the cell's resources towards the massive synthesis of lipids and of a large quantity of a minority of proteins. However, apart from small subsets of proteins such as the major milk proteins and lipid synthesis, there is no a sudden transcriptional switch around the time of parturition [20].

Gene ontology analysis suggests that much of the machinery for the secretory pathway is transcribed prior to lactation. Therefore, preparation of the mammary gland for lactation includes modifications of the transcriptional program, but the onset of lactation appears to be primarily controlled by post-transcriptional mechanisms [20, 25, 26].

Major milk protein genes: As a group, genes transcribed to synthesize major milk proteins are up-regulated sharply around the time of parturition. However, this represents a small subset of genes and the secretory pathway is likely devoted to the secretion of large amounts of a few proteins. For instance, highly expressed milk protein genes, such as the caseins, account for as much as 30% of total RNA. Concerning post-transcriptional mechanisms commented above, the mRNA of casein genes accumulates rapidly due to increased RNA stability in presence of lactogenic hormones [27]. Enhancement of translation of the mRNA of the β -casein protein occurs by lengthening the poly(A) tract, synergized by the activity of both prolactin and insulin signaling [28]. The rate of translation has also been found to be reduced by amino acid deprivation [29].

Fatty acid metabolism genes: Milk triglycerides (TG) are synthesized from substrates derived from exogenous (dietary) fats and from fatty acids that are synthesized *de novo* in the gland. These pathways converge at the point where short, medium and long chain fatty acids are esterified to a common glycerol backbone. As mentioned, the relative mRNA levels of the main TG synthesis enzymes begin to increase on average at about mid pregnancy and then remain elevated throughout lactation. This has been described for glycerol kinase (GK), which phosphorylates the glycerol imported from the plasma, the long chain acyl-CoA synthase (LACS4), which supplies the mammary epithelium with a pool of acyl-CoA, and for acylglycerol-3-phosphate-acyl transferase (AGPAT), the enzyme which acylates the sn-2 hydroxyl group of the glycerol backbone [26].

The mRNA levels for enzymes providing long chain polyunsaturated fatty acids (LC-PUFA) to the neonate are also significantly upregulated starting at mid pregnancy. These include Elovl-1, that elongates very long chain fatty acids, and a $\Delta 5$ fatty acid desaturase (FAD1) [30]. None of these genes respond to dietary lipids, since TG synthesis must remain approximately constant to maintain total milk lipid content. In contrast, mRNA for most enzymes of the β -oxidation pathway falls 2- to 3-fold at secretory activation, to allow all lipid substrates to be diverted towards milk fat synthesis. In addition, fatty acid synthesis is activated, particularly when the dietary supply is not enough to cope with the demand for milk synthesis. This process requires the coordination of multiple genes that control the flux of substrates, including enzymes, transporters, binding proteins and others. As happens in liver, sterol response element binding protein1 (SREBP1) seems to be the transcription factor

responsible for induction of the lipogenic genes in mammary gland such as fatty acid synthase (FASN), the stearoyl-coenzyme A desaturases (SCD1 and SCD2) and the mitochondrial citrate transporter (SLC25a1)[31].

Plasma membrane substrate transporters: The synthesis of lipid and lactose in the mammary gland begins with the transport of precursors or substrates from the plasma. The transport of glucose across the plasma membrane is important for both lipid and lactose biosynthesis. Therefore, a number of membrane transporters are induced to facilitate the transfer of glucose into the mammary gland. Glucose is conveyed into the cells either discretely by the family of GLUT transporters, which are dealing with facilitated transport, or coupled to a sodium-dependent mechanism (Slc5a1) [32-34]. The mammary gland also utilizes fatty acids mobilized from other metabolic tissues, specifically liver and adipose tissue. Fatty acids enter cells through both passive diffusion and by protein-mediated transfer mechanisms facilitated by transporter proteins, including fatty acid translocase (FAT/CD36), fatty acid binding protein (FABP) and caveolin [35-37]. Regarding the amino acids necessary for protein synthesis in the mammary gland, a number of transporter genes are also expressed in mammary gland, including the transporter ASC (specific for alanine, serine and cysteine), LAT1 and SLC3A2, with are specific for neutral amino acids, and the Tau or Slc6a6, specific for taurine. These transporters are not expressed at the same degree throughout time but its expression is able to respond to metabolic needs of the gland and to the protein intake of the mother, and may even be susceptible to hormonal changes [32].

Mineral binding/transport capacity: One of the nutritional properties of milk is the co-delivery of minerals, implying that the processes of lactation must include substantial ion transport capabilities. Gene ontology analysis also suggests that the increased transcription of genes associated with ion transport begins during pregnancy. Both zinc and iron transport systems are up-regulated in lactation (ceruloplasmin, ferritin heavy chain, lactotransferrin, sideroflexin 2 and transferrin receptor). However, some other genes associated with iron transport are also downregulated (ferritin light chain 1, hephaestin, hemochromatosis, sideroflexin 1 and solute carrier family 11 member 1 (Slc11a1)) [32].

Dietary Influence on Gene Expression and in Milk Composition

Environmental conditions, maternal status and dietary inputs may affect either positively or negatively the regulation of gene expression in mammary gland and therefore may affect the composition of breast milk. Subtle variations in the nutritional content and therefore, in milk quality, may not be critical for the success of the offspring but may contribute to perform specific imprinting in lactating neonates leading towards higher propensity to metabolic diseases in adulthood.

It has long been known that large inter-individual variations and changes in composition occur during the course of lactation. This goes together with the fact that breast milk volume and composition from the same individual may vary over the course of the day, during the course of suckling (difference between fore- and hind-milk) and considerably from day to day. In addition, changes in composition are greatest and occur most rapidly during the first week post-partum. The milk produced in the first few days after birth (colostrum) is higher in protein, vitamins A, B12 and K and immunoglobulins than mature breast milk but it is somewhat lower in fat content and hence in energy. Over the following week, the

composition of milk is transitional and, at about 10 days after birth, milk can be considered “mature” (see [38] for a review).

Nutrition during pregnancy directly affects mammary gland development, milk production and percentage of lactose, and the effects on growth of the offspring have been described in domestic animals [39]. In humans, the influence of maternal factors such as diet and body composition on milk composition has been studied and, although the postnatal feeding regime greatly determines infant growth trajectories, there is still a lack of substantial knowledge. For example, weight gain during the first week of life has been shown to be positively associated with later obesity and it is now clear that an earlier age of adiposity rebound is associated with later higher obesity risk [40, 41]. In this sense, the protective effects of breast feeding are documented and a potential role for milk leptin has been proposed [42-44]. However, in general there is a lack of association concerning the potential modulation of macronutrient milk composition by maternal diet, which is consistent with the buffering capacity of the mammary gland against fluctuations in maternal dietary intake or nutritional status [45-48].

In this respect, a number of studies in rats have shown that maternal undernutrition during lactation may influence body weight of lactating offspring, mainly associated with changes in protein and lipid milk concentration. Appropriate supply of protein in maternal diet is necessary to support protein load in milk and progeny growth. In this sense, protein-restricted rats show a deficient development of mammary glands and prolactin secretion, which results in lower protein concentration in milk. This is accompanied by long-lasting effects on liver and muscle mitochondria, and has been associated with the development of insulin resistance in later life. Similarly, energy-restricted dams have higher lipid milk content and, as a result, the offspring is heavier than controls in adulthood [49].

Concerning other important nutrients in milk, such as fatty acids, unbalanced $\omega 6$ relative to $\omega 3$ LC-PUFA intake in maternal diet has been pointed out as one of the factors contributing to the upward trend in childhood obesity. However, two recent systematic reviews have highlighted the paucity of robust data from both human [50] and animal [51] studies that could contribute to confirm the effect of a maternal diet supplemented with LC-PUFA on body fat mass in the progeny. Docosahexaenoic acid (DHA) is a biologically important fatty acid, present in high concentrations in the brain and retina, and accumulates rapidly in neural tissues during infancy. Therefore, sufficient provision of DHA is thought to be essential for optimal visual and neurologic development during early life. DHA levels in human milk vary considerably, mainly as a consequence of differences in maternal intake of DHA [52, 53] and DHA supplementation of lactating women increases breast milk DHA content [54, 55]. Milk micronutrients constitute the only source of vitamins and oligoelements for the lactating newborn. A general lack of knowledge about the effects of perinatal supplementation with individual minerals/vitamins or multicomplexes and future health outcome in offspring is still lacking [56]. Much attention has been devoted to folic acid because it is essential in the synthesis of DNA, especially during periods of rapid growth and cell division, and has a role in epigenetic mechanisms (see Micronutrients in the following section). Also, lactating women have higher folate requirements due to its draining from breast milk. Folate concentration in human milk is tightly regulated and not affected by maternal folate status except in clinically folate deficient mothers [57]. Something similar occurs with milk zinc (Zn), whose concentration is maintained over a wide range of dietary Zn intake [58-60].

Therefore, future research is needed to establish better evidence of the effects of maternal diet composition during lactation on health in both mother and offspring, particularly for those nutrients whose presence in milk is directly related to the maternal dietary content.

Human Milk Composition

Milk is a very complete food, at least for the first period of the infant's growth. In addition to its well known and characterized content in macro- and micronutrients, breast milk contains hundreds of components, as well as complex macromolecular structures, whose presence has not been fully described and provide benefits in ways we still do not understand, but as outlined above, those may stand out throughout life [24].

It is not the aim of this section to give a detailed description of milk composition, which can be found in many previous reviews. In this section, some of the potential compounds that could play a role in future health outcomes of progeny, particularly concerning modulation of the risk of obesity in adulthood, will be presented. A core of functional components, whose presence in milk is recently beginning to be recognized as beneficial to the newborn and go beyond simple nutritional purposes, will also be analyzed.

Breast-Milk Lipids

The lipid composition of human milk is conditioned by recent maternal dietary fat intake, internal fatty acid composition, fats mobilized from adipose tissue stores and *de novo* lipogenesis during lactation. The contribution differs between sources i.e. about one-third of milk fat linoleic acid (LA) originates from diet whereas the dietary contribution of arachidonic acid (AA) represents only 10%; the other 90% of human milk AA originates from adipose tissue [61].

TG are the main components in milk, but their fatty acid composition and structure is variable. For example, when consuming a low fat diet, specific unsaturated medium chain fatty acids (MCFA) are synthesized in the mammary gland, which are more easily absorbed by the offspring than longer ones because they do not require the carnitine transport process. In addition, human milk is characterized by high palmitic acid content taking part of TG in the specific sn-2 position. This structure is relevant because it affects bioavailability of other nutrients and improves fat and calcium absorption in the infant gut [3, 62, 63].

Milk Fat Globules and Lactosomes

Traditionally, milk lipids have been characterised by their mono-, di- and TG content and their fatty acid-associated composition because these neutral lipid represent around 99% of total milk lipids. However, milk contains a complex mixture of protein-lipid ensembles which have specific synthetic and secretion pathways and probably different nutritional/functional roles. Once lipids are synthesised in the mammary gland, its secretion takes place as lipid droplets of variable size, coated by proteins and polar lipids. According to the droplet volume, at least two different species can be described, MFG and lactosomes, which differ in their lipid and protein composition and appear to be secreted from different pathways. MFGs are

present in cream, which are rich in TG and have the biggest volume (5-10 μm). Lactosomes are found in the skim fraction and are rich in a variety of phospholipid species, almost devoid of TG and cholesterol, and have smaller size (≈ 25 nm). Thus, it has been suggested that MFG would constitute the main lipid source to provide energy to infant, while lactosomes would potentially offer immunomodulatory functionality independent of macronutrient supply [64-66].

Long Chain Polyunsaturated Fatty Acids

Human milk fat also contains LC-PUFAs. These fatty acids are membrane components that act as second intracellular messengers, modulate gene transcription and serve as precursors for eicosanoids and docosanoids (leukotrienes, prostaglandins, lipoxins and resolvins) synthesis. Because of the limited ability of infants to synthesize them from essential fatty acids, human milk practically constitutes as the unique LC-PUFAs source, and maybe for this reason colostrum has the highest concentration and it decreases with maturation of milk.

Approximately 30% of LC-PUFA milk content derives from the maternal diet, while the major part comes from maternal body stores, thus maternal long-term dietary intake influences milk fat composition [63, 67, 68].

In addition, endogenous synthesis of LC-PUFA in the mammary gland takes place depending on diet and nutritional status, which can be assessed by analysing desaturase and elongase activities in plasma. Obesity and metabolic syndrome have been associated with high $\Delta 9$ -desaturase and $\Delta 6$ -desaturase (Fads2) activities and low $\Delta 5$ -desaturase (Fads1) activity in plasma. Thus, obesity as well as maternal diet during lactation may influence milk fatty acid composition. On the other hand, elongase activity has been recently recognized as another key control point for LC-PUFAs synthesis. Specifically, the presence and the activity of Elov12 seem to be critical in understanding whether DHA synthesis can be increased by dietary means. In fact, supplementation of lactating women with DHA precursor has little effect on breast milk DHA content [50, 51, 53, 69-71].

Another aspect of concern is the fact that the increased consumption of vegetable oils (rich in $\omega 6$ PUFAs) in detriment of seafood intake ($\omega 3$ enriched) during the last decades has been associated with increased $\omega 6/\omega 3$ ratio in breast milk. In particular, a significant time-increase of LA content has been systematically reported in breast milk of women living in major industrialized countries of the Western world since the fifties, whereas α -linolenic (LNA) content has not changed in a parallel way, leading to a continuous increase in the LA/LNA ratio. Independently of mammary gland lipid metabolism, this ratio varies between 5:1 and 15:1 in breast milk of women in westernized countries. Such an imbalance of the precursor ratio may favour the conversion of LA to ARA to the detriment of the synthesis of eicosapentaenoic acid (EPA) and DHA from LNA. Consistent with the adipogenic role played by LA and the anti-adipogenic role played by LNA, the $\omega 6/\omega 3$ ratio is involved in the determination of body composition. In animal studies, α -linolenic acid (ALA) or $\omega 3$ LC-PUFAs supplementation during early development has been associated to an improvement in body composition of the offspring, being more noticeable in males. In humans, an enhanced maternal $\omega 3$ PUFAs status has been associated with lower childhood adiposity too. This obesity modulatory potential has been associated with the $\omega 3$ fatty acids potential to decrease serum leptin levels during suckling in offspring, even though milk leptin level is not affected, thus suggesting an endogenous effect on the regulation of its synthesis in offspring. In

addition, a high $\omega 6$ intake may favour transformation of inflammatory products (such as eicosanoids or prostaglandins) synthesized from AA that may contribute to the inflammatory status characterizing many metabolic diseases including obesity [50, 72-76].

Furthermore, the impact of increased dietary LA:LNA ratio maintained over generations (in combination with a high-fat diet) triggers a discrete and steady increase in inflammatory stimuli accompanied by enhancement of fat mass, which suggest the involvement of epigenetic mechanisms in transgenerational obesity [77].

Moreover, LC-PUFAs are molecules with the potential to modulate the levels of hypothalamic peptides responsible of the neuro-regulatory circuitry for the feeding behaviour control. In rats who are naturally susceptible to metabolic syndrome, an isoenergetic diet with higher EPA and DHA and lower LA increased hypothalamic expression of the anorexigenic peptide cocaine- and amphetamine-regulated transcript (CART) [78]. Therefore, LC-PUFAs may modify neuronal plasticity and leptin signalling pathway in hypothalamus.

Therefore, a number of evidences have led to the proposal that an imbalance in the $\omega 6/\omega 3$ ratio during critical stages of development may be one of the factors contributing to the upward trend in childhood obesity [50, 51, 79].

Other Fatty Acids

Conjugated linoleic acids: Other kind of human milk PUFA components are conjugated linoleic acids (CLA) and conjugated alpha-linolenic acids (CLNA). CLA is present in breast milk and increases in response to maternal intake [80]. Some of them, and in particular a 50% mixture of the two main isomers (c9,t11-CLA and t10,c12-CLA), exhibit hypolipidemic and antiobesity effects in animals and are used also in the management of human obesity [81-87]. The impact of CLA on body weight or composition of lactating infants has not been assessed.

Saturated fatty acids are often associated to unhealthy outcomes. However, they can also have beneficial roles in the body. Human milk contains butyric (C4:0, 0.4%), caproic (C6:0, 0.1%), caprylic (C8:0, 0.3%), capric (C10:0, 1.2%), lauric (C12:0, 5.8%), myristic (C14:0, 8.6%), palmitic (C16:0, 22.6%) and stearic (C18:0, 7.7%) acids. Butyric acid lowers inflammation processes in the intestine acting through specific receptors; caproic, caprylic, capric and lauric acids act as potent antimicrobiological agents across gastrointestinal tract; myristic acid, especially in the sn-2 position of milk triglycerides, is responsible to rise blood HDL levels and palmitic acid may promote lipoprotein metabolism in infants through peroxisome proliferator-activated receptor gamma coactivator-1 (PGC1) stimulation in the liver. Overall, saturated fatty acids from human milk, some of them being of short chain not usually present in other foods, seem to carry out biological functions of relevance in relation with metabolic disturbances [88].

Milk Sterols

Cholesterol is a major component of the brain, accounting for 2–3% of weight and 20–30% of all lipids in the brain. Human milk provides cholesterol between 10-20mg/dL, which is higher than the usual content in infant's formula. A systematic meta-analysis has shown that total blood cholesterol concentrations in breastfed subjects, compared with those in formula-fed subjects, were higher in infancy, similar in childhood, and lower in adult life. Thus, breastfeeding has been associated with lower blood cholesterol concentrations later in life and could be one of the potential factors contributing to the metabolic programming of healthy adulthood [63, 89-92].

Phytosterols: Breast milk also contains phytosterols (1.7 mg/dL) and their intake is followed by parallel changes in maternal milk and in both maternal and infant plasma [93]. This may be of importance in the context of high maternal intake of phytosterols, either from natural or from enriched food products. Dietary phytosterols are relevant because of their use as cholesterol-lowering agents to reduce incidence of cardiovascular disease (CVD). A recent systematic review and meta-analysis, though, has questioned evidences of association between serum concentrations of plant sterols and risk of CVD [94].

In general, the diversity of molecules involved in the term dietary lipids make them compounds with multiple potential roles in metabolism. They are potential signalling molecules involved in appetite and energy metabolism, metabolic sensors in the regulation of energy storage/oxidation, mediators of the control of gene expression, and so on. Therefore, specific milk lipids may be considered as important factors with the potential to contribute to metabolic programming during lactation.

Milk Proteins

Milk proteins have been studied in depth for decades and knowledge on immunoglobulin abundance in milk has allowed characterizing the protective and immunological role of milk in newborns. Recently, and thanks to available proteomic techniques, a novel set of proteins present in human milk have been found and are under characterization [95, 96].

Traditionally, milk proteins are grouped into three main fractions: caseins (80%) found in micelles in the form of colloidal dispersion; whey proteins found in the soluble fraction and mucine proteins associated with the MFG membrane (16%) and therefore most involved in lipid delivery. Milk protein content and composition is a changing medium that evolves along lactation together with newborn development.

Biological functions of milk proteins are mainly associated with immune response, cell proliferation/differentiation and lipid metabolism/nutrient transport.

Concerning the nutrient transport role, milk proteins provide an important source of amino acids to the rapidly growing breastfed infants, but in addition they may have other complementary roles. Therefore, casein and whey fractions are involved in facilitating the digestion and uptake of other nutrients in breast milk. Some proteins are able to bind nutrients and assist their delivery in the intestinal mucosa; this is the case for β -casein and lactoferrin, which help in the absorption of calcium and iron, respectively. Specific proteins have been found to assist degradation of mono-, di-, and triacylglycerols and cholesterol esters, TG, complex carbohydrates, biotocidin and glutathione. For example, γ -glutamyltransferase is a protein involved in transferring the glutamyl moiety of the milk glutathione to a variety of acceptor molecules, yielding cysteine as an available amino acid essential for redox homeostasis and neonatal growth/development. In addition, various inhibitors of proteases are present in milk, which have been proposed to limit the activity of pancreatic enzymes, reducing protein digestibility and allowing essential polypeptides to reach the intestinal tract. Some of these inhibitors have a dual role in regulating complement activation/inflammatory processes, supporting the cross-talk between nutrient delivery and immune response pathways/interactions. Furthermore, milk proteins are a source of biologically active peptides. A number of milk proteins are resistant against proteolysis in the gastrointestinal tract and contribute, in an intact or partially digested form, to the release of an arsenal of encrypted

bioactive peptides in the gut lumen. This cryptome, still poorly investigated, seems responsible for concretization of number of physiological activities with hormone-like activity [97].

In this review, two specific aspects of milk proteins will be addressed, those which have a latent functionality which arises after enzymatic cleavage in the intestinal tract and those which have a modulatory role, being substances that have the potential to act as mediators between mother and child and contribute to establish biochemical and physiological communication during lactation. The latter aspect may include hormones, growth factors, cytokines and even whole cells.

Bioactive Peptides in Milk Proteins

Milk proteins constitute the main source of bioactive peptides with a functional role in newborn development. Bioactive peptides are generated from milk proteins in the gastrointestinal tract. Due to the relative immaturity of intestinal epithelium in the newborn, peptides may pass through the epithelium and reach peripheral tissues with the potential to act locally or systemically. Bioactive peptides may influence immune system maturation and cognitive development, as well as a possible participation in the prevention of colonization by pathogens, promoting a positive microflora in newborn [24, 98].

The main milk proteins α 1-casein and β -casein have the capacity to liberate about 20.000 peptides each. Once liberated and absorbed, these bioactive peptides may exert physiological effects on the various systems of the body. Examples of milk bioactive peptides include caseinophosphopeptides, which have a role in the transport and absorption of minerals; glycomacropptides, which bind toxins; casoxins and casomorphins, which act as opioid antagonists and agonists respectively; isracidin, which has immunomodulatory effects and casoplatelin with antithrombotic activity. Other activities identified include angiotensin converting enzyme (ACE) inhibition, antihypertensive and antithrombotic effects, which have been extensively studied as potential dietary tools to be used in the treatment and prevention of cardiovascular disease [99, 100].

However, most of the studies deal with animal sources (fermented dairy products) and their impact in adult humans; whether breast milk has the same potential in newborns and/or conditions future phenotypic features is not known.

Opioid peptides are peptides like natural enkephalins that have affinity for the opiate receptor (as agonists or antagonists) and elicit tissular/cellular opiate-like effects. β -Casein gives way to a number of derived peptides with opioid agonist activity termed β -casomorphins. Other peptides with agonist opioid activity are derived from α -casein (exorphins) or α -lactalbumin (lactorphins). Casoxins (from κ -casein) and lactoferroxins (from lactoferrin) are milk derived peptides with opioid antagonist activity. β -casomorphins are the most characterized concerning their potential activity in newborn gut lumen. Interestingly, no absorption of β -casomorphins in adult intestinal tract has been reported. In contrast, passive transport of β -casomorphins across intestinal mucosal membranes does occur in neonates, which may lead to physiological responses such as an analgesic effect on the nervous system resulting in calmness and sleep in infants. Casomorphins, as opioid ligands, are able to modulate social behaviour, increase analgesic behaviour, prolong gastrointestinal transient time, exert antidiarrheal action, modulate amino acid transport and stimulate endocrine responses such as the secretion of insulin and somatostatin. Opioid-like milk peptides also play a regulatory role regarding appetite by modifying endocrine activity of the pancreas [101].

Modulatory Proteins

Human milk is a rich source of growth factors, several hormones (i.e. insulin and growth hormone) and neuropeptides (i.e. neuropeptide Y) with known functions in the cellular context. However, their presence in milk and biologically relevant functions need the assumption that they are not degraded in the gastrointestinal lumen but absorbed in a functional form. As mentioned above, this may occur and be particularly important in newborn infants. Furthermore, prolonged breastfeeding has been associated with a lower risk of obesity in adulthood in comparison to formula fed infants. The preliminary hypothesis associated this effect to the lower protein content of human milk compared to most infant formula (the early protein hypothesis) [102, 103]. Not only protein content but also the type of proteins present in breast milk are different from those included in formula milks. In this respect, leptin is one of the proteins with a hormone-like function, present in breast-milk and lost during the processing of cow milk, that has been most characterized for its role in prevention of adult obesity. Research in our group during the last few years has been specifically devoted to this field to show the potential role of breast-milk leptin in the prevention of adult obesity [43, 44, 104-107]. In this chapter, a specific section is dedicated to leptin in the context of metabolic plasticity. Below is a description of some other factors that, on the basis of actual knowledge, could also be involved in potential imprinting of newborns towards an adult obese/lean phenotype.

Adiponectin is the most abundant adipose-specific protein present in human milk and serum, circulating at a very high concentration in the latter. Obesity decreases circulating adiponectin and a reduction in adiponectin expression has been associated with insulin resistance. Adiponectin levels in human milk are high in comparison to other hormones, such as leptin and ghrelin. Maternal factors, including duration of lactation, ethnicity and adiposity have been associated with the concentration of adiponectin in breast milk. In contrast with serum levels, a positive association has been found between adiponectin concentration in milk and maternal adiposity. Since prolactin secretion is dampened in obesity and adiponectin is negatively regulated by prolactin, it has been suggested that diminished negative regulation by prolactin in heavier mothers could increase the concentration of adiponectin produced locally in breast tissue and secreted into milk [108]. Reports showing both direct and inverse relationships between milk adiponectin concentration and infant obesity exist [108, 109]. Therefore, more studies are needed to confirm the extent of the association between maternal milk adiponectin and children obesity, as well as the interacting inputs that may affect this relationship. Interestingly, serum adiponectin in breastfed infants is related to the adiponectin concentration in the milk being consumed, suggesting transport across the human intestinal mucosa. In addition, given the biological properties of adiponectin, its presence in breastmilk and the expression of adiponectin receptors in the gastrointestinal tract, it is feasible that milk adiponectin may affect infant growth and development.

Ghrelin has many endocrine and non-endocrine functions: it is involved in energy balance regulation, stimulates food intake and decreases energy expenditure. Ghrelin is an endogenous ligand of the growth-hormone secretagogue receptor (GHSR) and exerts a strong growth-hormone releasing action. Produced primarily in the stomach, it has potent orexigenic and adipogenic activities. Ghrelin is present in significant quantities in human breast milk and there is a direct relationship between milk fat content and ghrelin levels. Although some studies have suggested that milk ghrelin comes from maternal plasma [110], other authors have found evidence that ghrelin in breast milk is most likely synthesized and secreted from

within the breast [111, 112]. Furthermore, there is controversy concerning the relationship between plasma ghrelin and anthropometrical parameters at birth. In the first months of life, ghrelin could exert an important influence on growth, exerting its actions through the receptors found in the gastrointestinal tract [110, 112-116].

Resistin is a cytokine secreted by adipocytes and has been implicated in the development of insulin resistance in animal models, although its role in insulin sensitivity in humans has not been clearly demonstrated. In the perinatal period, resistin does not seem to be directly involved in the regulation of insulin sensitivity or adipogenesis. However, there is a decrease in resistin levels in both milk and serum of breastfeeding mothers, which correlates with hormone status (as prolactin and leptin) and with the concentration of the inflammatory marker C-reactive protein. In addition, resistin concentration has been found to be higher in the serum of breast-fed infants than in either breast milk or their mother's serum. Therefore, resistin, as other breast milk hormones, could be involved in the metabolic development of infants [117, 118].

Insulin is present in human milk in a higher concentration than in commercial fresh cow's milk or infant formula. Insulin content in breast milk is positively related to its concentrations in plasma, and tends to be greater in colostrum and fall gradually in the first weeks post-partum. Milk insulin appears to influence intestinal maturation in the developing gastrointestinal tract and in animal models and has been associated to activation of mechanisms that suppress the development of autoimmune diabetes [119, 120].

In conclusion, human milk contains a number of peptides relatively resistant to proteolysis and with hormonal/modulatory properties that could contribute positively to the performance of the neonatal gut and, if absorbed, to the newborn. Furthermore, receptors of these peptides are also present throughout the gastrointestinal tract. Therefore, adipokines and hormones involved in the regulation of energy balance and delivered through breast milk to the neonate suggest that maternal inputs are able to transmit timely information regarding food resources and environmental clues, and at the same time predispose the newborn towards a healthier profile.

Oligosaccharides in Human Milk

Human milk is a complex biofluid in which oligosaccharides are the third most abundant solid component (following lactose and lipids). Human milk oligosaccharides (HMO) constitute a heterogeneous group of soluble glycans containing N-acetylglucosamine with a degree of polymerization, and incorporate D-glucose, D-galactose, L-Fucose and N-acetylneuraminic acid residues. Lactose is found at the reducing end and can be elongated with up to 15 N-acetyl-lactosamine repeating units. Lactose or the poly-lactosamine backbone can be further sialylated and/or fucosylated. To date, more than 200 HMO have been identified in human milk and their profile indicate that human milk is unique in terms of complexity and content in oligosaccharides. In contrast, infant formula contains only trace amounts of less complex oligosaccharides. Levels of HMO range between 21 to 24 g/L in colostrum and 12 to 14g/L in mature milk. In contrast, the level of milk oligosaccharides in cow's colostrum is 20 to 30 fold lower than in human milk [121-125].

Whether these significant differences cause physiological distinctions between breast-fed and formula-fed infants remain speculative, but may very well be possible considering the putative physiological effects of HMO:

- HMO present in milk are non-digestible by the newborn, withstand the low pH in the gut and resist enzymatic degradation. Therefore, they may undergo bacterial fermentation in the colon and could be considered as prebiotics which modulate microflora ecology [126-129].
- HMO may serve as adhesion soluble ligand analogs, blocking pathogen binding to aerial and intestinal surfaces and protecting breast-fed infants against infections and diarrhoea [130, 131].
- HMO alter expression of glycosyltransferases and, as a consequence, change the glycome of cells [132].
- HMO are partially absorbed intact in the infant's intestine and appear in the urine of breast-fed, but not formula-fed, infants. Therefore, a protective role against urinary pathogens has also been suggested [133-135].

Prebiotic properties of HMO are subject of intensive research and initial characterisation of infant microflora is under development. Bifidobacteria isolated from infants are proficient at capturing and utilizing HMO as a sole carbon source showing metabolic interplay between different species. A model has been proposed in which bacteria such as *Bifidobacterium longum subsp. infantis* captures intact HMO, whereas *B. bifidum* secretes extracellular enzymes prior to translocating lacto-N-biose degradation products and *B. breve* utilizes HMO monosaccharides cleaved by extracellular enzymes secreted by heterologous members of the consortium. Accordingly, a mixed-species transcriptome of the breastfed infant microbiome is enriched for bifidobacterial carbohydrate utilization, suggesting that milk sugars are actively metabolized by phylotypes incapable of utilizing intact HMO under *in vitro* isolation [125, 136].

Therefore, HMO from human origin will contribute to define an infant's microflora different from that originated in formula-fed infants.

Using methods of high-throughput sequencing, it has been described that gut microbiota is able to rapidly shift its membership and representation at the gene content level in response to host adiposity and nutrient environment. Furthermore, specific composition of the gut metagenome has been associated with obesity predisposition in adulthood. Therefore, the potential of breast milk to modulate the composition of gut microflora in infants is highly relevant, taking into account that gut microbiota shapes the host metabolome and this may have an impact in the future phenotype of the infant [137, 138].

Infant Gut Microbiota

The human gut microbiota is dynamic and responsive to dietary changes. Nevertheless, several studies have revealed that microbiota of an individual is more similar over time than to other individual's. However, before the establishment of a stable and diverse microbiota in childhood, this may be different. The fetus is sterile *in utero* and it is rapidly colonized by environmental bacteria at birth and during vaginal delivery, and most of them are derived from the vaginal and fecal microbiota. In fact, the initial bacterial community, even in the

gastrointestinal tract (GI), depends strongly on delivery mode. Lactation is then able to selectively nourish genetically compatible bacteria in infants through its complex array of HMO. The initial microbiota is characterized by low diversity and mainly facultative anaerobic bacteria belonging to Proteobacteria and Actinobacteria (e.g. Bifidobacteria). The gut microbiota then becomes more diverse, and bacteria belonging to Firmicutes and Bacteroidetes are dominant. GI tract microbiota changes throughout life and therefore older adults have substantially different GI tract communities than younger adults. In addition, a number of evidence shows that specific changes in the gut microbiota characterize the obese state and associated metabolic diseases, including diabetes. Knowledge to counteract obesity development by target specific bacterial types with prebiotic compounds though is still insufficient [137, 139-144].

Body weight gain during pregnancy and maternal diet composition (fat and carbohydrate fractions, in particular) are main factors modulating the number of bifidobacteria and lactobacilli in the mother's gut and might be a possible determinant of postnatal obesity development [144, 145].

Diet may favour the development of specific phylum and therefore constitute a major factor in shaping gut microbiota. Gut microbiota may increase energy absorption from the gut by indirect mechanisms (e.g. gut transit time) or direct mechanisms: involving enhanced glucose uptake from the small intestine by a yet unidentified mechanism, fermenting oligo- and polysaccharides to short-chain fatty acids and by modulating lipid absorption. The increased levels of glucose, as well as short-chain fatty acids, can be used for *de novo* lipogenesis. All these metabolic adaptations in lipid metabolism are providing a driving mechanism towards increased adiposity [137, 138].

Thus, programming the gut microbiota in early life by promoting a healthy core microbiome, not necessarily at the level of microbial species, but rather at the level of collective function, may have beneficial effects on host metabolism later in life [121, 125, 137, 144].

Sialic Acid

Newborn infants undergo rapid growth and development, particularly regarding their nervous system. The rate of initial brain growth exceeds that of any other organ or body tissue and by 2 years of age, the brain is about 80% of adult weight. Rapid brain growth places exceptionally high demands on the supply of precursors and nutrients. Sialic acid is a structural and functional component of brain gangliosides and correlates with the amount of DHA and total LC-PUFA in the ceramide tail of brain gangliosides. Sialic acid may be a conditionally essential nutrient in infancy if demand outstrips the rate of endogenous synthesis [146-148].

Several studies show that children who were breast-fed as babies attain higher scores in intelligence tests than those who were bottle-fed [149, 150]. Interestingly, sialic acid present in milk has been shown to enhance brain development, cognition and memory in animals [148, 151].

Sialic acid, an important constituent of human milk, is the generic term for the family of N- and O-substituted derivatives of neuraminic acid. Sialic acid is a key monomeric building block of brain gangliosides and glycoproteins and is part of the diversity of HMO described above. Human milk contains relatively large amounts of sialylated oligosaccharides that are not found in significant quantities in bovine milk or infant formulas. The concentration of

sialic acid in human breast milk varies with genetic, geographic and dietary intake of mothers. The highest concentration of sialic acid is found in colostrum, with a gradual decrease as lactation progresses [152]. In human milk, most sialic acid (approximately 73%) is associated with HMO and this proportion remains fairly constant throughout lactation. In contrast, infant formulas contain most sialic acid bound to glycoproteins (70%). Both glycoprotein (21-28%) and glycolipid (3%) fractions of human milk contain lower levels of sialic acid than that of the free oligosaccharide fraction [146, 148, 152].

Gangliosides are not uniformly distributed within the human body. Ganglioside concentration in brain grey matter is 15 times that of large visceral organs such as liver, lung and spleen and 500 times greater than intestinal mucosa. The mammalian central nervous system has the highest concentration of sialic acid. The majority (65%) is present in gangliosides and glycoproteins (32%) with the remaining 3% as free sialic acid [153]. The finding that babies who had been largely breastfed had significantly higher concentrations of ganglioside-bound and protein-bound sialic acid in the gray matter of their frontal cortex than did the formula-fed infants supports the role of sialic acid and gangliosides in breast milk in human development [154].

Micronutrients

Milk contains micronutrients, compounds which are not synthesized by the human body and are represented by vitamins and minerals, which contribute to vital functions. Traditionally, the mineral fraction has been considered to be composed by macroelements (Ca, Mg, Na, K, P and Cl) and oligo- or microelements (Fe, Cu, Zn and Se). Macroelements are distributed according to their physical-chemical properties, found in a milk aqueous phase or also bound to other milk elements as micelles and carrier proteins (e.g. caseins) [155] or even associated with lipophilic components. Vitamins A, D, E and K are mainly located in the lipid phase and vitamins of group B and C in the aqueous phase.

A large number of micronutrients are either cofactors for enzymes or part of the structure of proteins (metaloenzymes), which are involved in the maintenance of genome stability, including DNA synthesis and repair (Zn, Mg), prevention of oxidative damage to DNA (Zn, vitamin C and E) and methylation status of DNA (folate, vitamins B2, B6 and B12).

In the context of this chapter it is of interest to analyse the potential role of breast milk micronutrients contributing to modify epigenetic marks and how this can have life-long consequences for the newborn, particularly on the potential imprinting of a healthy versus obese phenotype. As mentioned above, one of the most characterised epigenetic mechanisms is DNA methylation on the 5' position of cytosine residues.

Methylation is carried out by specific DNA- and histone methyltransferases, all using S-adenosylmethionine (SAM) as the methyl donor, whose availability is directly influenced by diet. SAM is formed from methyl groups derived from choline, methionine or methyl-tetrahydrofolate. Zn, Se, folic acid and vitamins B6 and B12 are all micronutrients involved in these steps. Furthermore, recovery of the methyl group to form again tetra-hydrofolic acid involves the additional participation of vitamin B2 [156].

Zinc has been identified as the first limiting nutrient in breast milk when anthropometric indicators of growth were correlated with Zn levels in healthy breastfed infants. The high Zn concentrations during the first week postpartum fall consistently as lactation progresses.

Unlike other mammals, the variations of Zn concentrations in breast milk are neither affected by dietary composition nor by maternal metabolism. Most of the Zn is found in the skim milk fraction, but significant amounts are present in the fat associated with the fat globule membrane; less than 4% is found in the casein. Cow milk Zn is associated with high molecular weight fractions, and Zn in human milk is associated with low molecular weight fractions. No decline in hair Zn concentrations of breastfed infants during the first 6 months of life has been found whereas bottle-fed infants had a significant decline, supporting the concept of superior bioavailability of Zn in breast milk [58, 157, 158].

