Physiology of milk secretion

Sandrine Truchet, PhD, Scientist, Cell Biologist \textsuperscript{a,}* , Edith Honvo-Hou\textsuperscript{eto, RT, Technician, Physiologist \textsuperscript{b}}

\textsuperscript{a} VIM, UR 892 INRA, Université Paris-Saclay, Jouy-en-Josas, France

\textsuperscript{b} GABI, INRA/AgroParisTech/Université Paris-Saclay, Domaine de Vilvert, 78352 Jouy-en-Josas, France

Article info

Article history:
Available online 31 October 2017

Keywords:
mammary gland
mammary epithelial cell
lactation
secretion
milk
breastfeeding

1

Introduction

Milk is a unique and complete nutritive source for the mammal neonate, also providing immune protection and developmental signals. Lactation is a complex process, proper to the mother and child dyad, and including numerous variables ranging from psychological aspects to the secretory functioning of the mammary epithelial cells, all contributing to a successful breastfeeding. This review gives an integrated overview of the physiology of lactation with a particular focus on cellular and molecular mechanisms involved in milk product secretion and their regulations.

© 2017 Elsevier Ltd. All rights reserved.
mammary epithelial cells (MECs) in order to provide milk of adequate composition and in sufficient quantity to the newborn.

**Mammary gland development**

The mammary gland (MG) is a dynamic exocrine organ that can undergo repeated cycles of growth, functional differentiation, and regression, closely intertwined with the reproductive processes. Indeed, mammary development begins during early fetal life, occurs only slightly during estrous cycles, while complete mammmogenesis only takes place during pregnancy to become fully functional after parturition to provide a nutritional support to the newborn. Once the child is weaned, the mammary tissue declines during involution and can re-differentiate if a new pregnancy starts (Fig. 1). All steps of the physiological development of the MG are tightly spatio-temporally coordinated, both by systemic hormones and local factors.

From about 7 weeks of gestation, the human MG develops from a single ectodermal ridge localized along the anterior body wall which extends from the epidermis into the underlying mesenchyme. Concomitantly, a loose condensation of mesenchyme extends sub-dermally to form the fat pad precursor. The ectoderm elongates to form a mammary sprout, invades the fat-pad precursor (10th-12th weeks), branches (13th-20th weeks), and canalizes to form the primary mammary ductal system (32nd weeks), which opens onto the area that gives rise to the nipple (Fig. 1, birth) [1,2].

After birth, the MG remains as a rudimentary network of small branching ducts ending in short ductules called terminal end buds (TEBs) lined by one to two layer of epithelial and one of myoepithelial cells. These structures regress at ~4 weeks postpartum along with a decrease in the secretion of prolactin (PRL) from the anterior pituitary gland of the infant. Until puberty, the growth of the breast is isometric. Of note, diet and/or metabolic pathologies such as diabetes may impair mammary development and subsequent lactation performance [3].

After a period of quiescence during childhood, the increase in ovarian estrogen secretion at puberty (8–12 years of age) stimulates the allometric growth of both the epithelial network and the adipose tissue within the MG (Fig. 1, puberty). Thus, while the increase in breast size is merely due to the enhanced deposition of adipose tissue, the mammary epithelium progressively elongates and further branches resulting in an extensive ductal network. These maturational changes occur in the course of ovulatory cycles and are then regulated by both systemic and locally released factors such as estrogen, progesterone, PRL, luteinizing hormone (LH), follicle stimulating hormone (FSH), growth hormone (GH) and epidermal growth factor (EGF) [4]. 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, ovarian progesterone stimulates lobulo-alveolar development, e.g. mitotic activity of the bilayered MECs, and opening of lumens in TEBs to form small alveoli [4]. TEBs generate new branches, twigs, and small alveolar structures which cluster around a terminal duct, forming a lobule (~11 alveoli/duct, Fig. 2B). Lobule formation occurs within 1–2 years after onset of the first menstrual period. Alveolar clusters grow and increase in complexity during each luteal phase and slightly regress with the onset of the

![Fig. 1. Mammary gland development. The postnatal development of female mammary tissue occurs in several steps regulated by hormones. At birth, the mammary epithelium consists of limited ducts. At puberty, high levels of circulating hormones stimulate both the proliferation of the MECs and the enlargement of the surrounding fat pad. At the onset of pregnancy, epithelial ducts elongate, branch and alveoli develop. During lactation, the mammary epithelium reach its maximal development containing numerous alveoli, which produce huge amounts of milk. Upon weaning, milk production ceases, the mammary alveoli regress (involution) and the mammary epithelium returns to a non-pregnant state.](image)
menses and the loss of hormonal support, thus leading to a gradual accretion of the epithelial tissue with each successive cycle. In women, 3 types of lobules have been identified based on the size of the composing alveolar buds and their differentiation state (see Fig. 1 in [1]). With increasing years, mitotic activity slightly decreases until ~35 years of age. Then, full differentiation of the MG is a gradual process taking many years, which is achieve only if pregnancy supervenes.

While in the non-pregnant adult woman connective and adipose tissues predominate and epithelial tissue is sparse, the onset of pregnancy induces progressive changes in both cellular and functional organization in the MG. Early pregnancy is characterized by growth due to a marked proliferation of both ductal and alveolar cells, concomitantly with the reduction of the fat pad. This leads to the formation of an extensive branched ductal system with a high number of alveoli of variable size and shape, gradually derived from TEBs (mammogenesis, Fig. 1, gestation). The surrounding stromal and myoepithelial cells provide essential cues for MEC survival, proliferation and differentiation. In newly formed lobules, the alveolar MECs not only increase in number due to active cell division but also increase in size, mainly because of cytoplasm enlargement. These modifications are regulated by numerous systemic hormones, including estrogen, progesterone, PRL, GH, insulin, glucocorticoids (GCs) and parathyroid hormone-related protein, as well as local factors such as insulin-like growth factor-1 (IGF-1), EGF and fibroblast growth factor (FGF), which are likely produced by the stromal cells [2,4]. Moreover, both MECs and stromal cells produce various extracellular matrix (ECM) components (e.g. proteoglycans, hyaluronan, fibronectin, and laminin), which are important for MG growth and function [5]. The definitive structure of the ductal tree is essentially settled by the end of the first half of pregnancy and further changes until parturition are chiefly continuation and accentuation of branching and alveoli formation. Hence, small amounts of secretion product (colostrum) can be observed in enlarged lumen of alveoli and milk ducts, signing the functional secretory differentiation of MECs (referred as lactogenesis I). In the last trimester, there is a reduced proliferation of new alveoli with a further increase in their size due to distension of their lumen (terminal differentiation of MECs) by accumulation of colostrum. In addition to progesterone, PRL and/or human placental lactogen (hPL) appeared to be involved in the final stages of secretory MECs growth and differentiation. Concomitantly with the increased metabolic activity of MECs, the mammary blood flow approximately doubles in volume during pregnancy and persists during lactation until weaning.

