



### SPLASH! milk science update

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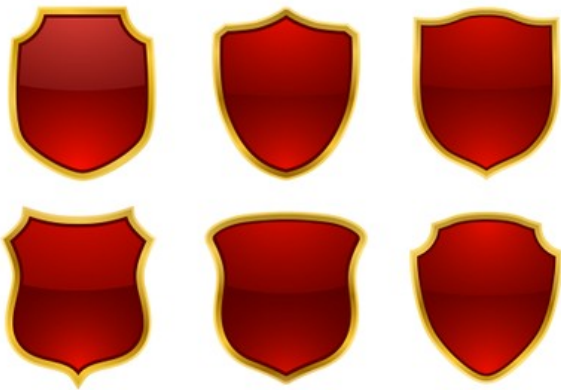
This month's newsletter contains several articles about mother's milk and immune responses. The topics include the role milk plays in [fighting viral infections in infants](#), the relationship between [guts, milk, and immunity](#), the role of [epigenetics in milk production](#), and the changes mother's milk can undergo in the [presence of an infection](#).

We hope you enjoy this month's issue!

## Virus-Fighting Milk Sugars

- **Complex sugars found in human milk have many antiviral properties.**
- **Different complements of sugars in milk alter the probability that virus-exposed infants become infected.**
- **High-throughput methods to elucidate these links look promising.**

For many years, researchers have known that breastfed infants gain some protection from certain viral infections. Occasionally, however, viruses like HIV, a kind of herpes called cytomegalovirus, and HTLV-1, which is linked to leukemia, are transmitted in breast milk from mom to babe. Explaining why infection occurs in some mother-infant pairs but not in many others remains a pressing question. Lately, a series of papers has implicated the complex and highly variable jumble of carbohydrates found in breast milk. In most cases these appear to protect infants from viral infection. But, on rare occasions, they may facilitate it.



Perhaps the most pressing case of all is HIV. When no antiretroviral drugs are available to lower their moms' viral loads, the guts of breastfed infants are washed in lots of virus particles many times a day. Breastfeeding is nonetheless advised because infants born HIV-negative in such circumstances stand only a 10-15% chance of picking up the virus this way—meanwhile they have many health benefits to gain from consuming breast milk instead of formula.

Lars Bode, a researcher working at the University of California, San Diego, has focused his attention on explaining what distinguishes the unlucky 10-15%. He and his team have partnered with researchers monitoring a cohort of HIV-infected mothers in Lusaka, Zambia's capital. By analyzing these women's milk and monitoring

which of the HIV-negative-born, breastfed babies went on to catch the virus, Bode has shown that different complements of oligosaccharides (HMOs) in breast milk skew the odds of infection [1].

All human breast milk contains some of the 200-odd known HMOs, but different women produce different ones in different amounts. Bode concluded that the more HMOs the better: higher overall concentrations of these sugars in breast milk reduced the chance that a baby would catch HIV from her mom. But some HMOs are more protective than others; milk that was low in a common HMO called 3'-sialyllactose (3'-SL) was particularly protective. The reverse was consequently also true: the women whose milk contained relatively high levels of 3'-SL were more likely to pass HIV to their babies.

The next obvious question is why different complements of HMOs alter the odds of viral infection. In another recent paper, Jason Iskarpatyoti of Vanderbilt University School of Medicine, in Nashville, Tennessee, and his colleagues studied how a virus called reovirus interacts with carbohydrates in human breast milk and in cow's milk [2]. Researchers call on reovirus when they seek a model mechanism of how viruses commonly infect the body.

Iskarpatyoti et al. tested the capacity of various milk components to bind two reovirus strains. The strains, T3D and T1L, have different routes of infection and therefore cause different symptoms. The key to explaining the results lies in

understanding how different carbohydrates in milk interact with a specific part of these viruses, called 'attachment protein sigma-1'. When reovirus strains infect a mammal, they first gain a footing on target cells by attaching this protein to a carbohydrate that protrudes from the cell's surface.