Besides the absolute levels of the different micronutrients, interactions between them are also relevant. Dietary Zn deficiency and a relative shortage of maternal Zn have been associated with neural tube defects in humans [159]. It has been suggested that in the presence of Zn deficiency, the administration of high-doses of folate increases the teratogenicity of such a deficiency [160]. The enzyme γ -glutamyl hydrolase is Zn-dependent and converts polyglutamates to monoglutamates, which is an important step in the absorption of folate. Therefore, the availability of folate is dependent on the glutamyl hydrolase activity, which is regulated by the concentration of Zn [161].

Selenium is considered an essential nutrient in humans. Increased Se requirements have been observed in pregnant and lactating women. Supplementation with different compounds, such as Se-enriched yeast and selenomethionine, significantly influenced selected indices of Se status, including milk concentrations [162]. It is an integral component of glutathione peroxidase, an enzyme known to metabolize lipid peroxides, and deficiency states have been described. Questions have been raised about the detrimental effects of high Se intake on dentition. Breast milk has around twice as much of Se as formula milk; accordingly, breastfed infants have greater intakes and higher serum levels of Se than formula-fed infants in the first 3 month of life [163].

Folate is an important B-group vitamin and participates in many metabolic pathways, such as DNA and RNA biosynthesis, DNA replication, repair and methylation, synthesis of nucleotides and some vitamins and synthesis and interconversion of amino acids. Pregnancy is associated to a marked acceleration in methyl transfer reactions, including those required for nucleotide synthesis and thus cell division, which is the basis for the substantial increase in folate requirements during pregnancy. Low maternal folate status has been associated with premature birth, low birth-weight and increased risk of neural tube defects in the offspring. Neural tube defects result due to incomplete development of the central nervous system and it is closely related to surrounding structures during the early stages of pregnancy. Folic acid is also important for lactating women due to the demands of breastfeeding on the mother's folic acid stores [164-166].

The generic term "folate" includes the complete group of all folic acid derivatives, including the polyglutamates naturally present in foods and folic acid, which is a synthetic folate form commonly used in food fortification and nutritional supplements. Natural food folates or pteroylpolyglutamates are hydrolyzed to pteroylmonoglutamate forms prior to absorption in the small intestine. The monoglutamate forms of folate, including folic acid, are transported across the proximal small intestine via a saturable pH-dependent process. Higher doses of folic acid are absorbed via a nonsaturable passive diffusion process. Milk is a well-known source of folate with 5-methyl-THF being the major form, which is present in both the free form and bound to folate-binding proteins. Folate-binding proteins from milk may act to increase efficiency of folate absorption, thus preventing its uptake by bacteria in the gut and

increasing absorption in the small intestine. Supplementation with folic acid in deficient mothers causes prompt increase in levels in the milk. When mothers and their infants were evaluated, folate levels were 2 to 3-times higher in the breastfed infants than in their mothers, and a correlation was seen between levels in the milk and in the infants' plasma [166-170].

Vitamin B2 or riboflavin is significant for the newborn in whose intestinal tract bacterial synthesis is minimal. Riboflavin is involved in oxidative intracellular systems, but also in maintenance methylation of DNA, synthesis of dTMP from dUMP and efficient recycling of folate as a cofactor of methylene-tetrahydrofolate reductase. Levels in human milk are lower than in cow milk (36 mg/dL and 175 mg/dL, respectively) and dietary supplementation with a multivitamin B complex in lactating woman increases riboflavin levels in the milk [171, 172].

Vitamin B6 (pyridoxine) forms the enzyme group of certain decarboxylases and transaminases involved in metabolism of nerve tissue. The supply of vitamin B6 is vital for DNA synthesis, since it is needed to form the cerebroside in the myelination of the CNS.

There are three natural vitamers of vitamin B6, namely pyridoxine, pyridoxamine and pyridoxal. All three must be phosphorylated and the 5'-phosphates of the first two vitamers are oxidized to the functional pyridoxal 5'-phosphate (PLP), which serves as a carbonyl-reactive coenzyme to a number of enzymes involved in the metabolism of amino acids. Hypovitaminosis B6 may often occur with riboflavin deficiency, because riboflavin is needed for the formation of the PLP.

Human milk has 12 to 15 mg/dL of vitamin B6 and cow milk has 64 mg/dL. The main form of vitamin B6 in human milk is pyridoxal, but pyridoxine is the major form of vitamin B6 fortification in infant formulas. Average maternal diets in several studies were consistently below the recommended levels of vitamin B6, which is reflected in lower milk levels. The accumulated stores of vitamin B6 during pregnancy are significant for the maintenance of adequate vitamin B6 status of infants during the early months of breastfeeding. For some infants, human milk alone without supplementary foods may be insufficient to meet vitamin B6 needs after 6 months of age [173, 174].

Vitamin B12 is a water-soluble vitamin and has long been recognized for its role as a covalently bound coenzyme for carboxylases. Vitamin B12 functions in transmethyations, such as in the synthesis of choline from methionine, serine from glycine and methionine from homocysteine. It is also involved in pyrimidine and purine metabolism and affects the metabolism of folic acid. Early studies reported that vitamin B12 is found in human milk in low concentrations and in cow milk it is 5 to 10-times higher. However, cow milk has little vitamin B12-binding capacity, which is substantial in human milk. Well-nourished mothers with balanced diets appear to have adequate amounts for their infants. Microbiologic assays have demonstrated that high concentrations of vitamin B12 appear in early colostrum but level off in a few days to those of serum [175-177].

Biotin has long been recognized for its role as a covalently bound coenzyme for carboxylases. More recently, evidence emerged that biotin also plays unique roles in cell signalling, epigenetic regulation of genes and chromatin structure. Biotin is attached to histones via an amide bond; some biotinylation sites have been identified in human histones and are functionally relevant. For example, biotinylation of K12 in histone H4 plays a role in gene repression, DNA repair, heterochromatin structures and repression of transposons, thereby promoting genomic stability. Importantly, biotinylation of histones depends on dietary biotin supply [178-186].

Most of the biotin is found in the skim fraction of milk, of which more than 95% is free as opposed to reversibly or covalently bound biotin. Biotin concentrations in human milk increases 5- to 30-fold from colostrum to transitional milk, and in mature milk the levels are between 20- to 50-fold higher than in plasma [187, 188].

Moderate biotin deficiency has been observed in up to 50% of pregnant women in USA. However, about 20% of the US population reports taking biotin supplements, producing supraphysiological concentrations of the vitamin in tissues and body fluids. Therefore, both biotin deficiency and supplementation are prevalent in the North American diet, which likely have effects on gene regulation and genome stability that go far beyond the classical coenzymatic role of biotin in metabolism [184].

Milk Exosomes Contain MicroRNAs

A new class of RNA regulatory genes known as microRNAs (miRNAs) has been found to introduce a whole new layer of gene regulation in metabolism. miRNAs are naturally occurring, small (around 22 nucleotides in length), single-stranded, non-coding RNAs. After being transcribed in the nucleus, exported to the cytoplasm and processed into a mature form, miRNA, in combination with a set of specific proteins (e.g. RNA-induced silencing complex (RISC)), is able to bind a target mRNA causing its degradation or blockage of its translation, in a process associated with the degree of homology between the miRNA and the target mRNA.

However, extracellular stable miRNAs exist in human body fluids, including plasma, saliva, urine and breast milk. Therefore, miRNAs can also be packaged and transported to the extracellular environment by different pathways [189]. In breast milk, miRNAs have been associated with exosomes, small endosome-derived vesicles (30-100 nm) containing a subset of specific proteins on their surface. Milk exosomes could have been originated either from macrophages or lymphocytes present in breast milk, from epithelial cells in mammary gland or captured from plasma by mammary gland. Loading of miRNAs into exosomes seems to be a selective process and specific proteins of the RISC complex would be included and delivered together, in order to enhance miRNA function in the target tissue [190]. The expression profile of milk miRNAs is significantly different at various lactation stages, indicating a regulatory process. Exosomes could stimulate target cells either via the transfer of bioactive proteins, lipids and genetic materials, or by binding to specific surface receptors. Evidence suggests that milk exosomes play a significant role in intercellular communication by transferring miRNAs to target cells and triggering downstream signalling events [191-194].

A recent report identified 602 unique miRNAs in human breast milk exosomes, showing that they are enriched in a subset of immune-related miRNAs that could contribute to the development of the immune system in lactating infants [195]. Because exosome miRNAs are quite resistant to harsh conditions (acidic pH and RNase digestion), miRNAs could reach the infants' gastrointestinal tract and be absorbed. In fact, a recent study has shown that specific plant miRNAs are found in human serum, acquired orally through food intake [196]. Therefore, although biological significance of miRNAs in milk exosomes remains uncertain, the maternal organism may cross-talk with the lactating infant via breast-feeding miRNAs, which may have the potential to modulate phenotype's infant.

Other Bioactive Compounds

Throughout the previous sections we have analysed the potential beneficial effects of some of the naturally present compounds in milk. This section is now devoted to examine whether natural bioactives, toxic compounds or drugs ingested by mothers are present in breast milk. In this case, these components are not synthesized in breast but transferred to breast milk, but in any case, they can also affect normal development and growth of offspring. A great variety of drugs, cosmetics, food ingredients and environmental contaminants are secreted with human milk as a result of either actual exposure or because of the accumulated body burden of the mother.

There is a lot of concern regarding safety of medications taken by the nursing mother and the risk to the infant. The presence of drugs or chemicals in milk may, if the concentration is high enough or if the infant is sensitive enough, interact at many possible physiological levels. There is special concern regarding the vulnerability of the developing central nervous system. Long term metabolic consequences, including newborn imprinting by medications is unknown.

Common Medications

Some of the most common drugs used by mothers in pregnancy and the safety for the newborn will be discussed. The term “safe” has often been applied as for use in lactation. However, the recommendation is made on the basis of the available studies and/or the extent of experience with drug use in lactation. The term can not be considered absolute and safety should always be interpreted in the context of the risk and benefits for the individual case. Reports of adverse effects frequently do not include a measure of the drug in milk but report events that occur during lactation.

Exogenous substances ingested by the mother regardless of route (oral, parenteral, air, skin) will appear in milk usually at concentrations below the mother’s simultaneous plasma concentrations. The maternal plasma concentration of the drug is an important determinant of how much drug is available for excretion into milk. Because diffusion occurs along a concentration gradient, high maternal plasma/serum levels will produce high milk levels. The serum/plasma concentration is determined not only by the maternal dose but also by the mother’s ability to metabolize the drug. Because the milk concentrations for most drugs are less than or equal to the maternal plasma concentration, the total exposure of the nursing infant is usually less than 1% of the maternal dose [197, 198].

Furthermore, there are significant differences in the functioning of drug metabolism pathways in the neonatal and young infant [199]. The changing levels of activities for the CYP isoform family may influence the exposure of the young infant to both the parent drug and perhaps to unusual metabolites not identified in adults. CYP1A2, responsible for demethylation of caffeine, reaches adult levels of activity by 4-5 months of age [200]. It is known in the young infant that measurable levels of a drug can be attained in the nursing infant after maternal administration. As an example, fluoxetine has a long half-life in the adult (4-6 days) and its metabolite even a longer one (4-16 days). Both compounds are found in maternal milk and in plasma and urine of the breastfed infants [201]. Although the effects of continuous exposure to such drugs are not known, one report suggests that infants receiving milk from mothers treated with fluoxetine, have decreased weight during the first 6 months [202].

Analgesic and anti-inflammatory drugs: Analgesics are commonly used in breastfeeding mothers early postpartum and for both chronic and acute conditions in the months thereafter. Paracetamol or paracetamol/codeine combinations are considered safe to use within the limits of 4 g paracetamol and 240 mg codeine daily. Ibuprofen and diclofenac are considered safe on the grounds that the drug concentration is below the limits of detection in breast milk (for a review see [203]).

Benzodiazepines: As a class, benzodiazepines are transferred to milk in low concentration and are considered safe as a single dose or for short-term use. However, benzodiazepines such as diazepam, clonazepam and lorazepam have moderate to long half-life and may cause sedation and failure to thrive. Temazepam has a low transfer into milk and is suitable for short-term use as a night time sedative (for a review see [203]).

Anticonvulsants: They have low to moderate transfer to milk and are considered safe to use, provided the infant is monitored closely [203]. However, the anticonvulsants primidone and carbamazepine inhibit biotin uptake into brush-border membrane vesicles from human intestine. Long-term therapy with anticonvulsants increases both biotin catabolism and urinary excretion of 3-hydroxyisovaleric acid. Phenobarbital, phenytoin and carbamazepine affect plasma transport, renal handling or cellular uptake of biotin. Therefore, biotin requirements may be increased during maternal anticonvulsant therapy [186].

Antimigraine drugs: To our knowledge, sumatriptan is the only contemporary antimigraine drug for which there is published data. Oral bioavailability of sumatriptan is very low; the amount of drug absorbed by breastfeeding infant is most probably negligible [203].

Antibacterial, antifungal and antiviral drugs: Antimicrobial drugs are commonly used in breastfeeding mothers. Most antibiotics transfer into milk in very low amounts and have the potential to modify gastrointestinal microflora, causing gastrointestinal symptoms such as diarrhoea. Concerning penicillins and cephalosporins, only trace concentrations have been detected in milk and they are generally considered compatible with breastfeeding. Clavulanic acid added to amoxicillin is transferred to milk, but no adverse effects have been reported, and its use in breastfeeding mothers is considered safe. Amphotericin and nystatin antifungals are both considered safe to use in breastfeeding mothers based on the minimal maternal oral absorption. Acyclovir (antiviral) is considered safe to use in breastfeeding mothers as is valaciclovir, a precursor rapidly converted to acyclovir [203].

Endocrine drugs: Carbimazole, an antithyroid drug that converts rapidly to the active metabolite methimazole, and propylthiouracil are both used to inhibit thyroxine secretion. Methimazole transfers into milk in low concentrations, but several studies have indicated no change in a breastfed infant's thyroid function. Thyroxine also transfers to milk in very low concentrations and is safe to use in lactation [203].

For oral antidiabetic agents there is sparse literature on the transfer of the sulfonylureas into milk. There are studies showing transfer of the drug in milk. However, the infants thrived well and had very low or undetectable levels in plasma. Therefore, metformin is reported to be "safe" during lactation because of low infant exposure [204].

Adequate maternal glucocorticoid concentrations in milk may be necessary to continue accurate modulation of the surfactant pulmonary system. Human milk contains hydrocortisone in concentrations ranging the needed levels for pulmonary maturation [198, 205, 206].

Psychotropic drugs: Psychotropic drugs appear in milk at low concentration. Citalopram and escitalopram are well tolerated during lactation with no reports of adverse events. Escitalopram is preferred in lactation.

Use of lithium during lactation is a difficult decision as the concentration in milk is around 40-50% of the level in mother's serum and in the infant's serum it is very similar to maternal serum. Olanzapine has been studied most extensively and there are no adverse events. However, some studies recommend that breast-fed infants must be monitored closely [203, 207].

Hypertension: All diuretics appear to be safe. Although some β -blocking agents appear to be quite safe, these are drugs that should be avoided. Calcium channel blockers (nifedipine, verapamil and diltiazem) are excreted in very small amounts in milk. No hypotensive episodes have been described in infants whose mothers take this drug [197].

Herbal/Natural Products

Research into herbal preparations and transfer into breastmilk is difficult as the active ingredients are usually not well specified as in therapeutic drug preparations. Nevertheless, use of herbal preparations is prevalent in some communities, and it has been reported that around 40% of lactating women had used a herbal galactagogue whilst breastfeeding [208, 209].

Galactagogues: The use of natural products for increasing milk production has a long history. The most frequently used products include silymarin (*Silybum marianum*), fenugreek (*Trigonalla foenum graecum*) and galega (*Galega officinalis*).

Silymarin is an extract characterised by the presence of flavanolignans (silycristine, silybin, isosilybin and silydianin). This is a well know medical plant whose extract is used mainly as a liver protective agent. Silymarin may also exert some galactagogue effects in lactating women, reflected by an increase in the quantity of milk production without affecting its main biochemical characteristics (proteins, sugars, lipids, water), although the mechanism of action is still unknown [210].

Fenugreek is used as a spice and a medicine. It is believed to have a number of therapeutic uses, including anti-inflammatory, reconstituent and galactagogic effects. The side effects most commonly reported are a maple-like smell of the urine, breast milk and perspiration, diarrhoea and the worsening of symptoms in individuals with asthma or hypoglycemia. The potential for transfer to milk or side effects in the infant are unknown, the dose necessary to obtain a galactagogic effect has not been defined and the data demonstrating the galactagogue activity are scarce [210].

Galega is another herb widely used as galactagogue in humans when it was discovered to increase milk production in cows. Galega has been shown to cause hypoglycemia. No recommended dose and no side effects are reported in the mother. Transfer in maternal milk is unknown [211].

Coffee: The consumption of coffee and caffeine seems to have potential effects on lactation and development. However, these data are from animals with a high dose of caffeine. The quantities of caffeine found in maternal milk vary with authors, but it appears clearly that caffeine does not change maternal milk composition and has a tendency to stimulate milk production. However, caffeine does accumulate in infants. So, the recommendation for nursing mothers is to limit the caffeine intake [212].

Theobromine, which is found in chocolate and cocoa, has been studied to evaluate its possible cumulative effects when taken with caffeine or theophylline. A small amount is detected in milk, with a potential dose to an infant after one chocolate bar (1.2 oz) of 0.44 to 1.68 mg. No theobromine was found in infants' urine.

Toxic Compounds

Alcohol: Beer and wine are standard beverages in many parts of the world and have been recommended to enhance lactation, especially when a mother is stressed with worldly chores.

When a lactating woman consumes alcohol, some of that alcohol is transferred into the milk. In general, less than 2% of the alcohol consumed by the mother reaches her milk and blood. Although alcohol is not stored in breast milk, its level parallels those found in maternal blood. That means that as long as the mother has substantial blood alcohol levels, the milk will also contain alcohol.

Alcohol peaks in mother's blood and milk approximately half an hour to one hour after drinking. Women should not nurse for several hours after drinking to avoid the possible adverse effects in the infant [213].

Nicotine: The consumption of cigarettes and nicotine exposure must be limited in nursing mothers. Epidemiological studies show a higher prevalence of obesity in children from smoking mothers and that smoking may affect human thyroid function. In animal models, it has been shown that maternal exposure to nicotine during lactation causes neonatal thyroid hypofunction and programs for overweight, hyperleptinemia and lower function of the pituitary–thyroid axis later in the offspring life [214].

Nutrition, Epigenetics and Developmental Plasticity

During critical periods of perinatal development, nutrition and other environmental stimuli influence developmental pathways and thereby induce permanent changes in metabolism and chronic disease susceptibility.

The biologic mechanisms underlying such “metabolic imprinting” are poorly understood, but epigenetic mechanisms are likely involved. Epigenetics is the study of mitotically heritable alterations in gene expression that are not caused by changes in DNA sequence. Therefore, transient environmental influences during development can cause permanent changes in epigenetic gene regulation.

The worldwide increase in the prevalence of obesity in recent decades has occurred too rapidly to be explained completely by genetic variation, suggesting the involvement of epigenetic mechanisms.

In this respect, maternal obesity during pregnancy and lactation may cause metabolic imprinting of neural networks in the offspring, perpetuating, or even amplifying, obesity susceptibility across generations [8, 9, 215, 216].

Role of Leptin Present in Maternal Milk in Obesity Prevention

Epidemiological data point that breastfeeding compared with infant formula feeding confers protection against several alterations later on in life and, particularly, against obesity and related medical complications [217-219]. In this sense, leptin has been identified as the specific compound that may be responsible for some of these beneficial effects during lactation [43].

Leptin is a hormone mainly produced by the adipose tissue that plays an important role in the central regulation of energy balance, decreasing food intake and increasing energy expenditure [220, 221]. This hormone is also produced by other tissues, such as stomach [222-225], placenta [226] and mammary epithelium [227], and it is naturally present in maternal milk [228, 229]. Placenta represents the main source of leptin for the fetus, while breast milk represents a continued source of leptin for the infants after delivery. Therefore, infants who are breastfed will continue to receive a significant amount of leptin from their mothers during lactation; conversely, formula-fed infants will not be exposed to exogenous leptin after delivery because these formula do not have leptin as an ingredient [230]. Moreover, leptin concentration in human milk is not uniform but varies significantly amongst individuals, and there is a positive correlation between leptin concentration in milk and maternal plasma leptin levels and adiposity [42, 229]. Thus, the amount of leptin supplied to infants through breast milk depends on the mother's adiposity. Lean mothers with very low plasma leptin concentrations produce milk with little or even no significant leptin, similar in this sense to infant formula [43].

Leptin Present in Maternal Milk is Bioavailable for Neonates

In rats, it has been shown that leptin supplied by milk or as a water solution during the suckling period can be absorbed by the immature stomach [228, 231, 232] and be transferred to the bloodstream [228, 232], suggesting that maternal leptin may play a regulatory role during development. In this sense, leptin administered orally during the suckling period has been shown to inhibit food intake without affecting body weight gain of neonate rats during this period [232]. Thus, exogenous leptin supplied by maternal milk could regulate short-term feeding in neonates and exert other biological effects at a time in which both the adipose tissue and the appetite regulatory systems are immature [232].

Results in humans also suggest that leptin present in maternal milk may be available for infants, since serum leptin in breastfed infants is higher than in formula-fed infants [233].

Milk Leptin May Program the Neonate for Lower Susceptibility to Overweight/Obesity

Indirect evidence of the role of breast milk leptin during lactation in humans has been obtained in independent studies [42, 234-236]. Miralles et al [42] showed a negative correlation between milk leptin concentration at 1 month of lactation and body weight gain of infants until 2 years of age, which was the period studied. Infants studied were from a group of 28 non-obese women which were breastfed for at least 6 months. Doneray et al [235] also found a negative correlation between leptin concentration in mature milk in a group of 15 mothers and BMI increase of infants during the first month of life. Schuster and collaborators [236] also found a negative association between breast milk leptin levels and infant weight

gain over 6 months of lactation. From these results, it could be concluded that moderate milk-borne maternal leptin appears to give moderate protection to infants from an excess of weight gain.

Direct evidence of the role of leptin during lactation in the prevention of later overweight/obesity has been obtained from animal studies [107, 237]. Oral administration of leptin at physiologic doses to neonate rats during the entire lactation period had later positive effects that prevented the animals from overweight and obesity and other metabolic alterations, which were particularly associated with feeding of a high-fat (HF) diet [107, 237]. Differences in body weight between control and leptin-treated animals could be explained, at least partially, by lower food intake and enhanced sensitivity to the central action of leptin [107, 237]. In fact, leptin seems to play a role during a critical window of development to ensure normal development of hypothalamic pathways in the arcuate nucleus, which are important because they convey leptin signals to brain regions regulating body weight, therefore altering the impact of leptin on energy homeostasis throughout life. Leptin-deficient (Lep^{ob}/Lep^{ob}) mice present an altered hypothalamic development characterised by a dramatic decrease in neuronal fibre density in hypothalamic structures involved in leptin signalling [238]. Leptin treatment of neonates with exogenous leptin rescued the development of these neural projection pathways, but did not reverse these neuroanatomical defects when the treatment was made in adulthood [238].

In addition, leptin treatment during lactation has lasting effects on the expression of hypothalamic factors involved in the control of food intake and regulated at the central level by leptin, particularly pro-opiomelanocortin (POMC), leptin receptor (LepR) and suppressor of cytokine signalling 3 (SOCS3), when animals are exposed to a HF diet. Briefly, animals supplemented with leptin during the suckling period are resistant to the decrease in LepR mRNA levels, which occurs in control animals when exposed to a HF diet. In addition, mRNA levels of SOCS3, a leptin-inducible inhibitor of leptin signalling and a potential mediator of leptin resistance in obesity [239], are lower in leptin-treated animals than in the controls, when fed either a normal-fat (NF) or a HF diet [107, 237]. Moreover, under HF diet conditions, leptin-treated animals show higher expression levels of the main anorexigenic neuropeptide regulated by leptin, POMC, while this increase is not found in control animals [107, 237]. These changes in POMC expression levels have been explained by changes in methylation of CpG sites in the POMC promoter [104]. Then, leptin treatment during lactation promotes epigenetic modification in the POMC promoter with lasting effects on food intake and body weight, particularly when these animals are exposed to a HF diet. This pattern of expression may contribute to the apparently improved capacity to regulate food intake, even when exposed to HF diets, thus helping to protect animals against excess weight gain in adulthood.

Therefore, leptin may be important during the lactation period in both regulating neonate food intake and affecting the developmental events involved in the control of energy balance in adulthood [43]. However, leptin exerts regulatory effects not only at the central level, but also peripherally, and leptin during the suckling period has also been shown to program a better response of the adipose tissue to a HF diet, by preventing the decrease of leptin receptor in internal depots and increasing the oxidative capacity of this tissue [240]. Therefore, the improvement of the peripheral action of leptin may be associated with a better handling and partitioning of excess fuel, improving the sensitivity of these rats to insulin

[237] and preventing other metabolic disorders related with HF-diet feeding, such as hepatic lipid accumulation [240].

Modulation of food preferences may also provide a mechanism through which obesity may be programmed [241]. Interestingly, supplementation of rats with leptin during lactation affected not only the amount of food intake in adulthood, but also affected food preferences [237]. At the age of 9 months, male control rats show a clear preference for fat-rich food, while no preferences were found in the leptin-treated group [237]. Taking into account that in humans, the failure to control obesity is generally associated with increased appetite and preference for highly caloric food, in addition to other factors such as reduced physical activity and increased lipogenic metabolism [242], changes in food preferences in favour of less caloric food could be of interest to prevent obesity, particularly when energy-dense foods are widely available as in our developed societies [43].

In summary, leptin may represent an essential nutrient during lactation in the protection against obesity and its metabolic-related disorders in later life. This fact opens a new area of research on both the use of leptin in the design of more appropriate infant formula as well as the identification of potential factors influencing leptin levels in maternal milk, aspects of great relevance as strategies for the prevention of obesity from early stages of development.

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Human Milk Oligosaccharides and Their Health Effects

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Abstract

Human milk contains high concentrations of all the nutrients required for infant growth and development. The essential proteins, fats, lactose, vitamins and minerals are present in milk in amounts optimal for the growth and development of the neonate. Additionally, human milk contains a broad range of nonessential components with clear health benefits for the newborn. The most remarkable beneficial components in human milk are free oligosaccharides, which comprise the third most abundant component. This abundance is extraordinary because these molecules are not digestible by the newborn's gastrointestinal system, yet they have been conserved and amplified in human milk during evolution.

Oligosaccharides in milk actively influence the bacterial species that colonize the infant's gut. These molecules support the growth of selected bifidobacteria within the intestine, thus achieving a protective microbiota dominated by beneficial bacteria (*prebiotic activity*). Milk oligosaccharides also possess anti-infective and anti-toxic activities as they inhibit pathogens from binding to intestinal cells. This inhibition occurs because some human milk oligosaccharides have functional residues in common with the glycan structures found on the gut mucosa. These glycan structures regulate cell-to-cell communication, and they are also used by enteric pathogens to bind to the mucosa and cause infection. Another therapeutic activity of sialylated oligosaccharides is their contribution to the cognitive development of the infant, as sialic acid is an essential nutrient for brain development and is used for sialylation of brain gangliosides. Since human milk oligosaccharides show extreme diversity in structure and may contain various functional groups, they also exert different bioactivities. The bio-therapeutic properties of human milk oligosaccharides, in particular those of fucosylated and sialylated oligosaccharides, are discussed as well as the analytical methods used to determine their composition and identify the potential for their commercial translation.

Keywords: Oligosaccharides; human milk; bioactivity; health effects

Introduction

Human breast milk is extremely important for newborns as it is their only source of nutrition. It contains all the nutrients required for their growth and development. The essential proteins, fats, lactose, vitamins and minerals are present in amounts optimal for the neonate. Additionally, human milk contains a broad range of nonessential biologically active components with clear health benefits for the newborn. Breast-fed infants compared with formula-fed infants have a lower risk of gastrointestinal disease, respiratory disease and other infectious diseases, and a better development of their gastrointestinal tracts and immune systems [1-5]. The biologically active components in milk include proteins such as immunoglobulins, lactoferrin and lysozyme, fatty acids, nucleotides, growth factors, hormones and oligosaccharides [1, 6].

Human milk is extremely rich in immunoglobulins (0.3-1.1 g/L (7-10)) of which more than 90% consist of secretory immunoglobulin A (sIgA) [4, 11]. Milk sIgA provides passive immunity against specific pathogens in the gastrointestinal tract [12]. As the immune system of the newborn is not yet able to produce sufficient levels of antibodies during the first weeks after birth [13], it is dependent on the passively obtained immunity via sIgA in the breast milk. These specific immunoglobulins are particularly resistant to proteolytic breakdown in the gastrointestinal tract [14] and, therefore, can prevent antigen binding and uptake at the mucosal intestinal surface [15-17].

Lactoferrin and lysozyme are proteins that are able to destroy undesirable bacteria or inhibit their growth [4]. Lactoferrin is effective against a broad range of pathogenic bacteria and fungi, such as *Escherichia coli*, *Salmonella typhimurium*, *Shigella dysenteriae*, *Listeria monocytogenes*, *Bacillus stearothermophilus*, *Bacillus subtilis* and *Candida albicans* (reviewed in [18]). Possible mechanisms of actions include the withholding of iron from iron-requiring pathogens and the production of the bactericidal peptide lactoferricin [11, 18]. Lactoferrin is specifically active against pathogenic bacteria and is not effective against the beneficial *Bifidobacterium* strains in the gut. Lysozyme kills bacteria by breaking down their outer cell wall and by working synergistically with lactoferrin [19, 20].

Other important compounds in human milk are triglycerides. They are a major energy source and, furthermore, are protective because free fatty acids and monoglycerides released from human milk triglycerides in the stomach have antibacterial, antiviral, and antiprotozoal properties (reviewed in [3] and [21]).

Nucleotides in human milk are thought to be bioactive as well, given that they potentially have a beneficial effect on immune function, iron bioavailability, lipid metabolism, intestinal microflora and gut maturation of the newborn (reviewed in [22-25]).

Various health-promoting growth factors and hormones are present in human milk. The most important of these growth factors are insulin-like growth factors and epidermal growth factors [11, 26]. They promote the development of the gastrointestinal tract by increasing epithelial cell renewal and accelerating the development of the intestinal mucosal barrier (reviewed in [27] and [28]).

The hormones that are present in human milk induce several immediate and long term health effects. Major functions involve immunomodulation and stimulation of maturation of specific organs like the gastrointestinal tract (reviewed in [17, 25, 26, 28]).

Finally, the most remarkable beneficial components in human milk are free oligosaccharides, which comprise the third most abundant component of the milk. This abundance is extraordinary because these molecules are not digestible by the newborn's gastrointestinal system, yet they have been conserved and amplified in breast milk during evolution.

Human Milk Oligosaccharides (HMO)

Breast milk has undergone constant evolutionary pressure, being the sole nourishment of newborns. Its components should maximize the health of the infant while the costs of lactation for the mother should remain as low as possible. Remarkably abundant components in human milk in this respect are complex oligosaccharides, since they are indigestible by the infant; their concentration is approximately 12 g/L [29-35]. These HMO are derived from lactose in the mammary gland where monosaccharides are added to this lactose core by several glycosyltransferases [36-38]. HMO are built out of five different monosaccharides: D-glucose (Glc), D-galactose (Gal), *N*-acetylglucosamine (GlcNAc), L-fucose (Fuc) and *N*-acetylneuraminic acid (NeuAc), a sialic acid. The lactose core is located at the reducing end (\sim Gal-(β 1,4)-Glc) and is elongated by Gal(β 1,3)GlcNAc (type I) or Gal(β 1,4)GlcNAc (type II) units [39]. The great structural diversity of oligosaccharides in human milk is provided by the addition of NeuAc and/or Fuc residues at the terminal positions.

The addition of sialic acid or Fuc residues to the oligosaccharide backbone is dependent on the activity of specific enzymes secreted by the mammary gland [29, 40]. Several sialyltransferases may add NeuAc units via α 2,3- or α 2,6-linkages to Gal or GlcNAc, producing acidic oligosaccharides. Addition of Fuc residues takes place via α 1,2-, α 1,3- or α 1,4-linkages. This fucosylation of oligosaccharides is dependent on enzymes (fucosyltransferases) encoded by the same genes (FUT2 and FUT3) that encode Lewis blood group types, meaning that the expression of specific fucosylated oligosaccharides in milk relates to the Lewis blood group type of the mother [4]. Around 80% of the Caucasian population is classified as secretor, and woman that belong to this blood type express α 1,2-fucosyltransferase [29, 40, 41]. Milk of these women contains α 1,2-fucosylated oligo-saccharides, which are biologically important components as will be discussed subsequently. Non-secretor mothers lack these oligosaccharides, because they are not able to express α 1,2-fucosyltransferase; [29, 40, 41] their milk contains either solely α 1,3- (Lewis negative mothers) or α 1,3 and α 1,4-fucosylated oligosaccharides (Lewis positive mothers) [29, 40, 41].

The most abundant oligosaccharides in human milk are fucosylated [41]. The four most abundant oligosaccharides in human milk are 2'-fucosyl-lactose (2'-FL), lacto-*N*-fucopentaose I (LNFP I), lacto-*N*-difuco-hexaose I (LNDFH I) and lacto-*N*-tetraose (LNT) (Figure 1) [42-46], with the first three HMO exclusively present in milk from secretors. Large variations in oligosaccharide content and composition can be observed throughout both lactation and between women [47-49].

a) 2'-FL,

b) LNFP I

c) LNDFH

d) LNT

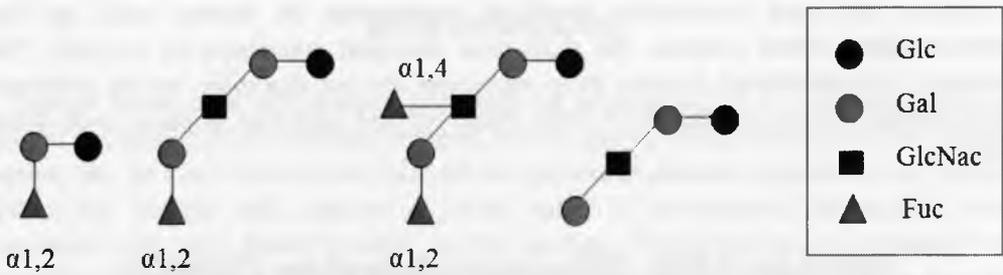


Figure 1. Schematic representation of the structures of the four most abundant HMO. a) 2'-FL, b) LNFP I, c) LNDFH I, d) LNT.

HMO and Health

The HMO are not digestible and, therefore, cannot nourish the infant [39, 50]. However, they are present as one of the main components in human milk, a product evolved to promote the health and survival of newborns. Consequently, it is likely that these structurally diverse components exert a variety of protective functions [50].

The main function of HMO is thought to be their ability to support a protective intestinal microbiota after passing undigested to the colon [51-54].

Beneficial Microflora

A beneficial microbiota in newborns, predominated by bifidobacteria, is known to support postnatal intestinal maturation, nutrient absorption and maturation of the immune system [55-60]. The species that predominantly colonize the healthy infant's intestine are the bifidobacterial species *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum* subsp. *longum* and *Bifidobacterium longum* subsp. *infantis* [39, 61-63]. Colonization by bifidobacterial species protects the infant because it makes the gut much less accessible to many types of pathogens via spatial inhibition and a lower pH [40]. Furthermore, bifidobacteria are protective because they are involved in fighting infections caused by bacterial pathogens [56, 62].

Consequently, it is believed that a bifido-dominated microflora decreases the risk of gastrointestinal diseases. Additionally, there is strong evidence that a beneficial intestinal microbiota during the first weeks of life is important for the development of the immune system [58, 60, 64].

The proposed mechanism is that specific bacteria interact with immune cells in the intestines, increasing cytokine and antibody production (reviewed in [65]). Finally, lower levels of *Bifidobacterium*-species during the neonatal period are associated with an increased risk of developing allergic diseases [66-68].

Stimulation of Bifidobacteria

The gastrointestinal tract of breast-fed infants compared with that of formula-fed infants contains more of the beneficial *Bifidobacterium* species and less of pathogenic bacteria such as *Clostridium difficile* and *E. coli* [15, 61, 69-71]. HMO selectively feed bifidobacteria while inhibiting pathogenic bacteria and toxins from binding to epithelial cells [51, 63, 72-74]. The specific ability of oligosaccharides in human milk to support bifidobacteria is attributed to the presence in their structure of GlcNAc residues, which are incorporated in the cell wall of the bacteria [75]. Especially *B. infantis* and, to a lesser extent, *B. bifidum* have metabolic capacities to effectively use a variety of HMO for their growth [51, 54, 76]; most of the other major bifidobacteria only grow on the HMO LNT [51-53]. However, a commensal activity has been proposed in which these other strains also use the monosaccharides provided by *B. infantis* upon degradation of more complex HMO [76].

Inhibition of Pathogens

In addition to stimulating a beneficial microflora, HMO furthermore inhibit pathogenic bacteria in the intestines [39, 77-85]. This inhibition occurs because some HMO have structures that are similar to glycan structures on the mucosa of the intestines [3, 4, 40, 41]. These glycan structures regulate cell-cell communication and bind to signaling agents such as hormones. Their epitopes are additionally used by enteric pathogens to bind to the mucosa, after which the pathogens can infect the target cell. As some HMO have the same epitopes as part of their structure, they can bind the pathogens and inhibit them from attaching to the intestinal cell glycans, thereby preventing the human body from being infected (Figure 2) [15, 86]. Especially oligosaccharides containing α 1,2-linked fucose [4, 15, 77-82, 87, 88] or terminal NeuAc α 2,3Gal residues [83-85] are able to block specific pathogens.

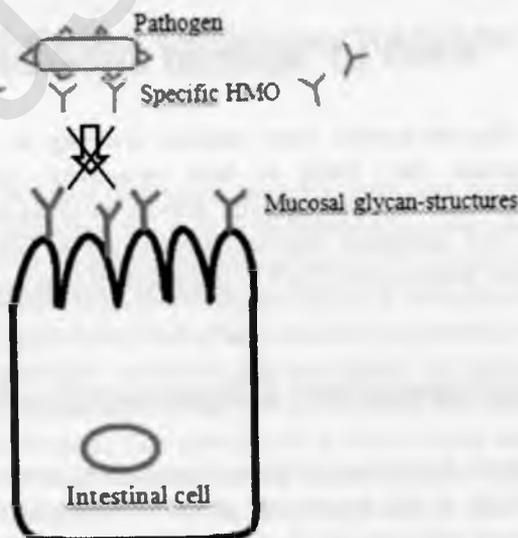


Figure 2. Inhibition of pathogen binding to glycans on the intestinal cell surface by specific HMO.