Secretory differentiation of MECs

Secretory differentiation of the alveolar MECs or lactogenesis starts around mid-pregnancy and has been divided into two successive phases: initiation or lactogenesis I and activation or lactogenesis II. These critical stages rely on variations of gene expression, structural and functional properties of alveolar cells, all of which being hormonally regulated [6,7].

During lactogenesis I, MECs differentiate morphologically and become competent to produce and secrete some milk components referred as colostrum [8], due to the activation of the expression of some milk protein genes and biosynthetic enzymes, as well as the production of lactose and accumulation of lipid droplets (LDs) [6]. However, production and secretion of milk components appear to be restricted to a limited number of alveolar MECs with incompletely developed secretory mechanisms. As colostrum is not removed by suckling, its components are reabsorbed into the blood through the paracellular pathway. At late pregnancy, milk secretion is inhibited by high plasma concentrations of progesterone and estrogen until parturition.

After parturition, the expulsion of the placenta results in a rapid withdrawal of progesterone [9], estrogen and hPL during the 4–6 days after birth, while PRL concentrations remain high in the presence of insulin and cortisol, thus triggering lactogenesis II [6,10].

Colostrum is produced during the first 4 days postpartum, followed by a 10–15 days period of transitional milk secretion, before copious production of mature milk (after 15 days) [8]. Milk composition is dramatically altered: sodium and chloride concentrations fall while those of lactose, immunoglobulins A (IgA), lactoferrin (LTF) and other components of mature milk increase. These changes are completed by 72 h postpartum and precede the increase of milk volume by ~24 h, accordingly to the terminal differentiation of alveolar MECs into lactocytes [11]. These changes result from substantial variations of milk protein genes (e.g. \( \alpha \)-lactalbumin) and biosynthetic enzymes (e.g. acetyl-CoA carboxylase and fatty acid synthetase) expression [12], supported by alveolar MECs
reorganization, including apico-basal polarization of organelles, expansion of mitochondria and RER, maturation of the Golgi apparatus, appearance of secretory vesicles (SVs) containing casein micelles and of numerous microvilli at the APM, increase in the number of bigger LDs and closure of TJs that blocks the paracellular pathway, to adapt to their high secretory state [13]. Moreover, there is an increase of transport activities for all substrates for milk production such as amino-acids, glucose and fatty acids, as well as ions. Indeed, with the closure of TJs, ions such sodium and chloride can no longer pass from the interstitial space into the lumen of the alveolus and then must be secreted by the cellular route.

Milk volume produced rapidly increases in the first 24 h postpartum, accordingly to the increase of both the frequency of breastfeeding and the volume consumed by the newborn, and stabilizes after ~1 month (~750–800 ml/day) to remain fairly constant up to 6 months postpartum [11].

Although not essential within the first hours after birth, milk removal by day 3 is critical for the establishment of a successful lactation. Both the time of the first breastfeeding and the breastfeeding frequency on day 2 were positively correlated with milk volume on day 5 postpartum, suggesting that milk removal soon after birth increases the efficiency of milk secretion. Once lactation is established, the volume of milk produced is merely determined by the baby’s appetite [14]. Indeed, the breast is rarely completely drained during a suckling (on average 67% of the available milk is consumed). Thus, in connection with the frequency and effectiveness of the drainage-filling cycle of the alveoli [14,15], there is a switch from endocrine to autocrine control and milk removal becomes the primary regulatory mechanism for galactopoiesis (milk synthesis) and to adjust milk volume to the requirements of the newborn. Milk can be stored for up to 48 h before the rate of milk synthesis and secretion begin to decrease. However, incomplete/inefficient milk removal or milk stasis induce multiple local effects on milk secretion: 1) an autocrine whey protein, termed ‘feedback inhibitor of lactation’ (FIL) regulates milk secretion according to frequency or completeness of milk removal in each MG [16]; 2) other factors such as osmolarity and mechanical stress [17] influence milk synthesis; 3) expression of the PRL receptors in MECs decreases, thereby uncoupling the stimulatory effects of PRL on milk synthesis; and 4) prolonged milk stasis triggers MECs apoptosis. Lactation is prolonged as long as milk is regularly removed from the MG [14].

Delayed and impaired lactogenesis

Some particular conditions may delay lactogenesis, including placental retention, caesarean section, diabetes or stress during parturition. Obese women are more likely to experience delayed lactogenesis II, potentially due to hormonal influences on milk production, increased difficulty attaining a successful infant latch to the breast, and/or socio-cultural factors [18].

Early milk removal, correct attachment of the baby to the nipple, as well as the frequency and the efficiency of suction are the main key conditions contributing to a successful breastfeeding. Therefore, irregular or incomplete removal of milk leading to breast engorgement may be due to a mother’s pathology such as an impaired milk ejection, inverted nipples or mastitis, as well as poor attachment and/or positioning, ineffective suckling, infrequent feeds of the infant. The best indicator of an adequate milk supply is the infant weight gain during the early neonatal period.