In the experiment, several HMOs bound this protein, proving that they mop up virus particles that would otherwise be free to infect cells. Curiously, the work draws attention to 3'-SL, as Bode's did. This HMO, along with two others (that go by the shortened titles, GD3 and GM3), stopped the T3D strain from infecting cells. Infection by the second strain, T1L, was prevented after the initial attachment stage, by an HMO known as GCB.

So that gives a clue as to how HMOs might generally impede viruses. But wouldn't it be helpful to generate many more results more quickly?

A high throughput method has indeed been tested. Ying Yu of the University School of Medicine in Atlanta, Georgia, and her colleagues have created microarrays of HMOs, which they have also interrogated with samples of known viral attachment molecules [3].

By studying where exactly these attachment molecules stick to their microarrays, Yu and her team can tell which viruses are inhibited by which HMOs: if the attachment molecule being tested sticks to, say, three different HMOs on the microarray, it means that these three HMOs probably block viruses that employ this particular attachment molecule in their infection strategies. The proof-of-concept of the method has worked, but the microarrays still need to be expanded to include more HMOs.

Thus the jigsaw puzzle pieces describing how breast milk sugars modulate the odds of infants catching different viral diseases from their moms are being assembled at a rapidly increasing pace. One day, when synthetic chemistry has developed the means to make more complex HMOs, these results might suggest antiviral additives for infant formulas—even for the milk of women who don't make certain ones. For now, though, the practical applications are more limited. In 2007, Asakuma et al. [4] reported that the concentration of 3'-SL decreases rapidly after the first two days of milk production. That might be a helpful detail for some HIV-positive moms.

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## From Mother's Gut to Milk

- **Babies receive antibodies specific to the mother's intestinal pathogens.**
- **Antibodies in the mother's gut travel to the mammary gland and enter the milk supply.**
- **In the mammary glands, gut-primed "digestible" IgA antibodies transform into "digestion-evading" secretory IgA antibodies.**
- **Lab mice with longer intestinal tracts produce milk with more sIgA.**
- **A mother's gut bacteria also travel to the mammary gland and enter her milk supply.**

In a world filled with harmful bacteria, viruses, and parasites, it seems quite paradoxical that a human infant would be born with an immature and inefficient immune system. That is, of course, until you realize the infant benefits from mom's immune system hard at work in mucosal surfaces. The process of transferring immunity, also known as passive immunity, begins during pregnancy with the transfer of Immunoglobulin G (IgG) cells from maternal to fetal circulation through the placenta. At birth, the mammary gland takes over, providing numerous types of immunoglobulins (antibodies) and other immune factors. But the mammary gland isn't working alone—the antibodies in milk are derived from antibodies

the mother produced in her own gut. This link between the mammarys and the gut, known as the entero-mammary pathway, means that infants are not just ingesting generic antibodies with their milk; they are getting antibodies specific to pathogens in their own environment.

### The gut-mammary connection

The lymphatic system is responsible for moving lymphocytes (cells of the immune system, including antibodies) throughout the body to help initiate and participate in an immune response. Lymph tissues are found throughout the human body, with the largest mass (up to 70% of the body's immune cells) found in the gastrointestinal tract (Jung et al., 2010). Called gut associated lymphoid tissue (GALT), this tissue stores antibodies and other immune cells (e.g., T cells, macrophages) and also contains specialized cells known as M cells that transfer pathogens passing through the gut to the lymph tissue so they may be recognized by antibodies.

In a nonlactating female, or in a male, pathogen recognition within M cells will stimulate an immune response that leads to the production of large numbers of immunoglobulin A (IgA) antibodies specific to the pathogen and their release into the gut mucosa, where the invader was first encountered, as well as the salivary glands and lymph nodes.

The scenario plays out quite differently in a lactating female. In addition to being directed back to gut mucosal surfaces and other lymphatic glands, IgA antibodies from GALT have a homing mechanism directing them towards the mammary gland (Blewett et al., 2008; Brandtzaeg, 2002; Goldman, 1993; Kleinman and Walker, 1979). The movement of IgA from the gut to the mammary gland is believed to be under the control of numerous hormones, including prolactin, estrogen, and progesterone, as well as chemokines, and cytokines (Boumahrou et al., 2012; Brandtzaeg, 2002). This results in the mammary gland becoming a part of the integrated mucosal immune system in a lactating female (Brandtzaeg, 2002).