Pathogen inhibition also occurs at parts of the body other than the intestines. Because HMO are secreted in urine of breast-fed infants, it is likely that small amounts of oligosaccharides are absorbed intact from the gastrointestinal tract and end up in the systemic circulation [89, 90]. In the urinary and genital tract, oligosaccharides may prevent infections by inhibiting bacteria and viruses from binding to their receptors [90]. Sialylated HMO furthermore inhibit adhesion of *Streptococcus pneumoniae* and *Haemophilus influenzae* to epithelial cells of the mouth and throat, which prevents respiratory tract infections and acute otitis media, a middle ear infection [73].

Immunomodulating Effect

In addition to their roles in enriching a beneficial microbiota and inhibiting pathogens, HMO are thought to have a direct effect on the immune system. Prebiotics perform various immune-stimulating effects—they can influence the production of pro- and anti-inflammatory cytokines in gut-associated lymphoid tissues, secondary lymphoid tissues and in the peripheral circulation (reviewed in [91]). The proposed mechanisms behind this modulating function are firstly, the production of short-chain fatty acids by gut-bacteria upon fermentation of the prebiotics, which can exert immunomodulating functions, and secondly, the direct interaction of prebiotics with receptors on immune cells in both the intestines and the systemic circulation (reviewed in [65] and [91]).

The immunomodulating role of prebiotic oligosaccharides is confirmed by the fact that supplementation with prebiotics reduces the occurrence of allergies [72, 92-95] and inflammation (reviewed in [96]). It remains unclear whether these reductions take place through the mentioned proposed mechanisms or via stimulation of beneficial gut bacteria that interact with immune cells in the intestines. The observed effects are not specific for HMO; most of the mentioned studies examined galacto- or fructo-oligosaccharides.

HMO of Special Interest

As human milk oligosaccharides show extreme diversity in structure and may contain various functional groups, they differ in their bioactivity. Some oligosaccharides are particularly interesting regarding their specific activity in promoting the infant's health. In general, fucosylated and sialylated oligosaccharides are of special interest, as will be discussed in this section. Additionally, LNT is an important oligosaccharide.

Fucosylated Oligosaccharides

As pointed out above, fucosylated oligosaccharides that contain α 1,2-linked fucose may have important bioactivity as this moiety has structural similarity to the epitopes on glycans of the intestinal mucosal cell surface [3, 4, 41]. Therefore, fucosyl-oligosaccharides may prevent pathogens from binding to the cell-surface receptors in the gut by functioning as receptor decoys, such that enteric pathogens bind to the milk oligosaccharide instead of the

receptor. Indeed, α 1,2-linked fucose-containing oligosaccharides of human milk inhibit specific pathogens, thereby protecting against diarrhea, the most common cause of infant mortality [41, 97, 98]. α 1,2-fucosylated oligosaccharides inhibit *Campylobacter jejuni* binding to the intestinal mucosa, which prevents bacterial diarrhea [77-79] and further protects the infant against stable toxin produced by *E.coli* [15, 87, 88]. This toxin inhibits chloride transport from the gut to the intestinal epithelial cell, which in turn inhibits electrolyte and water resorption from the gut, resulting in diarrhea [15, 88]. Finally, α 1,2-linked fucose inhibits binding of norovirus—a major calicivirus—to the cell surface, and in doing so, it prevents virus infections [80-82].

Not only the risk of diarrhea is decreased by α 1,2-fucosylated oligosaccharides, *Candida* infections are inhibited as well [99, 100], even as are recurrent urinary tract infections caused by *E. coli* colonization [101-103].

Besides their pathogen-inhibiting activity, α 1,2-fucosylated oligosaccharides function in long-term potentiation, a model for learning and memory. These oligosaccharides drastically increase long-term potentiation in rats [104]. α 1,2-fucosylated oligosaccharides are thought to modulate neuronal communication in the brain by regulating the structure and function of neuronal proteins [104, 105]. Although their precise roles are unclear and they have not yet been detected in brain tissue, fucosylated oligosaccharides may contribute to neuronal development and long-term memory storage [105, 106].

2'-FL is the most prevalent of the milk oligosaccharides [4, 41, 78, 107]. Because it is an α 1,2-fucosylated oligosaccharide, it can prevent diarrhea, especially diarrhea caused by *Campylobacter* [4, 15, 41, 77, 108-110]. Recently, Sotgiu *et al.* [107] demonstrated that 2'-FL has a specific immunomodulating function as well. The molecule inhibits macrophages by down-regulation of their cytokine production. This immunomodulating activity was also attributed to LNFP I. Other important molecules in human milk that contain α 1,2- fucose are LNDFH I and difucosyl-lacto-*N*-hexaose a (DFLNH a). They both protect against diarrhea [98], LNDFH I especially protects against diarrhea caused by caliciviruses [109, 110].

Sialylated Oligosaccharides

Pathogen Inhibition

The initial attachment of many pathogenic bacteria, fungi and viruses to endothelial and epithelial cells, which is necessary for their pathogenesis, is speculated to occur via the terminal sialic acid residue of receptors present on these cells. Oligosaccharides containing terminal NeuAc α 2,3Gal residues may, therefore, act like α 1,2-fucosylated oligosaccharides as decoys for these pathogens, preventing them from attaching to epithelial cells [83-85]. Sialyl-lactose (SL) is the most abundant sialylated oligosaccharide in human milk and has two isomers: 3'-SL and 6'-SL. Especially, 3'-SL, which contains the terminal NeuAc α 2,3Gal structure, inhibits various pathogens. The adhesion of *E. coli* to brain endothelial cells can be inhibited by 3'-SL [83]. *E. coli* infection is the most common cause of meningitis in premature babies and newborns [111]. NeuAc and sialyl-lactose also inhibit *Helicobacter pylori* from binding to epithelial cells [112, 113]. Infection with *H. pylori* is the major cause of a variety of gastrointestinal diseases [114]. *Pseudomonas aeruginosa* colonizes the mucus

in the respiratory tract in certain diseases, such as cystic fibrosis. Sialyl-lactose is a good inhibitor of the binding of this bacterium to mucosal cells [115]. Furthermore, NeuAc and sialyl-lactose inhibit the binding of *Aspergillus fumigatus conidia*—a pathogenic fungus responsible for various respiratory infections—to bronchial epithelium [84]. Influenza viruses, which cause common respiratory infections, are bound by sialyl-lactose, suggesting inhibition of these viruses by sialyl-lactose [116, 117]. Finally, polyomavirus, a tumor producing virus, is bound to cell surface receptors by NeuAc α 2,3Gal residues, suggesting an inhibitory role of 3'-SL [118-120].

Additionally, 3'-SL can change the glycome of intestinal epithelial cells. It induces modifications that may alter the ability of certain pathogens like enteropathogenic *E. coli* to adhere [121]. The anti-infective properties of sialylated oligosaccharides seem to be limited to oligosaccharides containing 2,3-bound sialic acid, since it is the terminal NeuAc α 2,3Gal residue that inhibits pathogens. Inhibition by 2,6-bound sialic acids like 6'-SL has rarely been described. The only reported information concerns the potential inhibition of influenza A by oligosaccharides containing 2,6-bound sialic acid; Gambaryan *et al.* [116] showed that both 3'-SL and 6'-sialyl-*N*-acetylactosamine (6'-SLN) are capable, in a competitive assay, of binding different phenotypes of influenza virus isolates from different hosts. Furthermore, human strains of influenza A virus preferentially bind to NeuAc α 2,6Gal residues on host cell receptors [122].

Immunomodulation

Because sialylated HMO stimulate the cytokine production of T cells in the blood, they may specifically play a role in the maturation of the immune system and prevent allergy development [64]. Sialylated HMO are also thought to be anti-inflammatory. [123, 124]. These HMO—reduce formation of platelet-neutrophil complexes that are associated with activation of neutrophils and thereby, they may reduce damage in several diseases [123]. Sialylated oligosaccharides also reduce rolling and adhesion of human leukocytes on activated endothelial cells [124], in that way reducing organ dysfunction and tissue injury in various diseases [125].

Brain Development

Sialyl-oligosaccharides in human milk are thought to be an exogenous source for sialic acid (NeuAc), which is used by the infant for sialylation of brain gangliosides [126]. This is thought to contribute to the intellectual development of the infant because sialic acid is an essential nutrient for brain development and cognition [127]. This hypothesis is strengthened by the results of several studies that showed higher intelligence scores for breast-fed infants compared with formula-fed infants [128-130]. Concerning immunomodulation and brain development, it is not clear if there is a difference in activity between sialyl-oligosaccharides containing α 2,3 and α 2,6-linkages, as published studies did not discriminate between different isomers, but examined only the total sialylated oligosaccharide fraction of human milk.

LNT

The small oligosaccharide LNT (Figure 3a) is of special interest, since it preferentially feeds the dominant bifidobacterial species—*B. breve*, *B. infantis*, *B. bifidum* and *B. longum* [48, 52, 53, 131, 132]—in the gut of breast-fed infants. These species are thought to contain specific enzymes that make them capable of releasing and using the type I building block lacto-*N*-biose I (LNB) that is present in LNT [52, 53].

LNB is the Gal(β 1-3)GlcNAc (type I) structure found in most human milk oligosaccharides as the unit elongating the lactose core, whereas, in milk of other species, LNB is mostly absent, with only Gal(β 1-4)GlcNAc being present [40].

The presence of oligosaccharides containing LNB probably provides a nutritional advantage for the bifido species, increasing their counts in the intestines. This in turn is favorable for the microbial environment in the intestines, improving the overall health of the host [55-59]. In addition to LNT, the LNB building block is present, among others in DFLNH, LNFP I and LNDFH I [132]. However, especially LNT effectively stimulates growth of bifidobacteria [51]. This might be because it is more accessible to the bacteria due to its smaller size.

The isomer of LNT, lacto-*N*-neotetraose (LNnT, Figure 3b) is thought to play a role in down-regulation of pro-inflammatory reactions [133]. This down-regulation facilitates the colonization of the gut by preventing an immune response toward the gut-bacteria. Molecules with terminal Gal(β 1-4)GlcNAc structures inhibit pro-inflammatory factors such as galectin-3 by binding them and preventing them from attaching to their ligands [133-135]. Since LNnT contains this structure, it may prevent or reduce inflammatory reactions [133].

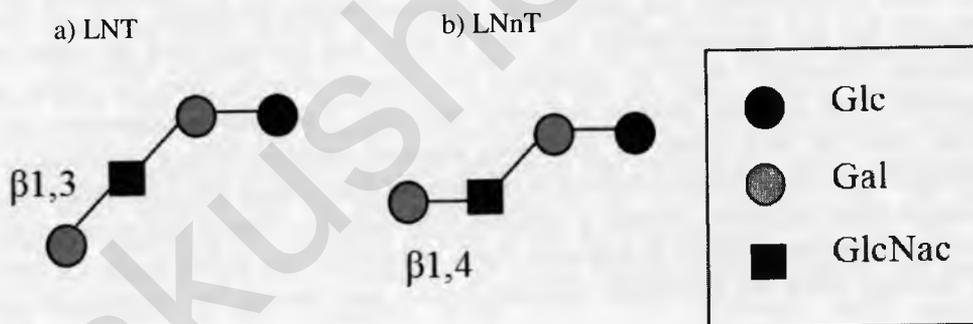


Figure 3. Schematic representation of the structures of a) LNT and b) LNnT.

Taking all of the information presented above together, it becomes clear that there are two groups of molecules that are extremely important for the health of newborns: α 1,2-fucosylated oligosaccharides because of their role in pathogen inhibition (preventing diarrhea), and sialylated oligosaccharides because of their role in pathogen inhibition (preventing among others respiratory infections), immunomodulation and brain development. Furthermore, LNT is of special interest because of its extraordinary ability in stimulating a beneficial microflora. Figure 4 gives a summary of the health effects demonstrated in the different studies to date.

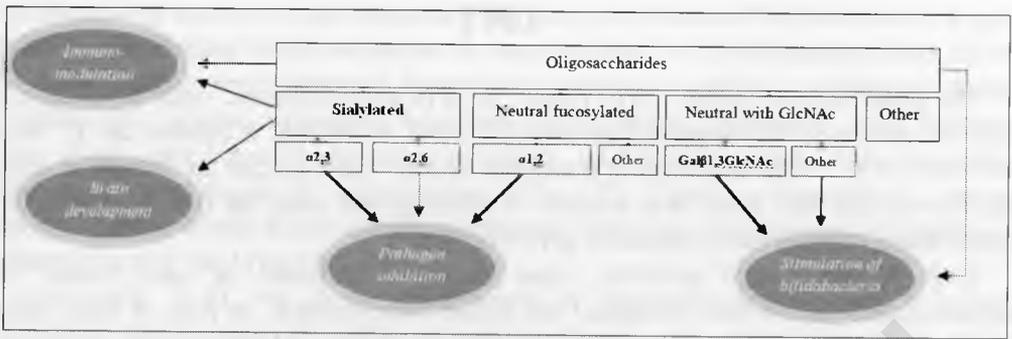


Figure 4. Health effects of oligosaccharides.

Extraction and Analysis of Oligosaccharides

Analysis of complex oligosaccharides in milk presents several difficulties because of the number and complexity of the structures. Extraction is a crucial step in the characterization and quantification process of oligosaccharides in milk as it is presently impossible to study oligosaccharides directly in milk.

Extraction of HMO

In order to characterize and quantify the oligosaccharides accurately, it is of utmost importance that the oligosaccharides are well isolated and purified from the other milk components. Extraction starts by separating the oligosaccharides from the other milk components, such as lipids, proteins and small molecules. These other components can be removed from the milk serum by employing physical methods [39]; a combination of centrifugation and liquid/liquid extraction is effective for this purpose [74, 136]. Additionally, the sample might be passed through a carbon-8 (C8) cartridge. During this reversed-phase solid-phase extraction, residual hydrophobic lipids and proteins are bound by the C8 bonded silica cartridge while the hydrophilic oligosaccharides pass through the column unretained [137-139].

The next step involves the purification and concentration of the oligosaccharides, e.g., the removal of salts, mono- and disaccharides, reagents and remaining proteins [39]. Packer *et al.* [140] developed a method using solid phase extraction (SPE) employing graphitized carbon cartridges (GCC) and showed that it can be used for purification of oligosaccharides, giving complete recovery (90–97%).

During SPE-GCC, the oligosaccharides are bound by the highly-selective, porous graphitized carbon, while monosaccharides and other substances pass through the cartridge unretained [141]. The mechanism of carbon interaction with carbohydrates is poorly understood, but probably involves both absorption and hydrophobic interaction [140, 142-144]. Successively, the oligosaccharides can be eluted from the column in fractions by applying different concentrations of acetonitrile in water, with or without trifluoroacetic acid to elute acidic or neutral oligosaccharides, respectively [140, 145].

Analytical Characterization of HMO

The method most often used to analyze HMO, since the systemic characterization of HMO started in the 1950s, is high-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection [31, 44, 45, 146-150]. However, this method does not reveal the structure of the molecules because it is not directly compatible with mass spectrometry (MS), so different methods are applied to completely elucidate the structures. Fast atom bombardment-MS and nuclear magnetic resonance were established as widespread techniques for this purpose [151-153].

A newer, more sensitive and accurate method for characterization of milk oligosaccharides is Matrix-Assisted Laser Desorption/Ionization (MALDI) coupled to time-of-flight (TOF), Fourier transform ion cyclotron resonance (FT-ICR). Other mass spectrometric methods based on separation by microfluidic HPLC Chip have also been used with success. Those methods allow separating with a good resolution different isomers with identical chemical composition.

MALDI-MS

The success of MALDI lies in the fact that it is an extremely sensitive method for ionizing medium-size molecular weight compounds without fragmenting them [154]. The molecules are transferred into the gas phase, making them measurable by a detector [155]. The sample is incorporated into a suitable matrix, which heavily absorbs the applied laser energy and disintegrates after energy deposition. This ablation of the matrix produces a dense plume containing matrix neutrals as well as matrix radicals, electrons and hydrogen atoms [156]. The plume expands into the vacuum, and within this plume, analyte molecules can be ionized with high efficiency, transferring them to the gas phase without excitation and fragmentation [157-159]. The presence of the matrix ensures a controllable energy transfer to the analyte, inducing a uniform and soft desorption and causing the analyte molecules to remain intact because they do not directly absorb the laser energy [156]. The exact mechanism underlying the ionization process is not fully understood [154, 156, 160]. The mechanism probably involves protonation or charge transfer between matrix and analyte during collisions in the expanding plume [155, 157]. Although highly accurate, reproducibility of MALDI is low because of ion suppression and combined crystallization of the matrix and the analyte [161, 162].

The mass analyzer mostly used in combination with MALDI is the TOF analyzer, in which the ionized molecules are accelerated to a defined kinetic energy. The time it takes for them to travel a fixed distance is dependent on their m/z ratio, which subsequently can be calculated [163]. The TOF analyzer couples very well with pulsed ion sources like MALDI and has an extraordinary high mass range (out to 100,000 m/z) [164]. FT-ICR MS is also commonly used in combination with MALDI. In this analyzer, the ions enter a cell that is located inside a magnetic field, causing them to move in a circular motion (cyclotron motion). An oscillating electric field perpendicular to the magnetic field causes excitation of packets of ions of the same m/z , resulting in an alternating current between the detector plates. These currents are subsequently converted to mass spectra by Fourier transformation [165]. The

strength of FT-ICR is its extremely high resolution, higher than any other form of MS [164]. Furthermore, the ions can be fragmented in the FT-ICR, allowing structural elucidation of the molecules [166].

High Pressure Liquid Chromatography (HPLC)-MS

Currently, HPLC-MS using graphitized carbon columns coupled to MS has emerged as the preferred method for HMO characterization [148, 167-170]. During HPLC, a pump moves the oligosaccharide-containing solution together with a liquid mobile phase through a densely packed graphitized carbon column, allowing the oligosaccharides to bind to the graphitized carbon and separating them based on the strength of their interaction with this stationary phase. The stronger the oligosaccharides bind to the column, the later they elute from it. When applying gradient elution, the composition of the mobile phase is changed to increase the affinity of the oligosaccharides for the mobile phase and decrease their elution time. This speeds the analysis and decreases peak widths [154].

The newest HPLC technique, nano-HPLC, has extremely high sensitivity and reproducibility and only uses minute amounts of sample in the order of 1-2 μ l [48, 136, 148, 171]. Commercially available HPLC chip devices integrate enrichment and separation columns and contain a nanospray tip used in electrospray mass spectrometry [146, 148]. The enrichment column ensures an extra purification of the sample by trapping only the molecules of interest; the separation column separates these molecules based on their interaction with the stationary phase and the nanospray tip produces a spray of protonated ions ready to be analyzed for their mass [172].

The integration of these components eliminates dead volumes and reduces the possibility of leaks [146]. When these chips are coupled with high performance MS, such as the high accuracy quadrupole time-of-flight (QTOF) mass analyzer, HMO can be rapidly analyzed in a precise and reproducible manner [48, 136, 171, 173]. The ions produced by the nanospray tip enter the QTOF mass analyzer, where they are accelerated by an electric field and travel towards a detector.

Since all ions are given nearly identical energy, the time it takes for them to reach the detector is dependent on their m/z ratio, which subsequently can be calculated like in a normal TOF analyzer. In the MS/MS mode, the precursor ions strike collision gas molecules generating product ions, which are subsequently analyzed for their m/z ratio [163]. These product ions give information about the fragmentation pattern of the precursor ions, enabling their identification. The availability of the HPLC-chip/QTOF has contributed to the identification of over 200 neutral and acidic HMO of which over 80 are now fully characterized also from the structural point of view [35, 148, 151, 152, 171, 174-177].

Quantification of HMO

To date, less attention has been paid to the quantification of the oligosaccharides in human milk and contradicting results are obtained. The total concentration of HMO in term milk ranges between 2 and 21 g/L, which is a large range [29-33]. When values from different

studies are compared, caution should be paid because there is no routine method used in these studies. Every study uses its own extraction and separation techniques, standard sets and calculations. Both HPAEC and HPLC are dependent on external standard substrates to accurately determine the concentration of specific oligosaccharides in the sample. The quantification of an oligosaccharide is done by comparing the ratio of its own area to the area of a known amount of its standard. Another method is to make a calibration curve with different concentrations of the standard and then to use this curve to calculate the amount in the sample. Standards are only available for a limited number of known oligosaccharides and are expensive, so typically only 10 to 20 different oligosaccharides have been quantified [31, 44, 45, 147-150, 167, 170].

Quantitative comparison of oligosaccharides after HPLC-MS is also performed by comparison of the relative peak areas under the curve without external standards. However, Fong *et al.* [167] found that different oligosaccharides have different ionization efficiencies and hence, different MS responses on an equimolar basis. Comparing peak areas of different molecules does consequently not provide an accurate measure for quantification.

When HPAEC is used for quantification of HMO, comparison with internal standards such as stachyose is sometimes made to calculate the quantity of individual oligosaccharides [44, 178]. The concentration of the oligosaccharide is calculated by comparing its peak area with that of the internal standard. Recently, Albrecht *et al.* [147] developed a method for the characterization and quantification of oligosaccharides using capillary electrophoresis with laser-induced fluorescence detection and MS. By derivatizing the oligosaccharides with the fluorescent aminopyrene trisulfonic acid in a mole-based manner, the oligosaccharides were quantified using the internal standard xylose.

Gas Chromatography (GC)

An alternative and more affordable method, is to hydrolyze the oligosaccharides quantify the amount of the individual monosaccharides. GC may then become a rapid and inexpensive method for quantification of these monosaccharides [179, 180], and could be use to gain complementary information about the the identity of the monosaccharides of some isoforms that Mass Spectrometry cannot distinguish e.g. between Glc and Gal (mass 162.0528), or GlcNAc and GalNAc (mass 203.0794).

In GC, molecules are separated based on their interaction strength with the stationary phase on the column, as during HPLC separation. The stronger this interaction, the more time a molecule will spend in the column and the bigger its retention time will be. The difference with LC is that the mobile phase in GC is a carrier gas and, therefore, only volatile molecules can be separated and analyzed.

Instead of applying a gradient in the composition of the mobile phase, a temperature gradient is often applied to increase the affinity for the mobile gas phase, thereby, speeding the analysis [154].

To analyze monosaccharides using GC, they first must be derivatized to volatile molecules. Silylation, in which each acidic hydrogen is converted to a trimethylsilyl group to make the molecule volatile [181] is the most widely used derivatization technique for carbohydrates [182].

Infant Formula and Oligosaccharides

Oligosaccharides have been gaining a lot of interest in the infant formula industry. Although exclusive breastfeeding during the first six months of life is a global public health recommendation set and promoted by the World Health Organization [183], there may be some reasons that alternative feeding is needed. These reasons include insufficient health of the mother, inability of the baby to suckle, separation of mother and baby and personal preferences and beliefs. The best alternative to breastfeeding by the mother is to feed the baby breast milk from a human-milk bank [184]. When this is not an option, a commercial infant formula prepared in accordance with the *Codex Alimentarius* standards is the best alternative to mother's milk [185]. Since the development of the first infant formula in 1876, enormous efforts have been made to produce formulas that closely match the composition of human milk [186].

The Food and Drug Administration regulates the nutrient content in commercially available formulas in the USA. They must contain protein, fat, carbohydrates (lactose, sugars or starch), nucleotides, vitamins (A, C, D, E, K, thiamin (B1), riboflavin (B2), B6, B12), minerals (magnesium, iron, zinc, manganese, copper), linoleic acid, niacin, folic acid, pantothenic acid, calcium, phosphorus, iodine, sodium chloride and potassium chloride [187]. The addition of oligosaccharides is not obligated. However, these prebiotics are of great importance for the infant's health, as discussed above.

In comparing infant formula to human milk, HMO-research has hitherto focused on the sialyl-oligosaccharide content. As pointed out above, a sufficient intake of sialic acid by newborns is important, and although the percentage of sialylated oligosaccharides is higher in bovine milk, the total quantity of oligosaccharides in bovine milk is lower than in human milk. Therefore, infants fed bovine milk-based formula might have a lower sialyl-oligosaccharide intake compared with breast-fed infants. Martín-Sosa *et al.* [188] indeed found that infant formula did not contain significant amounts of sialyl-oligosaccharides, and Carlson [47] found that bovine milk-based formula contained significantly less sialyl-oligosaccharides as compared with human milk. Wang *et al.* [189] examined the sialic acid content of formulas and showed that the sialic acid content of most formulas is less than 25% of that found in human milk. They also observed that 70% of the sialic acid in formulas is bound to glycoproteins, whereas in human milk, most is part of free oligosaccharides. The lower sialic acid content in formula results in significantly lower levels of sialic acid in brain and saliva of infants fed formula than in that of breast-fed infants [190-192].

Potential for Commercial Translation

Bottle-fed infants receive significantly less health promoting oligosaccharides than breast-fed infants, therefore, they are more vulnerable to several diseases. The health of these infants could be improved by incorporation of complex and bioactive oligosaccharides in infant formula. Because human milk is not widely available, it cannot serve as a source of HMO for addition to formula. Because of their complex structure and variability, it is not yet feasible to reproduce HMO [34, 39, 189], although the technology for their chemical and chemoenzymatic construction has advanced in the past few years [193]. A simple prebiotic

oligosaccharide mixture was developed as a compromise and added to some formulas to imitate the prebiotic activity of human milk [194]. The mixture consisted of short-chain galacto-oligosaccharides (GOS) and long-chain fructo-oligosaccharides (FOS) in the ratio 9:1. Several clinical trials showed that supplementation of formula with this GOS/FOS mixture increased counts of *Bifidobacterium spp.* in the infant's intestine and resulted in stools similar to those of breast-fed infants [195-201]. Arslanoglu *et al.* found that the addition of the GOS/FOS mixture to formula reduced the number and recurrence of infections in infants during the first six months of life [72]. A decrease in the incidence of atopic dermatitis for babies at risk is reported with GOS/FOS formula-supplementation [94].

FOS and GOS might, however, not be able to bind pathogens and prevent their adhesion to the epithelial cells because they do not resemble the structures of epithelial cell surface glycans [202, 203]. Furthermore, simple oligosaccharides are fermented more easily than complex oligosaccharides; therefore, they do not selectively stimulate bifidobacteria, but nonspecifically enrich a broad range of bacteria in the gut [71, 204-207].

Supplementation of infant formula with molecules extracted from other sources, like mammalian milks, and that better mimic HMO structures would be beneficial for the infant. Bovine milk oligosaccharides offer an interesting alternative in this respect; their structures are quite similar to those of HMO, suggesting a comparable protective role [177, 208]. Unlike human milk, bovine milk is widely available and, therefore, more suitable as a source for oligosaccharide extraction. However, the quantity of total oligosaccharides in bovine milk is lower than in human milk and the oligosaccharide-composition of the two milks are not identical [188]. Human milk has a high content of complex fucosylated oligosaccharides [42-45], whereas they are only present at the trace level in bovine milk, which contains mostly sialylated oligosaccharide [168, 173, 188, 209]. Bovine milk contains only small amounts of bovine milk oligosaccharides, therefore its potential as an oligosaccharide source for supplementation of infant formula is limited.

In bovine colostrum, the concentration of oligosaccharides and in particular of the bioactive sialylated oligosaccharides is exceptionally high [49, 168, 210], around one gram per liter [42, 210, 211]. This might be important in providing extra protection to the infant while its immune system and intestinal epithelial barrier are still maturing.

Bovine colostrum is processed and sold as health-promoting supplements by several companies worldwide. This production of bovine colostrum-based functional products involves membrane filtration processing to concentrate the whey proteins, leaving the permeate (liquid passed through the semi-permeable membrane) containing the low-molecular weight compounds of colostrum as a by-product. Because oligosaccharides have low molecular weights (between 500 and 1,500 Da) and are soluble in water, they are expected to be present in this low-molecular weight fraction (LMWF) of colostrum. The potential presence of concentrated oligosaccharides in a waste stream makes colostrum LMWF an extremely interesting product for uses in formulae and fortifier supplementation.

Another potential source of natural milk oligosaccharides are dairy processing by-products. Barile *et al.* [212] recently discovered 65 oligosaccharides in bovine cheese whey permeate, of which several had the same composition as HMO. Whey permeate is currently a waste-stream of whey protein concentrate production and might, therefore, serve as an inexpensive source of oligosaccharides. Compared with the LMWF of colostrum, this waste-stream is more widely available; however, the concentration of oligosaccharides in the stream is lower and may require more sophisticated techniques for a profitable commercial recovery.

In addition to their use in supplementation of infant products, another application of HMO-like structures might be their use in the pharmaceutical industry. HMO have been shown to selectively increase the growth of the beneficial bacteria *B. infantis* and *B. bifidum* [51, 54, 76], they might be of use in the development of symbiotic supplements, in which probiotics and prebiotics are used in combination to manipulate the gut microbiota of infants and adults [213]. The idea is that the addition of these complex oligosaccharides to probiotic formulations of their target *Bifidobacterium spp.* could improve the survival of these strains in the gastrointestinal tract, resulting in an improved microflora of the host. Studies examining symbiotic preparations containing the prebiotics FOS, soy germ powder and resistant starch gave promising results (reviewed in [214]), and further research is needed to evaluate the potential of the application of complex milk oligosaccharides in symbiotic mixtures with bifidobacteria.

Conclusion

Oligosaccharides in human breast milk are of special interest as they promote the health of neonates in various ways. Besides the stimulation of a healthy, bifidobacteria-dominated gut microflora, attributed to GlcNAc-containing structures, inhibition of pathogens by specific fucosylated and sialylated oligosaccharides occurs upon ingestion of the mother's milk, which prevents several infections and diseases. Extraction and analysis methods developed specifically for milk oligosaccharides allow the determination of their exact structure and concentration in various potential sources, and assessment of their potential use in infant formula and nutraceutical industries.

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The Emerging Role of Micro-RNAs in the Lactation Process

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Abstract

Micro-RNAs (miRNAs) are small RNA molecules known to participate in important regulatory mechanisms through the targeting of mRNAs by sequence specific interactions, leading to specific inhibition of gene expression. Ongoing studies have revealed the role of miRNAs in the regulation of mammary gland development but a role in lactation is not yet completely clear. Recently, the identification of significant quantities of selective miRNAs in the milk of a number of mammals, together with the recent characterisation of plant food miRNAs in the blood of people, have precipitated an investigation of the potential role of miRNAs in the regulation of the lactation process. This investigation should include both the process of milk production by the mother and the post-partum development of the young. In order to examine the role of milk miRNAs in the lactation process, we propose a comparative framework for the analysis of lactation. We review mammalian lactation diversity and animal models of lactation and recent literature on milk miRNA.

We also perform comparative and functional analysis of milk miRNAs and, discuss the function of milk miRNAs as informative markers of both lactation status and maternal physiology, as well as information carrying signals facilitating the timely delivery of maternal development signals to the young.

Keywords: milk, microRNA, lactation, mammary gland, neonate, development

Introduction

MicroRNAs (miRNAs) are genomically encoded small non-coding RNAs, 18 to 26 nucleotides long, that regulate the genetic information flow by controlling translation and stability of mRNAs. Recent research has led to a rapidly expanding set of important biological mechanisms and functions associated with miRNAs. This has revealed their broad post-transcriptional inhibitory effect on gene expression, and subsequent effects on the control of differentiation, metabolism, cell morphology, polarity, migration, signal transduction, cellular communication, organogenesis, epigenetic and developmental processes (Inui, Martello et al. 2010; Chen, Liang et al. 2012). These discoveries point to a new layer of regulatory mechanisms directed at the fine-tuning of genome expression in a tissue and time specific manner. A broader picture is emerging where miRNAs appear to act during the dynamic events of cell-lineage decisions and morphogenesis, linking dynamic physiological process with gene expression in response to mechanical and environmental changes. Advances in the understanding of miRNA biogenesis, target recognition and participation in regulatory networks have demonstrated their importance in lineage decisions of progenitor cells or organogenesis, and future discoveries in this area are likely to reveal developmental regulation and disease mechanisms related to miRNAs (Small and Olson 2011). The mammary gland is the main organ directly involved in the lactation process. The repeating developmental cycle of the mammary gland, comprised of successive cell growth, differentiation, metabolic activity, apoptosis and remodelling implies a process highly controlled by precise temporal gene regulation. Below, we review the current knowledge regarding the role of miRNA during mammary gland development.

The identification of high levels of a large number of miRNAs in the milk of eutherian mammals (human, mouse and cow) over the last two years (Chen, Gao et al. 2010; Hata, Murakami et al. 2010; Kosaka, Izumi et al. 2010; Zhou, Li et al. 2012), and the recent observation that exogenous miRNAs consumed from plant foods may directly influence gene expression in animals (Zhang, Hou et al. 2012), raise questions about the use of miRNAs as biomarkers and their putative role in mammary gland physiology together with their potential effects on growth and development of the young. Here, we explore these aspects including the idea of miRNA signalling by milk. After reviewing mammalian lactation diversity, we focus on the unique reproduction strategy of marsupials and monotremes. These animals have a short gestation and a relatively long lactation during which the newborn undergoes a significant part of its development (corresponding to intrauterine development in the eutherian lineage). During lactation marsupials also exhibit extensive mammary gland development, and changes in milk composition which can be correlated with developmental stages of the suckled young. Thus marsupials and monotremes are valuable animal models to study the potential role of milk miRNA in the lactation process, and potentially, to better understand the role of miRNA in signalling developmental process in the young.

Molecular Function of miRNAs

miRNAs are small non-coding RNA from 18 to 26 nucleotides, which recognise mRNAs, by partial sequence complementary to specific target sites in association with protein

complexes. This miRNA-mRNA target recognition process brings about either the degradation or translation inhibition of targeted mRNAs, leading to the post-transcriptional sequence specific silencing of expression of the gene (Valencia-Sanchez, Liu et al. 2006). Due to its short sequence and partial recognition of the complementary mRNA target sequence, any miRNA typically targets a large number of genes. Currently, it is thought that over one thousand miRNAs potentially regulate more than 60% of the human genome (Gunaratne, Creighton et al. 2010). Therefore miRNAs have rapidly emerged as important regulators of gene expression to control metabolic and developmental processes, including the lactation cycle.

miRNA Biogenesis

The biogenesis of miRNA is presented in figure 1. Most miRNAs are encoded in the genome within RNA polymerase II dependant non-coding transcription units (Lee, Kim et al. 2004). The promoter organization of miRNA genes is similar to coding genes, encompassing TATA box sequences, initiator elements, GpC islands and histone modification marks (Corcoran, Pandit et al. 2009). The primary (pri-miRNA) transcript is capped with 7-methylguanylate at the 5' end and carries a polyA tail at the 3' end. Often these genes encompass several miRNAs in clusters of miRNA families. Other miRNAs may be found within introns of protein coding genes (Cai, Hagedorn et al. 2004). Thus miRNA gene expression is likely integrated within the same machinery controlling mRNA expression, including transcription factors, enhancers, silencers and chromatid modifications. Pri-miRNA transcripts adopt secondary stem loop structures due to the presence of imperfect complementary sequences encompassing the miRNA. The post-transcriptional maturation of the kilobase range pri-miRNA into ~22 bp miRNAs involves two subsequent cleavage steps. The first cleavage occurs in the nucleus where the Drosha complex, composed of at least 20 proteins, hydrolyses the RNA at the base of the stem loop to release a stem loop pre-miRNA. Nuclear export of pre-miRNA to the cytoplasm is facilitated by Exportin 5 and Ran guanosine triphosphate-dependent (RanGTP) double strand RNA binding protein (Bohnsack, Czaplinski et al. 2004; Corcoran, Pandit et al. 2009).

In the cytoplasm the pre-miRNA is recognised by the Dicer complex composed of the RNase III enzyme Dicer in association with TAR RNA binding protein TRBP. The endonuclease Dicer cleaves the pre-miRNA to release double strand RNA about 22 nucleotides in length. The guided strand corresponding to the miRNA is then loaded into the RNA-induced silencing complex (RISC) to function as a mature miRNA in the miRISC complex while the other strand, called either the passenger or star strand (miRNA*) is usually degraded.

The level of miRNA* present in the cell is generally significantly lower relative to the corresponding miRNA, but widespread regulatory activities have also recently been associated with passenger strand miRNA* (Okamura, Phillips et al. 2008; Yang, Phillips et al. 2012). In cases where there is a higher proportion of passenger strand present in the cell, the nomenclature miRNA-3p/miRNA-5p is used instead of miRNA/miRNA*. miRNA-3p is the miRNA derived from the 3' arm of the precursor miRNA. whereas miRNA-5p is the miRNA derived from the 5' arm of the precursor miRNA.

Biogenesis of secretory miRNA

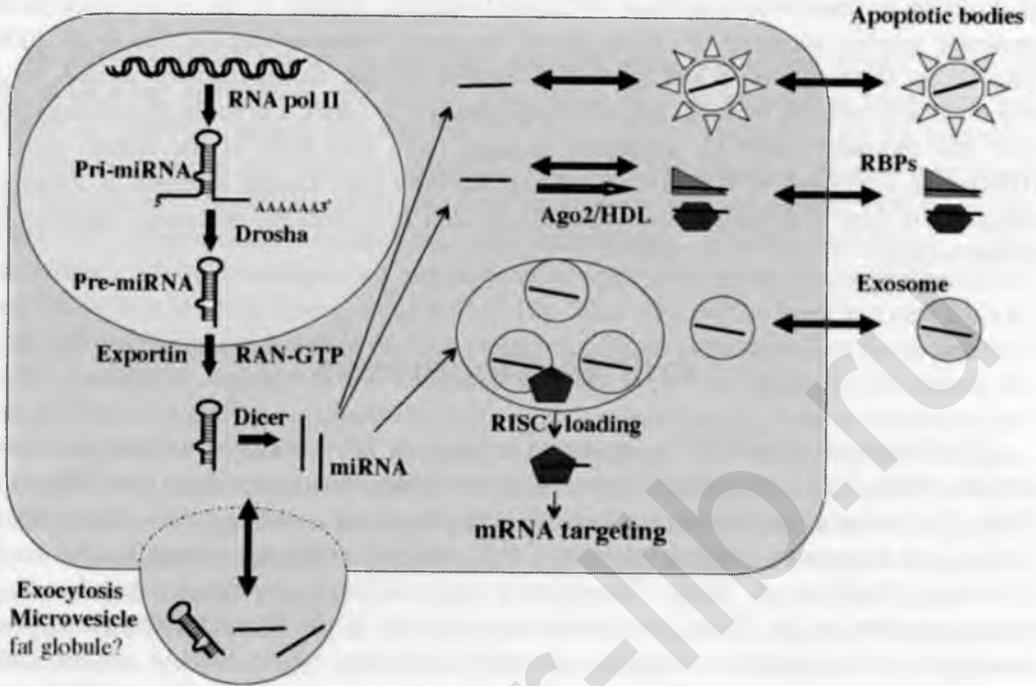


Figure 1. Biogenesis of micro-RNA.