**Fig. 2.** Anatomy and functional organization of the lactating mammary gland. A) During lactation, the mammary epithelium is organized in lobes containing numerous lobules and connected to lactiferous ducts, which drain milk towards the nipple. B) Each lobe contain numerous lobules formed by several alveoli, which are the milk secreting units. C) The alveolus is defined by a monolayer of polarized alveolar mammary epithelial cells (MEC) arranged around a lumen, where milk is secreted. The alveolus is connected to the lactiferous duct formed by a bilayer of MECs. Alveolar MECs contact the BM, a specialized ECM and are surrounded by contractile myoepithelial cells. Mammary alveoli are embedded in a stroma containing collagen, endothelial cells, adipocytes and fibroblasts. D) Milk products are secreted in the lumen of the alveolus by various pathways in polarized MECs. Transport of plasma components and sometimes leukocytes through the paracellular pathway (1) occur only during pregnancy, early lactation before the closure of TJs, and inflammation or during infusion. Membrane transporters (2) allow the direct movement of ions, water and glucose across the BPM and the APM of the MEC. Some plasma proteins such as Igs and PRL reach the lumen after crossing the MEC by vesicular transcytosis (3). Milk proteins, lactose, calcium and other components of the aqueous phase of milk are transported in secretory vesicles (SVs) and released after exocytosis (4). LDs are formed in the endoplasmic reticulum (ER) and grow during their transport to the apex where they are released as milk fat globule (MFG) by budding, being enwrapped by the apical plasma membrane of the MEC (5). BV, blood vessel; M, mitochondrion; Myo, myoepithelial cell.
Effects of lactation on reproduction

Lactation has a marked effect on fertility in breastfeeding women. After parturition, the systemic levels of LH and FSH, both controlled by the pulsatile release of gonadotropin-releasing hormone (GnRH), are low due to the suppression of the hypothalamic—pituitary axis by placental steroids. While fertility returns approximately 6—9 weeks postpartum in non-breastfeeding women [19], in breastfeeding women GnRH secretion is suppressed by various factors such as maternal nutrition, PRL levels [20] and the suckling stimulus [21] and appears to be highly correlated with the breastfeeding pattern, e.g. the frequency and duration of suckling. The inhibition of GnRH release results in the disturbance of pulsatile LH secretion, which in turn suppresses ovarian activity. In addition, the increased sensitivity of the hypothalamo-pituitary system to the negative feedback effects of ovarian estrogen after parturition also contribute to the suppression of fertility during lactation.

Post-lactational changes and involution

At the end of lactation, when regular removal of milk ceases, the MG enters a tissue-remodeling process known as involution [22]. Early after weaning, the epithelial architecture is maintained by the recent exposure to elevated systemic hormones, e.g. PRL, GCs and IGF-1, which are also critical survival (anti-apoptotic) factors for MECs. During this first phase of involution or “reversible phase”, the MG can revert to a state of milk production if the suckling stimulus occurs again [23]. However, extended milk stasis in the ducts and alveolar lumen, concomitantly with PRL and GC withdrawal due to the absence of suckling, leads the MG to enter an “irreversible phase” (or phase 2 of involution) and to become unable to return to lactation without being re-stimulated by pregnancy levels of hormones [23]. Milk stasis directly inhibits milk protein synthesis and secretion through both mechanical stretch and local production of various pro-apoptotic factors such as serotonin (5-hydroxytryptamine, 5-HT), LTF, Interleukin (IL)-6 family of cytokines, transforming growth factor-β (TGFβ) and α-lactalbumin. These factors lead to the inhibition of milk production by inducing the desensitization of MECs to lactogenic hormones. The fine balance between survival factors (PRL, GC, IGF-1) and cell death factors (5-HT, ILs, TGFβ, Vitamin-D receptor, IGF-binding protein-5) regulates the coordinated, multifocal and asynchronous processes resulting in a massive epithelial tissue regression (~80%), mainly via apoptosis and autophagy of MECs. TJs gradually breakdown and the ECM is progressively remodeled by the action of both the matrix metalloproteases (MMPs) and the plasminogen system [24]. Loss of attachment-dependent survival through integrins signaling (e.g., anoikis) together with pro-apoptotic signals leads to the elimination of MECs, collapse of acinar structures and narrowing of the tubules, while myoepithelial cells remain relatively well-organized during involution around residual ductal buds [25]. In addition to immune cells present in the MG at all stages of development [26], surviving MECs play a major role in the clearance of residual milk and cell debris as they engulf casein micelles, MFGs and apoptotic cells [27]. They also release anti-inflammatory cytokines which limit the action of the recruited leukocytes and neutrophils during early involution. Macrophages, local acute-phase response activation, and a late B-lymphocyte response complete the clearance of cell debris. These events ultimately lead to rapid regression of the epithelial tissue resulting in a rudimentary ductal tree morphologically similar to a virgin MG with some persisting alveoli (Fig. 1, involution). Concomitantly, pre-adipocytes re-differentiate (adipogenesis) and colonize a major part of the MG, while the vascular tissue is also remodeled [28].

Menopausal breast

After menopause, accompanied by an almost complete cessation of ovarian estrogen and progesterone production, the breast undergoes a slight regression. Nulliparous and parous breasts appear quite identical with only minimal quantitative differences in the proportion of lobule subtypes. However, nulliparous women exhibit a higher incidence of breast cancer than parous women and differentiation is suggested to protect the MG against carcinogenesis [29].