### The right man for the job

When gut-primed IgA antibodies reach epithelial cells of the mammary gland, they change their structure into secretory IgA (sIgA): two IgA molecules attached by a joining chain and then combined with a secretory component. The structural change is critical for their success; digestive enzymes cannot degrade sIgA, and the digestive tract is exactly where these antibodies are needed.

Their success also depends upon their ability to recognize the target pathogens. Pathogen recognition permits them to bind to, and therefore block, enteric pathogens attempting to attach to the surface of the infant's gut. And herein lies the beauty of the entero-mammary pathway: mother and infant share the same epidemiological environment, and thus the sIgA transferred from mother to infant will be directed towards the very pathogens the infant is likely to encounter.

The link between gut and mammary is critical for the transfer of specific protection of the infant's gut. Numerous studies have identified milk sIgA antibodies directed against enteric pathogens, including *E. coli*, *Campylobacter*, cholera, shigella, giardia, and salmonella (Blewett et al., 2008; Goldman, 1993; Hoshower, 2005; Lonnerdal, 2003; Ruiz-Palacios et al., 1990). All of these pathogens would be novel to the infant immune system. Even if it were fully functional, the immune system's first encounter with a pathogen is always slower and less efficient. This is why we vaccinate; the vaccination introduces the pathogen to the body in a weakened (or dead) state, and the resulting immune response creates numerous memory cells. During the next encounter with the pathogen, the memory cells are able to quickly recognize and attack and thereby prevent infection. In the same way, milk sIgA provide the infant with the mother's memory cells and act as a vaccination against a variety of pathogens directed at the gastrointestinal tract.

While certainly important for infants in developed countries, the presence of these antibodies may be life saving for those living in environments with a high pathogen load. Diarrheal diseases (which includes *E. coli*, *Campylobacter*, cholera, shigella, giardia, and salmonella) are responsible for 22% of all deaths for children under the age of 5, or approximately 5 million children a year (Brandtzaeg, 2002; Labbock et al., 2004). Several studies have demonstrated a protective effect of breastfeeding against diarrhea, with both direct and indirect evidence that the protection is conferred by milk antibodies (Labbock et al., 2004; Ruiz-Palacios et al., 1990).

## More guts, more glory

Like humans, lactating mice demonstrate a link between gut IgA and milk sIgA, therefore providing an animal model for which to test hypotheses about the mechanisms involved with transferring immunity from mother to offspring. Of particular interest are factors that may increase milk sIgA content. Boumahrou et al. (2012) took advantage of an inbred strain of mice that have elongated intestinal tracts (called PRM/Alf mice) to investigate whether having more gut surface area, and thus presumably more GALT, would increase the quantity of IgA transferred to the mammary gland and subsequently increase the amount of sIgA in the mother's milk.

They found that, compared to control mice, the PRM/Alf mice had a greater number of gut-derived IgA in the mammary gland and a corresponding increase in milk sIgA. The two breeds of mice did not differ in the concentration of IgA or sIgA in maternal plasma, nor in the concentration of a cytokine thought to be integral to the transfer of IgA from the gut to the mammary gland (called CCL28). Thus, the difference appears to be the result of a greater number of IgA migrating to the mammary gland directly from the gut, which Boumahrou et al. hypothesize is the result of a higher source of precursor cells available from the elongated intestine of the PRM/Alf mice strain.

Increasing the length of the gastrointestinal tract is not a viable option for human females interested in increasing the sIgA content of their milk. But these results are still applicable for understanding the entero-mammary pathway in humans. Boumahrou et al. clearly demonstrate that the production of sIgA in the mammary gland is dependent upon the availability of IgA solely from the gut, as opposed to IgA from maternal circulation (plasma). Variation in milk sIgA in humans has been linked to stress, maternal age, and immune status (Groer et al., 2004). This comparative study in mice suggests these factors may influence pathogen recognition by M cells in GALT or the ability of M cells to transfer IgA from gut to mammary.