The RISC complex is made of a number of proteins, including members of the Argonaute (Ago) family, which encompass 8 proteins in the human genome (Ago1-4 and PIWI1-4) (Sasaki, Shiohama et al. 2003; Hock and Meister 2008). Ago2 is considered to be a critical component for the functionality of the RISC complex as it is the only protein carrying RNase activity necessary for the degradation of the target mRNA recognised by the loaded miRISC complex due to partial complementarity to the loaded miRNA. RISC proteins have been located in two subcellular compartments called P-bodies or GW-bodies (Gibbins, Ciaudo et al. 2009). However miRISC associated with mRNA can also be localised with membrane fractions from the Golgi apparatus and the endoplasmic reticulum (ER) (Cikaluk, Tahbaz et al. 1999). P-bodies carry decapping activity. They are dispersed in the cytoplasm, not associated with any other identifiable cell structure, enhanced during cellular stress and are believed to be associated with miRISC degradation (Liu, Rivas et al. 2005; Gibbins, Ciaudo et al. 2009).

More interestingly, GW-bodies are closely associated with membranes in the multivesicular body (MVB), a key subcellular structure of the endoslysosomal pathway where the sorting of cellular and endosomal components takes place for either recycling or targeting toward lysosome or exosome excretion pathways (Lemmon and Traub 2000). GW-bodies appear to play an important role in the loading of miRNA into the functional miRISC complex (Lee, Pressman et al. 2009). They also possibly have a direct role in mRNA targeting by miRNAs since mRNAs and miRNAs are highly enriched in MVB membrane fractions and, blocking turnover of MVBs into lysosomes by loss of the tethering factor HPS4 enhances siRNA and miRNA mediated silencing in *Drosophila* and humans, also triggering over-accumulation of GW-bodies (Li, Rusiniak et al. 2004). Conversely, blocking MVB

formation by ESCRT (Endosomal Sorting Complex Required for Transport) depletion results in impaired miRNA silencing and loss of GW-bodies (Lee, Pressman et al. 2009). GW182, a protein of the RISC complex associated with P-bodies, ubiquitinated proteins and membrane of the MVB plays an important role in the localisation and turnover of the miRISC complex in the MVB, and it has been suggested that GW182 may act as a lock by binding to AGO and inhibiting the loading of miRNA (Gibbins, Ciaudo et al. 2009). Thus, the functionality of miRNAs seems to be closely associated with their activation in the MVB of the endosomal pathway and this is probably relevant to miRNA function and the secretion of miRNA in general, and in milk in particular.

Mechanisms of miRNA Action

The relatively recent discovery of miRNA in 1993 identified the key role of lin-4, encoding a small non-coding miRNA, in the control of larval development of the nematode through its inhibitory control of lin-14 gene expression (Lee, Feinbaum et al. 1993). The mechanism of action of miRNA loaded miRISC on gene expression is mediated by the recognition and binding of miRISC to mRNAs in a sequence specific manner. While in plants the recognition has a high degree of sequence specificity, in animals sequence complementarity between miRNA and mRNA preferentially covers the seed region of the miRNA within nucleotide positions 2 to 8 and more extensive variation exists in the 3' sequence of the miRNA beyond these seed positions. This has greatly impaired the development of miRNA target recognition algorithms for the investigation of miRNA function in animals. The seed region facilitate the nucleation of the miRNA-mRNA hybrid but a better understanding of the role of the 3' sequence in the context of AGO protein binding in the RISC complex may help improving sequence based target prediction methods (Wang, Li et al. 2010). Once bound to the mRNA, miRISC may influence its expression by different mechanism, including inhibition of translation elongation, co-translational protein degradation, competition of cap structure, inhibition of ribosomal subunit joining or polyA tail deadenylation and decapping (Eulalio, Huntzinger et al. 2008).

miRNA Secretion

A diversity of miRNAs has been identified in cells and, more recently, body fluids including milk, milk vesicles and milk exosome fractions (Chen, Gao et al. 2010; Hata, Murakami et al. 2010; Kosaka, Izumi et al. 2010). In addition serum has been shown to contain protein bound miRNAs, including proteins such as AGO2 and high-density lipoprotein HDL (Arroyo, Chevillet et al. 2011; Vickers, Palmisano et al. 2011). MiRNAs do not seem to simply diffuse passively out of the cells where they are produced, but may instead be selectively exported inside vesicles (Wang, Zhang et al. 2010). One important property of secretory miRNAs is that they are very stable in acidic conditions and are protected from RNase digestion (Mitchell, Parkin et al. 2008; Kosaka, Izumi et al. 2010). Secreted miRNA profiles do not fully overlap with cellular miRNA profiles suggesting some selectivity in miRNA secretion in an energy dependant manner (Wang, Zhang et al. 2010). In cow milk, there is no clear correlation between serum and milk miRNA contents and a large enrichment

of miRNA is observed in milk compared to serum, indicating selectivity in diffusion of miRNAs through the epithelial barrier as well as the high miRNA secretory activity of the lactating mammary gland (Weber, Baxter et al. 2010) (Hata, Murakami et al. 2010). These studies indicate the importance of mammary gland miRNA biogenesis on milk composition.

There are at least four different pathways by which miRNAs are secreted leading to different secretory particles: protein-miRNA complex secretion, microvesicle budding, exosome and apoptotic body secretion (figure 1). The secretory pathways were first recognised from the identification of miRNAs in cell culture media or serum (Turchinovich, Weiz et al. 2011). The secretion of miRNA complexed with HDL is regulated by neutral sphingomyelinase, in a manner opposite to exosome secretion suggesting that these secretory pathways are different. It was shown that HDL can deliver miRNA signals to recipient cells and this was demonstrated to be scavenger receptor BI-dependent (Vickers, Palmisano et al. 2011). In addition, variation in serum HDL miRNA composition can be associated with familial hypercholesterolemia (Vickers, Palmisano et al. 2011). The interaction of miRNA with HDL is thought to involve interaction between miRNA and lipids. In the serum it has been claimed that the majority of circulating miRNAs are found in the form of a protease sensitive complex with the AGO2 protein, while only a small proportion of serum miRNAs are associated with microvesicles (Arroyo, Chevillet et al. 2011).

Microvesicles and exosomes are often named circulating microvesicles. However, while they are both surrounded by lipid membranes, these secretory vesicles are produced by two distinct pathways. Microvesicles (also called microparticles or ectosomes) vary in size from 50 to 1000 nanometers in diameter and are produced by budding and fission of the cellular plasma membrane (Keller, Sanderson et al. 2006; van Niel, Porto-Carreiro et al. 2006). This process that is regulated by calcium, involves local lipid organization of the cell membrane as well as reorganisation of the cell structural scaffold. Contractile molecules play a direct role in bringing opposite parts of the membrane together to allow budding of small vesicles sequestering part of the surrounding cytoplasm inside the vesicle, including RNA (pre-miRNA, mRNA, miRNA) and cellular proteins. Exosomes are about 100 nm in diameter and are formed inside the cell. Endocytic invaginations of cell membranes form intracellular endosomes. Endosomes then segregate within the MVB structure which later fuses with the plasma membrane to release its exosome content. As we have seen above, miRNA biogenesis appears to be highly integrated with the MVB and inhibition of MVB turnover has profound effects on miRNA function.

Apoptotic bodies, also called apobodies, are small membrane vesicles produced by cells undergoing cell death by apoptosis. The formation of apoptotic bodies is thought to prevent the release of free, potentially toxic or immunogenic, cellular content by dying cells in order to avoid inflammation or autoimmune reactions as well as tissue destruction. Apoptotic bodies are actively taken up by phagocytosis by phagocytes and other cells for degradation. They have been shown to carry nucleic acids and have been implicated in the transmission of viral DNA (Halicka, Bedner et al. 2000).

In the mammary gland the secretion of milk miRNA may not be limited to these three mechanisms. It is likely that milk fat globules (MFG) also contain miRNA as it has been shown that fragments of cytoplasm are incorporated in the MFG during exocytosis of lipid droplets and that cellular RNA can be recovered from MFG fractions (Maningat, Sen et al. 2009). Profiling of miRNA in these different fractions will further our understanding of the selective excretion of milk miRNA and how these compartments differentially reflect the

physiological activity of the mammary gland. This will potentially allow the development of new and powerful markers of mammary gland physiology, enable the identification of new potential milk miRNA signalling pathways, and permit the evaluation of the impact of the environment on the lactation process.

Significantly, in human milk the majority of miRNAs are found in vesicle or exosome fractions with higher abundance of immune related miRNAs during the first 6 month of lactation (Kosaka, Izumi et al. 2010; Zhou, Li et al. 2012). Our unpublished results also show a large exosomal milk miRNA fraction in the milk of other species.

The Emerging Role of miRNAs in Cellular Communication

The mammalian genome is currently estimated to encode more than 1400 miRNA species. A diversity of miRNAs has been identified in cells and more recently, body fluids including milk (Hata, Murakami et al. 2010; Kosaka, Izumi et al. 2010). At the same time, a new hypothesis about the role of miRNA in inter-cellular communication has also been suggested in which secreted miRNAs serve as signalling molecules (Chen, Liang et al. 2012). Human blood cells and cultured THP-1 cells selectively package miR-150 into actively secreted microvesicles and deliver miR-150 into human microvascular endothelial HMEC-1 cells (Zhang, Liu et al. 2010). The elevated exogenous miR-150 effectively reduced c-Myb expression and enhanced cell migration in these cells. Moreover, intravenous injection of THP-1 cell culture purified microvesicles significantly increased the level of miR-150 in mouse blood vessels. Microvesicles isolated from the plasma of patients with atherosclerosis contained higher levels of miR-150 and were more effectively promoting cell migration than microvesicles from healthy donors (Zhang, Liu et al. 2010). Communication between endothelial and smooth muscle cells through circulating miRNA-143/145 has also been reported, providing a novel treatment approach for arteriosclerosis based on circulating miRNA microvesicles (Hergenreider, Heydt et al. 2012). In plants, circulating miRNA-165/166 species have been shown to specify the radial position of xylem vessels in roots of Arabidopsis and miRNAs provide signals that are transported from the shoot to the root (Furuta, Lichtenberger et al. 2012). miRNAs have also been implicated in oocyte-somatic cell communication in the ovary (Hawkins and Matzuk 2010).

These results demonstrate that cells can secrete miRNAs and deliver them into recipient cells where these exogenous miRNAs can regulate target gene expression and recipient cell function. This is further supported by a ground-breaking study, which recently showed that exogenous miRNAs consumed from plant foods pass through the intestinal mucosa into the plasma and are delivered to specific organs where they directly influence gene expression in animals (Zhang, Hou et al. 2012). These observations suggest that miRNA concentrations in body fluid compartments may be at least partially under biological control. This points to a possible role of milk miRNA as new markers or essential players of mammary gland function as well as potential agents communicating signals of maternal origin to the young during lactation. The challenge is now to fully understand the extent of specific biological function of miRNAs. The possibility for such mother-young signalling may open new perspectives toward the understanding of a healthy start in life.

Lactation Evolution and Origin of the Mammary Gland

Evolution of Lactation

The nourishment of the young with copious milk secretion by the mammary gland during lactation, is an important aspect of mammalian reproductive physiology. The naming of the class Mammalia by Linnaeus in 1758 (Linnaeus 1758) implicates lactation as a dominant character of mammalian species.

Lactation is an essential period of the mammalian reproduction cycle. Young mammals are totally dependent upon milk for survival and the nursing of the young during lactation may have facilitated the development of affectivity and learning in man (Peaker 2002). Thus, lactation is an important aspect of mammalian evolution. Fossil and molecular analysis place the appearance of early mammals on the synaptid branch of the tree of life during the end of the Triassic period (from 210 to 166 million years ago) but, a complex lactation system was already established in these ancient mammals as confirmed by comparative genome analysis (Ofstedal 2002) (Lefevre, Sharp et al. 2010). For example, in the egg-laying monotremes, milk cell cDNA sequencing has shown the highly conserved organization of casein genes in all mammalian genomes suggesting that the establishment of concerted expression of caseins and whey proteins in the mammary gland predates the common ancestor of living mammals 166 My to 240 Mya (Lefevre, Sharp et al. 2010). Thus, it is recognised that lactation gradually evolved into a complex system during the Triassic, between 320 and 200 million years ago along other mammalian characters such as the development of an integument, endothermy and fur.

The exact adaptive pathways by which the mammary gland and lactation may have been gradually established during synapsid evolution are controversial due to both the the lack of direct fossil evidence for mammary gland development or parental care, and the absence of living representatives of these early lineages.

In contemporary mammals, lactation is a complex process involving morphological, physiological, biochemical, ecological, and behavioural adaptations, which could not have arisen suddenly, and a simpler process must have taken place at the origin of lactation and gradually evolved into a more complex system. Unfortunately, little evidence of simpler intermediary processes has survived through therapsid evolution to be observed today. The survival advantage that could be gained through the neonate's chance consumption of a new secretion from the cutaneous glands of a parent has been a challenge to the theory of evolution.

Darwin himself has addressed this in the second edition of "On the Origin of the Species" (1872) (Darwin 1872), only to be misled by hypothesising a common origin for the uterine secretion of fish that give birth to live young and milk produced by the mammary gland. It is now recognized that mammary glands instead evolved from a glandular integumentary system (Blackburn, Hassen et al. 1989; Ofstedal 2002).

Origin of the Mammary Gland

The structure of the mammary gland is similar to skin glands, but there is some controversy regarding the exact type of ancestral gland from which it evolved. Blackburn has suggested that the mammary gland evolved through the combination of different skin gland populations into new functional units (Blackburn 1993) but, Oftedal argued that the mammary gland most likely derives from an ancestral apocrine-like gland associated with hair follicles (Oftedal 2002), an association that is still observed in features of mammary gland development of monotremes and marsupials.

This suggests that lactation has coevolved with hair in the cynodont lineage, possibly first in the ventral patch, where it played a functional role in the nursing of eggs through protolactal secretory glands.

Lactation could have preceded the appearance of endothermy and fur and may have played a direct role in their evolution. The exact pathways of mammary gland evolution remain speculative. However, it is now accepted that mammary glands likely derived from ancestral skin glands, probably of the apocrine type, and that these glands acquired a functional role in the nursing of the egg or the young of mammalian ancestors by providing protolactal secretions.

Scenario for the Evolution of Lactation

Several hypotheses have been advanced for the selective value of primitive protolactal secretions including thermoregulation of eggs and young through evaporative cooling (Haldane 1965) or heat transfer from the mother (Bresslau 1907), prevention of desiccation of eggs (Haldane 1965; Oftedal 2002), maintenance of proximity between the mother and the egg or the young glue (Gregory 1910), pheromones operating as a milk precursor (Duvall 1983) and, protection of the egg via secretions with antibacterial activity (Blackburn 1985). While the exact original drivers of the selective advantage of lactation remain elusive, it seems possible that the secretion by chance of a primitive molecule of immunoprotective, nutritional, or communicative value may have provided selective advantage to favour the incremental establishment of the lactation process.

The true origin of lactation and the timing of the evolution of its most primitive form remain speculative, but by 200 Mya, a complex lactation system with a highly differentiated mammary gland specialized in the production of complex milk secretions, including a large number of milk-specific proteins, had evolved (Lefevre, Sharp et al. 2010).

During subsequent mammalian radiation, a variety of lactation strategies have been adopted in different lineages to account for the diversity of environmental and behavioural adaptations found in extant mammalian species.

It is likely that miRNA have contributed to these processes and the study of their participation in the lactation process may enable new insights on the evolution of lactation.

miRNAs in Mammary Gland Biology: Mammary Gland Development

The mammary gland, a mammalian specific organ, is an exocrine tubulo-alveolar gland whose function is to produce milk; a complex mixture of carbohydrates, proteins and lipids, essential for the development and health of the new-born (Picciano 2001). This gland is composed of an epithelium network surrounded by stroma. The epithelium consists of a layer of secretory luminal cells (ductal and alveolar cells) directly in contact with the lumen and a layer of myo-epithelial cells in contact with the basement membrane.

The stroma is further subdivided between the periductal stroma, mainly composed of fibroblasts and collagen-rich extracellular matrix, which directly surrounds the ducts, and the fat pad composed of adipocytes, fibroblast, endothelial cells and mast cells, which play a role of support for the gland.

The mammary gland differs from all the other organs, as its development mainly occurs after birth, undergoing important developmental cycles under control of female reproductive hormones and local factors (oestrogens, progesterone, etc.) (Medina 1996). Indeed, even though the mammary anlage is established during foetal development, at birth, the mammary gland is rudimentary, mainly composed of short ducts restricted to the nipple region. Ductal elongation and branching is only mainly achieved during puberty; proliferation taking place at the end bud area, localised at the ducts apex. During pregnancy, dramatic alveolar differentiation occurs. During lactation, the alveolar cells start producing milk while the myoepithelial cells allow the ejection of milk from the alveoli lumen into the duct. Upon weaning, the cessation of suckling induces a rapid down regulation of milk protein gene expression, followed by an alveolar epithelial cells death concomitant with the collapse and remodelling of the mammary gland, returning the gland to a virgin-like stage. This is the involution phase (Stein, Salomonis et al. 2007).

miRNA in Mammary Gland Development

Although numerous studies have addressed the distribution of miRNAs in breast cancer, and more generally cancer where it is believed that the power of miRNA profiling is to allow a better classification of tumors, few studies have yet been conducted during normal mammary development (Silveri, Tilly et al. 2006; Sdassi, Silveri et al. 2009). Even though the role of miRNAs in mammary gland development remains generally unclear and no miRNA has been found to be mammary gland specific so far (Liu, Calin et al. 2004), it has been reported that some miRNAs present a specific expression pattern during the different stages of mammary gland development. Early experiments examining about 10% of the human miRNome have revealed a breast specific signature composed of a least 23 miRNAs (Silveri, Tilly et al. 2006; Sdassi, Silveri et al. 2009). Similar profiling studies in mice have estimated that up to half of all miRNAs tested were expressed at different stages of mammary gland development and cloning experiments have identified miRNAs, which are differentially expressed during organogenesis (Wang and Li 2007; Avril-Sassen, Goldstein et al. 2009). Other reports have described miRNA signatures at a more comprehensive set of mammary

gland development stages, including lactation and gestation (Wang and Li 2007). 21 miRNA were specifically down regulated (*mmu-miR-292-5p*, *mmu-miR-290*, *mmu-miR-126-3p*, *mmu-miR-138*, *mmu-let-7g*, *mmu-miR-106a*, *mmu-miR-10b*, *mmu-miR-142-3p*, *mmu-miR-152*, *mmu-miR-183*, *mmu-miR-195*, *mmu-miR-291a-3p*, *mmu-miR-376b*, *mmu-miR-409*, *mmu-miR-431*, *mmu-miR-376a*, *mmu-miR-467a*, *mmu-miR-30e*, *mmu-miR-1*, *mmu-miR-221*, *mmu-miR-129-5p*) and 17 up regulated (*mmu-miR-434-3p*, *mmu-miR-30d*, *mmu-miR-129-3p*, *mmu-miR-365*, *mmu-miR-483*, *mmu-miR-345*, *mmu-miR-542-5p*, *mmu-miR-381*, *mmu-miR-291a-5p*, *mmu-miR-291b-5p*, *mmu-miR-133b*, *mmu-miR-484*, *mmu-miR-133a-133b*, *mmu-miR-324-5p*, *mmu-miR-375*, *mmu-miR-434-5p*, *mmu-miR-361*, *mmu-miR-146b*) in the Gestation/Lactation stage compared to the Virgin/Involution stage. In 2009, Avril-Sassen *et al.*, undertook to characterize each murine mammary developmental stages by specific miRNA clusters (Avril-Sassen, Goldstein *et al.* 2009). Globally, they found that one third of miRNAs were expressed intermittently throughout development and were absent in one or more stages, with a general decrease during lactation and involution. They therefore identified seven expression clusters with distinct complex temporal expression profiles. While some clusters are characterized by decreased expression during lactation and involution (miR-196a/b and miR-203), others are highly expressed during lactation and early involution (among them miR-141, miR-200a, miR-429 and also miR-146b, miR-210 and multiple members of miR-148 and miR-181 family), or during puberty, maturity and early gestation (first five days) such as members of let-7 family. Interestingly, miRNAs belonging to the same cluster often encompass identical seed-sequences, which suggests common evolutionary origin and common targeting of gene expression. Expression clusters are often located inside genomic miRNA clusters and can be co-expressed in polycistronic transcripts. Thus, it is likely that some miRNAs expressed during puberty and gestation could have a role in proliferation and invasion during mammary gland development, while miRNAs expressed during lactation and early involution could have a role in the regulation of innate immune response and inflammation, as it has been observed for miR-146b (Monticelli, Ansel *et al.* 2005). Inversely, miRNAs up-regulated during lactation and early involution could be associated with increased contact between epithelial cells during the early reversible stages of involution and prevent involution during lactation. This is the case for miR-200 family members, known to inhibit epithelial to mesenchymal transition; a process similar to the loss of contact between epithelial cells during the transition from lactation to involution (Gregory, Bert *et al.* 2008). These observations that each stage of the mammary gland development cycle is characterized by a particular miRNAs expression profile suggest that miRNAs play an important role in mammary gland biology.

One direct functional proof that miRNAs participate in the control of mammary gland development was provided by Ucar *et al.* (Ucar, Vafaizadeh *et al.* 2011) when they showed how the miR-212/132 family was necessary for epithelial-stroma cell communication during mammary gland pubertal development. Using miR-212/132 null mice, the authors observed that while the embryonic and pre-puberty mammary gland development are normal, ductal outgrowth and invasion of the fat pad are impaired during puberty. Interestingly, during gestation, the lobulo-alveolar differentiation and the milk secretion were successful, even though restricted to the nipple area. By performing transplantation experiments, they showed that miR-212/132 family was required by stromal cells to maintain a low expression of the matrix metalloproteinase 9 (MMP9) in the periductal stroma. This proteinase is responsible for the degradation of the collagen, which acts as a reserve of transforming growth factor

TGF β , maintained in an inactive form (Annes, Munger et al. 2003). Thus, in miR-212/132 null mice, MMP9 escapes the down-regulation of gene expression inhibited by these miRNAs, and degrade collagen, leading to the release of activated TGF β . This activation induces the inhibition of epithelial cell proliferation, responsible for the ductal outgrowth impairment. Thus, by restricting the expression level of MMP9, miR-212/132 family prevents the anti-proliferative effect of TGF β activation on epithelial cells, allowing a normal fat pad invasion and the establishment and maintenance of a functional epithelium network during puberty. These observations demonstrate how miRNAs participate in the maintenance of a proper balance in the complex regulatory mechanisms of mammary gland development by fine-tuning gene expression of key effectors.

In 2009, Tanaka *et al.*, (Tanaka, Haneda et al. 2009) identified miR-101a as a potential mediator of mammary gland development, and notably for the involution process. In this study, the up-regulation of miR-101a observed at the onset of involution suppressed the expression of the cyclooxygenase-2 (cox-2), a protein mediating cell proliferation (Liu, Chang et al. 2001). Interestingly, this decrease of cox-2, known to happen during involution (Tanaka, Haneda et al. 2009), suggests a decisive role for miR-101a in this process. This supposition was reinforced by the negative correlation between miR-101a and β -casein gene expression, a cell differentiation marker also down-regulated during involution (Liu, Chang et al. 2001) in a process shown to be related to cox-2 repression.

Recently, Cui *et al.*, (Cui, Li et al. 2011) highlighted a role for miR-126-3p in mammary gland development via the regulation of the progesterone receptor, mammary epithelial cells proliferation and beta-casein gene expression. This miRNA was found to be down-regulated during pregnancy and lactation, probably in order to allow side-branching and alveogenesis mediated by progesterone and other hormones during gestation, and beta-casein gene expression during lactation. Finally the regulation of lactoferrin by miR-214, an important protein with immune and apoptotic function has also been reported (Liao, Du et al. 2010).

These initial studies highlight the importance of miRNA in normal development processes associated with the lactation cycle and further studies are essential to identify direct targets and functional mechanisms associated with the regulation of miRNA gene expression to fully understand the normal development processes in the breast.

Milk miRNAs

Recent studies using high throughput sequencing techniques have reported the presence of a large number of miRNAs in bovine and human milk. Hata et al. reported 6 bovine milk miRNAs from milk derived exosomes using real time PCR (Hata, Murakami et al. 2010). Chen *et al.* reported the purification of RNA from cow milk and colostrum, including some mRNA, accounting for less than 1% of the total RNA (Chen, Gao et al. 2010). In addition they reported a high proportion of miRNA and ribosomal RNA (50% of total RNA each) and identified 245 miRNAs in fresh milk (Chen, Gao et al. 2010). Amongst these, 47 miRNAs were present in cow milk but not identifiable in serum, while serum contained 162 other miRNAs not detected in milk. 108 miRNAs were significantly more highly expressed in cow colostrum than in mature milk while only 8 other miRNAs showed higher expression in mature milk compared to colostrum. Expression of another 129 miRNAs remained unchanged

from colostrum to mature milk. In total 105 miRNAs were reported to be milk specific or milk enriched. Seven miRNAs were consistently found in both colostrum and mature milk (miR-26a, miR-26b, miR-200c, miR-21, miR-30d, miR-99a, and miR-148a). These milk specific miRNAs, apparently constantly expressed throughout lactation, were proposed as potential biomarkers for the quality control of raw milk and other milk-related products. Using a PCR assay panel for the quantitative profiling of these miRNAs was shown to be sensitive enough to accurately estimate milk concentration in milk dilutions or formula milk powders for children, highlighting the low miRNA concentrations in formula milk compared to fresh milk and the value of PCR panels for new milk quality control assays.

In human milk *Kosaka, et al.*, reported RNA concentration of 10-200 ng/ml in milk and identified 281 miRNAs in human milk using miRNA microarrays (Kosaka, Izumi et al. 2010). They showed that highly expressed miRNAs were enriched for immune related functions. MiR-146b is present specifically in milk compared to plasma, whereas miR-181 and miR-155, which are highly expressed in milk, are also present at similar levels in plasma. MiRNAs were also found to be associated with microvesicle fractions purified by centrifugation. In a more recent study using sequencing, *Zhou, et al.* reported the presence of 602 unique miRNAs in breast milk exosomes and estimated exosomal RNA concentrations in milk around 160-280 ng/ml (Zhou, Li et al. 2012). The ten most highly expressed miRNAs accounted for 62 % of the total miRNA content (miR-148a, miR-30b, let-7f, miR-146b-5p, miR-29a, let-7a, miR-141, miR-182, miR-200a, miR-378). The majority (8) of these miRNAs was also found in cow milk and was reported to be milk specific or milk enriched.

Milk Exosome miRNAs

As discussed above, exosomes are tiny membranous vesicles (~30-100 nm in diameter) that are generated in the MVB and can mediate intercellular communication (Wang, Zhang et al. 2010). They are complex structures composed of a lipid bilayer containing trans-membrane proteins. These vesicles can affect the physiology of recipient cells by binding to receptors (Lässer, Eldh et al. 2012). They are secreted by various tissues and are present in different body fluids (such as amniotic fluid, blood, malignant ascites fluid, milk, saliva and urine)(Weber, Baxter et al. 2010; Zhou, Li et al. 2012). There is a link between exosome biogenesis and miRISC loading and exosomes are known to carry mRNA and miRNA molecules (Valadi, Ekstrom et al. 2007; Hata, Murakami et al. 2010) (figure 2).

The characterisation of the relatively large amount of RNA in human milk exosome fractions suggests that a large proportion of milk miRNAs are contained in exosomes (Zhou, Li et al. 2012).

We have obtained similar results with the milk of other species. It will be interesting to analyse further the difference in miRNA composition of exosomes derived from milk in comparison to whole milk, fat or whey fractions. As exosomes may be able to travel longer in the infant digestive systems this may be relevant to the differential functional targeting of milk miRNA. *Hata et al. (2010)* demonstrated the uptake of milk exosomes by cultured cells and the transfer of 6 bovine miRNAs and milk specific genes into these cells, suggesting the possible transfer of genetic information encoded by RNA between mother and child through milk exosomes (Hata, Murakami et al. 2010).

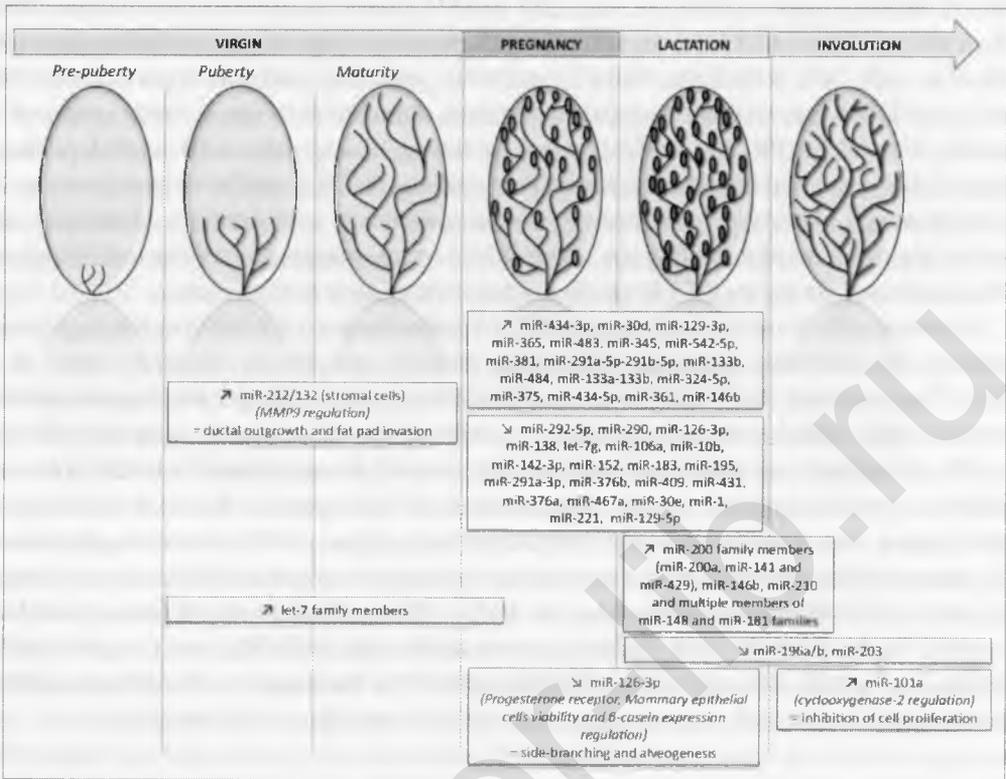


Figure 2. Overview of micro-RNAs involved in mammary gland development.

Milk miRNA Stability

The RISC effector protein AGO2 binds post-transcriptionally to miRNAs and increases their stability. Milk miRNAs have been shown to withstand harsh environmental conditions including prolonged room temperature, multiple freeze-thaw, RNase digestion and even boiling (Chen, Gao et al. 2010). Human breast milk miRNAs were able to withstand acidic (pH 1) solution for 1 hour (Kosaka, Izumi et al. 2010). Like mRNAs, miRNAs possess differential stability (Bail, Swerdel et al. 2010).

Using mouse embryonic fibroblasts following *Dicer1* ablation the average half-life six miRNAs was 119 hour (Gantier, McCoy et al.). However cellular miRNA can be degraded at different rates. When cells are grown at low density or cells are detached by trypsinization or EGTA treatment, mature miR-141 is rapidly downregulated while miR-200c from a common primary transcript (pri-miR-200c~141) remains unaffected.

This differential decay of mir-141 was shown to involve sequence specific RNase activity (Kim, Yeo et al. 2011). These studies show that miRNA can withstand the acidic environment of the gut and enter cells to target specific cellular pathways, possibly including early development of the young.

Immune Related miRNAs in Milk

Exosomes are well known communication channels between immune cells (Admyre, Johansson et al. 2007). Four out of the top ten most highly expressed miRNAs in human breast milk exosomes (miR-148a-3p; miR-30b-5p; miR-182-5p; and miR-200a-3p) have designated immune-related functions (Zhou, Li et al. 2012).

In total, 59 well-characterized immune related miRNAs were enriched in breast milk exosomes including T- and B- cell related miRNAs (miR-181a-b, miR-155, miR-17) and innate immune system related miRNAs such as miR-146b, miR-92 and miR-125b. These observations are consistent with the report that breast milk-derived exosomes can increase the number of Foxp3+ CD4+ CD25+ regulatory T cells in infants and that human breast milk miRNAs may induce B-cell differentiation (Admyre, Johansson et al. 2007).

Comparative Analysis of Milk miRNAs

Comparative approaches allow a more detailed analysis of the functional evolution of specific molecular components of lactation, emphasising the ancient origin of the essential components of the lactation system at the molecular level and, the evolutionary paths that have allowed the adaptation of a variety of lactation strategies during mammalian radiation (Lefevre, Sharp et al. 2010).

Despite major differences between plant and animal miRNA biogenesis and mechanisms of action, there is support for a common origin of miRNA in the common ancestors, which are most likely associated with the appearance of multicellularity. However miRNA may originate from different evolutionary mechanisms leading to the formation of expressed hairpin RNA structures (Liu, Okamura et al. 2008) and rapid rates of miRNA evolution have been observed (Nozawa, Miura et al. 2010). While some miRNA are highly conserved, showing purifying selection indicating their important functionality, other more recent miRNAs seem to evolve neutrally in search of novel functions. The identification of conserved miRNAs, both in terms of miRNA structure and expression levels in milk will potentially allow the characterisation of miRNA essential for the lactation process. Towards this end, we can compare datasets from bovine and human milk, although one has to be cautious about the different preparations used to obtain these datasets as the bovine data are from raw milk while the human data are from milk exosomes (Chen, Gao et al. 2010; Zhou, Li et al. 2012).

The results are also compounded by normalisation issues, reference database versions, and bioinformatic annotation pipelines. Annotated on line data were retrieved from the depository for the bovine milk miRNA study, and raw data from the human study were annotated using the online deep sequencing analysis pipeline tool (Huang, Liu et al.). Data were normalised by estimating proportions, taking the average of the four human individual samples from human milk exosome study. Available data on human milk exosomes and cow milk miRNA contents are compared in figure 3a, showing the log normalised expression plot of cow versus human milk RNA after averaging values for the 4 individual human samples. In total we were able to identify 172 common miRNAs representing a large proportion (70%) of all miRNA identified in the smaller cow dataset (245 miRNAs).

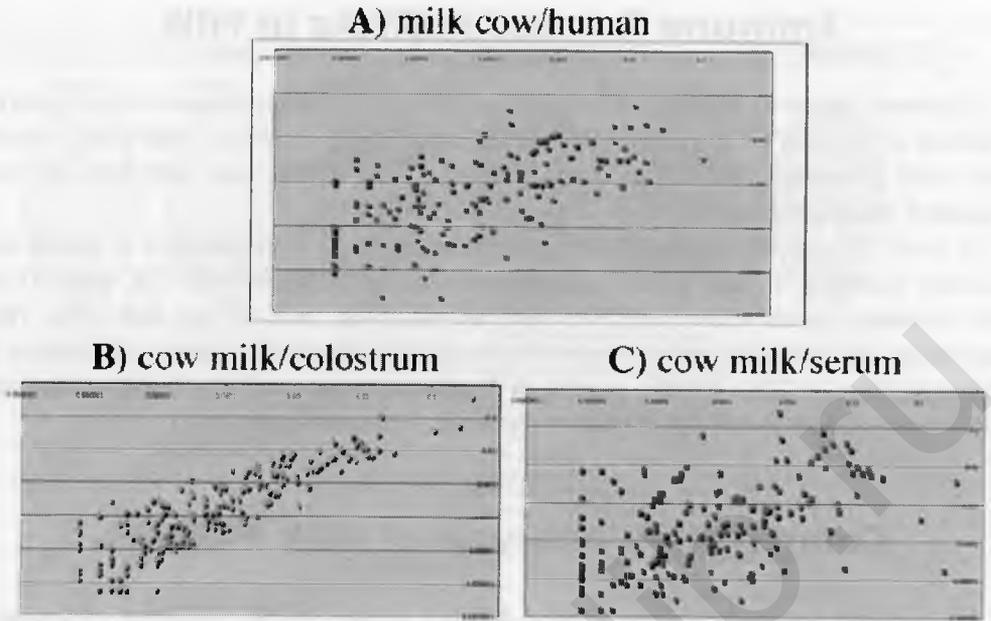


Figure 3. Comparative analysis of milk micro-RNA. 3a) miRNA levels of bovine versus human milk on the logarithmic scale. 3b) Bovine milk compared to bovine colostrum. 3c) bovine milk versus bovine serum.