Functional anatomy of the lactating breast

During lactation, the MG consists of a highly branched tubulo-alveolar glandular epithelium (or parenchyma) (Fig. 1, lactation), embedded in a stroma of both connective- and white adipose-tissue,
and supported by a loose framework of fibrous connective tissue referred as Cooper's ligaments (Fig. 2A) [30]. There is a decrease in the amount of adipose tissue relative to glandular tissue (ratio ~1:2), which is not correlated to milk production or storage capacity, and the size and weight of the breast increases. Human breast consists of 15–20 lobes, the size of which is highly variable, subdivided into lobules containing between 10 and 100 alveoli or alveoli (~0.12 mm in diameter), which are the basic secretory units producing milk (Fig. 2B). Alveoli are clustered around ductules connected to the interlobular duct of the lobules that coalesce to form larger ducts, which are drained towards the nipple by a lactiferous duct (1.2–2.5 mm in diameter) that only dilate during milk ejection (no storage, only transport) [31]. The significant variation in lobule size observed may reflect the difference in secretory activity from lobule to lobule. Moreover, growth and differentiation of MECs can occur in the same lobule, concomitantly with milk production. Each alveolus is surrounded by contractile myoepithelial cells responsible for milk ejection [32] and an extensive capillary network [28]. In addition to their role in milk ejection, myoepithelial cells also regulate mammary development through secreting various growth factors [4], spatially restrict MECs to form ducts during puberty, and act as tumor suppressors. Alveoli are embedded in a connective-tissue stroma containing adipocytes, fibroblasts and some plasma cells, which produce the Igs found in milk, as well as non-cellular components such as collagen and proteins of the ECM (Fig. 2C). Lymph is drained by two main pathways: the axillary nodes and the internal mammary nodes, which mostly drain the medial and lateral portions and the deep portion of the breast, respectively. The lymphatic network transports lipid-soluble nutrients (e.g., vitamin K and lipids) to the lactocytes, while the lymph nodes, which contain leukocytes (mainly lymphocytes and macrophages), provide an immune defense system to the MG in response to bacteria or foreign material. The MG contains only few internal innervations. Nerve fibers associate with the major duct system and are rather sparse in the region of the smaller ducts, areola, and nipple [33]. Sympathetic nerves are associated with the arteries but not the alveoli and there is no parasympathetic innervation of the MG. However, sensory nerves present in the nipple are critical for initiating the afferent neural pathway of the milk ejection reflex. As there is no motor innervation of the mammary epithelium nor the myoepithelial cells, milk production and ejection are independent of the neural stimulation.

In the alveoli, the secretory MECs form a sealed epithelial monolayer upon the closure of their apical adherens- and tight-junctions (TJs), which segregate the lumen from the interstitial space, thus preventing paracellular transport (Fig. 2D). TJs also delimit the apical (APM) from the baso-lateral (BPM) plasma membranes of MECs, thus contributing to the establishment and the maintenance of the functional asymmetry (polarity) of MECs required for the vectorial secretion of milk [34]. The basal side of alveolar MECs contacts myoepithelial cells and the basement membrane (BM), a specialized ECM, which separates the epithelium from the stroma and the vascular system. The BM results from the secretion by both stromal cells and MECs of specific ECM components further assembled in a 100 nm thick matrix at the basal surface of the mammary epithelium [5]. Integrins are hetero-dimeric ECM receptors localized on the BPM of MECs and mediate cell—matrix adhesion and regulate various aspects of MEC development and function through integration with other signals [5]. Integrin/BM interaction leads the formation of focal adhesion centers integrating both the assembling of the cytoskeleton and cell survival signals. Integrins signaling is thus involved in the establishment of the apico-basal polarity (e.g. apical side speciation) of MECs and lumen formation during pregnancy, enables PRL signaling through its effectors Jak2 and Stat5 to activate milk protein genes during lactation, as well as remodeling of the mammary tissue during involution [35]. Moreover, the composition and stiffness of the BM change during MG development according to the variations in the ratio of the mammary cell types. Therefore, there is a critical interdependency of tissue architecture and cell fate for the spatio-temporal regulation of both mammary development and function [35]. Thus, by transducing both biochemical (survival, differentiation and functional) and biophysical signals (changes in cell shape or membrane tension through cytoskeletal changes), integrins determine the fate of MECs [35,36].

The APM of MECs borders the lumen of the alveoli, where milk product are released. As their principal function is to produce and secrete huge amounts of milk to feed the newborn, the intracellular organization of MECs reflects their highly secretory state. Indeed, the cytoplasm of alveolar MECs is filled with an extensive rough endoplasmic reticulum (ER) network, enlarged Golgi apparatus, and contains numerous mitochondria and SVs containing casein micelles. Lactose is synthesized in the
Golgi, and is transported with casein micelles into the SVs towards the APM. Secretory MECs also produce LDs emerging from the ER by accumulation of neutral lipids and which grow during their transport before being released as milk fat globules (MFGs) by budding (Fig. 2D) [37].

**MECs secretory routes**

After reaching the MG through the blood stream or the lymph system, nutrients and other components used to synthesize milk constituents diffuse in the interstitial space and reach the BPM of MECs. Depending on their molecular nature, they enter MECs and are secreted in milk by several routes. Most of the transport pathways are tightly regulated and coordinated, so that sufficient milk of adequate composition is available for the newborn, even during inadequate food intake by mothers.

**Paracellular pathway**

Molecules can enter milk through paracellular or transcellular pathways (Fig. 2D), which are affected by the functional state of the MG and regulated by hormones, growth factors and probably mechanical constrains. While the mammary epithelium is leaky before lactation, the direct bi-directional paracellular exchanges of molecules between the interstitial space and the alveolar lumen (Figs. 2D and 1) is inhibited during the first days of lactation after the closure of TJs triggered by the hormonal changes [13]. Consequently, large trans-epithelial concentration gradients are established and maintained for ions and macromolecules between blood and milk.

**Transcellular pathways**

After TJs closure, the composition of the milk reflects the highly coordinated functioning of four main transcellular pathways in MECs, which operate to produce milk components from blood-borne and interstitial molecules [6,38]. Many transporters (Fig. 2D and 2) are involved in the transfer of ions, glucose, amino acids and water are present on both the BPM and the APM [39]. Transcytosis (Fig. 2D and 3) allows the transport numerous components originating from the bloodstream or the stroma, such as Igs, albumin, transferrin, insulin, PRL, estrogen, cytokines and lipoprotein lipase [40].

Endogenously produced constituents such as major milk proteins, oligosaccharides, lactose, secreted through the exocytic pathway (Fig. 2D and 4) [41], while lipids (mainly triglycerides) are secreted by a specialized budding process (Fig. 2D and 5) [37].

**Membrane transporters.** Water is drawn across the alveolar MECs in a transcellular manner, driven by an osmotic gradient largely created by the lactose content of the milk. Water is transported by small transmembrane proteins of the aquaporin (AQP) family. AQP are quite ubiquitous and, in addition to water, may also facilitate entry of gases such as CO2, NO and ammonia within cells. Various AQP have been identified in the MG of various species including human, and localized in MECs, endothelial and myoepithelial cells. For example, AQP3 is localized in the BPM of alveolar MECs and may participate in the regulation of milk isotonicity by diluting milk components. The permeability of AQP is strongly dependent on the molecular weight of the osmolytes they are exposed to. Moreover, the activity of AQP could be up-regulated after their rapid membrane translocation in response to hormones [42].