### Other travelers on the entero-mammary pathway

sIgA antibodies may not be the only milk components derived directly from the maternal gut. Several studies have demonstrated the presence of bacteria in human milk (Grönlund et al., 2007; Martin et al., 2003), and a study by Perez et al. (2007) suggests that in lactating females, intestinally derived bacteria travel to the mammary gland and ultimately end up in milk using the same cellular pathway utilized by IgA cells. They compared the ribosomal DNA (rDNA) sequences of microbiota in milk, maternal feces and infant feces and identified common bacterial signatures from mother/infant dyads. They call this process bacterial translocation and suggest it imprints upon or programs the immune system of the infant gut to detect harmful from helpful bacteria. Usually bacterial translocation would result in sepsis (bacteria in the bloodstream). But Perez et al. speculate the migration of these bacteria takes place within intestinally derived cells (perhaps mononuclear phagocytes) and therefore has no effect on maternal health.

It can have huge impacts on offspring health, however. Like the infant's immune system, the gut is also naïve and requires time to learn which bacteria should be tolerated and which should be attacked. Bacterial imprinting of the infant gut through bacterial translocation allows the infant to fast track the process of recognizing bacterial patterns from the environment (Perez et al., 2007).

This study is the first to suggest a programming effect as well as a connection of milk bacteria to the entero-mammary pathway. The authors were unable to identify the mechanisms by which bacterial components were mobilized from the maternal gut and then transferred to the mammary gland but do hypothesize it could be the same intracellular pathway (e.g., the lymphatic system) used by IgA molecules. Nevertheless, it is intriguing to think of antibodies and components of the harmful bacteria they are directed against traveling together from maternal gut, to mammary, and finally to milk, all with the ultimate goal of boosting infant immunity.

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## The Epigenetics of Milk-Making: Why a Few Atoms Matter

- **Epigenetics relies on chemical changes to DNA.**
- **DNA modifications are found around many genes.**
- **Mammary glands use epigenetics during development.**
- **Milk genes are primed for action by changes to epigenetic chemistry.**

Last month in *SPLASH!*, we learned that early life conditions can influence a cow's future milk production (see [Katie Hinde's article](#)). But how does this happen? Why does the amount of energy available to a female fetus or calf influence how much milk her mammary gland produces later in life?

As if modern genetics was not complicated enough, epigenetics, and now epigenomics, has emerged as a field in its own right; but what is epigenetics exactly and how does it have an effect? From a mechanistic point of view, epigenetics refers to chemical changes that occur to DNA that modify the usual way in which a region around a gene operates. But what about the functional consequences of these changes?

### Epigenetics affects hereditary characteristics



The most amazing concept in epigenetics is that the chemical changes that occur around DNA may be passed on to the next generation (1). We have known about some of these changes for decades. For example, imprinted gene modifications, meaning genes whose expression is controlled exclusively by the parent that contributed that copy of the gene, are produced in offspring. (PEG is an example of an imprinted gene that may influence lactation performance (2).) Exposure to a range of events, particularly during early life, can also lead to these chemical modifications (3). They can even result from physiologically traumatic events in adults. In addition, the same mechanisms that can cause epigenetic changes when an organism is under duress may be utilized under normal circumstances where tissues are changing and growing. The mammary gland is unique in this respect, with its natural cycle of growth and regression during pregnancy and lactation.

### What are these chemical modifications?

DNA is packed into the nucleus of every cell (except mammalian red blood cells). The packaging requires complex mixes of proteins, sugars, and some small biological chemicals. There are two molecular complexes especially important for understanding DNA packaging and epigenetics: histones and methyl groups. Histones are proteins around which DNA coils for storage until it is needed. And for DNA to be maintained in that tightly packaged state, methyl groups must be attached to the histones. Methyl groups are small groups of atoms that consist of one carbon atom and three hydrogen atoms (methyl groups are what give methane its name). If information within DNA needs to be accessed, for DNA replication during cell division, for example, the methyl groups must be removed from the histones to allow uncoiling of the DNA.