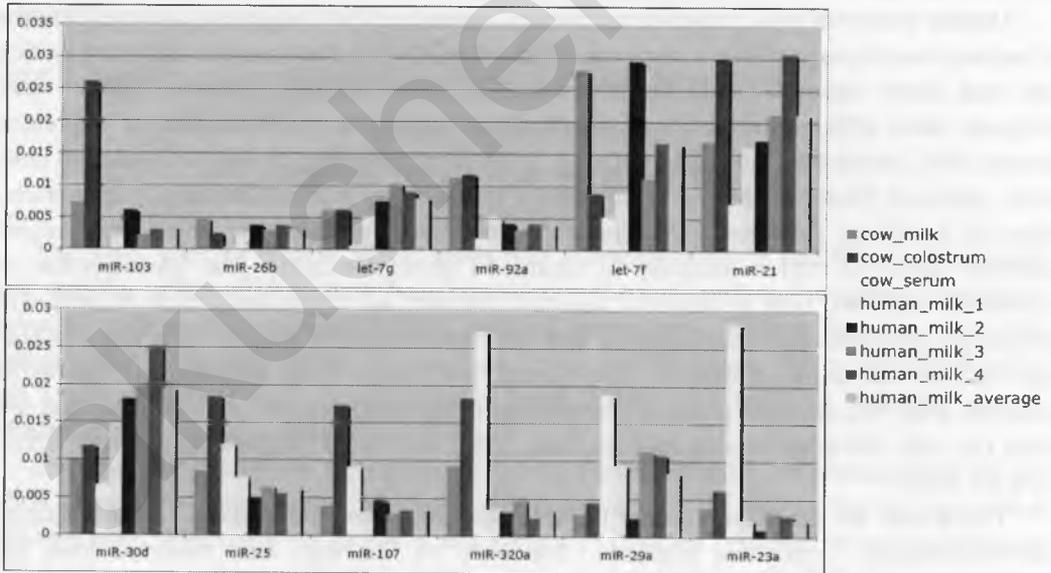


Figure 4. Milk content profiles of candidate micro-RNAs markers that are highly expressed at comparable levels in bovine and cow milk. Proportion of total miRNA in milk colostrum and serum from cow and in four human subjects plus their average.

In contrast to the relatively strong correlation ($R^2 \sim 0.9$) between cow colostrum and milk from the same dataset shown in figure 3b with the grouping of the data points along the diagonal, the comparison of human and cow milk does not show this trend as the data is spread on each side of the diagonal. If we compare cow milk and cow serum, also from the

same dataset as shown in figure 3c, we find a similar result with perhaps a larger spread of data-points, confirming a large difference in milk and serum miRNA content and, possibly, only a slightly better correlation between human and cow milk (R^2 0.35 versus 0.3). This analysis shows a large overlap of miRNA populations present in human and cow milk but a relatively poor correlation in apparent global expression levels between the species. However, it is always possible to identify a subpopulation of miRNAs present at concentrations within an arbitrarily close range. For example 64 miRNAs have concentrations with less than five-fold difference between cow and human milk, including 5 of the 7 miRNAs previously proposed as milk-enriched quality markers in cow studies (mir-26b, mir-21, mir-30d, mir-26a and mir-148a). 46 miRNAs are within a 3-fold difference, including four quality-markers candidates; mir-26b, mir-21, mir-30d, mir-26a. The most highly and consistently expressed miRNAs in this group can be identified as let-7f, miR-21, and miR-30d, representing each 1 to 2% of the total milk miRNA content, followed by miR-92a, miR-320a, miR-25, miR-103, let-7g, miR-26b, miR-107, miR-23a and miR-29a with expression level estimates between 0.1 and 1%. These miRNAs emerge as potential candidates for conserved, perhaps universal, milk markers. Normalized expression profiles of these miRNAs in cow milk, colostrum, serum and human milk exosomes are presented in figure 4. It is interesting to note that relative concentrations in serum and milk are very variable. MiRNA levels also vary between human individuals and often overlap between one of the individual human milk sample and cow milk. This shows that individual variation can also be relatively large and should be taken into consideration, as noted previously. Additional controlled studies in the same and other species will be needed to fully address this issue and allow the validation of conserved lactation candidates or universal milk markers. This will also allow further identification of specific milk miRNAs together with the influence of physiological, dietetic or environmental conditions on their expression in milk. Other studies are also needed to integrate miRNA and mRNA gene target expression datasets in order to further explore the potential benefits of miRNAs in milk. It is however becoming clear that the potential for miRNA research to open such a novel window of observation provides a new approach for the analysis of lactation offering exiting opportunities.

Food miRNA in Milk

It was recently reported that plant food miRNAs can be found in the blood of people and that plant food miRNA can regulate gene expression in the host, demonstrating that miRNA can be horizontally transferred between species through the digestive system (Zhang, Hou et al. 2012). These surprising results show the potential for the functional transfer of milk miRNA from the mother to the child. They also raise the logical question of whether or not plant food miRNA ingested by the mother may also be transferred into milk. To address this possibility, we have revisited published data on the sequencing of short RNA in human milk exosomes. A number of candidate plant miRNAs were identified at variable levels between individuals, including; MIR-159, a plant miRNA highly conserved from moos to flowering plants (Li, Li et al. 2011), at frequency 10^{-4} in 3 out of 4 human subjects, and MIR-168a, a major plant miRNA previously identified in human serum at comparable frequency 10^{-3} in 2 out of the 4 human milk samples (Zhang, Hou et al. 2012). This result supports the notion that

food miRNA may be transferred into milk. Additional experiments will be needed to confirm the true plant origin of candidate plant food miRNAs by chemical analysis. Contrary to animal miRNAs, plant miRNAs are commonly methylated on the 2' OH position of the ribose ring of the last 3' terminal nucleotide. This modification renders plant miRNAs resistant to oxidation and it is possible to use this property to confirm the plant origin of particular miRNAs (Zhang, Hou et al. 2012).

Lactation Diversity: A Treasure Chest for Milk miRNA biology

Lactation, an important characteristic of mammalian reproduction, has evolved for over 200 million years by exploiting a diversity of strategies across mammal lineages. Comparative genomics and transcriptomics experiments have now allowed a more indepth analysis of the molecular evolution of lactation and have emphasized the ancient origin of the essential components of the lactation system at the molecular level (Lefevre, Sharp et al. 2009).

We have also reported milk cell and mammary gland transcriptomics experiments revealing conserved milk proteins and other non-coding RNA components of the lactation system of monotreme, marsupial, and eutherian lineages, confirming the ancient origin of the lactation system and providing useful insight into the function of specific milk proteins in the control of lactation (Lemon and Bailey 1966; Nicholas, Wilde et al. 1995). These studies illuminate the role of milk in the regulation of mammary gland function and the regulation of growth and development of the young beyond simple nutritive aspects (Green, Griffiths et al. 1983). In a similar manner, comparative studies of milk miRNA deployed over a larger spectrum of mammalian lineages, promises to allow a more detailed analysis of the functional evolution of specific miRNAs involved across the lactation process.

Monotremes

Monotremes have diverged from other mammals at least 166 Mya and are regarded as the best living representative of early mammals with a more primitive prototherian lactation system. These egg-laying mammals are now confined to Australia and New Guinea and display a relatively long lactation cycle compared to gestation and egg incubation with reported changes in milk protein content. These animals excrete milk from a series of ducts opening directly on the surface of the ventral skin patch of the areola. Hatchlings are highly altricial and depend completely on milk as a source of nutrition during the period of suckling. Much of the development of the monotreme young occurs after hatching and before weaning and the role of the milk in this process needs to be examined. Genomics and transcriptomics approaches have recently enabled the molecular analysis of cells in milk from monotremes (Sharp, Lefevre et al. 2007; Warren, Hillier et al. 2008; Lefevre, Sharp et al. 2009). In a similar way, the study of monotreme milk miRNA may allow the identification of conserved miRNAs corresponding to the most ancient pathways of their biogenesis and function. We are currently collecting and testing milk from these animals to engage these studies.

Marsupials

Marsupials present one of the most sophisticated lactation programs yet described. These animals are found on the Australian and American continents and have diverged from eutherian about 140 Mya. After a short gestation, marsupials give birth to highly altricial young that are totally dependent upon the progressive changes in milk composition for normal growth and development during the extended lactation period (Tyndale-Biscoe 1987; Brennan, Sharp et al. 2007; Nicholas, Sharp et al. 2012). Under certain conditions, some species of macropods such as the tammar wallaby (*Macropus eugenii*) produce milk of different composition from adjacent mammary glands, a phenomena called concurrent asynchronous lactation, demonstrating the importance of local control of the lactation program including major shifts in the relative milk content in carbohydrates, lipids and proteins as well as the phase specific expression of major milk proteins (Nicholas, Wilde et al. 1995). Thus, the tammar wallaby (*Macropus Eugenii*), with an extensively studied lactation system, represent one of the most interesting model to analyse the variation of milk miRNAs during the lactation cycle in order to identify specific miRNA expression that may be correlated with the demand of the young or mammary gland development. Preliminary results from our lab indicate that tammar milk contains significant amount of miRNA and profiling experiments are ongoing.

Eutherians

Eutherians are the most common mammals and are the only mammals surviving on all continents except Australia and America. By contrast to monotremes and marsupials, eutherians have invested in extended intrauterine development of the young and produce a milk of relatively constant composition, apart from the initial colostrum. However, there are interesting adaptations of the lactation system within Eutherian species. For example, the pinniped family of marine mammals present the most diverse lactation strategies. There are three families of pinnipeds with very different lactation characteristics; phocids (true seals), odobenids (walrus) and otariids (sea lions, fur seals). The walrus has the lowest reproductive rate of any pinniped species, with extended lactation for at least 2 years. Phocid seals have adopted a fasting strategy of lactation whereby amassed body reserves of stored nutrients allow fasting on land during continuous milk production over relatively short periods (Oftedal et al. 1987). Finally, otariid seals have adopted a alternating strategy of short periods of copious milk production on shore, and extended periods of maternal foraging at sea while the young remain on shore (Bonner 1984). Thus, the study of milk miRNA in the pinniped family may allow the analysis of the role that miRNAs may have played in the rapid adaptation of the lactation system in this family. For example, preliminary studies have implicated mir-21 in the maintenance lactation of the fur seal during extended foraging periods (Buscara, unpublished).

Comparative approaches have started to allow a detailed analysis of the functional evolution of specific molecular components of lactation and rapid advances in genome sequencing of a number of mammalian species have provided invaluable resources for the comparative evolutionary analysis of genes and, more recently, miRNAs involved in

lactation. There is much to learn about the genetics of lactation from the rich natural resource of animal diversity through the comparative analysis of gene expression in a variety of lactating mammalian lineages. Non-invasive milk sequencing approaches will enable a still broader exploration of lactation diversity to facilitate a broader understanding of the role of miRNAs in the lactation process.

Conclusion

MiRNAs are established mediators of mammary gland development and have recently emerged as new and powerful biomarkers. Milk miRNAs provide a fascinating new way to look at the lactation process, creating novel opportunities for the bioinformatic analysis of milk and gain a unique and deeper insight into lactation and the role of milk. Milk is so far the body fluid found to have the highest concentration of miRNA and is a good source of natural exosomes. This observation alone should promote milk as a rich source of miRNA and rapidly raise the interest of lactation research and milk related industries. During the last two years, a number of reports have shown the great potential of miRNA to be used as biomarkers for milk quality, and suggested that miRNA are new signalling molecules for the immunoprotection or the development of the human infant. Rapid advances in miRNA sequencing combined with the natural resource of mammalian lactation diversity, provide a fantastic platform to address the role of miRNA during lactation. We have started to analyse milk miRNA in animal models with extreme adaptation of lactation, and are developing bioinformatics tools for the comparative analysis of gene expression (Church, submitted) or the creation of genome-wide miRNA target databases (Kumar, submitted) with associated data mining tools to mine a growing number of milk miRNA datasets for evolutionary conserved or newly adapted miRNA functionalities for innovative applications of milk miRNA.

A number of important questions on miRNA biology in the lactation process are only starting to be addressed. First, the distribution of miRNA in different milk fractions (whey, fat, cells, microvesicles and exosomes) and their biosynthetic origin need to be resolved. This will allow further understanding of milk miRNA biogenesis, and the development of more informative assays of the quality and the origin of milk and milk products. Preliminary studies have highlighted the major contribution of exosomes in milk miRNA content. Second, statistical analysis needs to be conducted to assess individual variability in relation to genetic variation. Third, more detailed longitudinal studies of how milk miRNA content may change during the lactation cycle are necessary. Preliminary reports in eutherians have revealed similarities and differences between colostrum and mature milk, but complementary studies in marsupial or monotreme species with large and gradual changes in milk composition will provide the most interesting models to study the link between milk miRNA and mammary gland or infant development. Fourth, the effect of physiological and disease conditions of the mother on milk miRNA composition need to be evaluated. This could lead to innovative assays to improve lactation and milk quality. Fifth, the passage of plant food miRNA into milk needs to be confirmed. Ultimately, proof for the direct role of milk miRNAs in mammary gland and infant development will most likely require the construction and analysis of transgenic lines of animals with modified miRNA expression. Finally the effect of purified

milk miRNAs and exosomes fractions in cell culture systems and other biological models should be evaluated further for the functional analysis of milk miRNA signals. Further research in these and other areas will rapidly result in the characterisation of the emerging role of miRNA as biomarkers and signalling molecules of the lactation process and beyond. Milk miRNAs may contribute to the apparent advantage of breastfeeding for the health of the young and the health benefit associated with the consumption of dairy products. The identification of milk miRNA signals would potentially provide new opportunities for the maintenance of good health, and the prevention and treatment of disease.

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Lactation: Natural Processes, Physiological Responses and Role in Maternity

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Abstract

Lactation is of paramount importance to the survival, development, and growth of mammalian species. The mammary gland development starts during fetal life. The ductal development is particularly associated with puberty, and the alveolar development is associated with proliferative activity in the luteal phase of the reproductive cycle and in the early stages of pregnancy that leads to formation of the milk secreting unit. Parturition and lactation are two processes that are closely coordinated. Profound changes in several key hormones, such as progesterone, estrogen, prolactin, cortisol, placental lactogen, and insulin, occur early in pregnancy and parturition. These prepare and assure milk production by the mammary gland after delivery. For milk ejection, a neuro-hormonal reflex leading to the contraction of the myoepithelial cells surrounding the alveoli is stimulated by the action the oxytocin. After ceasing the stimulus for lactation, involution by apoptosis leads to regression of the mammary gland to the quiescent non-lactating state. Besides these physiological responses, the hormones can also influence cognitive functions and maternal behavior. Lactation is also important for establishing an affective linkage between mother and baby. This review will focus on natural processes of mammary gland development, on the hormonal control of lactation and on the role of breastfeeding in maternity.

Keywords: Lactation, Mammary gland, Development, Physiology, Hormones, Maternity

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Introduction

The primary function of the mammary gland is to provide nutrition for the young in the form of milk protein and fat. However, there are other benefits that are provided by lactation, such as the provision of immune factors that are secreted into the milk, which provide protection from infection, and also the close contact that occurs between mother and infant during nursing, which might have developmental benefits.

Natural Processes of Mammary Gland Development

Mammary glands are a modified and highly specialized type of sweat gland (Pandya; Moore, 2011). The human breast is a dynamic organ that does not go through all developmental stages unless a woman experiences pregnancy and childbirth. The course of breast development can be described in distinct phases beginning with the fetal phase and progressing through the neonatal/prepubertal and postpubertal phases. Development of the breast can then proceed through a number of lactation cycles (pregnancy, lactogenesis and involution) (Gueddes, 2007).

The control of mammary growth is a complex process involving factors intrinsic to the gland (local control) or the whole animal (systemic control) as well as external influences such as environment, climate and diet (Knight; Peaker, 1982).

Fetal Development

During the fetal period, the development and growth occur in both sexes (Ross, Pawlina, 2005). At about five or six weeks' gestation, a thickened ectodermal ridge (milk line) is situated longitudinally along the anterior body wall from the axillary to the inguinal regions. During the 7th and 8th weeks of gestation, this structure disperses into individual lens-shaped placodes (one pair in humans and five in the mouse, for example) at the future sites of the glands (Figure 1). Each placode then develops into a ductal tree that is embedded into a fat pad, and an external nipple. Between the 10th and 12th weeks, epithelial mammary buds begin to develop as solid down growths of the epidermis into the underlying mesenchyme. The cells migrate as the result of reciprocal epithelial and mesenchymal interactions (McCave et al., 2010; Pandya; Moore, 2011)

In the human embryo, the mammary ridges regress as the embryo develops, except for small portions that persists in the pectoral regions (2nd– 6th rib), which forms the mammary glands. Supernumerary gland is rare, but may develop anywhere along the milk lines, these glands either mature into mammary glands (polymastia) or remain as accessory nipples (polythelia) (Gueddes, 2007; Robinson, 2007).

Each primary mammary bud originates several secondary mammary buds, which develop into lactiferous ducts (Figure 2). The parenchymal branching occurs from the 13th to 20th weeks. The canalization of these ducts is induced by placental sexual hormones. This process

continues until fullterm birth, when there are 15-19 lactiferous ducts formed. (Moore; Persaud, 2007; Gueddes, 2007)

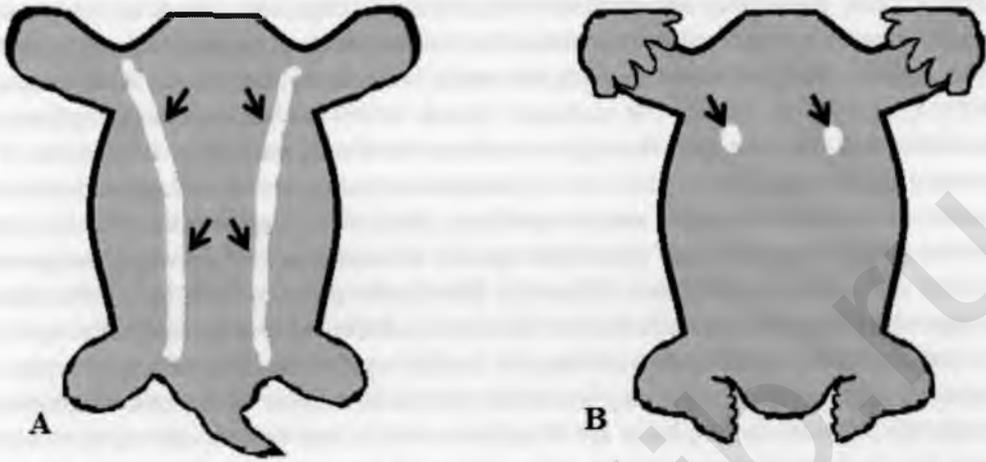


Figure 1. Diagram representing the development of mammary glands. A. ventral view of a 28 day embryo, showing the mammary ridge or milk line (arrows). B. Similar view of a six weeks embryo, showing a pair of mammary placodes (arrows).

Major lactiferous ducts develop, opening into a shallow mammary pit, which during infancy transform into a nipple. At birth the nipple is inverted and elevates above the skin during childhood. If this elevation does not occur, it gives rise to an inverted nipple. The fibrous connective tissue and the fat tissue of mammary glands, as well as the smooth muscle of nipple and areola, develop from the surrounding mesenchyme (Moore; Persaud, 2007; Pandya; Moore, 2011).

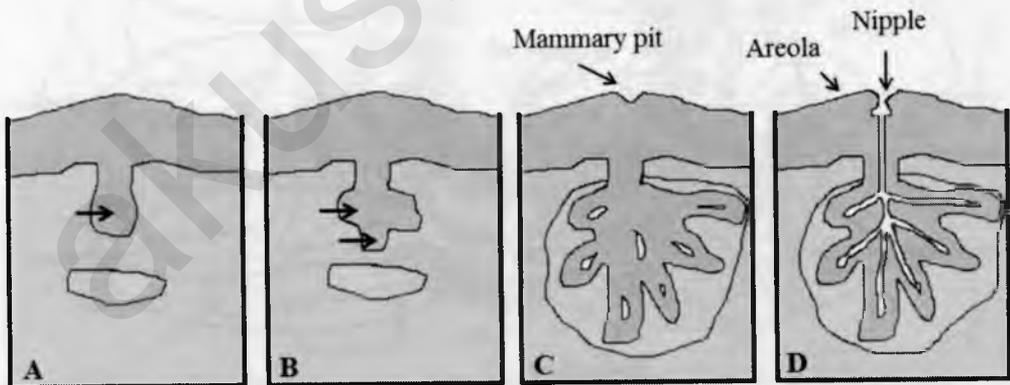


Figure 2. Overview of embryonic mammary development. Ectoderm (blue) invaginates through dermal mesenchyme (pink) in direction to the fat pad (yellow). Primary mammary bud (arrow) (A) originates several secondary mammary buds (arrows) (B). C and D: Canalization and formation of lactiferous ducts.

Neonatal and Prepubertal Development

The rudimentary mammary glands from newborns boys or girls are identical and usually enlarged. These glands may also produce some secretion. Popularly known as witch's milk, this secretion can be observed in most infants by the first week of postnatal life, and lasts for up to 6 weeks. This is attributed to the pro-lactation hormones present in the fetal circulation at birth. (Hovey et al., 2002). The mammary glands of newborns contain only rudimentary ducts with small club-like ends that regress within a short time after birth. Regression of the mammary gland usually occurs by 4 weeks postpartum and coincides with a decrease in the secretion of prolactin from the anterior pituitary gland of the infant (Hovey et al., 2002; Gueddes, 2007). The mammary gland is composed of numerous cell types that compose an epithelial and a stromal component (Figure 3). The ductal epithelium consists of two distinct cell types: luminal epithelial cells that line the ducts and lobules, and myoepithelial cells that form the contractile network surrounding the luminal epithelium. Separating the epithelial component of the mammary gland from the stroma is a layer of basement membrane. Immediately adjacent to this layer are fibroblasts, which interact with the epithelium and secrete factors that influence epithelial cell proliferation and migration. The adipocytes in the stroma comprise the major cell type of the mammary fat (Aupperlee, 2008).

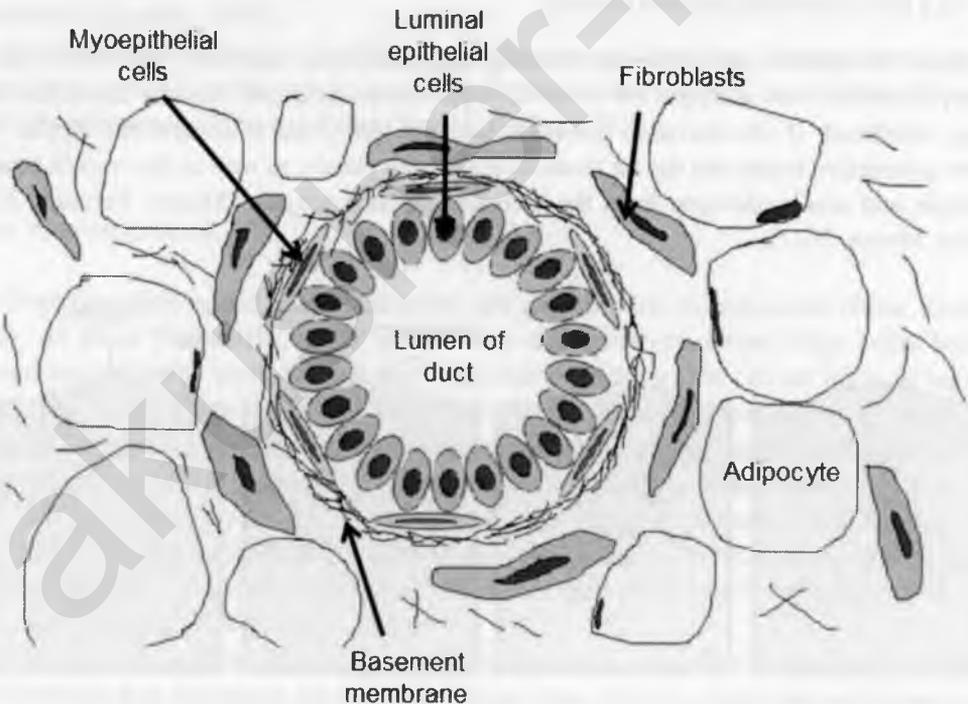


Figure 3. Mammary gland cell types. In this cross section of a mammary gland duct, the luminal epithelial cells line the lumen of the duct and are directly surrounded by a layer of contractile myoepithelial cells. The epithelium is separated from the stroma by a layer of basement membrane. Fibroblasts secrete the basement membrane and are located adjacent to the basement membrane. Adipocytes fill the majority of the fat pad. Not shown, but also present in the mammary gland, are cells found in blood and lymphatic vessels or nerve bundles and wandering cells of the immune system. Source: modified from Aupperlee, 2008.

Puberty

In man, there is little additional development of mammary glands after birth, remaining rudimentary. In women, the glands go through several changes during the pubertal phase. This development depends on the variations of ovarian hormones serum levels during each menstrual cycle, which induce changes in the morphology of the secretory portion of the mammary gland (Ross, Pawlina; 2005).

In the inactive gland, the glandular component is sparse being most constituted of ductal elements. During the menstrual cycle, the inactive breast goes through minimal cyclical changes. In the beginning of the cycle, the ducts appear as solid cords without lumen. However, at ovulation, the secretory cells become higher, the ducts are canalized, and there is an accumulation of small amounts of secretion. (Ross, Pawlina; 2005).

In general, little or no true lobulo-alveolar development occurs before first pregnancy. This period is essentially one in which a framework is laid down, within which the specialized secretory cells will be able to proliferate (Figure 4). At puberty, the increase in breast size is mainly caused by the increased deposition of adipose tissue within the gland. However, progressive elongation and branching of the ducts creates a more extensive ductal network. The major site of growth is the bud-like structures at the end of the ducts, and these form the terminal duct lobular units or acini (Gueddes, 2007). During the follicular phase of the menstrual cycle, the lobules are small, with few alveoli, and there is low mitotic activity. During the luteal phase, the lobules and alveoli develop with open lumens and mitotic activity is at its greatest. From day 27 to menstruation, these changes regress.

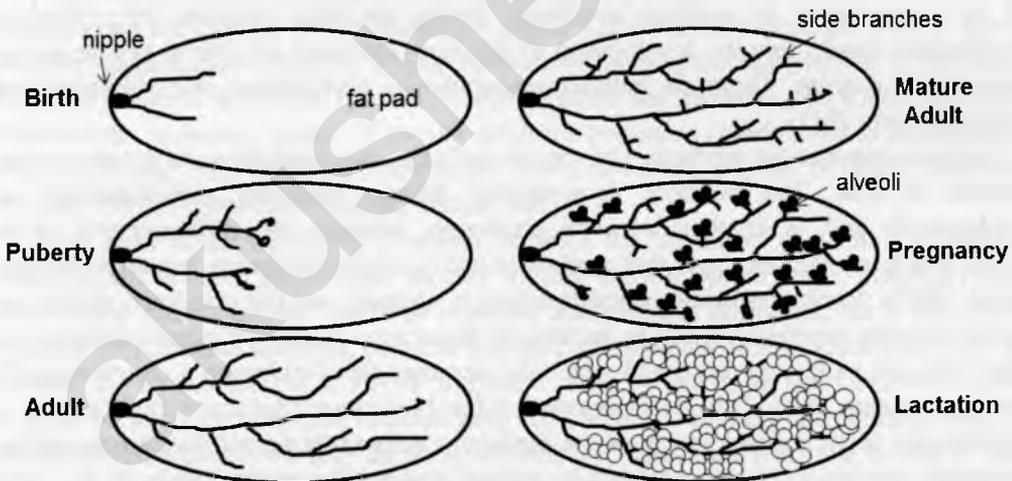


Figure 4. With the onset of puberty, ovarian hormones promote ductal development. In the adult mammary gland, the ductal epithelium has formed a network of primary, secondary, and tertiary ducts that extend to the limits of the fat pad. In the mature adult mammary gland, successive ovarian cycles induce the formation of side branches that increase in number over time. During pregnancy, the increased levels of hormones drive proliferation and alveologenesis. The mammary gland achieves full differentiation during lactation, when the mature luminal epithelial cells produce and secrete milk into the alveoli that travels through the ductal network to the nipple. Upon removal of the suckling stimulus, involution occurs and the mammary gland regresses to a pre-pregnant-like state. Source: Modified from Aupperlee, 2008.

However, the degeneration of the epithelial growth is not complete, and some of the follicular growth remains until the next cycle. With increasing years, there is a relative decrease in mitotic activity until about 35 years of age, when breast development plateaus (Geddes, 2007). Whereas the gross anatomical changes that occur during puberty have been well documented, the sequence of events at the cellular level is less well understood. Changes occur in both the epithelium and the stroma. In the stroma, there is an increase in the amount of fibrous and fatty tissue, with the adult nonlactating breast consisting of 80% or more of stroma. Indeed, the extension of ducts is preceded by proliferation of connective tissue, and the fatty tissue is thought to inhibit the growth of the epithelium. The ducts elongate and branch dichotomously (Howard; Gusterson, 2000).

Pregnancy, Lactation, and Involution

During the onset of pregnancy the breast completes development. At this time, the breast enlarges with increases in volume and density. Clinically, the breast enlarges, superficial veins dilate, and the nipple areola complex darkens. During the first trimester, the stromal elements of the breast (specially connective and fat tissue) are gradually replaced by the proliferating glandular epithelium. And plasma cells, lymphocytes and eosinophils infiltrate the connective tissue. Glandular cells proliferate through mitotic divisions; the ducts elongate and branch and the alveoli develop (Ross; Pawlina, 2005).

In more advanced stages of pregnancy, the alveolar development increases, the stromal proliferation declines and the breast size is increased due to the hypertrophy of secretory cells and the accumulation of secretion in alveoli. During the third trimester, the epithelium differentiation results into the development of secretory cells that are able to synthesize and secrete milk proteins. Oxytocin induces myoepithelial proliferation and differentiation. (Pandya; Moore, 2011)

Lactation requires the production of specific cells that can synthesize and secrete copious amounts of milk. The hormone progesterone induces extensive side-branching and alveologensis and, in combination with prolactine, promotes the differentiation of the alveoli, which are the structures that synthesize and secrete milk during lactation (Watson, Khaled, 2008). Involution occurs when lactation is weaned, and the glandular, ductal, and stromal elements atrophy resulting in decrease in breast size. Mammary gland involution is a highly complex multi-step process in which the lactating gland returns to a morphologically near pre-pregnant state. This developmental stage is characterized by a high degree of epithelial cell death, redevelopment of the mammary adipose tissue and tissue remodelling. Postpartum involution is characterized by wound healing-like events (Stein et al., 2007; O'Brien et al., 2012).

Menopause

During menopause the breast regresses and the ductal and glandular elements involute resulting in the breast predominantly containing fat and stroma. As well with aging, there is an overall reduction in the number of ducts and lobules. Over time, there is a progressive decrease in the fat and stromal elements resulting in breast shrinkage and loss of contour. The

suspensory ligaments of Cooper relax with time and eventually result in breast ptosis (Pandya; Moore, 2011).

Studies that were undertaken before the more regular use of oral contraceptives and hormone replacement therapy indicate that involution of the breast begins in the premenopausal period due to a decline in ovarian function. Regressive changes occur in both epithelial structures and in the stroma. Morphometric analysis has shown that the amount of epithelial tissue in the breast declines steadily from the third to the sixth decade, with a corresponding decline in the lobular volume, with no correlation between the amount of epithelium and the number of previous pregnancies (Walker, Martin, 2007).

The Hormonal Control of Lactation

Milk secretion is an important and essential hormonally regulated process of the reproductive cycle that reflects an integrated action of endocrine, neuroendocrine, and behavioral mechanisms. The physiology of lactation involves development of the mammary gland from the fetal to the adult stage, its development during pregnancy, and postpartum. In the adult, the lactation cycle can be divided into four consecutive stages known as mammogenesis, lactogenesis, galactopoiesis, and involution, all of which are characterized by strict hormonal control (Whitworth, 1988; Svennersten-Sjaunja; Olsson, 2005).

Studies *in vivo* in laboratory and farm animals have indicated that three categories of hormones are involved in the regulation of lactation: [1] hormones from the ovary (estrogen and progesterone), pituitary (prolactin and oxytocin) and the placenta (steroids and placental lactogen) that act directly on the mammary gland; [2] metabolic hormones such as growth hormone, adrenocortical steroids, thyroid hormones (specially triiodothyronine – T₃), insulin, and gastrointestinal hormones (gastrin, cholecystokinin and secretin) which also have direct effects on the mammary gland; [3] locally produced hormones (synthesized in the mammary gland) including GH, prolactin, parathyroid hormone-related peptide (PTHrP), and leptin (Forsyth, 1991; Svennersten-Sjaunja; Olsson, 2005).

From birth to the beginning of sexual maturity, the mammary gland is a rudimentary ductal system in most mammalian species. During the embryonic stage, mammary gland development doesn't depend solely on systemic and maternal hormones, but is also locally regulated by paracrine signaling between neighboring epithelial and mesenchymal cells. For example, the local secretion of PTHrP triggers a series of outside-in and inside-out positive feedback between epithelial and mesenchymal cells, so that mammary bud epithelial cells can proliferate and sprout down into the mesenchymal layer until they reach the fat pad to begin the first round of branching (Hens; Wysolmerski, 2005).

However, at the onset of puberty, drastic hormonal changes in the females, i.e. enhanced estrogen secretion, allow further development of the mammary ductal tree and alveoli (Rillema, 1994). The crucial role played by estrogen in mammary development was evidenced in rodents that underwent ovariectomy prior to puberty and showed inhibition of mammary growth, which was then restored by the administration of estrogen (Forsyth, 1989; 1991). Despite the importance of estrogen to initiate mammary development at puberty, it seems that its action depend on the presence of other hormones, such as prolactin, since the administration of estrogen failed to exert the expected effects on the development of the

mammary gland in hypophysectomized prepubertal animals, suggesting a synergistic involvement of the pituitary hormones (Rillema, 1994).

Furthermore, several growth factors, including insulin-like growth factor I (IGF-I), epidermal growth factor (EGF), mesenchyme-derived growth factor (MGDF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), pituitary-derived mammary growth factor (PMGF), the transforming growth factor α (TGF α) and the transforming growth factor β (TGF β), are believed to participate in the hormonal regulation of mammary development by probably facilitating the development of parenchymal and stromal tissues in the mammary gland through autocrine, paracrine and/or endocrine mechanisms (Daniel; Silberteiu, 1987; Rillema, 1994).

In non-pregnant females, the connective tissue and adipose tissue predominate over the glandular tissue while during gestation and postpartum the glandular tissue becomes gradually predominant. In the human, for instance, the first trimester of pregnancy is characterized by hyperplasia of ductal and secretory structures, resulting in an increase in the number of alveoli. Later in pregnancy, there is a marked hypertrophy of alveolar cells and a decrease in the relative amount of adipose and fibrous tissue. Within two days of delivery, the alveoli become distended with milk followed by the beginning of an intense activity. After weaning, most of the secretory elements undergo apoptosis and the gland regresses nearly to the prepartum state, showing an increasing predominance of connective tissue (Glasier; McNeilly, 1990; Neville, 2006).

Having established some aspects of the developmental stages of the mammary gland, the hormonal and paracrine regulation of proliferation and differentiation in pregnancy and during milk secretion will be addressed below.

Mammogenesis

Mammogenesis is the process of growth and development of the mammary gland that begins at puberty under the influence of estrogen and is completed during the third trimester of pregnancy (Beesley; Johnson, 2008). The endocrine control of mammogenesis has been studied in some mammal species, such as the rat and farm animals, in which an integrated participation of ovarian and adrenal steroids, GH and prolactin was shown to be necessary for the production of milk (Glasier; McNeilly, 1990). The effects of ovarian steroids were evidenced in *in vitro* cultures of breast tissue that responded to estrogen with ductal development and to progesterone with lobulo-alveolar proliferation (McNeilly, 1977). Furthermore, the injection of estrogen and progesterone were shown to be ineffective in hypophysectomized animals, suggesting the involvement of pituitary hormone(s) in mammary development during pregnancy (Daniel; Silberstein, 1987).

During early pregnancy of human females, the production of most maternal estrogen and progesterone occurs in the corpus luteum, whose function is maintained by placental chorionic gonadotropin (Saxena, 1983). After a period of 8 to 10 weeks of gestation, the placenta becomes the dominant source of estrogen and progesterone synthesis, converting precursors of fetal and/or maternal origin into these steroids (Simpson; MacDonald, 1981). Total estrogen excretion increases from 20 – 20.000 ng/day and combined with the uninterrupted and rising concentration of progesterone and prolactin cause the breast to exhibit increased water, electrolyte and fat content (Beesley; Johnson, 2008). The rising

plasma estrogen levels stimulate the ductal tree growth and the differentiation of the epithelial cells, acinar and myoepithelial elements. Much of the fatty tissue of the breast is replaced by the acinar-ductal system that becomes organized into functional lobular-alveolar-ductal units surrounded by hypertrophied myoepithelial cells. Additionally, estrogen stimulates the synthesis and release of prolactin from the pituitary gland, whose presence seems to be necessary for estrogen to exert its biological effects on the mammary gland. This was evidenced in hypophysectomized animals which showed little mammary gland growth when treated with estrogen and progesterone. The mammary response was restored, however, when prolactin (and growth hormone), along with estrogen and progesterone, was injected in hypophysectomized rats. During pregnancy the serum prolactin levels increase from approximately 19 ng/mL in the non-pregnant state to approximately 20 – 200 ng/mL at term (Rigg et al., 1977; Neville, 2001; Beesley; Johnson, 2008;).

In ruminants, estrogen also stimulates secretion of IGF-I from stromal cells of the udder and thereby causes growth of epithelial cells (Svennersten-Sjaunja; Olsson, 2005). Its biological effects on the mammary gland are predominantly mediated by the estrogen receptor α (ER α) and not by ER β (Clarke, 2000). ER α is found in the epithelial and stromal compartments of the mammary gland (Hovey et al., 2002), however, in the human (Bartow, 1998) and heifer (Capuco et al., 2000) stromal cells, ER apparently are not expressed. Moreover, the activation of ER α during ductal development has been shown to induce proliferation of murine mammary epithelium through stimulation of the expression of growth factors, such as IGF-I, which are also probably locally secreted by stromal cells (Forsyth, 1996; Hovey et al., 2002).

Progesterone is another important hormone for mammary growth, which, in the presence of estrogen and prolactin, stimulates acinar proliferation and inhibits lactose synthesis. The increased concentrations of estrogen and progesterone before delivery inhibit the secretory effects of prolactin on mammary alveolar epithelium (Beesley; Johnson, 2008). During pregnancy, progesterone stimulates the synthesis of a 65-kD pregnancy-specific mammary nuclear factor (PMF), which has been shown to bind to two specific sites of the β -casein gene promoter region and inhibit transcription of this gene. In fact, one molecular event involved in the initiation of lactation postpartum seems to be the decreased level of PMF due to loss of placental progesterone (Lee; Oka, 1992).