Membrane transport pathway (Fig. 2D and 2) relies on the concerted activity at both the BPM and the APM, as well as in cellular membranes, of various transporter proteins, allowing transcellular transfer of ions, trace elements, glucose and amino acids from blood to milk. Ion transporters or channels for sodium, potassium and chloride are found on the BPM and the APM of MECs, while calcium, phosphate, iodide and citrate transporters appear to be limited to the BPM [39,43]. Sodium and potassium are also actively transported by Na+/K+ ATPase pumps localized in the BPM but not APM of MECs. Active transport of calcium and trace elements including iron, zinc, copper, selenium, iodide, fluoride, and manganese have also been described in MECs but the underlying mechanisms have not been fully characterized. Moreover, the activity of some of these transporters, such as Ctr1 and ATP7A for copper and Zip3 for zinc, has been shown to be up-regulated by PRL, which induces their targeting to the BPM. Adequate supply of trace elements from milk is crucial to ensure neonate survival.
and both their uptake from blood and release in milk are tightly regulated by MECs, so that trace element concentrations remain remarkably stable, independently of the mother’s diet [44].

As human milk contains up to 8 mM of calcium, large quantities of calcium are transported from the blood, which contains ~3 mM of calcium, before being concentrated in milk [11]. The presence of calcium channels has been described in the BPM and some intracellular membranes of MECs [44,45], while the intracellular compartmentalization of calcium depends on cytoplasmic binding proteins. In milk, calcium is found associated with casein micelles (~20%), free ionized or non-ionized (~32%) or complexed to inorganic anions such as phosphate and citrate (~46%).

In addition, the expression and/or the activity of some ion transporters may be hormonally regulated. For example, potassium uptake [39] and chloride transport may be up-regulated by PRL via phosphorylation of transporters, while the expression of the sodium/iodide symporter is regulated by PRL and OT [43].

Plasma-derived glucose is a substrate for several key metabolic processes in MECs, including fatty acid and amino acids synthesis, triglyceride esterification, and is the obligate precursor for lactose synthesis. Hence, several types of glucose transporters (predominantly GLUT1) are found at both the BPM and the APM of MECs, as well as on Golgi, where lactose is synthesized from UDP-galactose and glucose, and SVs membrane. Lactogenic hormones such as PRL control both the expression of glucose transporters and their activity through translocation from intracellular sites to the BPM [46].

As amino acids are building blocks of proteins, large amounts of these precursors are required to support milk protein synthesis in the lactating MEC. Both sodium-dependent and -independent amino acid transporters are present at the BPM of MECs, but their presence at the APM remains unclear, although milk contains some amino acids. Amino acid transport has been shown to be modulated by PRL and milk stasis [47].

**Transcytosis.** After their receptor-mediated endocytosis at the basal side of MECs, some interstitial molecules enter milk through the transcytic pathway (Figs. 2D and 3). After endosomal maturation, these molecules are transported alone or in complex with their receptor to the APM of MECs, where they are secreted by exocytosis, while their receptor are degraded or recycled back to the BPM. Transcytosis has been described for IgA, insulin, PRL, serum albumin, transferrin, IGF-1 and low-density lipoprotein. Of note, the fusion of some transcytic vesicles with SVs may occur in the apical area of MECs before the exocytosis of their content [40].

**Protein secretion pathway.** Milk proteins are synthesized in a classical secretory pathway (Fig. 2D and 4), beginning with the transcription of their genes into mRNA, then translated in proteins and folded in the rough ER. Major milk proteins, namely caseins (α-, β-, γ-, and κ-caseins) also undergo post-translational modifications [48], mostly in the Golgi, associate with calcium and phosphate to form supramolecular structures called casein micelles (~140 nm in diameter), which are packed in SVs. SVs also contain water, LTF, oligosaccharides, and high concentration of lactose, phosphate, calcium and citrate. SVs are vectorially transported via microtubules and fuse with the APM, then releasing their content into the lumen of the alveolus by exocytosis (Fig. 2D and 4). Interestingly, this pathway may be regulated by lactogenic hormones at several levels. Indeed, independently of activating casein gene expression, PRL exerts a secretagogue effect on the last steps of apical transport and possibly the exocytosis of caseins through the production of arachidonic acid [49]. On the other hand, after binding to its cognate receptor in MECs, OT has been shown increase the number of SVs and to accelerate their transport towards the APM [50].

The molecular machinery responsible for membrane fusion has been characterized in many cell types, particularly in neuronal cells, and more recently in MECs [51]. SNARE (Soluble N-ethyl-maleimide-Sensitive Factor Attachment Protein Receptor) proteins mediate specific fusion of transport vesicles with target cellular membranes. To do so, the vesicular SNARE (v-SNARE) binds to cognate SNAREs located on the target membrane (t-SNAREs), thus forming a tripartite SNARE complex that promotes the fusion of the vesicle with the target membrane (Fig. 3A and B). The whole process of exocytosis is highly regulated by numerous proteins working in close association with the SNARE complex. In MECs, specific SNAREs have been observed associated with the APM, SVs and MFGs during lactation [51]. On the other hand, several studies have shown that SNARE proteins are the targets of
arachidonic acid in different neuroendocrine cell types [52]. Thus, it is tempting to speculate that the SNARE proteins may be the target effectors of arachidonic acid produced in response to PRL, then providing a link between signal transduction, secretagogue effect and exocytosis in MECs (Fig. 3C), [53]. Moreover, the expression of some SNARE genes has been found to be regulated by PRL [54]. In MECs, the expression of some genes encoding SNAREs involved in casein exocytosis (e.g. SNAP23 and VAMP8) is strongly up-regulated during lactation, [51] and our unpublished results, potentially in response to lactogenic hormones [55].