## Chemical changes affect gene activation

DNA that does not unpack and consequently makes its control sites inaccessible prevents the activation of genes in that region. The epigenetic changes may therefore influence a wide range of functions by affecting the level at which a gene is turned off or on. The chemical changes mean that when the DNA is called upon to produce proteins, it may result in much less protein production, or perhaps protein synthesis is shutdown all together. Clearly this has downstream consequences by affecting the function(s) that the protein is responsible for driving. This could be a change in the way in which a mammary gland develops, or how an animal grows, or even its health over an entire lifetime.

## So how can this affect lactation and milk?

The mammary gland develops under hormonal influences at puberty and then undergoes major developmental changes again during pregnancy to become a fully functional secretory gland capable of producing milk packed with highly nutritious components. The primary solid components that give milk its nutritious value are proteins, lipids, and carbohydrates. The production of each of these depends on gene activation in the mammary tissue. So, anything that affects gene activation can influence the production of milk components.

## Epigenetic changes switching milk genes on and off

A recent study by Monique Rijnkels and her colleagues (4) used mice as a model system to understand these epigenetic changes in the mammary gland. The study utilised the most advanced methods for detecting epigenetic marks on DNA. They focused on the major milk protein genes and found that when they tracked the chemical signatures over time, a dynamic process existed. First, there were changes that occurred at puberty that prepared the DNA close to the milk protein genes for future action. During pregnancy further changes occurred so that regions in the DNA that switch on the milk protein genes opened up. Then, with the onset of lactation, these genes were primed to respond to the signals that turned them on, which are mostly generated by hormones. When lactation ceases, the mammary gland undergoes involution and the patterns of epigenetic marks resets.

These results tells us that there are multiple levels of control influencing mammary gland development and milk production in many species, including dairy cows (5). The interesting aspect of the epigenetic impact is that how the cow's mother was managed during her pregnancy and the nutritional state of the cow as a heifer may affect the cow's epigenetics. Conscientious farmers will be paying ever-increasing attention to these details in the future.

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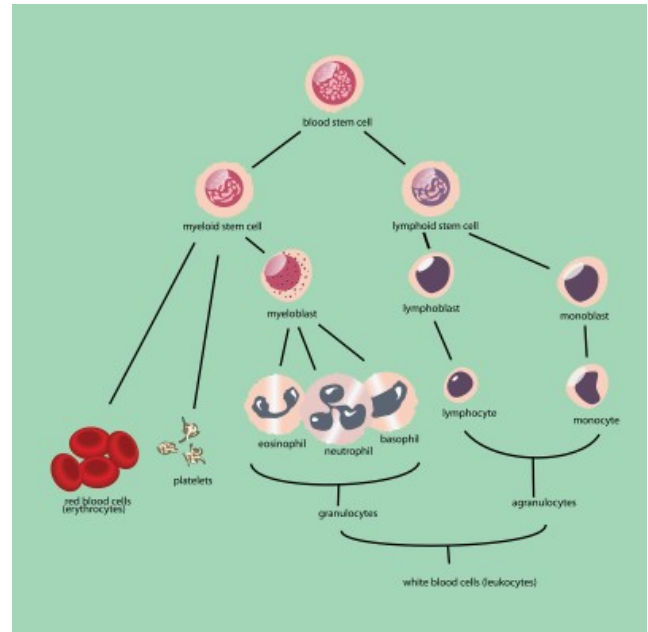
## Protective Cells in Breast Milk: For the Infant and the Mother?

- Human milk contains immune cells for infant development and protection.
- Colostrum and maternal blood have different distributions of immune cells, suggesting selective migration of immune cells into milk.
- Unlike other species, mature human milk contains few immune cells when mom and baby are healthy.
- Both maternal and infant infections stimulate a rapid immune cell response in breast milk.
- Human milk immune cells provide a diagnostic opportunity to assess maternal health.