The physiological functions of progesterone depend on the expression of a progesterone receptor (PR) which is present in two isoforms (PR-A and PR-B) encoded by a single gene. PR-deficient mice are infertile and an experiment with ovariectomized prepubertal PR-deficient mice and wild-type mice treated with exogenous estrogen and progesterone showed that they both exhibited similar ductal morphology, whereas PR-deficient mice did not present the expected side branching development and alveologenesi under steroid treatment (Ollivier-Bousquet; Devinoy, 2005). Studies have demonstrated that PR is selectively localized in the mammary epithelium but not in the stroma, and its expression gradually decreases at the end of gestation until it is completely lost in the final phase of differentiation of the secretory epithelium (Lydon et al., 2000). Although progesterone is necessary for the transition from ductal to lobulo-alveolar morphology, estrogen (17 β -estradiol) indirectly stimulates lobulo-alveolar formation as well because it can also induce mammary PR expression via ER (Atwood et al., 2000)

Besides the sex steroid hormones, local growth factors have been shown to modulate survival and apoptosis of the mammary gland. Moreover, *in vitro* studies have demonstrated

that insulin is necessary for estrogen, progesterone and prolactin to stimulate the growth of mammary epithelial cells, whereas the specific effects of placental lactogen on mammogenesis have not yet been fully elucidated. It has been suggested that the human placental lactogen (also known as human chorionic somatomammotropin) may stimulate mammogenesis directly or by competitively inhibiting prolactin receptors in the mammary gland during pregnancy to delay lactogenesis until after delivery (Beesley; Johnson, 2008).

Lactogenesis and Galactopoesis

By the third trimester of pregnancy, the first milk, called colostrum and whose protein composition differs considerably from that of mature milk, appears in the acinar glands, reflecting the beginning of protein synthesis under the influence of prolactin. (Chettertor, 1978; Whitworth, 1988; Rillema, 1994; Beesley; Johnson, 2008). Upon parturition, the mammary gland has gone through a considerable lobulo-alveolar growth to the extent that the alveolar cells become biochemically differentiated, acquiring the capacity to secrete milk (Lamote et al., 2004). Before delivery, the mammary acinar epithelium is kept in a presecretory state until the abrupt fall in plasma estrogen and progesterone concentration. In the first few days postpartum (4 – 5 days), these hormones concentrations are less than normal follicular levels and the epithelium transition from presecretory to a secretory state is complete. At the time of delivery, withdraw of progesterone stimulates milk secretion and the initiation of this process is called lactogenesis while its maintenance promoted by suckling or milking is known as galactopoesis (Lamote et al., 2004; Beesley; Johnson, 2008).

The endocrine control of milk production during lactogenesis and galactopoesis is done by the lactogenic hormones prolactin and growth hormone, which are responsible for the transition from a proliferative to a lactating mammary gland. Distinction in hormonal requirements, however, can be made among different mammalian species. For example, in ruminants (cow, goat, and sheep) the influence of growth hormone dominates over prolactin during galactopoesis whereas in other species like rodents and humans the influence of prolactin dominates over growth hormone during galactopoesis as well as during lactogenesis. Studies have demonstrated that GH is not indispensable for lactogenesis in mice and humans, as growth hormone receptor (GHR) knockout mice and human dwarfs with mutations in either growth hormone or GHR can lactate (Rimoin et al., 1968; Zhou et al., 1997; Rosenbloom et al., 1999; Lamote et al., 2004). In rats and mice, the optimal combination of hormones for maintaining the synthesis of milk components is insulin, glucocorticoid, prolactin and thyroid hormones (Rillema, 2004). Indeed, *in vitro* studies with mammary tissues showed that insulin, corticoids and prolactin are essential to maintain milk production (Topper; Freeman, 1980) while cortisol replacement was necessary for maintenance of lactogenesis in adrenalectomized animals (Cowie; Lyons, 1959; Gross et al., 1980). Furthermore, high levels of prolactin does not seem essential for lactogenesis in all species because milk formation occurs in cows, with a delayed time course, when prolactin secretion is suppressed with bromocriptine, a dopamine analog that effectively prevents prolactin secretion and inhibits lactogenesis (Akers et al., 1981; Neville et al., 2001).

In the human female, the beginning of lactogenesis takes 2 – 5 days, a time necessary for complete secretory maturation of the acinar epithelium. Before parturition, the impairment of lactogenesis by progesterone seems to involve a competitive inhibition of cortisol binding to

an intracellular receptor, preventing its synergistic action with prolactin to initiate milk production. Therefore, with progesterone withdrawal after delivery, cortisol binding occurs and lactogenesis is allowed to proceed. Studies have shown that the administration of progesterone in the immediate postpartum period inhibits lactogenesis, but once the secretory maturation of the acinar epithelium is completed, progesterone is ineffective in inhibiting milk production (Beesley; Johnson, 2008).

Prolactin and cortisol are considered essential for lactogenesis whereas growth hormone, insulin and thyroxin appear to play facultative roles. Prolactin acts directly on the mammary gland by binding to specific receptors on the surface of the alveolar epithelium and activating various transcription factors (Lamote et al., 2004). The binding of prolactin to its receptor causes receptor dimerisation, activation of the Janus kinases (Jak2), phosphorylation of the receptor, phosphorylation and dimerisation of signal transducers and activators of transcription, Stat 5a and Stat 5b, followed by their translocation into the nucleus. It was demonstrated that the absence of protein kinase Jak2 in the mammary gland of Jak2-deficient embryos transplanted into wild-type recipient animals impaired mammary gland development (Shillingford et al., 2002). A study using mice deficient in prolactin receptor (PRLR) and GHR has shown that epithelial PRLR is required for development and milk protein gene expression during pregnancy, whereas stromal but not epithelial GHR is required for functional mammary development (Kelly et al., 2002; Ollivier-Bousquet; Devinoy, 2005). Upon binding to its receptor, prolactin stimulates the synthesis of messenger RNA (mRNA) molecules that participate in the production of milk proteins and enzymes. This is the case of casein, an important milk protein, whose synthesis is initiated by synergistic action of prolactin and cortisol (Beesley; Johnson, 2008). An earlier study, however, showed that prolactin also functions synergistically, but not independently, with insulin in stimulating casein mRNA accumulation in *in vitro* cultures of rat mammary tissues (Kulshi et al., 1983).

Several studies have shown that without prolactin, lactation does not occur. Animals that underwent hypophysectomy or exhibited postpartum necrosis of the pituitary gland, impairment of the hypothalamic-pituitary axis functioning, and ingestion of dopamine agonists, such as bromocriptine or L-dopa, did not lactate. Contrary to what occurred during pregnancy, prolactin levels are not maintained by estrogen. In fact, after delivery, there is a rapid decrease in prolactin concentration, followed by normal nonpregnant levels at approximately 7 weeks postpartum in both lactating and nonlactating mothers. Nevertheless within 15 minutes of nipple suckling during nursing, prolactin secretion takes place, peaking at levels of 100 – 200 ng/ml during the first week, 25 – 250 ng/ml during the second to fourth weeks, and less than 20 – 40 ng/ml thereafter (Beesley; Johnson, 2008).

During nipple stimulation, sensory receptors produce afferent impulses that reach the spinothalamic tract in the spinal cord, ending in the mesencephalon neurons. From this point, the impulses are transmitted to the hypothalamus, resulting in the inactivation of the prolactin-inhibiting factor (probably dopamine) and thereby allowing the secretion of prolactin.

In addition to releasing prolactin, suckling stimulation of nerve terminals in the nipple or teat activates impulses that travel to the neurosecretory units of hypothalamic paraventricular and supraoptic nuclei, stimulating the synthesis of oxytocin, a hormone also required for lactogenesis, which is then transported to the posterior lobe of the pituitary gland and released into the bloodstream (Lincoln; Wakerly, 1974; Whitworth, 1988; Crowley; Armstrong, 1992; Svennersten-Sjaunja; Olsson, 2005; Neville, 2006; Beesley; Johnson, 2008).

In the lactating woman, the release of oxytocin but not prolactin becomes a conditioned response that needs only visual stimulation or conscious thought (Beesley; Johnson, 2008). Actually, oxytocin can be released even before the onset of suckling and the spontaneous ejection of milk often occurs in response to associated stimuli such as the sound of the baby crying (McNeilly et al., 1983). Oxytocin binds to specific receptors on the mammary myoepithelial cells surrounding the alveoli, causing their contraction and ejection of milk from the lactiferous ducts into ducts and subalveolar sinuses of the breast to be then removed by suckling (Neville, 1983, Whitworth, 1988; Neville et al., 2001; Beesley; Johnson, 2008). Oxytocin was also shown to increase the extracellular calcium influx, which caused phosphorylation of the myosin light chain in rat mammary myoepithelium cells, thus facilitating contraction (Olins; Bremel, 1984, Neville, 2006).

Involution

Upon arrestment of the natural course of lactation, the mammary gland undergoes involution, exhibiting a gradual regression to return to a state of development slightly in advance of that which existed at the beginning of the first gestation (Lamote et al., 2004). Like other stages of lactation, involution is also under hormonal control. For instance, *in vitro* studies have shown that apoptosis of epithelial cells in the mammary gland is related to decreasing levels of prolactin, growth factor and IGF-I (Talhouk et al., 1992; Quarrie et al., 1996; Wilde et al., 1997; Svennersten-Sjaunja; Olsson, 2005).

Several factors appear to act as inducers of involution, such as weaning or cessation of milking, which at first leads to the interruption of the release of galactopoetic hormones. In consequence, milk stasis and reduction of milk secretion and gene expression responsible for milk production occur (Lamote et al., 2004). Another factor involved in mammary involution is the feedback inhibitor of lactation (FIL), which is believed to play a role in the decrease in milk synthesis and functional differentiation of secretory cells at milk stasis. In addition, FIL has been shown to exert an inhibitory effect on protein synthesis by directly interfering with casein and lactose production and by exerting an indirect effect on cell differentiation by inhibiting synthesis of lactogenic hormone receptors on secretory cells (Knight et al., 1998; Lamote et al., 2004).

In rodents, proteolysis of the extracellular matrix and loss of epithelial cells by apoptosis are events observed in the mammary gland as involution progresses. This response, however, may vary between species. For example, the speed of involution in ruminants is slower than that of rodents, and in bovine mammary gland there is limited mammary tissue regression even at the end of the dry period prior to calving. Furthermore, apoptosis and changes in gene expression are major events at four days after interruption of milking in rodents whereas minor changes are observed only seven days after cessation of milking in cows (Capuco; Akers, 1999; Wilde et al., 1999; Lamote et al., 2004).

In vivo experiments evidenced that systemic lactogenic hormonal levels, such as prolactin and growth hormone, drop when cessation of milking occurs. Prolactin is known to inhibit the pro-apoptotic insulin-like growth factor binding protein-5 (IGFBP-5) mRNA expression.

Therefore, the decreased concentration of prolactin favors the IGFBP-5 expression in epithelial cells and, as a result, more IGF-I is removed by IGFBP-5, preventing it from suppressing apoptosis and delaying involution (Wilde et al., 1997; Accorsi et al., 2002; Lamote et al., 2004).

Growth Factors and Lactation

Growth factors are proteins that are needed by many organisms for proper growth and development, which may act in an autocrine, paracrine, or juxtacrine manner. They bind to receptors on the cell surface, and thereby activate cellular proliferation and/or differentiation. They also function as growth inhibitors and may be involved in cell death. In the mammary gland, growth factor-related paracrine signaling has been found to be involved in embryonic development, ductal elongation during puberty, and differentiation of mammary epithelial cells in early pregnancy (Dunbar; Wysolmerski, 1999; Foley et al., 2001; Hens; Wysolmerski, 2005; Neville, 2006).

One group of growth factors known to play a significant role in the mammary gland is the epidermal growth factor family (EGFs), including EGF, betacellulin and epiregulin. They bind with varying degrees of affinity to the epidermal growth factor receptor (EGFR) and exhibit direct mitogenic effects. Studies in mice revealed that signaling by EGFR is critical for ductal development (Lamote et al., 2004). Besides EGF, another normal ligand for the EGF receptor is TGF- α . *In vitro* and *in vivo* studies have indicated that EGF and TGF- α are involved in normal and abnormal mammary development. For example, local implants of EGF have been shown to stimulate lobuloalveolar development in virgin mice deprived of estrogen and progesterone (Vonderhaar, 1987, Forsyth, 1991) and to restart duct growth in the growth-arrested mammary glands in ovariectomized mice (Coleman et al., 1988, Forsyth, 1991). TGF- α was also shown to exert similar effects and it is believed that both growth factors probably stimulate the synthesis of basement membrane components such as laminin and type IV collagen, whose synthesis hold up seems to prevent normal mammary development *in vivo* (Forsyth, 1991). An *in vitro* study with rat mammary gland evidenced that TGF- α stimulated epithelial cell proliferation both in the presence or absence of EGF (Lamote et al., 2004).

EGF is also found in human and mouse milks at higher concentrations than that in plasma whereas TGF- α concentration is much lower than that of EGF in human milk (Connelly; Rose, 1988; Read, 1998; Forsyth, 1991). Both EGF and TGF- α appear to be produced locally in the normal mammary gland, as evidenced in the mouse mammary gland, in which mRNA for EGF precursors were present at low concentrations in pregnancy and increased levels in lactation (Brown et al., 1989, Forsyth, 1991).

The insulin-like growth factor I and II (IGF-I and IGF-II), receptors (type I – higher affinity for IGF-I, and type II – higher affinity for IGF-II) and binding proteins (IGFBP-1-6) play important roles in the organism by stimulating growth and development. The synthesis of IGFs in the mammary gland has been reported in many species (Lamote et al., 2004) and both types of IGF receptors are found in normal mammary tissue from rabbits, sheep, cows, and breast cancer cell lines and human primary breast cancer (Forsyth, 1991). Moreover, the activity of IGF-I is controlled by IGFBPs, which display opposite roles, i.e. some induce while others inhibit the stimulatory effect of IGF-I (Lamote et al., 2004). For instance, it was

suggested that growth hormone normally stimulates synthesis of IGF-I and that prolactin optimizes IGF-I action by suppressing the actions of IGFBP-5, which is an inhibitor of the IGF-I action, playing a regulatory role, as mentioned earlier, in mammary involution (Svennersten-Sjaunja; Olsson, 2005).

IGF-I at physiological concentrations exert stronger stimulatory effects than IGF-II on DNA synthesis and/or increase in cell number in cultures of normal mouse, rat, bovine and ovine mammary epithelial cells (Forsyth, 1991). It has been suggested that IGFs are also involved in the functional differentiation of mammary cells (Prosser et al., 1990). Moreover, both IGFs are present in milk, being found in high concentrations in the colostrums of cows and pigs, falling drastically after delivery to lower than blood concentration (Malven et al., 1987; Simmen et al., 1988). However, in human colostrums and milk, IGFs concentrations are much lower and EGF appears as the major mitogen (Read, 1988).

In fact, evidence shows that IGF-I levels tend to decrease during gestation, being relatively low during lactogenesis and lactation, and exhibiting increased levels during involution (Evans-Storms; Cidlowski, 1995).

Several studies have indicated the local systemic action of substances that exert inhibitory as well as stimulatory effects on the normal mammary gland. For example, the members of the transforming growth factor β (TGF- β 1, 2 and 3) family have been characterized as local apoptosis inducing and growth inhibiting factors which have been found in the mammary gland of many species, such as the mouse, sow, cow and goat (Forsyth, 1991; Atwood; Ikeda, 1995; Plath et al., 1997; Motyl et al., 2001; Wareski et al., 2001). In the mammary glands of virgin female mice, TGF- β 1 inhibited ductal elongation without affecting the stroma (Daniel et al., 1989). This inhibitory effect is probably associated with an accumulation of type I collagen and chondroitin sulphate, two important components of the extracellular matrix present at the tip of the ductal growth points, which creates a locally nonpermissive environment for growth, resulting in cessation of DNA synthesis and regression of the end bud (Daniel et al., 2001; Silberstein et al., 1990). In the human, normal mammary epithelial cells obtained from reduction mammoplasties exhibited growth arrest and altered morphology in response to TGF- β 1 (Hosobuchi; Stampfer, 1989).

During gestation, TGF- β plays a role in alveolar development and functional differentiation, albeit it also inhibits secretion of milk proteins. The inhibition is interrupted though after delivery, allowing lactation to begin, while during the dry period, TGF- β appears to support remodeling of the mammary gland (Daniel et al., 2001). It has been demonstrated that TGF- β 1 expression increased in the virgin mice, however, during gestation, its expression declined, disappearing during lactation and increasing again during involution (Daniel et al., 2001). Similarly, both TGF- β 2 and TGF- β 3 were expressed at increasing levels in the ductal and alveolar epithelium during pregnancy, falling rapidly at lactation (Daniel et al., 1989; Nguyen; Pollard, 2000; Pollard, 2001). In addition, increased expressions of TGF- β 3 were observed at involution, most likely participating in epithelial apoptosis and remodeling of the gland (Nguyen; Pollard, 2000).

In summary, lactation is the result of complex and synergistic responses regulated by endocrine and neuroendocrine mechanisms that culminates in milk secretion. The hormones classically involved in mammary growth and development such as estrogen, progesterone, prolactin, growth hormone, adrenal corticoids, and triiodothyronine produce effects on epithelial cell proliferation at least in part through growth factors, which have been shown to modulate survival and apoptosis in the mammary gland (Figure 5).

During gestation, steroid hormones and placental lactogen are produced by fetoplacental lactogen unit followed by maternal prolactin release due to fetoplacental estrogen stimulation. They all promote structural growth of the mammary gland in preparation for lactation. After delivery, prolactin also plays an important role in the initiation and maintenance of milk secretion, although the induction of its release does not rely on fetoplacental estrogen stimulus but changes to neurogenic stimulus of suckling, which ensures that prolactin release mechanism remain responsive to future suckling stimuli. The suckling stimulus is also related to postpartum infertility, whose duration depends on nursing frequency and may reflect the lactational hyperprolactinemia effects on neuroendocrine mechanisms controlling gonadotropin secretion.

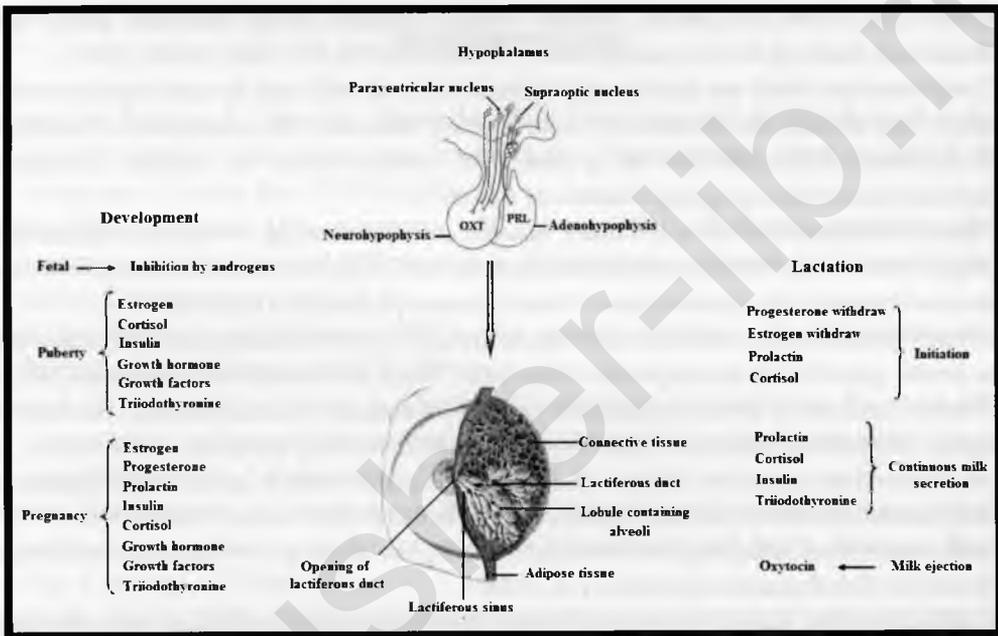


Figure 5. Hormonal control of mammary development and lactation. Postpartum, suckling of the nipple initiates impulses that reach the paraventricular and supraoptic nuclei in the hypophalamus, stimulating the posterior pituitary (neurohypophysis) to release oxytocin (OXT) and the anterior pituitary (adenohypophysis) to release prolactin (PRL).

Role of Breastfeeding in Maternity

Breastfeeding is the first relational behavior of the person, which deeply binds the baby to his mother (Bottorff, 1990). There are many emotional advantages that can be observed in this process.

The breastfeeding allows mother and baby to have a rich interaction, which facilitates the establishment of a link between both, leading to mutual satisfaction, thereby offsetting the rupture and the emptiness generated by the sudden separation during childbirth. The baby realizes that its manifestations (crying, facial expressions and babbles) are gradually understood by his mother. These repeated attempts favor the perception of itself as someone

confident, with an identity of its own and who is loved by the caretaker (de Bie et al., 2010; Quiqley, 2012).

Some studies show that when the baby licks the breast, the oxytocin released by mother provokes light somnolence, euphoria, increased pain tolerance and also increased love for the child (Klaus, 1998).

Breastfeeding is considered one of the pillars for the promotion and protection of the health of children all over the world. The superiority of human milk as a source of food, protection against diseases and affection, makes the experts from all over the world recommend exclusive breastfeeding. The World Health Organization (WHO) and the United Nations Children's Fund (UNICEF), stated that: "all women should be enabled to practice breastfeeding exclusively, and all babies should be breastfed exclusively from birth to four to six months age. After this period, children should continue being breastfed, along with complementary foods up to two years or more" (WHO & UNICEF, 2009, WHO, 2012).

Complementary foods are nutritionally inferior to breast milk and its early introduction in the baby's diet reduces the duration of breastfeeding, interferes the absorption of nutrients found in breast milk, and can be a source of contamination of children, increasing malnutrition rates in this age group (Saldiva et al., 2007).

There is evidence that there are many benefits of breastfeeding to women's health. It is confirmed by the lower risk of ovarian and breast cancer, of hip fractures by osteoporosis and also its contribution to the greater space of time between pregnancies (Rea, 2004).

Several epidemiological studies show the advantages of breastfeeding and of human milk for the health, growth and development of the child. Made in different socio-cultural realities these studies confirm the lower incidence or severity of illnesses such as diarrhea, bacteremia, meningitis, respiratory infections, otitis media, botulism, urinary infection, enterocolitis, as well as the possible protective effect of human milk against sudden infant death syndrome, insulin-dependent diabetes mellitus, Crohn's disease and allergic diseases (Rea, 1998).

Some researchers found that the breastfed infants grow faster at the beginning of life than the infants fed with formulas (Spyrides et al., 2008).

As already stated, health professionals agree that human milk provides the most complete form of nutrition for infants, including premature and sick newborns. However, there are rare exceptions when human milk is not recommended. The situations which breastfeeding is not advisable can be caused both by maternal and neonatal factors. One neonatal factor is if the infant is diagnosed with galactosemia, a rare genetic metabolic disorder. Among the maternal factors, are mothers who: have been infected with the human immunodeficiency virus (HIV); are taking antiretroviral medications; have untreated, active tuberculosis; are infected with human T-cell lymphotropic virus type I or type II; are using or are dependent upon an illicit drug; are taking prescribed cancer chemotherapy agents, such as antimetabolites that interfere with DNA replication and cell division; are undergoing radiation therapies; however, such nuclear medicine therapies require only a temporary interruption in breastfeeding (American Academy of Pediatrics, 2001).

Conclusion

In this chapter, three aspects of lactation are reviewed. First, the mammary gland development is characterized, since embryonic/fetal stage until adult, lactating and involuting gland. Second, the physiology of lactation is described including hormones and other factors involved in the control of mammary gland development and lactation. And finally, the chapter describes the role and importance of breastfeeding for babies and mothers, including the nutritional and protective actions, as well as, the importance of breastfeeding for the development of an affective bond between mother and baby.

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Wnt Signaling in Lactation: A Balancing Act

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Abstract

The mammary gland is unique in that most of its development and dynamic morphogenesis occurs postnatally in response to changes in the hormonal milieu. Multiple components of the Wnt/ β -catenin signaling pathway have been implicated in mouse mammary gland development. It is clear that the Wnt proteins themselves are important regulators of numerous stages throughout postnatal mammary gland development. Mouse models have been developed to analyze whether β -catenin stabilization, expression of pathway components, or expression of Wnt/ β -catenin target genes is sufficient to disrupt mammary gland development and lactation. In addition to the role of β -catenin in the Wnt signaling pathway, it is also a major component of the adherens junctions and as such, has a role in maintaining epithelial integrity, which is essential for lactation.

Multiple components of the Wnt pathway are known to have cross-regulation with other signaling pathways involved in the lactogenic phenotype. Furthermore, investigation into the regulation of Wnt pathway components has demonstrated that hormones, such as progesterone, can regulate components of the Wnt pathway. Through various mouse models of mammary gland development, it has become clear that the Wnt/ β -catenin signaling pathway is a critical regulator of normal mammary gland development. Interestingly, the specific roles of Wnt pathway regulators and components in lactation have given us insight to alterations that occur in breast tumor development.

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Introduction

The mammary gland is a unique organ that undergoes both embryonic and extensive postnatal development with most changes occurring in the postnatal phases as a response to alterations in the hormonal milieu. During puberty and estrous, ductal morphogenesis occurs in the mammary gland. However, most of the development takes place during pregnancy with a rapid proliferation of the mammary epithelium. Alveolar morphogenesis, including side-branching, occurs during pregnancy, and can be defined by multiple phases: proliferation, secretory initiation, and secretory activation. These phases of pregnancy-mediated mammary gland development are also defined as lactogenesis I (secretory differentiation) and lactogenesis II (secretory activation). The inactive alveoli are stimulated by the decrease in progesterone and sustained expression of prolactin to initiate active milk secretion. This leads directly to lactation, which is the functional phase of mammary gland development and is marked by the differentiation of these cells into milk-producing and secretory alveolar structures. Importantly, lactation is marked by the full differentiation of the mammary epithelial cells with minimal proliferation or apoptosis. Upon termination of lactation, the mammary gland reverts to a virgin-like morphology through the regulated process of remodeling and apoptosis in the involution phase of mammary gland development.

Lactational insufficiency can present as impaired alveolar differentiation, deficient synthesis and/or secretion of milk proteins, and deficits in letdown [1]. As such, there are a variety of manifestations of defective lactation in mouse models, though the most common are pup weight and survival, and milk protein production. Fostering experiments are particularly useful to assess whether there is a maternal or suckling deficiency with the mother or pups, respectively. Other parameters that denote a lactational defect include: mammary gland weight, which indicates growth during pregnancy and correlates to function during lactation; morphology of the mammary gland through either whole mount staining or histology (i.e., at the initiation of lactation, the epithelial cells flatten and the nuclei become more basal); the presence of adipocytes, which are typically lost during functional lactation; RNA expression of milk-related genes or a ratio of epithelial to adipocyte genes; and milk composition and volume [1]. To specifically address the gene expression profiles related to lactation, an elegant study by Rudolph, et al., used microarray analysis to look at gene expression changes in FVB/n mice during pregnancy, lactation, and forced weaning/involution. This work demonstrated that adipocyte-specific genes, collagens, lipid degradation genes, and proteasome components are downregulated during lactation [2]. As discussed above, this is indicative of the decrease in adipocytes in the mammary gland during lactation. There are also genes that are upregulated during lactation, and these include milk proteins, such as caseins, whey acidic protein (WAP), α -lactalbumin, West-mead DMBA8 nonmetastatic cDNA 1 (WDM1), and genes involved in lipid synthesis [2]. Strikingly, global alterations in the Wnt/ β -catenin signaling pathway were observed, with a late-pregnancy/early lactation increase of two pathway inhibitors, namely secreted frizzled-related protein 1 (SFRP1) and adenomatous polyposis coli (APC) [2, 3].

The Wnt/ β -catenin pathway is involved in embryonic and tissue development, stem cell maintenance, induction of the epithelial-to-mesenchymal transition (EMT), and the promotion of tumor initiation and progression. Because of these vast activities, the Wnt pathway is tightly regulated to prevent inappropriate activation. Briefly, the Wnt pathway is maintained

in an inactive state by the β -catenin destruction complex, comprised of APC, Axin, casein kinase 1 α (CK1 α), and glycogen synthase kinase 3 β (GSK3 β). Together, this complex regulates the phosphorylation of β -catenin, which is subsequently targeted for proteasomal degradation (reviewed in [4, 5]). Wnt/ β -catenin signaling can be activated through multiple components that act at specific points in the pathway, including expression of the Wnt proteins, downregulation of secreted Wnt inhibitors, stabilization of β -catenin, or inactivation of the destruction complex through mutation of any of its components, such as APC. Ultimately, pathway activation prevents β -catenin degradation and promotes its translocation to the nucleus where it binds to members of the T-cell factor/lymphoid-enhancer factor (TCF/LEF) family of transcription factors. The complex of β -catenin/TCF activates gene expression of multiple targets, including *cyclin D1*, *c-myc* and *Limb-Bud and Heart (LBH)*, the last of which was recently identified in the mammary gland [6] (for a complete list of target genes, see The Wnt Homepage at <http://www.stanford.edu/~rnuisse/wntwindow.html>).

The Wnt/ β -catenin pathway has been implicated in all stages of mammary gland morphogenesis, including both embryonic and postnatal development (reviewed in [7]). In this chapter, we will focus specifically on the role of the Wnt/ β -catenin pathway during lactation. We will first discuss components, including the Wnt proteins that are upstream of the destruction complex and have a role in the development and differentiation of the fully functional mammary gland. This will be followed by a review of the role of the destruction complex proteins in lactation. The role of β -catenin stabilization or loss of function will be reviewed separately, encompassing both its role in the Wnt/ β -catenin signaling pathway and its important function as a member of the adherens junction. Finally, the role of select Wnt/ β -catenin target genes in lactation will be examined.

Upstream of the Destruction Complex

While Wnt-4 expression is required for branching and alveolar development during pregnancy, Wnt-4 expression is lost during lactation [8, 9]. In fact, expression of most Wnt proteins (Wnt-2, -4, -5a, -5b, -6, -7b, and -10b) is decreased significantly or absent during lactation, suggesting that in fully differentiated mammary epithelial cells, Wnt is not required for the secretory function (10-14). Interestingly, while most Wnt protein levels drop to being undetectable during lactation, Wnt-6 maintains a low level of expression during lactation [12]; however, the functional importance of Wnt-6 expression in the mammary gland has not yet been delineated. Wnt-2 is expressed in virgin development but decreased at the onset of pregnancy, and absent during lactation [13]. The reduction of Wnt-2 expression coincides with reduced proliferation, suggesting that Wnt-2 may be required for mammary epithelial cell proliferation. Similarly, Wnt-5a and Wnt-5b are expressed during pregnancy, but are significantly downregulated during lactation [10, 12, 13]. As such, decreased Wnt expression could be used to identify the fully differentiated state of a lactating mammary gland [12].

Although Wnt expression is normally suppressed during lactation, it is clear that proper Wnt protein regulation is critical to the lactational phenotype. MMTV-Wnt-1 mice, expressing Wnt-1 in mammary epithelial cells, were not able to nurse their pups, as evidenced by lack of pup survival 24 hrs after birth. Pups were able to survive if they were fostered to

wild-type mothers, suggesting that the maternal influence was the critical determinant of pup survival [15]. A similar phenotype of decreased pup survival was observed in MMTV-Wnt-5a mice [16]. There was no effect on alveolar development or apoptosis, which eliminates premature involution as a possible explanation for the decreased pup survival. In addition, differentiation and milk production were normal. Interestingly, there was a failed responsiveness to oxytocin to regulate milk ejection, with a concomitant increase in p-cannexin 43, which closes the junction to prevent milk ejection [16], supporting a mechanism by which Wnt-5a mediates lactation. In contrast to the MMTV-Wnt-1 and -Wnt-5a mice, mammary-specific overexpression of Wnt-10b resulted in an early lactogenic phenotype during pregnancy, but with the retention of a functional mammary epithelium and the ability of the females to nurse their pups [11]. Mammary knock-out of Wnt-10b, however, results in decreased mammary ductal formation [17]. Collectively, these data suggest that the various Wnt proteins have distinct roles in the process of mammary gland development, and specifically lactation.

In accordance with the lack of Wnt expression during lactation, the Wnt antagonist SFRP1 is upregulated during lactation [2]. Mammary-specific overexpression of the related SFRP4 (MMTV-SFRP4) results in precocious lactation; however, their pups still exhibited a decrease in body weight early in life. Offspring were rescued in fostering experiments utilizing wild-type females [18]. These results demonstrate the complexity of the Wnt/ β -catenin signaling regulation specifically in the mammary gland, in that SFRP1 and SFRP4 appear to have contradictory functions during lactation.

The Destruction Complex

The β -catenin destruction complex is comprised of APC, Axin, GSK3 β , and CK1 α . While GSK3 β and CK1 α may influence mammary development in the phase of lactation, the majority of literature surrounding the destruction complex proteins in lactation have focused on APC and Axin. Similar to the Wnt proteins, there is temporal regulation of APC, as APC is upregulated during lactation [2, 3], suggesting that the *APC* gene may be under the control of lactogenic hormones. This appears to be physiologically relevant given that *Apc*^{Min/+} mice with germline heterozygous mutation of *Apc* have inhibition of lobuloalveolar development and have lactational defects [3]. In addition to the increase in mRNA levels, we observed an altered localization of APC protein during lactation from the basolateral surface in glands from pregnant and virgin animals to the apical surface of alveolar structures during lactation. Our observation that when the *Apc*^{Min} allele is backcrossed onto the FVB/n genetic background, the lactation phenotype is alleviated (unpublished observations), is consistent with the identification of modifiers that regulate mammary tumor development in different genetic backgrounds of the *Apc*^{Min/+} mouse [19]. It is possible that the same could be true of normal mammary gland development. Conditional loss of *Apc* in differentiated mammary epithelial cells using the β -lactoglobulin (BLG)-cre:*Apc*^{580S/580S} model demonstrated developmental dysfunction during virgin development, but also resulted in pups that did not thrive. It was further found that during lactation, there were multiple metaplastic nodules that increased with subsequent rounds of pregnancy and lactation [20]. Another model, WAP-cre:*Apc*^{CKO/CKO} mice showed an inability to properly nurse their pups [21], but the mechanism

was not further investigated. Taken together, these data support a role for APC in maintaining the lactation phenotype.

As mentioned above, we identified that *Apc* expression is temporally regulated with highest expression during lactation [3]. The expression pattern of another destruction complex protein, Axin, during normal mammary gland development is unknown. Doxycycline (DOX)-inducible overexpression of Axin in the mammary gland resulted in impaired lobuloalveolar development and premature post-partum apoptosis consistent with early involution [22]. Glands from the Axin mice contained minimal amounts of milk, suggesting that there was a deficiency in terminal differentiation of the mammary epithelial cells corresponding to a decrease in casein expression [22]. Interestingly, this phenotype in the Axin-overexpressing mice is similar to the phenotype observed with *Apc* mutation [3], suggesting that when the destruction complex proteins are altered in the mammary gland, the result is abnormal lactation. From these data, it is clear that the absolute level of destruction complex proteins is equally important to their presence or absence. It is also possible that Wnt-independent activities of APC and Axin contribute to these phenotypes. For example, the lactational defect in *Apc*^{Min/+} mice is not accompanied by robust Wnt pathway activity but is associated with defects in polarity and morphogenesis [3].

β-CATENIN

β-catenin is the major effector of the canonical Wnt pathway. Therefore, much attention has been given to the stabilization of β-catenin in normal mammary gland development. There are multiple models of N-terminal deletions resulting in stabilized β-catenin that will be discussed herein: overexpression of a mutant β-catenin by deletion of the first 89 amino acids (MMTV-ΔN89β-catenin); an N-terminal truncation, amino acids 1-57, of β-catenin specifically in basal epithelial cells (K5-ΔN57-β-catenin); and the MMTV-β-catenin-ΔN90 mice [23], which do not have a lactational phenotype and will not be further discussed. In addition to the transgenic models overexpressing mutant stabilized β-catenin, another approach is the conditional stabilization of endogenous β-catenin using the *Catnb*^{+Δex3} mice either crossed to WAP-cre (to induce stabilized β-catenin in differentiating alveoli) or MMTV-cre (to target the ductal epithelium).

MMTV-ΔN89β-catenin mice have precocious alveolar development and delayed involution [24], suggesting that stabilization of β-catenin is responsible for the differentiation that occurs during lactation. In fact the mammary glands involute to a mid-pregnancy appearance as opposed to the glands from wild-type animals that involute to a virgin morphology [24]. These β-catenin mutant animals are able to nurse their first litter, but their ability decreases with subsequent litters [25], most likely due to development of hyperplasia. Interestingly, the mechanism is unclear, as milk protein production was increased in mammary glands in the absence of pregnancy hormones and cyclin D1 [25]. To address whether stabilization of β-catenin could rescue the phenotype of progesterone receptor (PR)-null mice, crosses were generated of the two alleles [26]. Interestingly, in the presence of PR, the MMTV-ΔN89β-catenin cells were able to advance to lactogenesis I, which involves differentiation. In contrast, the absence of PR permits the MMTV-ΔN89β-catenin cells to

advance to lactogenesis II (secretory activation) [26]. In addition, the alveoli from PR^{-/-};MMTV-ΔN89β-catenin mice were significantly larger than those from the PR^{+/-};MMTV-ΔN89β-catenin mice. Furthermore, late milk protein transcripts were increased in the PR^{-/-};MMTV-ΔN89β-catenin mice, indicating an advanced stage of lactation [26]. These data demonstrate that while β-catenin is required for differentiation of the mammary epithelium, the hormonal milieu influences the role of β-catenin in the developing mammary gland. To assess the cell-type specific role of β-catenin, keratin-5 (K5)-ΔN57-β-catenin mice, expressing a stabilized β-catenin in the myoepithelial cells were generated [27]. These mice have persistent luminal cell proliferation during lactation, supporting crosstalk between the two mammary epithelial cell layers, and that the mechanism of β-catenin in this cell population is through enhanced proliferation. Inappropriate β-casein expression was observed during pregnancy suggesting a precocious lactation phenotype; however, there was no change in β-casein, WAP, or α-lactalbumin specifically during lactation [28].