Lipid secretion pathway. MECs import, synthesize and store lipids as LDs which are mainly formed by accumulation of triglycerides between the two leaflets of the ER and coated with some specific proteins (Fig. 2D and 5) [37,56]. Precursors of triglycerides include acetate, β-hydroxybutyrate, acetoacetate, fatty acids, glycerol, and monoacylglycerides, which are taken up by MECs, as well as ketone bodies. Free cholesterol also associates with LDs [57]. LDs are thought to grow by fusing with each other during their apical transport, and are released by budding, enwrapped by APM, as MFG [37]. Although proteins such as butyrophilin (BTN1), adipophilin (PLIN2) and xanthine oxidase (XOR) appear to play a critical

![Diagram](image-url)
role in this unique secretory process, the molecular mechanisms of MFG release have not been fully deciphered [58]. Moreover, it occasionally results in the inclusion of a cytoplasmic crescent in the MFG, thus virtually enabling any cellular components to reach milk. The MFG is a major energy source for the newborn and also contains numerous enzymes, immunomodulatory factors, such as lactadherin/MFG-E8 and BTN1. Lipid secretion is regulated by hormones such as PRL [59] and OT through mechanical deformation of MECs upon myoepithelial cells contraction [60].

**Secretory pathways coupling**

As the release of milk constituents involves at least two distinct mechanisms (e.g., exocytosis and budding, Fig. 2D, 4 and 5) synchronized at time of suckling, it is likely that common activation switch and/or molecular effectors may exist to coordinate their activities. On the other hand, because of their large size (~4 μm in diameter) and their high number, the membrane surface needed to enwrap the MFGs could exceed that of the APM of MESCs. Thus, at time of suckling, there is both membrane supply and loss at the APM of MESCs, due to SV fusion and MFG budding, respectively. Various data also reinforce the possibility of coupling of these two processes: 1) the association of SVs with the APM and the basal part of the budding MFG (Fig. 3A) has been extensively described, 2) some SNARE proteins are localized at the interface between SVs and the budding MFG [51] (Fig. 3B) and 3) the membrane supplied by the fusion of a high number of SVs with the APM is used to enwrap the MFG [57,61]. As depicted in Fig. 3C, a possible scenario could be that, in response to PRL (secretagogue effect), local production of arachidonic acid, potentially from neutral lipid core of the MFG, stimulate membrane fusion through interacting with SNARE proteins [40,53]. Both heterotypic (SVs with APM) and homotypic (SVs with SVs) fusion may then occur (Fig. 3C), leading to the coordinated release of milk products. Furthermore, this would also partly balance the membrane loss caused by MFGs release, concomitantly with the efficient resealing of the APM (Fig. 3D). Recently, the final expulsion of MFGs has been shown to occur after OT-mediated contraction of the myoepithelial cells [60]. This observation is compatible with the above scenario and suggest that milk secretion processes are spatio-temporally coupled and regulated by both hormonal and mechanical factors.

**Hormonal regulation of milk secretion**

As MG growth and differentiation, lactation is regulated by hormones, but also by interactions between the MG and the central nervous system. PRL signals through the JAK2/STAT5 pathway to regulate the expression of target genes, and also stimulates lipid synthesis and exocytosis. On the other hand, OT is rapidly released in response to suckling and induces the contraction of myoepithelial cells surrounding the alveoli, thus triggering milk ejection [6].

**Prolactin**

PRL is a pleiotropic hormone produced by the anterior pituitary which is involved in homeostasis, reproduction, and lactation. During the MG development and differentiation, PRL exerts morphogenic effects, while during lactation this hormone displays lactogenic effects by stimulating milk protein and lactose synthesis and secretion, as well as other metabolic processes in MECs. PRL is thus required to maintain milk yield, but also for alveolar MECs survival and maintenance of tight junctions (TJs) [6,13]. During pregnancy, the serum PRL level slightly increases from ~10 ng/mL in the non-pregnant women up to ~200 ng/mL at term [62]. In the course of lactation, levels of circulating PRL gradually decrease to return to ~10 ng/mL after ~6 months postpartum. PRL is episodically released in response to suckling to reach a peak in concentration in the blood 45 min after the beginning of breastfeeding, for up to 75 min in duration [63]. However, while the amount of PRL released is related to the intensity of nipple stimulation, plasma PRL concentration does not appear to be directly correlated with the volume of milk produced. Interestingly, in serum and milk, several molecular forms of PRL are found, which arise from PRL processing such as cleavage [64]. Whether this molecular heterogeneity can account for the various effects of PRL remains unclear. For example, while binding of the 23-kDa PRL to its cognate receptor on the BPM of MECs stimulates milk protein genes transcription, the internalization of the
PRL/PRL receptor complex enables the transcytosis of PRL to the lumen, which is required for milk protein secretion [40].

Oxytocin

As soon as it begins, suckling is detected by mechanoreceptors of sensory nerve terminals in the areolus of the nipple which send afferent cholinergic impulses to the paraventricular nuclei and supraoptic nuclei in the hypothalamus, that in turn stimulate the pulsatile release of OT, a nonapeptide hormone, from the posterior pituitary [65]. Once in the bloodstream, OT reaches the MG where it interacts with specific G-protein-coupled receptors localized on myoepithelial cells, and induces their asynchronous contraction. As OT receptors are also present in MECs [66], this hormone may also exert direct effects on the secretory activity of MECs (Fig. 3D) [50,60]. Milk is then expelled out of the alveoli into the ducts and lactiferous sinuses. Contraction of the myoepithelial cells also shortens and widens the ducts, thus increasing the intraductal pressure and consequently the milk flow rate, ultimately leading to milk ejection from the nipple. Thus, OT mediates the milk ejection reflex (or let-down reflex), which is essential for the efficient removal of milk from the breast. As OT is released in a pulsatile manner, there are several ejections of milk during a feeding [31]. The number of ejections is significantly correlated to the volume of milk consumed but not to the duration of the feeding [31]. Suckling also causes an inhibition of the release of LH-releasing hormone by the hypothalamus that results in the inhibition of ovulation and a natural form of birth control.