MMTV-cre-mediated stabilization of β-catenin during puberty and pregnancy demonstrated that the females were unable to lactate due to squamous metaplasia filling the mammary gland [27]. This demonstrates that the suppression of β-catenin signaling is required for proper differentiation into secretory epithelial cells. In contrast, WAP-cre-mediated stabilization of β-catenin, which stabilizes β-catenin in the differentiating alveoli, results in transdifferentiation and the development of epidermal structures. Females are able to lactate with their first litter, but after multiple rounds of pregnancy and lactation, they are unable to lactate [27]. Furthermore these animals (WAP-cre;Catnb^{+Δex3}) display a spectrum of phenotypes with the primary difference being the degree of transdifferentiation [29]. In less differentiated tissues, STAT5 expression is maintained, leading to the development of functional alveoli and expression of milk genes. In contrast, in highly transdifferentiated tissues, STAT5 is absent and there is an increase in squamous-related cytokeratin expression [29]. Combined these data suggest that stabilized β-catenin in the mammary gland maintains a lactation-related gene expression profile, and that β-catenin turnover is required for proper differentiation.

In contrast to stabilizing β-catenin, another model uses mammary-specific expression of the β-eng dominant negative construct, in which the *Drosophila* engrailed repressor domain replaces the C-terminal region of β-catenin. This results in the inhibition of β-catenin signaling while retaining the cell-cell adhesion properties of β-catenin [30]. These animals show reduced lobuloalveolar development and fail to nurse their pups, suggesting that the β-catenin survival signal is required for lobular progenitors that later differentiate into alveoli. There was also a decrease in epithelial content during lactation with fewer alveolar structures. The pups died within 12 hours of birth with no milk in their stomachs, but were able to thrive when fostered to wild-type mothers [30].

As β-catenin has multiple binding partners aside from the interaction with the destruction complex, modulation of these proteins may also mediate mammary gland development. One example is the interaction of β-catenin with E-cadherin, α-catenin, and p120-catenin at the adherens junction. MMTV-H2E-cad, a transgenic model of mammary-specific over-expression of the cytoplasmic domain of E-cadherin that binds β-catenin, results in disruption of epithelial cell polarity, premature differentiation, and early milk protein synthesis ([31] and reviewed in [32]). Although no difference in litter size was observed, survival was reduced in

pups from transgenic females. The milk protein levels were equal, but triglycerides were low early in lactation [31]. While α -catenin loss is embryonic lethal, mammary-specific deletion of α -catenin results in a decrease in differentiation and milk production. Loss of α -catenin results in an involution-like morphology at parturition (33), consistent with either a disruption in apoptosis and remodeling or the lack of functional alveolar structures. Interestingly, p120-catenin null mammary epithelial cells are shed during early development allowing the gland to develop normally [34]. It is possible that if p120-catenin knock-out was complete, the gland would neither develop nor lactate [34]. Therefore, an inducible, conditional knock-out of p120-catenin may be informative to clearly define the role of p120-catenin during lactation. In addition to the well-characterized junctional components, there is a pool of the *fps/fes* protein tyrosine kinase that co-localizes with the adherens junctions.

Fps/fes is upregulated, with a concomitant increase in kinase activity, during pregnancy and lactation. Despite this, milk protein levels were not altered in *fps/fes*^{-/-} females; however, offspring from *fps/fes*^{-/-} mice were significantly smaller than those from the wild-type counterparts [35], indicative of a deficiency in the quantity of milk produced.

In addition to the adherens junction, both β -catenin and APC bind to the glycoprotein mucin 1 (MUC1). MUC1 normally is expressed apically in the lactating mammary gland, where it co-localizes with APC [3]. APC and MUC1 also co-localize in normal and tumor mammary gland sections from the MMTV-Wnt-1 mouse model [36]. Overexpression of MUC1 specifically in the mammary gland results in “atypical histology.” Interestingly, mammary glands from multiparous MMTV-MUC1 females have a failure to induce involution and dedifferentiation [37].

These data suggest that MUC1 overexpression results in a maintenance of the lactational state of the mammary gland. β -catenin and MUC1 interact in the lactating mammary gland as well as in tumors that develop in the MMTV-MUC1 mice [37].

Wnt/ β -Catenin Target Genes

As discussed above, activation of the Wnt/ β -catenin pathway results in β -catenin translocation to the nucleus where it binds to the TCF/LEF family of transcription factors to regulate target gene expression. These target genes are involved in normal functions, including development and EMT, as well as disease states, such as cancer development. A full list of Wnt/ β -catenin target genes can be found on the Wnt homepage (http://www.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes). Here, we will focus on the lactational phenotype of two well-characterized Wnt/ β -catenin target genes, *cyclin D1* and *c-myc*, and a recently identified Wnt/ β -catenin target gene in the mammary gland, *Limb-Bud and Heart (LBH)* [6].

Amongst other phenotypes, *cyclin D1*^{-/-} mice have a reduction in acinar structure and a lactation failure [38]. Mechanistically, cyclin D1 deficiency results in attenuated total and phosphorylated STAT5, which is a key transcription factor involved in milk protein expression. Both α - and β -casein and WAP, which are early and late milk products, respectively, are reduced with cyclin D1 deficiency [39]. Based on studies where cyclin D1 was knocked out in the MMTV- Δ N89 β -catenin model, it is suspected that cyclin D1 plays a role in the differentiation step of alveolar progression [25]. The β -catenin wild-type animals

with mutant cyclin D1 (*cyclin D1*^{-/-}) had decreased pup survival only during the first two litters [25]. Interestingly, loss of cyclin D1 accentuates the phenotype of stabilized β -catenin, such that the *cyclin D1*^{-/-}; MMTV- Δ N89 β -catenin females resulted in 100% pup death [25]. Given the severe phenotypes in the mammary gland associated with cyclin D1 ablation, it is evident that cyclin D1 is a key regulator of alveolar development.

Although *c-myc* is generally expressed at low levels during lactation, mammary glands isolated from WAP-cre;*c-myc*^{fl/fl} mice have slower proliferative rates and delayed differentiation [40]. In addition, the quantity of the milk available is decreased; however, the quality of the milk was not altered. Coinciding with the alteration in milk quantity in the WAP-cre;*c-myc*^{fl/fl} mice, females were able to nurse their pups completely when smaller litter sizes were used [40]. Overexpression of *c-myc* using the WAP-promoter results in incomplete alveolar development and decreased milk protein expression. Interestingly, epithelial cell proliferation is increased during pregnancy and remains elevated through lactation, suggesting that *c-myc* overexpression results in blocked differentiation and the inability to develop the secretory alveoli required for lactation [41].

Given that pups born to MMTV-*c-myc* females die shortly after birth [42], the importance of *c-myc* at multiple stages of mammary gland development was investigated using the DOX-inducible MMTV-*rtTA*;TetO-*c-myc* model. When DOX was administered during virgin development and pregnancy, the pup survival remained equal to that in MMTV-*c-myc* animals. It was further found that *c-myc* activation during days 12.5-15.5 of pregnancy prevents lactation through a mechanism involving precocious lactation and subsequent premature involution, via activation of Stat5 and Stat3, respectively [43]. These data indicate that the precise timing and level of *c-myc* expression is critical for normal mammary gland development and function.

LBH was recently identified as a Wnt/ β -catenin target gene in the mammary gland [6]. Interestingly, the expression of *LBH* mimicked that of the Wnt proteins in that *LBH* is expressed in virgin, pregnant, and involuting mammary glands, but is downregulated during lactation.

As with the lack of Wnt protein expression in the lactating mammary gland, this suggests that *LBH* is not expressed in fully differentiated and functional mammary epithelium and may be important in the regulation of proliferation and/or restructuring of the mammary gland [6].

Conclusion

Together, the data presented here support the model that there is a critical balance of both inhibitors and activators of the Wnt/ β -catenin signaling pathway to regulate mammary epithelial cell fate and differentiation. Expression of most activators of the pathway is reduced during lactation, whereas inhibitors of the Wnt/ β -catenin pathway are activated during lactation. Collectively, this model suggests the Wnt/ β -catenin pathway is required for the termination of proliferation during pregnancy and the maintenance of the fully differentiated and functional mammary gland.

Despite this working model, genetically engineered mouse models (GEMs) either overexpressing or conditionally inactivating some components of the Wnt/ β -catenin signaling pathway have demonstrated the overt complexity of this process. One specific example is that

the mutation of *Apc* and the overexpression of Axin, both critical components of the destruction complex, result in lactational insufficiency [3, 20-22]. Interestingly, even specific overexpression of the Wnt proteins, specifically Wnt-1 and -5a compared to Wnt-10b, result in different outcomes in lactational capacity [11, 15, 16].

In contrast, two models that mimic pathway inhibition, mutation of *cyclin D1* and DOX-inducible mammary-specific Axin expression have a similar phenotype of lactation failure [22, 38]. However, these are mechanistically different, in that the *cyclin D1*^{-/-} phenotype is a result of inhibited proliferation, and the phenotype downstream of Axin overexpression involves both decreased proliferation (through cyclin D1 loss) and an increase in apoptosis. Furthermore, it is clear that the timing of expression is critical to the role of various components of the pathway.

For example, the DOX-inducible *c-myc* overexpression model implies a very specific, 72-hour timeframe during pregnancy in which *c-myc* expression attenuates the lactation potential [43]. Finally, as demonstrated by the studies of β -catenin stabilization, the cell type in which gene expression occurs also significantly impacts the function in regulating mammary gland development [24-29].

As we have gleaned much information from mouse models over the years in regards to the role of Wnt/ β -catenin pathway components, there are still details to be teased apart, including the timing, cell-type specificity, and the role of individual components all need to be investigated further.

Lastly, it is apparent that in the breast the importance of β -catenin is two-fold in that it drives the Wnt signaling pathway, and also is a critical component of the adherens junction.

Both of these functions of β -catenin are important in the process of mammary gland functional development. In addition to the *in vivo* GEM models described here, *in vitro* models of mammary gland differentiation have proven to be useful in the dissection of the molecular mechanisms involved in these processes.

For example, *in vitro* assays have shown that Wnt-3a blocks differentiation in response to prolactin, insulin, and glucocorticoids [44]. SFRP4, which is normally only expressed during involution, prevents the Wnt-3a inhibition [44].

A combination of both *in vivo* and *in vitro* approaches will aid future dissection of the Wnt/ β -catenin signaling pathway, as well as other signaling molecules, in mammary gland development and specifically in the differentiated state of lactation.

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Chapter VI

Ultrasound during Lactation

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Abstract

Breast milk is the 'gold standard' of infant nutrition providing not only nutrition for optimal growth but immune protection as well. Many women initiate breastfeeding however few continue to breastfeed for the recommended 6 months (WHO). Management of lactating women is predominately experience-based therefore lack of diagnostic tests and evidence-based treatment is likely to contribute to early weaning. Ultrasound imaging is not routinely used as a diagnostic tool during lactation however new research suggests that is a promising modality capable of identifying both breast and infant sucking pathologies. Imaging of the non-lactating breast is well established however little imaging is performed during lactation.

Ultrasound during lactation is relatively simple provided settings are optimized to accommodate the increased amount of glandular tissue. Furthermore an understanding of the growth of the breast during pregnancy and changes during lactation as well as lactation pathology enhance diagnoses. Ultrasound can also be utilized to confirm normal function of the lactating breast. While sufficient milk must be synthesised for the optimal growth of the infant it must also be released during breast feeding or breast expression by the milk ejection reflex.

Increasing duct diameter and visualisation of milk flow at milk ejection confirms that the reflex is intact. A successful lactation depends upon the infant's ability to remove milk from the breast. Infant tongue action can be visualised during both breast and bottle feeding. Recently this technique has been employed to assess infants with oral anomalies such as ankyloglossia.

It can also be applied to the infants of mother experiencing pain during breastfeeding to determine if compression of the nipple is a contributing factor. Ultrasound techniques have also been developed to image swallowing in both breast and bottle fed infants but have not yet been used extensively to identify swallowing pathology.

Introduction

The composition of breastmilk is tailored for the optimal growth and development of the human infant. Furthermore breastmilk contains enzymes and other factors that facilitate the digestion and absorption of nutrients. For example lactoferrin is a multifunctional protein in milk that both assists with the absorption of iron as well as protects the infant from infection via an antimicrobial action. Breast milk confers many benefits to the infant in both the short term; protection from infection, development of the infant gut and long term; decreased incidence of obesity, type 2 diabetes, asthma and some cancers however it also has many maternal health benefits. Maternal advantages include contraction of the uterus after birth, quicker return to pre-pregnancy weight as well as long term affects such as decreased risk of osteoporosis, breast and ovarian cancer. The understanding of the importance of early infant nutrition by women is confirmed by the high numbers of women who initiate breastfeeding. Unfortunately many women do not experience a successful lactation with only a small proportion continuing to breastfeed for the recommended 6 months (WHO). Treatment of lactation difficulties is largely experience-based with few diagnostic tests at the clinician's disposal.

While ultrasound imaging is a common diagnostic test for numerous pathologies it is not routinely requested during lactation. New research, however, suggests that diagnostic ultrasound may be beneficial for particular lactation pathologies.

Breast Imaging

Imaging modalities are constantly evolving allowing the improved visualisation of breast anatomy and pathology. Breast assessment of the non-lactating breast in particular has improved in the universal areas of mammography and ultrasound and the addition of Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) is often employed in difficult cases to gain extra information. In contrast little attention has been focused on the lactating breast during both normal and abnormal presentation. Several considerations of the lactating breast should be made when choosing an appropriate imaging modality. The lactating breast is not well suited to mammography due to both predominance of glandular tissue and the secretion of breast milk [1] that subsequently increases the radio-density making interpretation difficult [2]. Ductograms (radiographs taken after the injection of radio-opaque contrast media into a nipple duct) do not illustrate the ductal system in its entirety and only few studies have examined lactating women. The invasiveness of this procedure risks the introduction of pathogens into the breast and thereby increasing the possibility of mastitis making an inappropriate method for the lactating breast.

To date both Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) have had little to offer in elucidating pathology other than breast cancer in the lactating breast [3].

However it is likely these modalities may provide more useful information in the future. In the past ultrasound investigation of the lactating breast has been limited for the same reasons as mammography; increased density of glandular tissue and the accumulation of milk [4]. More recently, however, malignancies have been confirmed during pregnancy and lactation with both mammography and ultrasound [5].

Ultrasound has undergone enormous technical advances over time that have improved the resolution of the images dramatically thus allowing imaging of very small structures within the breast. Ultrasound has the added advantage of being non-invasive and consequently allow the breast to be examined without distortion.

It follows that ultrasound should be the initial modality of choice for investigation of the lactating breast[6] however this requires a sound knowledge of breast anatomy and pathology as well as development of imaging techniques unique to lactation.

Breast Anatomy

Text book descriptions of the gross anatomy of the human mammary gland are based on Cooper's dissections of the breasts of women that died during lactation [7]. The breast is comprised predominantly of secretory and adipose tissue supported by Cooper's ligaments. The secretory tissue is comprised of alveoli that are grouped into lobules and lobes. The ductal system drains the alveoli with the ducts converging into larger ducts culminating in a main milk duct prior to entering the nipple. Conventional texts describe the main milk ducts beneath the areola as 15 to 25 expanded 'sac-like' structures (lactiferous sinuses). Recently studies of the lactating breast using high-resolution ultrasound [8] showed fewer main ducts (mean 9; range 4-18) which is in agreement with observations during pumping [9] (mean 5; range 1-17) and the dissection of a lactating breast (4 patent ducts) [10]. Interestingly Cooper found 7-12 patent ducts in a cadavers of a women that had died during lactation although he could cannulate up to 22 ducts [7].

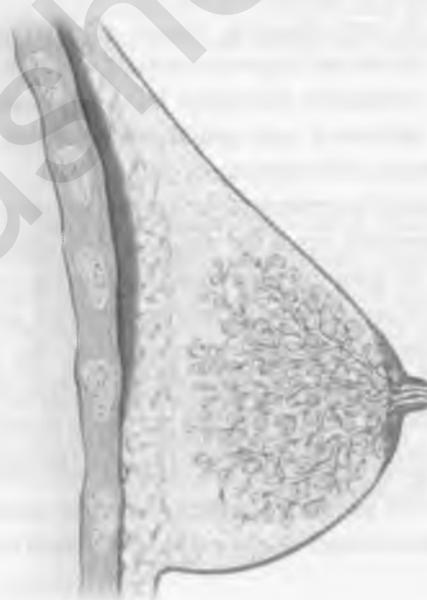


Figure 1. Anatomy of the lactating breast illustrating relatively small main milk ducts that branch rapidly under the areola.

In addition the typical sac like 'lactiferous sinus' was not observed. Instead branches draining glandular tissue immediately below the nipple often merged into the main collecting duct (average 2mm in diameter) very close to the nipple (Figure 1) [8]. An additional study showed that the milk ducts in the lactating breast distend substantially at milk ejection [11] suggesting the primary role of the ducts is the transport of milk rather than storage of large volumes of milk. It is often assumed that the lactating breast is comprised predominately of secretory tissue (glandular) and indeed ultrasound observations made throughout pregnancy confirm the growth of secretory tissue although 20% of women at 6-12 weeks gestation have proportionally more adipose tissue in their breasts [12]. There is currently no simple method of quantifying the volume of a particular tissue in the breast however a semi-quantitative measurements made with ultrasound in Caucasian mothers showed the ratio of secretory and adipose to be 2:1 in the lactating breast. However, it is notable that there is a wide variation between women with some having up to half of the breast comprised of adipose tissue in and others up to eighty percent of the breast was composed of glandular tissue[8].

Ultrasound of the Lactating Breast

Ultrasound Equipment and Settings

The highest resolution ultrasound imaging is critical for accurate examination of the breast. In particular the near field should be optimized in order to detect very superficial structures and pathology. An electronically focused linear array with a frequency of 7-12 MHz with multiple focal zones is appropriate for this examination [13].

However in the case of large lactating breasts a 5MHz probe may be useful to both increase penetration of the breast and improve focusing at depth. Other available features that will improve imaging are continuous electronic focusing, broad bandwidth and short pulse width. In addition coded harmonics and spatial compounding improve contrast resolution providing more detailed images of the structures of the breast.

Ultrasound settings should be optimised to the individual woman. The slope of the time compensation curve is dependent upon the volume of secretory tissue in the breast. Care should be taken to not set the gain too high thereby reducing the visualization of both small structures such as milk ducts and the discrimination between adipose and glandular tissue or set too low a gain thereby altering the appearance of the fat to anechoic rather than hypoechoic. One or two focal zones appropriately positioned will improve resolution of the image. The power setting should be high enough provide a complete view of all of the tissues of the breast from the skin to the pectoral muscle [6, 13]. This may need to be increased in the case of very dense or large breasts [14].

Scanning Technique

A combination of patient positions may be useful to image the lactating breast. The routine posterior oblique position serves to even out the breast and bring the internal structures more parallel to the ultrasound beam thus reducing artifacts. However the upright

position may be utilized to examine the entire breast in women with very large breasts. During scanning moderate compression of the breast with the transducer scanning is commonly employed to improve image resolution and visualize the deeper portions of the breast [14, 15] however milk ducts in the lactating breast collapse under mild/moderate pressure. Therefore if the ductal system is to be examined only enough compression for good contact should be used.

Palpable abnormalities of the breast require targeted ultrasound regardless of whether the patient is lactating or not. Furthermore comparison of the corresponding area in the contralateral breast may be useful. Should no changes in the breast tissue be detected further investigation using either CT or MRI imaging may be useful to exclude malignancy. Scanning planes used to examine the entire breast include dividing the breast into quadrants and performing transverse and longitudinal scans in each quadrant. Radial and antiradial scans can be employed to interrogate the ductal anatomy in the nipple-areolar region and this approach is becoming increasingly popular, as it encourages a more anatomical approach to the ductal system of the breast [2, 14, 16].

Special care should be taken in the nipple areolar region where distortion of the ultrasound beam may cause posterior shadowing obscuring the tissue behind the nipple and areola (3,17). The use of multiple scanning angles and approaches and the application of extra gel will ensure this area is thoroughly assessed. Grading of the proportion of adipose and secretory tissue is subjective and ranges from 1 to 4 with grade 1 representing a breast comprised of mainly adipose tissue and grade 4 predominantly secretory tissue. It is assumed that the lactating breast is composed mainly of secretory tissue due to extensive proliferation in pregnancy however Ramsay and colleagues (ref) have shown a similar variation in lactating women with a range in adipose tissue comprising from half of the breast to very little of the breast. This finding also supports the concept that the size of the breast is not reflective of the amount of milk produced or stored within the breast.

Nipple

Examination of the nipple may be necessary in women where the obstruction of milk flow is suspected. For example nipple piercing, nipple/breast surgery for inverted nipples and breast reduction may result in scarring or severing of milk ducts leading to a reduction in milk supply due to the inability to remove substantial volumes of milk from the breast. The nipple tends to increase in size during pregnancy and lactation and therefore is easier to scan compared to women that are not lactating. The aim is to orientate the nipple ducts so that they are perpendicular to the ultrasound beam to improve visualization.

This can be achieved with the mother in the upright position or by supporting the underneath of the nipple with one or two fingers and scanning the top side of the nipple [14]. Warm gel is necessary to avoid contraction of the nipple muscle and facilitate scanning.

Normal Ultrasonic Appearances of the Lactating Breast

The structures of the lactating breast display similar ultrasonic features as the non-lactating breast with some exceptions (Table 1).

Adipose Tissue

Generally the adipose tissue is hypoechoic and occasionally isoechoic with respect to the hyperechoic glandular tissue. The subcutaneous fat appears as a hypoechoic layer beneath the echogenic skin lines.

The amount of fat interspersed between the secretory tissue is highly variable with larger breasts generally comprised of more fatty tissue than smaller breasts although the proportion of fatty tissue increases with age. The retromammary fat appears as a hypoechoic layer above the pectoralis muscles which have a fibrillar pattern (Figure 2).

Table 1. Ultrasonic appearances of the structures of the lactating breast (adapted from Geddes 2009)

Structures of the Lactating Breast	Lactating Breast	Measurements
Adipose tissue	Hypoechoic, variable Large breasts often contain a large proportion of adipose tissue Located in subcutaneous, retromammary and secretory regions	Mean adipose to secretory tissue ratio 2:1
Secretory tissue	Hyperechoic, echogenicity varies according to volume of milk in the breast	As milk is synthesized the depth of the tissue increases
Milk Ducts	Hypoechoic Echogenic flecks within the ducts correspond to milk fat globules Echogenic walls may be visible Easily compressible	Resting state – 2mm (1-10mm) Distend at milk ejection
Nipple	Mid grey echogenicity compared to secretory tissue Nipple ducts hypoechoic tubular structures	Nipple ducts range from <1mm to 2mm
Skin	Hyperechoic Thicker in the areolar region	
Coopers Ligaments	Hyperechoic	
Lymph nodes	Hypoechoic periphery with an echogenic hilum	
Arteries and Veins	Hypoechoic	

Secretory Tissue

The echogenicity of the secretory tissue increases as more milk is synthesised and increasing volumes are stored within the breast. Milk synthesis is continuous and therefore between breastfeeds/expressions the breast fills with milk and becomes more firm.

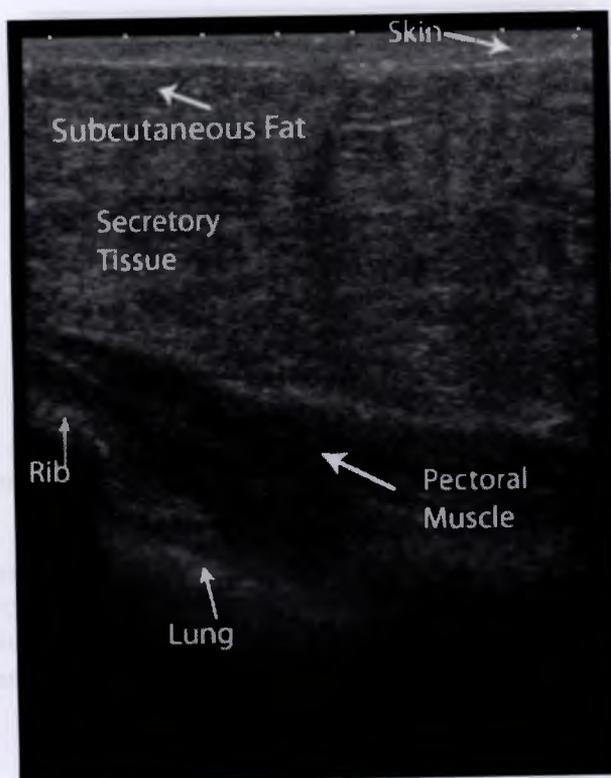


Figure 2. Ultrasound image with depth adjustment to show the secretory tissue. This lactating breast is composed of predominately secretory tissue with little subcutaneous fat.

When large amounts of milk are stored in the breast both breast size and tension may limit the compression that can be exerted with the transducer impeding adequate penetration of the breast. In these cases it may be necessary to partially empty the breast by asking the mother to either breastfeed or express milk to improve imaging. Each of the lobes of the breast are unable to be recognised as distinct entities due to their intertwined nature [17], however, the pattern of glandular tissue is observed more clearly by ultrasound than by mammography in lactation [18].

Milk Ducts

In the non-lactating breast ducts appear as small (2-3mm; range 0.6 to 4mm) hypoechoic branching structures that are generally non-compressible [14]. Colour Doppler Imaging is a useful tool to distinguish ducts from vessels and highlight any vessels within an intraductal lesion. The milk ducts of the lactating breast are also small hypoechoic branching structures (2mm; range 0.9 to 10mm) that branch close to the nipple (Figure 3) and are easily compressed (Figure 4).

The main milk ducts do not display large sinus like reservoirs beneath the nipple [8]. Furthermore they do not store large amounts of milk (2.5 mL; range 0-30mL) instead at milk ejection the ductal system expands to accommodate milk flow.

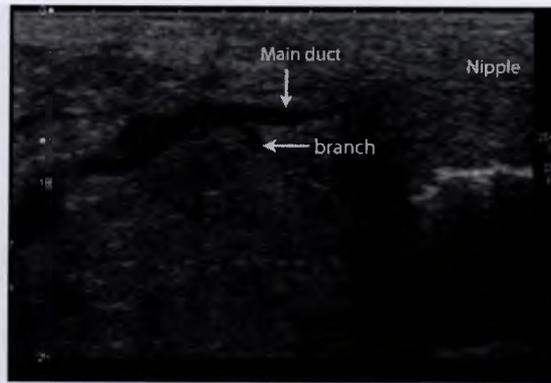


Figure 3. Ultrasound image displaying a milk duct in the lactating breast. Note the main branch is small (1.18mm) in its resting state (pre milk ejection). The first branch is identified 3.5mm from the base of the nipple.

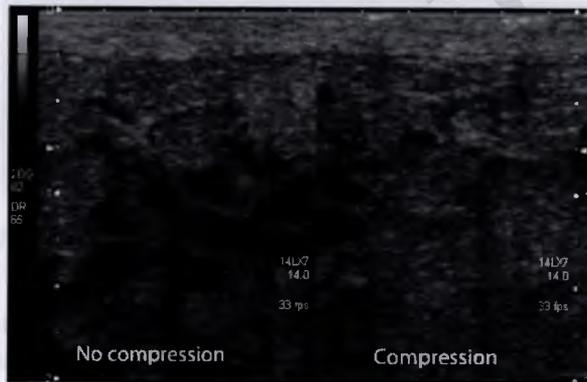


Figure 4. Ultrasound image of the milk ducts in the lactating breast in cross-section with no compression and under compression the ducts are completely obliterated.

Nipple

The nipple is composed of numerous muscle fibres that appear as mid level grey echoes on ultrasound. Nipple ducts are represented as small hypoechoic tubular structures ranging from barely perceptible up to 2mm in diameter (Figure 5). The application of cool gel has been shown to reduce the diameter of ducts within the nipple (Kent et al, in press) therefore warm gel is recommended for scanning the nipple.

Cooper's Ligaments

Cooper's ligaments are fibrous structures that traverse the parenchyma from the deep to the superficial fascia that support the breast tissues. The ligaments appear as echogenic bands that cross obliquely from the posterior part of the breast to the skin.



Figure 5. Ultrasound image of the nipple of a lactating woman. The nipple ducts are long hypoechoic tubular structures.

The curved nature of the ligaments reflects the beam causing posterior shadowing and thus obscuring structures behind the ligaments. Using multiple angulations of the transducer and increasing pressure will assist in reducing this artifact [19, 20]. The superficial fascia of the breast appears as a thin echogenic line below the skin and parallel to its surface [20].

Blood Flow to the Lactating Breast

Two major arteries, the Internal Mammary Artery (IMA) and the Lateral Thoracic Artery (LTA) supply most of the blood to the breast. The IMA supplies the breast via the posterior and anterior medial branches while the Lateral Thoracic Artery supplies the lateral portion of the breast by means of the lateral mammary branch. Branches of both the intercostal arteries and the thoracoacromial artery also contribute to the vascularisation of the gland [21]. Blood flow doubles during pregnancy by approximately 24 weeks of gestation and then remains constant throughout lactation [21, 22]. As with the non-lactating breast there is a wide variation between women in the proportion of blood supplied by each artery with little evidence of symmetry between breasts. The superficial veins of the breast also become more prominent during pregnancy and lactation [23].

The 24-h mammary blood flow required to produce one litre of milk in women is similar that of other species (500:1). Although no relationship between blood flow and milk production has been demonstrated in women to date. Mammary blood flow has been shown to be markedly reduced in a gland that is synthesising little milk compared to one producing a volume of milk within the normal range [23].

Ultrasound Doppler Technique

The dominant mammary branch of the IMA can be located by positioning the transducer in a transverse plane alongside the sternum and making a sweep scan from the second to the sixth intercostal space using Colour Doppler imaging.

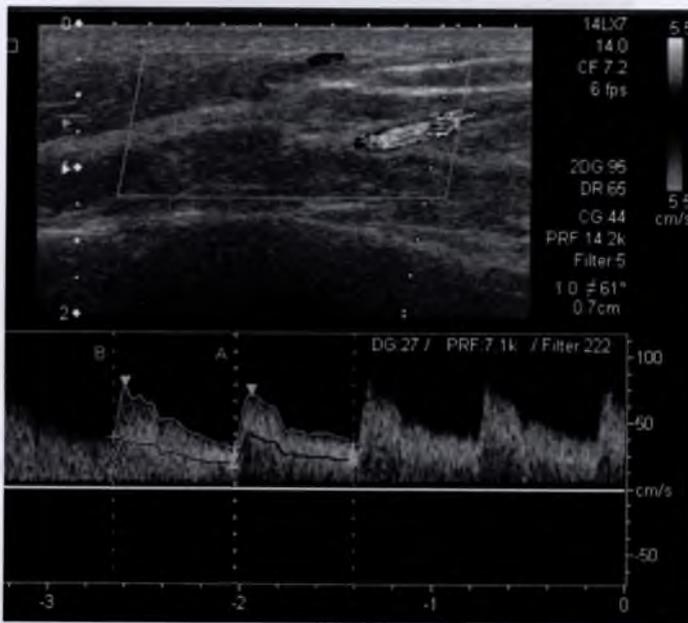


Figure 6. Doppler ultrasound of the anterior branch of the Internal Mammary Artery of a lactating woman.

The IMA and IMV appear as a circular hypoechoic area between the rib spaces deep to the pectoral muscle. Rotation of the probe until the long axis of the branch of the IMA is imaged so that Doppler measurements can be taken [24, 25] (Figure 6). Measurements are performed near the origin of the IMA. The mammary branch of the LTA is found superior to the lateral breast near the axilla. Generally settings for Colour Doppler are similar to those typically used for low flow vessels.

Normal Ultrasonic Appearances and Blood Flow Parameters

Both the arteries and veins of the breast can be easily identified and assessed with Colour Doppler ultrasound. Changes in blood flow to the breast occur with milk ejection. Flow initially decreases by approximately 50% prior to milk ejection and then subsequently increases in the next 1-2 minutes [26].

It is possible for spontaneous milk ejections to occur during scanning which may momentarily affect Doppler measurements. Common signs of milk ejection include leaking of milk from the nipple, sensations in the breast of pins and needles, pain and/or pressure and occasionally maternal feelings of warmth, thirst and nausea [27, 28].

Little research has been carried out with regard to normal mammary blood flow parameters of the IMA and LTA in the both the lactating and non-lactating breast. However the diameter of the IMA is increased during lactation (1.8mm compared to 0.2mm) as is mean velocity (39 cm/s compared to 19 cm/s) resulting in a higher flow volume (85 mL/min compared to 45 mL/min) [37 and geddes et al,]. The LTA has an average diameter of 1.3mm, mean velocity of 24 cm/s and flow volume of 45 mL/min during lactation [23].

Lymphatics of the Breast

The majority of the lymph in the breast is drained to either the axillary [29] or internal mammary nodes [29, 30]. The axillary nodes are believed to receive more than 75% of the lymph from both the medial and lateral portions of the breast [31], while the internal mammary nodes receive lymph from the deeper portion of the breast [32].

As expected however there is a large number of variations in the pattern of drainage of lymph from the breast with less common pathways demonstrated such as direct drainage to the supraclavicular nodes [33], passage through the interpectoral nodes [34] or lymph nodes within the breast parenchyma [33].

Normal Appearances of the Lymphatics of the Breast

Mammary nerves and normal lymphatics are not commonly visualised on ultrasound, however when the lymphatics are dilated due to either inflammation such as mastitis or malignant invasion they become visible as very thin hypoechoic lines traversing both parallel and perpendicular to the skin in the subcutaneous tissues [2]. Lymph nodes are demonstrated in the breast and axilla as well defined oval masses with an hypoechoic hilum and hypoechoic cortex [35].

Pathology of the Lactating Breast

Ultrasonic features of pathology of the lactating breast are summarized in Table 2. While breast changes during pregnancy and lactation alter the consistency of the breast persistent, focal lumps are not considered normal and should be investigated appropriately. Furthermore if women with pre-existing lesions prior to lactation experience any noticeable changes in these areas timely examination is warranted.

In these instances ultrasound is the first investigation of choice. Mammography is generally contra-indicated due to difficulties with both compressing the breast adequately and limited diagnosis due to the increased radiodensity caused by the substantial proliferation of secretory tissue and the presence of milk.

Focal masses of the lactating breast demonstrate the same ultrasonic features as the non-lactating breast. However masses in the lactating breast have the potential to obstruct the flow of milk by compressing adjacent milk ducts particularly if the breast becomes very full of milk.

Masses specifically associated with lactation include galactocele (dilated terminal ducts that are filled with milk), blocked ducts (tender lump ranging from the size of a pea to a large wedge shaped area believed to be caused by milk stasis as a result of causes changes in infant feeding pattern, mechanical obstruction, scarring or infection), abscess (a complication of mastitis) and lactating adenoma (tumour that develops from lactocytes lining the alveoli).

Diffuse pathologies that affect the lactating breast most commonly include engorgement (rapid increase in milk production at Day 3-5 post-partum) and mastitis (infectious or non-infectious inflammation of the breast).

**Table 2. Ultrasonic appearances of pathologies of the lactating breast
(adapted from Geddes 2009)**

Pathology	Ultrasonic appearances
Cyst	Sharp margins with thin smooth walls Anechoic centre Posterior enhancement Edge shadowing Absence of internal vascularity (Figure 7)
Fibroadenoma	Well defined or occasionally ill-defined Range of echogenicity from homogenous to heterogenous Generally no posterior enhancement Posterior shadowing if internal calcification is present Internal vascularity (Figure 8)
Abscess	Wide, indistinct, hypoechoic margins Range of echogenicity – from predominantly echo-free to heterogenous Posterior enhancement due to internal fluid No internal vascularity Vascularity may be increase in tissue surrounding the abscess
Malignancy	Irregular, ill-defined margins Heterogenous echogenicity Stellate shape +/- posterior shadowing Internal vascularity
Galactocele	Acute – range from anechoic and simple to mainly anechoic with diffuse echoes and multiloculated. Sub-acute – central echoes of mild to moderate intensity Chronic – diffuse echogenicity ranging from moderate to highly echogenic or heterogenous. Can be simple or multilocular +/-fat-fluid level Pressure from transducer may cause the internal contents to move Absence of central vascularity Wall vascularity
Blocked Duct	Focal – similar in appearance to an acute galactocele, non-compressible Diffuse – area of increased echogenicity associated with a palpable lump/area Occasionally a hypoechoic rim surrounds a more echogenic central region
Lactating Adenoma	Well circumscribed to ill-defined margins Echogenicity ranges from hypoechoic to hyperechoic and may be homogenous or heterogenous Posterior enhancement or acoustic shadowing Internal vascularity may be detected
Engorgement	Increased echogenicity of the secretory tissue due excess milk stored in the breast Severe engorgement may exhibit ultrasonic signs akin to mastitis
Mastitis	Acute phase: there may be no obvious ultrasonic changes in echogenicity of breast tissues Skin may thicken and appear increased in echogenicity Cooper's Ligaments and stromal fibrous tissue decrease in echogenicity. Increased blood flow associated with areas of inflammation (Figure 9,10) Advanced stages: Skin thickening is prominent Distinction between different breast tissues disappears Breast thickness increases

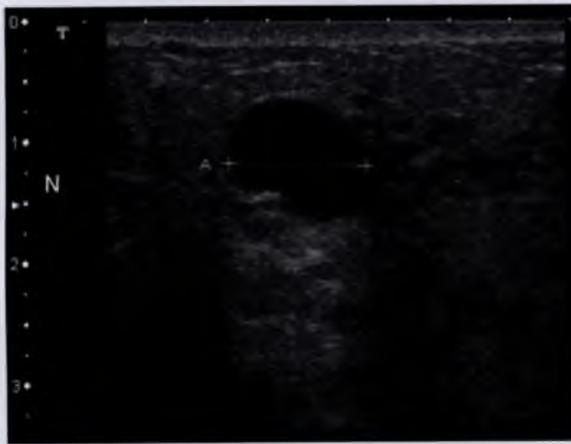


Figure 7. Ultrasonic image of a cyst in a lactating breast.