There is also a significant psychological component in the let-down reflex, as OT release also occurs in response to such stimuli as the sight or sound of the baby [67]. In addition to mediate the milk ejection reflex, OT also has significant roles on the central nervous system for the psychological integration of the interactions between the mother and the suckling neonate, and in maternal behavior. Furthermore, physical and psychological stress or pain of the mother has been shown to decrease milk output through the inhibition of OT release [68]. However, responses to stress seem to be reduced, e.g. plasma levels of adrenocorticotropic hormone (ACTH), cortisol, and epinephrine are significantly decreased in lactating women stressed with graded treadmill exercise as compared to those found in non-lactating women [69]. OT release is likely to be involved as its pulsatile release in response to suckling is accompanied by a decrease in plasma ACTH and plasma cortisol levels in lactating women.

Milk composition

The composition of milk varies between and within species and is specifically and ideally adapted to the needs of the neonate mammals to properly develop. Indeed, milk composition varies according to gestation, time postpartum and even during suckling.

During pregnancy, pre-colostrum contains high concentrations of protective Igs, lysozyme, and LTF, sodium, chloride, and low concentrations of casein, lactose, potassium, citrate, calcium, and phosphate. Colostrum persists for 4 or 5 days after parturition, followed by transitional milk for a further 5 days until mature milk is produced [11].

Mature milk is a complex emulsion of fat and aqueous fluid containing proteins (~3.5%), sugars (~7%), lipids (~4%), minerals (~0.5%) and water, constituting a unique complete nutritive source for the newborn.

Milk protein fraction includes four major proteins [70], e.g. α-lactalbumin and LTF (an iron-binding immunomodulatory protein with antibacterial properties), which are the most nutritionally important, caseins, and Igs (IgA for up to 10% of human milk protein, IgM and IgG). Igs provide passive immunity to the newborn and also serve as part of the immune system of the MG [71].

The aqueous fraction of milk (or whey) contains serum albumin, some hormones (e.g. PRL and insulin, leptin and adiponectin), growth factors (EGF, IGF-1, Ghrelin, and TGF), cytokines, lysozyme (a heme peroxidase with antibacterial and anti-oxidant properties), more than 30 enzymes (including lactoperoxidase which oxidizes bacterial components; proteases, protease activators, nuclease, glycosidases, amino-acid oxidases), vitamins, non-protein nitrogen, nucleotides, as well as minerals (sodium, potassium, chloride, citrate, calcium, magnesium, free phosphate, trace elements), and water. Growth factors such as EGF may regulate the intestinal growth, while hormones may modulate metabolism and body composition of the newborn [72]. Factors with antimicrobial activities play important roles in protecting both the gastrointestinal tract of the newborn and the mother’s breast...
Of note, the sodium concentration in breast milk within the first 3 days postpartum may be predictive of lactation success, particularly in some mothers at high risk for insufficient milk supply [74]. Indeed, high concentrations of sodium in milk are found in some clinical situations such as mastitis, inhibition of PRL secretion, and premature birth [75].

Milk also contains various types of carbohydrates, mainly lactose, a disaccharide unique to milk, glucose, galactose, and oligosaccharides, which display substantial protective effect against a variety of pathogens [76].

In human, the fat (MFGs) accounts for ~4% of milk volume and contributes for up to 50% of the energy content. MFGs mainly contain triglycerides as well as a variety fatty acids, cholesterol, phospholipids, and steroid hormones (GCs, progesterone and estrogen) [77]. Fat is the most variable fraction as its fatty acid composition varies with the maternal diet, and even during suckling. Bioactive lipids such as prostaglandins (PGs, including PGE2, PGD2, PGF2, PGI2), and thromboxane A2 are, which are synthesized from arachidonic acid by cyclooxygenases, are also present in milk and may exert protective effects.

Extracellular vesicles (EVs) such as exosomes (40–100 nm diameters) have been identified in milk. Exosomes are vesicles formed in the multivesicular bodies (MVBs) derived from the endocytic pathway. During MVBs biogenesis, cargos, such as proteins, lipids, non-coding RNAs including micro-RNAs (miRNAs), and mRNAs are sorted into internal vesicles (e.g. exosomes), which are released into milk after exocytosis of the MVBs. In addition to play a role in nutrition, exosomes may participate to cell-to-cell communication, regulate developmental and immune processes, intestinal microflora, as well as cellular metabolism and gene expression after ingestion by the newborn [78].

Human milk is particularly rich in miRNAs, which are potentially involved in infant protection and development. MiRNAs are small non-coding RNA molecules that regulate gene expression at the post-transcriptional level, modulating important cell functions such as cell cycle, metabolism, proliferation, differentiation, apoptosis, and immune response [78].

Some cells such as MECs, macrophages, neutrophils, lymphocytes and stem cells are also found in milk [79,80].

Human milk has also been identified as the first probiotic food as it contains a large microbial community including more than 200 phylotypes. Although not clearly established, these bacteria may be present on the mother’s skin or may come from the maternal intestine after reaching the MG via lymph and/or blood circulation [81]. In addition to enrich the intestinal flora of the newborn, milk bacteria could influence the long-term microbiota composition and activity, thus playing a key role to prevent various diseases such as allergies, disorders, and metabolic syndrome [82,83]. Therefore, breast milk not only functions as a nutritive source but also delivers both developmental and immune modulatory factors to the newborn.

Although the gross composition of mature human milk appears fairly constant with only slight changes for major components with stage of lactation, there are declines in the total fat content of the milk between 1 and 2 months, in the concentration of protein between 1 and 6 months [84], and in the concentration of calcium between 4 and 6 months. In addition, subtle variations occur in some constituents, such as fatty acids, vitamins, selenium and iodide, according to the maternal diet [3]. Indeed, although the total fat content of breast milk appears unaffected by diet, the proportions of some fatty acids, e.g. omega-3 and omega-6 polyunsaturated fatty acids (PUFAs) vary substantially with the mother’s diet [77]. These variations may have important consequences due to the positive correlation between the quantity of omega-3 PUFAs in the mother’s diet and the infant brain development [85]. The fat content of milk is also known to increase with the duration of breastfeeding in proportion to the emptying of the alveoli [86]. Thus, even if the storage capacity influences fat concentration in milk, it does not affect the total amount of fat consumed by the child [87]. While the concentration of lactose shows no significant change with stage of lactation, variations in milk glycans, e.g. complex oligosaccharides free or covalently bound (glycolipids, glycoproteins, glycopeptides, and glycosaminoglycans), have been observed both between lactating women and during the course of lactation, according to the newborn’s needs. These complex glycostructures are important dietary factors during early life as they regulate multiple functions [88]. However, the growth rate of breast-fed babies is related to the total amount of milk they consume, rather than the concentration of fat, protein, or lactose [89].
Maternal nutrition affects both the quantity and quality of milk, which vary among countries, and lactation requires adjustments of maternal metabolism to adapt to the energetic demands of breastfeeding [90]. As the milk production is almost entirely regulated by the infant demand, the maternal metabolism can be increased up to 20% of the metabolic output of the mother. This can be achieved by an increased food intake or increased weight loss to compensate for the metabolic needs to produce milk.

**Breastfeeding patterns**

Milk production works according to the ‘use it or lose it’ principle and current recommendations are to feed babies on demand [91]. Indeed, babies feed according to their appetite and the mother’s milk production is regulated to match the baby’s needs. Although PRL stimulates the synthesis of milk proteins, it does not control the amount of milk produced once lactation is established. In fact, the quantity of milk produced is correlated to the draining efficiency of the suckling and is accordingly up-regulated if the breast is well-drained [87]. Moreover, the efficient draining of the breast appears to be more important than the frequency of feeding to stimulate milk production. According to its appetite, a baby drains the breast one or more times per day but on average takes only 67% of the available milk [15]. Therefore, the feeding frequency appears significantly increased for mothers with low storage capacities. Milk contents in proteins and lactose also seem to have more influence on the frequency of feeding, which is independent of the volume of milk consumed, than the quantity of lipids or the calorie value of the meal [92]. Furthermore, the fat content of milk is related to the degree of “fullness” of the breast: the more the breast is filled with milk, the more the fat content of milk is low, while conversely, the more the breast is drained, the more the fat content of milk is high.

**Storage capacity**

During exclusive breastfeeding, the lactating breast has a limited capacity (from 80 to 600 ml) to store milk, which varies to adapt to the child’s needs [15]. Storage capacity also varies from one breast to the other, independently of the ability to produce enough milk, but potentially affecting the feeding frequency. This may be related to the frequency and the efficiency of milk removal and to the local negative feedback regulation of milk secretion occurring when alveoli and ducts are filled with milk. As supplementary feeds are introduced, the milk storage capacity decreases along with the reduction of milk production [87].

**Extended lactation**

The World Health Organization recommends exclusive breastfeeding for the first 6 months of life, and partial breastfeeding into the second year [91]. When lactation is extended beyond 6 months, a significant decrease of the mammary tissue occurs gradually accompanied with a slight decline of the volume of milk produced and changes of its composition [93]. Breast returns to its preconception size after ~15 months of lactation.

**Breastfeeding and associated outcomes**

Human milk is an optimal food for newborns as it contains both nutrients and bioactive compounds which contribute to both the short and long-term health benefits that have been reported to be directly correlated with the duration of breastfeeding. Breast-fed infants experience fewer and shorter infections, exhibit different growth patterns, have different gut microflora, show better cognitive development and even face differences in the risk of chronic diseases, such as obesity, type 1 and type 2 diabetes and cardiovascular disease. Breastfeeding also appears to be protective against sudden infant death syndrome, the risk of diarrhea, respiratory infections, and malocclusion, but does not seem to provide a protection towards either eczema or food allergy [94,95]. Breastfeeding outcomes are also related to mother genotype, phenotype, diet, disease, and lifestyle [90].

Human milk is also recommended to feed preterm infants as it significantly reduces complications associated with prematurity such as necrotizing enterocolitis, retinopathy of prematurity, broncho-
pulmonary dysplasia and late-onset sepsis and promotes brain development and neurocognitive outcome [96].

Extended breastfeeding has also beneficial effects for the mother as it leads to birth spacing due to longer periods of amenorrhea, reduces risk of developing a longer-term diabetes type 2, overweight/obesity, and leukemia [97]. However, breastfeeding seems not to have a protective effect towards hypertension and/or hypercholesterolemia [97]. Extended breastfeeding also reduces the incidence of ovarian and breast cancer. Numerous studies suggest that high parity is associated with a decreased risk of developing breast cancer but that lactation itself, even extended, contributes no extra protection. The incidence of breast cancer appears to be reduced among pre- and post-menopausal breastfeeders, but a direct relationship between the duration of lactation and the reduction in the risk is found only for women with premenopausal cancer. Nonetheless, the mechanisms by which lactation could protect against breast cancer are not clearly identified although they probably involve the hormonal changes associated with breastfeeding and their effects on both the breast and the inhibition of ovulation [29].

Conclusion

Numerous aspects of the lactation process still remain to explore and to understand. The emergence of more efficient approaches to decipher mammary development, secretory functioning and milk composition, together with the integration of multi-scaled data from clinical trials to cellular biology, should highlight new aspects of breastfeeding and help to improve both mother and child’s health, as well as infantile formulas.

Conflict of interest

The authors declare that there are no conflicts of interest.

Practice points

- Breast development and function under physiological and pathological conditions.
- Milk composition and influence of maternal and environmental factors.
- Consider important aspects to help mothers who want to breastfeed their newborn.

Research agenda

- Meta-analysis of clinical data.
- Integration of multi-scaled data, from populations to cell biology.
- Elucidate what and how milk can transfer information to the child and study of trans-generational effects.
- Milk composition and microbiota outcomes in neonate’s development, and in short- and long-term pathologies.
- Intra-vital imaging to decipher molecular mechanisms of breast development and milk secretion.
- Pre-term alimentation and improvement of infantile formulae.

References


Talbot AW, Burneau JD, B臀ngoye RD. A hypo-osmotically induced increase in intracellular Ca2+ in lactating mouse mammary epithelial cells involving Ca2+ influx. Pfugers Arch 1997;433:609–16.


Watson CJ. Involution: apoptosis and tissue remodelling that convert the mammary gland from milk factory to a quiescent organ. Breast Cancer Res — BCR 2006;8:203.


Watson CJ. Involution: apoptosis and tissue remodelling that convert the mammary gland from milk factory to a quiescent organ. Breast Cancer Res — BCR 2006;8:203.