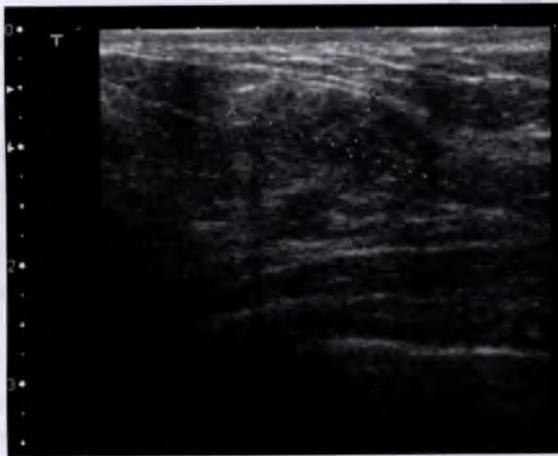


Figure 8. Ultrasound image of an isoechoic fibroadenoma in a lactating breast.



Figure 9. Ultrasonic images of the superficial tissues of a left mastitic breast and the normal contralateral breast. The left breast has marked skin thickening and the subcutaneous tissue are heterogenous compared to the right breast.



Figure 10. An image of a left mastitic breast showing the breast from the skin to pectoral muscle. Note the loss of demarcation between tissues and the increased attenuation of the sound beam with depth.

Ultrasound of Milk Ejection

Ultrasound has the unique ability of visualising movement and flow real-time. This characteristic has allowed it to be used to identify milk ejection in the lactating breast during both breastfeeding and breast expression.

Milk Ejection

The alveoli of the lactating breast are lined with lactocytes (secretory mammary epithelial cells) and these cells synthesise milk. In women most of the milk is stored in the alveoli until demanded by the suckling baby. Stimulation of the nipple by either the infant or the breast pump causes milk ejection usually within 90 seconds. Neural impulses are transmitted to the hypothalamus initiating oxytocin release from the posterior pituitary gland into the bloodstream.

Oxytocin causes the myoepithelial cells that surround the milk filled alveoli to contract and force the milk into the milk ducts [27, 36]. Oxytocin has a short half life, therefore milk ejection is a transient occurrence lasting between 45 seconds and 3.5 minutes [37-39]. Subsequently oxytocin is released in a pulsatile manner with multiple ejections usually occurring during either a breastfeed or pumping session [38, 39]. Continued milk synthesis relies on effective emptying of the breast and since only small volumes of milk (approximately 2.7mL; range 0 to 10.3mL) can be removed prior to milk ejection [40].

The milk ejection reflex is critical to a successful lactation [41]. Maternal sensations of milk ejection include tingling, pins and needles, pain or pressure in the breast, milk flow from the breast as well as nausea, warmth or thirst [27, 28].

During breastfeeding the infant may slow its sucking rate as a result of milk ejection where as during pumping jets of milk can be observed. Methods for the detection of milk

ejection such as serial sampling of maternal blood to detect oxytocin [38] and measurement of intra-ductal pressure changes by the cannulation of a milk duct through a nipple pore [42] are both invasive and stressful. Stress may impair milk ejection by inhibiting the release of oxytocin and cannulation of a milk duct increases the risk of infection of the breast. Ultrasound however is a simple means of confirming milk ejection during either breastfeeding or breast pumping [43].

Ultrasound Technique for Detection of Milk Ejection in the Lactating Mother

The aim of this examination is to identify milk duct dilation and/or milk flow that occur at milk ejection due to the increased positive pressure within the duct. Therefore the monitoring of a milk duct must be carried out during either a breastfeed or pumping session. The mother should be seated and be allowed to either breastfeed or pump in the position she would normally adopt.

Prior to beginning breastfeeding/pumping a milk duct in the un-suckled/non-expressed breast must be identified and then monitored for the session. Milk ducts larger than 1mm in diameter tend to be easier to monitor for long lengths of time. The longest axis of the portion of the duct to be monitored is achieved by rotation of the probe. Gain settings are increased so that the duct is not completely anechoic and this allows milk flow to be visualised as well as duct dilatation.

Colour Doppler Flow Imaging [2] is useful to ensure that blood vessels are not mistaken for milk ducts. In contrast to scanning the breast for abnormalities limited pressure should be applied as milk ducts are easily obliterated and too much pressure may inhibit duct dilatation at milk ejection. In this context it is useful to test compression levels to ensure the duct is not already partially compressed prior to commencing the scan. It is important that the ultrasound technician adopts a comfortable position for the duration of the scan, as the transducer must be held still for up to 20 minutes if one intends to detect multiple milk ejections throughout the session.

Prior to beginning the scan it is prudent to ask the mother to limit her movements to reduce movement artefact as well as indicate if she senses milk ejection so that this can be marked on the scan for later analysis. It is also highly recommended that the entire scan be recorded to allow retrospective analysis particularly in cases where duct dilatation is minimal [44].

Ultrasound Appearances of Milk Ejection

Although many of the structures of the lactating breast are similar in appearance to that of the non-lactating breast, particular features should be noted such as the small size of the ducts (2-3mm; range <1 to 10mm) and their easy compressibility. In addition the internal lumen of ducts may contain small echogenic flecks that most likely represent large fat globules in the milk [8]. The variability in echogenicity of the milk can be accounted for by the relationship of fat content to the volume of milk in the breast.

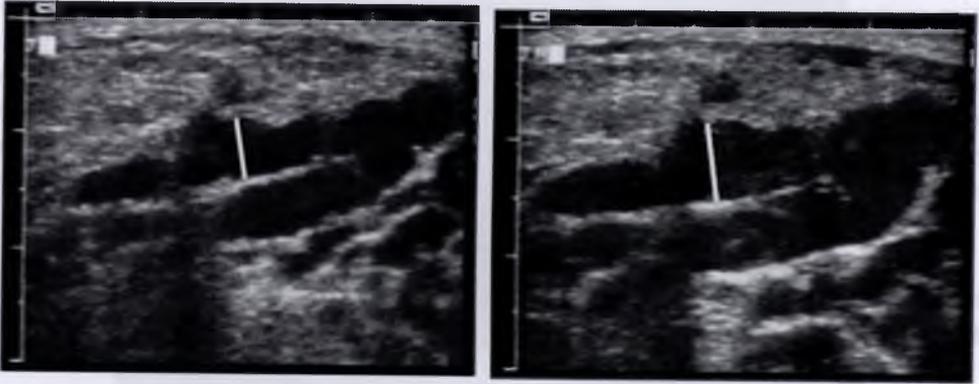


Figure 11. Milk duct diameter in the non-suckled breast was 5.6 mm and increased to 7.1 mm at milk ejection.

Milk of breasts that are drained is higher in fat compared to those that are relatively full of milk [45]. At milk ejection an increase in milk duct diameter and/or milk flow is visualized as small echogenic flecks moving towards the nipple. Degree of milk duct dilation is highly variable between women with some exhibiting large increases and others negligible increases (Figure 11). Reverse flow of milk is often observed in the second half of milk ejection and the duct decreases in diameter. This is due to both the reduction in myoepithelial contraction and the lack of storage of milk in the ducts beneath the nipple and areola. Monitoring of an entire breastfeed/expression session will often display multiple milk ejections as transient increases and decreases in duct diameter. Studies have shown wide ranges in the number of milk ejections (1 to 12 milk ejections) between women during breastfeeding and expression [11, 43].

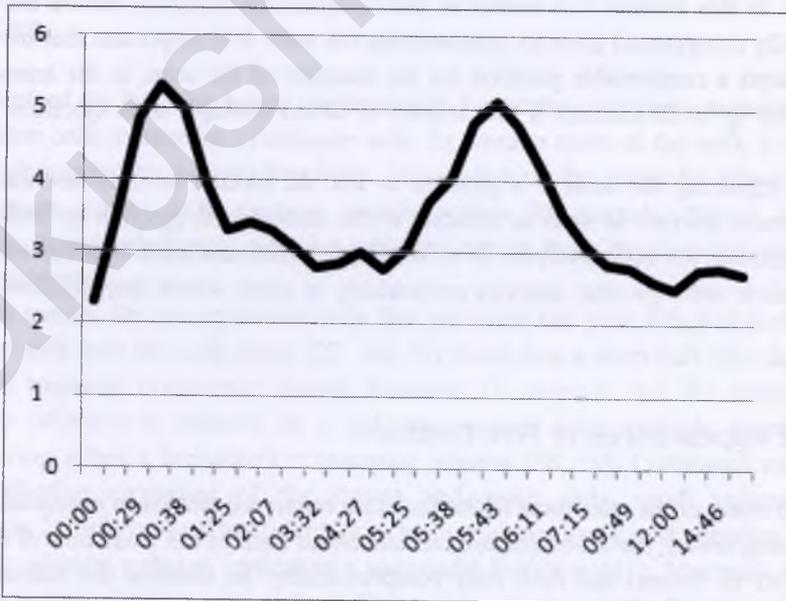


Figure 12. Two increases and decreases in duct diameter in the non-expressed breast of a lactating woman represent 2 milk ejections during a 15 minute breast expression with an electric pump.

Analysis of Ultrasound for Milk Ejection

Retrospective analysis of milk ejection entails numerous serial measurements of the monitored duct (3 to 20 seconds) to accurately detect multiple milk ejections. Plots of duct diameter against time facilitate evaluation of the number and duration of the milk ejections. The duration of milk ejection has been estimated from the beginning of an increase in duct diameter to the beginning of the next increase in duct diameter [43] (Figure 12).

Clinical Relevance of Monitoring for Milk Ejection

Although milk ejection is essential for the removal of milk and continued milk synthesis there is a lack of tests that confirm a normal milk ejection reflex. An acute increase in milk flow rate during expression has been associated with milk ejection imaged as an increase in duct diameter by ultrasound and this method may be useful to confirm milk ejection in pump dependant women. Unfortunately a proportion of women are unable to express sizeable volumes of milk, therefore ultrasound imaging would provide an alternative means of confirming a normal milk ejection reflex.

Infant Sucking Dynamics during Breastfeeding

A successful lactation depends also on the ability of the infant to remove sufficient milk from the breast. Therefore like the breast, understanding and interpreting sucking pathologies which may impact lactation and feeding ability is necessary.

Current protocol for assessing sucking abnormalities, normally involves only external observations of the infant, and monitoring position and attachment of the infant to the breast. Sucking problems are generally attributed to poor attachment of the infant to the breast, and therefore advice focuses on encouraging the infant to open its mouth wide enough, and draw sufficient breast/nipple into the mouth [46, 47]. However for a large proportion of infants, breastfeeding problems remain unresolved despite advice on positioning and attachment. Using ultrasound technology as a diagnostic tool to assess sucking, enables a health professional to more objectively assess tongue movement and position of the nipple within the infant's mouth during feeding.

Newborn Oral Anatomy

Key differences are observed between infant and adult oral anatomy (figure 13). The infant's tongue occupies most of the oral cavity, and is in contact with the gums and palate. The mandible is shorter and retracted compared to the adult. The hard palate is short and relatively wide, with little arching compared to the adult, where the hard palate is considerably arched and is positioned more superiorly within the skull [48]. Rugae (transverse folds) on the hard palate help the infant maintain nipple position in the oral cavity. The infant also has prominent buccal fat pads located between the buccinator and masseter

muscles. These fat pads are believed to provide stability during sucking, and prevent the cheeks collapsing when negative pressure is applied [48]. In the infant the larynx sits more superiorly, the pharynx is smaller and the epiglottis faces the tongue base and soft palate. The high placement of the larynx allows the infant to breathe through the nasal cavity. In addition the larynx elevates during swallowing and therefore separates the bolus from the airway and foodway [48, 49]. The epiglottis and soft palate are in close contact, preventing bolus in the oral cavity and oropharynx from entering the airway. Upon swallowing this contact is broken when the soft palate elevates and the epiglottis folds down [48, 49].

Theory of Sucking Dynamics

Oral feeding is common to all newborn mammal species [50]. It requires a complex interaction and coordination of the jaw, hyoid bone, tongue, palate, pharynx and, larynx to coordinate rhythmic patterns of sucking, swallowing and breathing [49, 51].

Newborns exhibit two types of sucking patterns, nutritive sucking (NS), where milk is consumed; [52] and non-nutritive sucking (NNS) that is characterised by a series of regulated bursts and rest periods [52]. NNS movements are more commonly observed towards the end of a breastfeed [53], where as NS bursts are longer and occur mainly at the beginning and middle of a breastfeed [53].

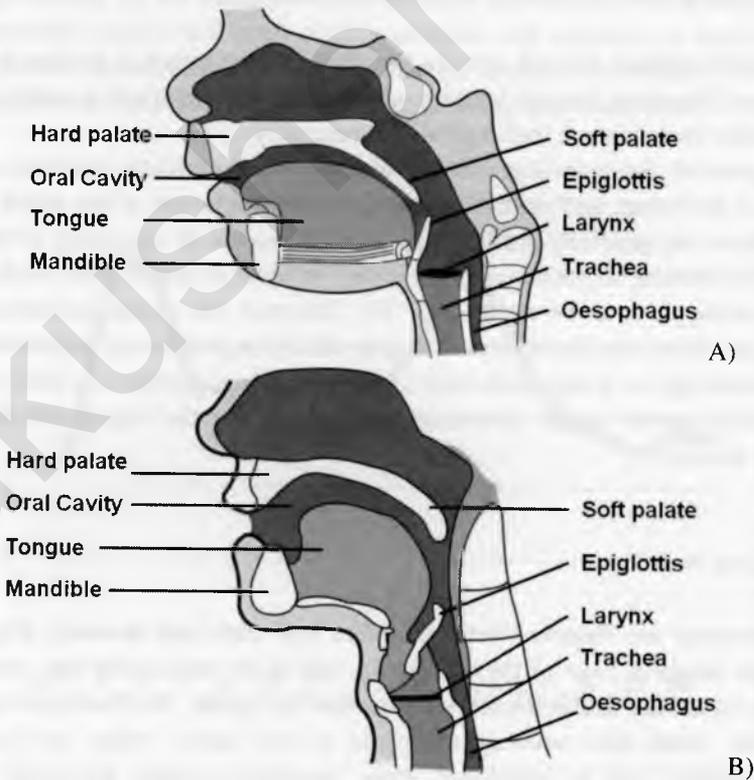


Figure 13. Midsagittal section of the A) Infant and B) Adult oral anatomy during sucking and swallowing.

The function of the tongue is of major importance during infant sucking: it must remove milk from the breast and safely clear the milk bolus to the pharynx [54], yet exactly how the tongue functions during sucking is still the subject of considerable debate. Two theories describing the sucking mechanism exist: the Stripping Action and [55-61] the Intra-Oral Vacuum [62-66]. The stripping action theory suggests the primary sucking mechanism of milk removal is the compression of the breast by the infant's lower jaw which is then followed by a peristaltic tongue movement squeezing milk from the nipple [56].

The stripping theory was based on the presence of lactiferous sinuses that were thought to act as reservoirs for milk, however as mentioned earlier the entire ductal system dilates at milk ejection to accommodate the flow of milk and most of the milk in the breast is stored within alveolar portion. As the distal portion of the tongue depresses a vacuum is created drawing the nipple backwards in the mouth and the sinuses refill with milk. Vacuum at this point is considered a secondary mechanism of milk removal. Since milk ducts have been shown to store only small amounts of milk [8], this theory required reinvestigation. In contrast to the stripping action theory, the intra-oral vacuum theory emphasises the creation of an intra-oral vacuum (negative pressure) to be the primary mechanism of milk removal [62]. The movement of the tongue inferiorly away from the palate is believed to create negative pressure, assisting in milk flow from the nipple [67-70].

Normal Sucking Dynamics

An intra-oral vacuum is created during breastfeeding by term infants [54, 65, 71, 72]. Studies investigating tongue movement using real-time ultrasound found that no milk was evident when the tongue was raised. The nipple was compressed and the distal portion of the tongue rested against the hard palate (2, 33). When the tongue moved downward, the nipple moved forward and milk flow into the intra-oral space was observed (figure 14). These findings suggest the negative pressure generated from the tongue's downward movement was responsible for milk flow and not compression of the nipple [54, 65, 71, 72].

Geddes et al [65] using simultaneous ultrasound imaging and intra-oral vacuum measurements, showed a baseline vacuum (-64 ± 45 mmHg) is generated when the distal tongue was in contact with the palate. As the tongue lowered nipple diameter increased and the nipple moved closer to the hard-soft palate junction and a cyclic vacuum (peak vacuum: -145 ± 58 mmHg) was applied (figure 15). In the tongue up position, the distal tongue did not indent the nipple. Similar vacuums (mean -50 mmHg, peak -197 mmHg) to Geddes et al [65] have been shown in term infants during a normal breastfeed [73]. To provide further evidence to support this theory, Geddes and colleagues compared vacuum patterns during breastfeeding and feeding from an experimental teat which was designed to only release milk when the infant applied a vacuum (sucking) [74]. This design differed from typical teats, where milk is usually released when the bottle is inverted and/or with compression of the teat (positive pressure).

The authors showed during both the breastfeed (b) and feed from the teat (t) the mechanism of milk removal was similar. As the distal tongue was in contact with the palate a baseline vacuum was applied (b -67 ± 25 mmHg, t -12 ± 13 mmHg) and as the tongue lowered the nipple/teat diameter increased and the nipple/teat moved closer to the hard-soft palate junction and a cyclic vacuum was applied (peak b -118 ± 46 mmHg, t -28 ± 19 mmHg). This

provided evidence that vacuum is necessary to remove milk from the teat in a similar manner to the breast, as well as support for the intra-oral vacuum theory [74]. Another study using ultrasound during breastfeeding devised a method of measuring the nipple to provide an indirect measurement of tongue movement. Key anatomical features were measured such as nipple diameters at 2,5,10 and 15mm along the length of the nipple, the tip of the nipple to the hard-soft palate junction distance and the extent of inferior movement of the tongue (depth) [75]. The authors found when the tongue was raised, all nipple diameters significantly increased along the length of the nipple, except for at 10 and 15mm which was similar.



Figure 14. Midsagittal image of the infant oral cavity and mother's nipple and breast during a scan. The suck cycle A) At the start and end of a suck cycle. The tongue (T) is up in apposition with the hard palate (HP) and soft palate (SP). The nipple (N) is situated a short distance from the hard/soft palate junction (HSPJ) B) The middle of the suck cycle. The infant's tongue has moved downwards away from the palate. At the same time vacuum is applied and milk flow (flecks) (MF) is visible in the posterior oral cavity. Note the nipple also has moved closer to the hard/soft palate junction.

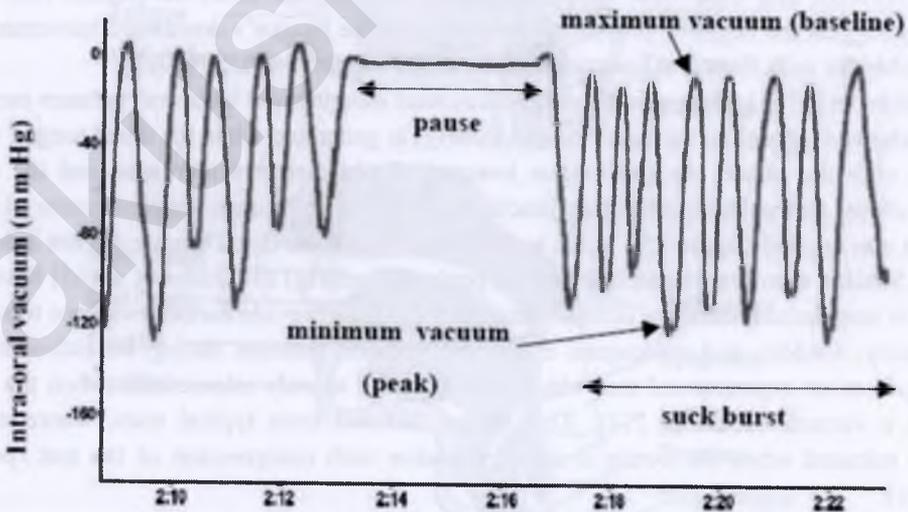


Figure 15. Vacuum trace during a breastfeed. Baseline vacuum (maximum pressure); corresponds to when the distal tongue was in contact with the palate on ultrasound) and Peak vacuum (minimum pressure); corresponds to when the tongue is lowered on ultrasound) are outlined.

Similarly to Geddes et al, when the tongue lowered, all nipple diameters increased, the depth measurement increased and the nipple moved closer to hard-soft-palate junction. All diameters were significantly different from each other except for 5 and 15mm. The authors suggested the nipple diameter at 15mm did not increase as much compared to the other diameters as the anterior portion of the tongue had already begun to rise, in order to maintain a seal to the breast as well as assist in clearing the bolus from the oral cavity before the next suck cycle. The measurements show the nipple diameters changed along the length of the nipple, and increased from tongue up to tongue down, which not consistent with a peristaltic action where one would expect indentation of the nipple would occur at one point on the nipple, and the areas adjacent to that point would be expanded [56].

Ultrasound Imaging

Ultrasound Equipment

Submental ultrasound scans of the midline of the infant's intraoral cavity can be performed successfully during breastfeeding and bottle-feeding [54, 65, 76]. A long-handled endocavity transducer no lower than 7MHz (to allow high frequency detailed ultrasound imaging) [71] with a curved 1.5-cm face is the most appropriate transducer for scanning. This probe has advantages over other shorter bulkier transducers, in that the infant is not adversely disturbed and is able to feed in its normal position. The small convex face ensures good skin contact with the infants chin and provides a wide 160 degree, panoramic view of the hard and soft palate [65, 71].

Scanning Technique

The breastfeeding dyad is normally scanned whilst the mother is comfortably seated and using the cradle hold (figure 16). The cradle hold is a common feeding position [77], and enables the best visualisation of the oral cavity and access to the infant. Submental scans along the midline of the infant's oral cavity enable imaging of the mother's nipple in the oral cavity, the tongue, hard palate, soft-palate, hard-soft palate junction as well as milk bolus. Other scanning planes have previously been investigated, however they provide substantially less information when compared to the midsagittal submental plane.

The transbuccal plane involves placing the ultrasound transducer on the infants cheek. This plane allows one to distinguish lateral compression and elongation of the nipple or teat, however provides little or no information on tongue movement or palate shape [54, 78]. The coronal plane (transverse submental view) allows observation of nipple compression, although as with the transbuccal plane tongue movement, palate shape or nipple elongation cannot be visualised [54, 78]. The transducer is placed gently underneath the infants chin with the handle of the transducer lying along the infants body and it is rotated until a midsagittal view of the nipple is displayed. Requirements for an optimal image is that the nipple is displayed at its maximum length and width, the hard/soft palate junction is visualised, as well as the upper surface of the tongue [35, 75]. The transducer can be held with two hands, one

supporting the distal end of the transducer, and the other the proximal end: to ensure good skin contact for optimal imaging [76]. The probe may require manipulation to maintain a midsagittal view when the infant moves [35, 75].



Figure 16. Midsagittal submental scanning during a breastfeed using the cradle hold.

Table 3. Ultrasonic appearance of the infant's oral cavity during breast and bottle-feeding

Structures during feeding	Ultrasonic Appearance
Tongue	midgrey, internal echogenic structures due to muscle fibres and interfaces between the muscles, echogenic surface/interface with nipple and soft-palate
Hard palate	thin echogenic curvilinear structure†
Soft palate	midgrey, echogenic superior border, moves downward as the tongue is depressed
Hard-Soft Palate Junction	space between the edge of the echogenic hard palate and mid-grey soft palate, junction is clearer as the tongue and soft palate move downwards during sucking
Milk Bolus	hypochoic fluid echogenic flecks (fat globules of milk)
Nipple/Teat	nipple: grey, hypochoic, (1.5-2mm in length) teat: grey, hypochoic*
Milk duct	hypochoic tubular structure within the nipple

Average settings previously used were; gain: 55 db, dynamic range: 60 db, frequency: 8.8 MHz however these may change depending on the type and model of the machine. Additional changes to gain, dynamic range, and time gain may be required to optimise image quality during scanning particularly in the case of a very large infant [65]. Furthermore lowering the frequency of the probe from 8.8 MHz to 7.3 MHz or 5MHz maybe necessary to improve imaging at depth in larger and older infants [65]. Applying two focal zones at the hard palate, and the other at the nipple-tongue border will narrow the ultrasound beamwidth and therefore improve image resolution.

Normal Ultrasonic Appearance of the Oral Cavity during Breastfeeding

The hard-palate (bone) appears as a thin echogenic curvilinear structure superior to the nipple and extends approximately 5-10mm beyond the nipple tip [65, 72] (see figure 2). The soft-palate appears as a mid-grey structure of which 10 to 20mm may be well visualized, with an echogenic border superior to the tongue. The nipple is displayed as a mid level echogenic (grey) structure. Within the nipple, milk ducts appear as hypoechoic (black) linear structures. Since the tongue is a muscle, it has an attenuation coefficient level between fat and fluid, and is visualised as a midlevel grey structure, with an echogenic (white) border at the tongue surface. Internally echogenic lines may be seen that represent muscle fibres and the interfaces between the muscles [65, 72] (table 3).

Measurement of Tongue Movement and Nipple Diameters Using Ultrasound Imaging

To systematically investigate infant sucking dynamics, a valid measurement tool is required to assess tongue movement, nipple diameter and nipple placement within the oral cavity. At the same time the reproducibility and operator reliability of ultrasound assessment is a common concern amongst users, and must be established before performing assessments. Recently, McClellan et al [75] developed and tested the validity and reliability of a measurement protocol to assess infant sucking. Two raters assessed 5 key anatomical sites on pre-selected ultrasound frames, when the tongue was up (TU) and when the tongue was down (TD) during a suck cycle.

The nipple diameter at 2,5,10 and 15mm from the nipple tip, the distance from the nipple tip to the hard soft palate junction and the depth of the tongue were measured using Screen Calipers, V.3.2 (Iconico Inc) (figure 17).

This study confirmed a high inter reliability (between examiners) and no measurement bias between measurements for each examiner (intra-reliability). However as expected the more experienced examiner was more consistent, highlighting the need for familiarisation with the ultrasound technique. The authors noted some measurements were more difficult to measure. The nipple to hard-soft palate junction measurement was the most variable measurement and often due to imaging artefact making the distal edge of the hard palate appear artificially longer. In several cases the nipple tip was not well delineated when milk was present, most likely due to the increased echogenicity of the fat globules in the milk [8].

The authors suggest assessing video clips in a frame-by-frame manner in order to correctly identify anatomical landmarks [75].



Figure 17. Example of key anatomical measurements on ultrasound imaging during sucking. Ultrasound Measurements made at 2,5,10 and 15mm along the nipple (red), nipple to hard-soft palate junction (green) and hard palate to tongue surface (yellow) when the tongue was up (A) and the tongue was down (B).

Application of Ultrasound to Investigate Sucking Dynamic Pathology

Currently ultrasound imaging of the intra-oral cavity of the breastfed infant is used for research however it shows much promise as a diagnostic tool in cases where feeding difficulties are not resolving with conservative management.

Nipple Pain

In the early stages of lactation many women find breastfeeding difficult, confusing and painful [46] and this is reflected in high early weaning rates (before 6 months) [79]. Traditionally studies and clinical protocol have focused on positioning of the infant, the attachment of the infant to the breast and nipple infection as the causes of nipple pain, however positioning and attachment advice is offered based on observation only [72]. Correction of these factors does not resolve a proportion of women's pain, indicating a need for more objective investigations. In a randomised trial, one-to-one counselling on positioning and attachment was investigated. Pain was significantly reduced after day two or three post-partum, however this difference was not evident at six weeks, three months and six months [80]. Another study assessing positioning and attachment, found no significant differences in nipple pain after counselling on body position, head position and other breastfeeding attributes of the baby. It is likely that standard positioning and attachment may not always be sufficient in reducing or preventing persistent nipple pain or increasing breastfeeding duration and this is reflected in low rates of breastfeeding [79].

It is suspected that one cause of nipple pain is due to infants exerting high intra-oral vacuums. Gunther argued that in order for the nipple to stay in the mouth, the baby must use suction [81]. Negative pressures of up to -200 mmHg by a 2-day-old baby of a mother reporting nipple pain were recorded. Gunther suggested vacuums of this level were capable of causing minor capillary damage to the skin of the nipple. Others have suggested that if the infant is not satisfied after the cessation of milk flow, they will maintain a high vacuum (similar to that of non-nutritive sucking) with the purpose of stimulating milk flow and that this vacuum level could cause nipple trauma [82].

More recently infants of breastfeeding mothers who report nipple pain have displayed differences in the vacuum applied to the breast when sucking [72]. This study found that infants of mothers reporting nipple pain exhibited stronger baseline and peak vacuums, and sucked faster than infants' of mothers not experiencing pain. This evidence supports Gunther's observations that high vacuums should be considered as a cause of nipple pain. Observations during ultrasound scans of mother's with nipple pain, suggest a proportion of cases have compression features, or unusual tongue movement. Studies are currently under progress investigating this.

Ankyloglossia

Ankyloglossia refers to the presence of a sublingual frenulum which due to its decreased length, minimal elasticity and/or the attachment being too distal from the tongue or too close to/on the gingival ridge, results in restricted movement of the infants tongue [83]. Usually infants are able to extend their tongue past the lower gum line, however ankyloglossia restricts tongue protrusion and tongue mobility [83] (figure 18).

The prevalence of ankyloglossia in infants ranges between 4.2% [84] and 10.7% [85] depending of the diagnostic criteria. Not all infants with ankyloglossia have feeding difficulties, however infants with these difficulties have shown poor weight gain, speech and feeding problems in later life, and poor oral hygiene [86].

Ankyloglossia can also inhibit the mothers breastfeeding experience, considering up to 80% of mothers breastfeeding infants with ankyloglossia experience persistent nipple pain during the early stages of lactation [87]. To treat ankyloglossia, a health professional may release the lingual frenulum by incision or 'snipping' of the frenulum. This is a simple and effective procedure and does not involve anaesthesia for infants under the age of four months [83]. Recently ultrasound has been used to investigate ankyloglossia [88].

Ultrasound pre and post frenulotomy (within 7 days), milk intake, pain scores and LATCH scores (5 part screening tool for newborn assessing latch, audible swallowing, type of nipple, comfort, and hold) were assessed. Post-frenulotomy milk intake over 24 hours, transfer rates, LATCH and pain scores improved.

Ultrasound scanning pre-frenulotomy identified two types of nipple distortion. Infants, who placed the nipple close to the hard-soft palate junction, compressed the base of the nipple. Scans post frenulotomy confirmed the nipple distortion had resolved, but for one infant. Other infants in the study seemed to place the nipple further away from the hard-soft-palate junction (figure 19), and compressed the tip of the nipple almost to a pointed end. Likewise scans confirmed compression either resolved or improved post-frenulotomy (figure 20).



Figure 18. Ankyloglossia. Infant exhibits a heart shaped tongue tip with an attached lingual frenulum.



Figure 19. Submental ultrasound image of an infant with ankyloglossia. Compression of the base of the nipple is obvious as the infants tongue is lowered.



Figure 20. Submental ultrasound image of the same infant post frenulotomy showing almost no compression of the base of the nipple as the infants tongue is lowered.

Another small group of infants were not unevenly distorting the nipple, however post-frenulotomy the nipple placement improved moving, slightly closer to the hard-soft-palate junction. In a case-series, Geddes et al [89] measured vacuum levels and sucking with ultrasound in infants successfully feeding with ankyloglossia. Ultrasound imaging showed two of the infants were unevenly distorting the nipple, one compressed the base and applied strong peak vacuums, and the other compressed the tip of the nipple and applied weak baseline vacuums. Another two infants applied strong baseline vacuums.

This case series showed infants with ankyloglossia may be able to obtain a sufficient amount of milk to maintain adequate growth without causing nipple pain, despite compressing the nipple and applying vacuums outside a normal range [89]. Further investigation of both intra-oral vacuum and tongue movement using ultrasound is essential. Nonetheless ultrasound appears to be a promising modality to assess abnormal tongue movement, ankyloglossia and nipple distortion, which could contribute to nipple pain and early weaning.

Swallowing

Normal swallowing is essential for successful coordination of the suck-swallow-breathe reflex in both term and preterm breastfeeding infants. Infant dysphagia may be due to anatomical causes such as; laryngomalacia, ankyloglossia and cleft-lip palate, or neurological such as; prematurity, cerebral palsy and muscular disorders [90]. These abnormalities may have a significant impact of the breastfeeding dyad and require investigation during the early stages of lactation. Infant's presenting with symptoms including gagging, aspiration, failure to thrive and general feeding difficulty may be recommended for a swallowing examination. Infant swallowing can be examined using a range of methods include flexible endoscopic evaluation [91] and manometry [92], which are both invasive and costly. Videofluoroscopy (modified barium swallow), has remained the gold standard method to image bottle-feeding infants with feeding difficulties. This method provides visualization of the three phases of swallowing in two-dimensions, however there are concerns about radiation dose to the infant, and it's not possible to perform this procedure during breastfeeding [91, 93]. Recently ultrasound has been used to identify swallows during breastfeeding in a research setting, and appears to be a promising modality in the investigation swallowing pathologies.

There are three phases of swallowing; the oral, pharyngeal and esophageal phases. The oral phase involves the infant removing milk from the breast and propelling the bolus towards the back of the oral cavity. To achieve this the breastfeeding infant lowers the tongue and creates a vacuum that results in a milk bolus filling the oral cavity. The upward movement of the tongue during each suck cycle assists in propelling milk bolus towards the pharynx [65, 76]. After a sufficient amount of milk has accumulated in the pharynx, a swallow is triggered [90, 94].

The swallow begins with the elevation of the hyoid and the posterior and lateral pharyngeal walls contracting, closing of the velopharyngeal isthmus, the larynx elevates while the laryngeal muscles of the vocal cord contract and epiglottis folds downwards to prevent bolus entering the airway and resulting in laryngeal closure [90, 94]. The bolus moves by a pharyngeal wave/peristalsis as well as changing pressure gradients in the pharynx. As the bolus moves past the airway, the cricopharyngeus muscle relaxes allowing the bolus to move into the oesophagus.

Once the milk has moved completely into the esophagus, the esophageal phase begins with cricopharyngeus closure and peristalsis moves the bolus into the stomach [90, 94].

Ultrasound Imaging

Ultrasound Equipment

A long-handled endocavity transducer can be used to image swallowing during breastfeeding. This transducer provides a panoramic view of the pharynx and upper esophagus to the clavicle. The transducer frequency should be relatively high (5-10MHz).

Scanning Technique

The transducer is positioned initially along the midsagittal line of the infant's neck. The probe is then moved backwards toward the infant's spine and angled toward the anterior neck. Some rotation of the probe may be required to visualise the longitudinal axis of the pharynx and esophagus and observe milk bolus moving along the pharynx towards the esophagus, using the carotid vessel as a window. Adjustments to gain and dynamic range may be required based on infant size and image quality. The depth is adjusted to approximately 5cm [76].

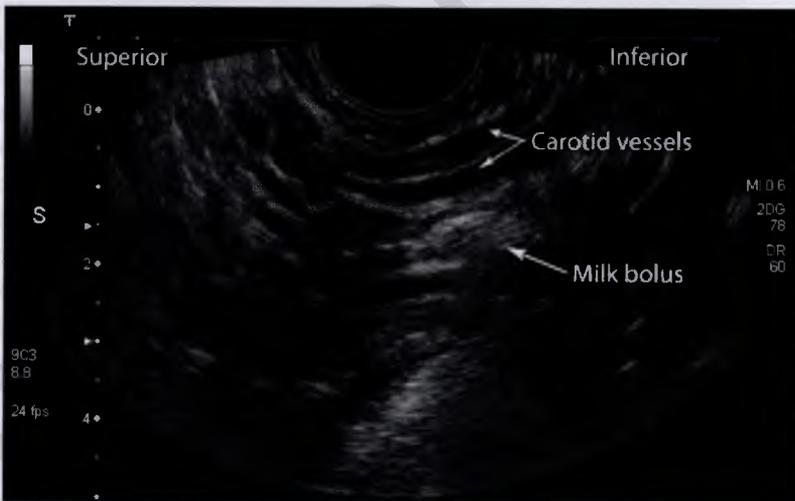


Figure 21. Ultrasound image of the lateral neck of a breast feeding infant showing the echogenic milk bolus in the oesophagus.

Normal Ultrasonic Appearance during Swallowing

Imaging of the infants neck during breastfeeding displays the carotid vessels anteriorly as black tubular vessels. The pharynx and esophagus are situated posterior to the carotid vessels, and are displayed as a midgray echogenicity. The internal walls of the pharynx are of higher echogenicity compared to the outer walls. The milk bolus in the pharynx appears echogenic, due to the fat content of the milk [11], and moves inferiorly towards the esophagus.

Intermittently reverberation artefacts may be observed as white reflections behind the milk bolus and are likely to be due to a small amounts of swallowed air [76].

Validation of Ultrasound Technique to Investigate Swallowing

To determine the accuracy of the ultrasound technique described above, the authors compared the technique, to respiratory inductive plethysmography (RIP) during breastfeeding. Using RIP (Respirtrace QDC, SensorMedics, Yorba Linda, CA, USA) a respiratory band was placed around the infant's thorax and another placed around the abdomen, and their outputs were recorded to a Power Lab (ADI Instruments, Sydney, Australia).

Inspiration was displayed as an increase in voltage and expiration a decrease in voltage, while a swallow was identified as a cessation of breathing, or a flat line on the trace. Both ultrasound and RIP measurements were synchronised and therefore directly comparable.

A flat line on the respiratory trace coincided with movement of the milk bolus in the pharyngeal area on ultrasound. Ultrasound detected 388 swallows, and this corresponded with 379 swallows detected by RIP ($R^2=0.987$). Ultrasound imaging of the infant's neck to detect swallows, enables visualization of the movement of milk bolus through the pharyngeal region during breastfeeding in both an accurate and safe manner. This method has only been employed in a research setting, but has the potential to be used in a clinically to assist in identification of infant swallowing difficulties during breastfeeding.

Conclusion

Few diagnostic tests are available to the clinician to assess women and infants that are experiencing breastfeeding problems. Ultrasound imaging is being used extensively to monitor the lactating breast as well as infant tongue movement and swallowing during breastfeeding in a research setting.

With increasing use in the clinical setting, ultrasound has the potential to provide more accurate diagnoses of the lactating breast and breastfeeding infant. These examinations could provide information to the clinician that will enable better support of mother-infant dyads to achieve a successful lactation.

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