Babies are well protected and nourished while still in the mother's womb, but what happens after they are born when they are suddenly exposed to a challenging environment full of new and invasive bugs? The mother steps in again by providing breast milk. This magical dynamic fluid contains not only the necessary nutrients for the optimal growth of the infant, but also activated immune cells. Two breakthrough studies show that these immune cells selectively migrate into colostrum<sup>11</sup> and milk<sup>5</sup>.

### Which cells are in milk?

Breast milk has long been known to contain maternal cells<sup>3</sup>; what is less well understood is the proportion of different milk cell types, their significance for the mother and the infant, and factors influencing them. Typically, breast milk is thought to harbor epithelial cells and immune cells. Recent breakthroughs are showing that breast milk cellular composition is more heterogeneous than previously thought and that amongst the different cell types there are stem cells<sup>2, 4</sup>. Stem cells, however, do not comprise the majority of breast milk cells. Earlier reports from the last century suggested that the dominant cell type in human milk was the immune cell (leukocyte). A closer look at these studies reveals that they mostly examined colostrum and milk in the early lactation period. Moreover, the health status of the mother and the baby was not taken into consideration in all of the studies. So, the question remained unanswered, is the immune cell the dominant cell type in mature human milk, and what is its significance?



In an effort to shed some light on this area, we examined a population of breastfeeding mothers and infants both when they were healthy and when they had an infection<sup>5</sup>. Since it had been suggested as early as 1953 that immune cell populations in breast milk often share morphological traits with epithelial cell populations<sup>6</sup>, we used flow cytometry to examine expression of the pan-immune cell marker CD45. This ensured that only immune cells and all immune cells were identified and counted. The results were fascinating and certainly unexpected. Human colostrum contained a significant number of immune cells (up to 70% of the total milk cells), consistent with the greater infant immunological needs in the early postpartum period. However, within the first two weeks after birth, breast milk immune cell numbers decreased to a low baseline level of 0-2% of total cells, which was maintained throughout lactation when both the mother and baby were healthy.

Considering the cellular content of human milk, which ranges approximately 10,000 to 13,000,000 cells/ml<sup>2</sup>, 0.1% of immune cells correspond to 10 to 13,000 immune cells/ml milk, while 2% of immune cells correspond to 200 to 260,000 immune cells/ml milk. And given that breastfed babies normally consume 470-1350 ml of breast milk daily, they ingest thousands to millions of immune cells from their mothers every day. Thus, although the proportion of immune cells relative to other cells in mature human milk is low under healthy conditions, the total number of immune cells ingested by the breastfed infant is significant. What is more, it has been shown that the baseline level of immune cells in human milk is higher in exclusively breastfeeding mother-infant dyads, which may be associated with the lower incidence of infections that is observed in this group<sup>5</sup>. This further supports the importance of exclusive breastfeeding in the protection of the baby.

### Cell populations change when either mother or baby gets sick

During periods of infection of either the mother or the baby, immune cell levels increased significantly in breast milk, but returned to baseline upon recovery. Infections examined ranged from breast-specific infections such as mastitis, which stimulated the most extreme immune cell response in breast milk (comprising up to 95% of total cells!), to other infections of the mother or the baby, that included the common cold, gastrointestinal infection, vaginal thrush, urinary, eye or ear infection.

It is remarkable that a breast milk immune cell response was elicited by an infant infection whilst the mother remained asymptomatic. And although this response was small in comparison to breast infections, it was nevertheless consistent<sup>5</sup>. Similar results were recently reported by another study showing that infant fever stimulated a breast milk immune cell response, which returned to a low baseline level after recovery<sup>7</sup>. This was accompanied by a parallel biochemical response of increased levels of TNF $\alpha$  and lactoferrin, although this was borderline significant and not as consistent as the immune cell response. We reported similar results for breast milk lactoferrin and immunoglobulins<sup>5</sup>. It is extremely interesting that immune cells in breast milk rapidly respond to either maternal or infant infection, suggesting a role in the protection of the mother as well as the infant.

### And the mechanism is...?

These findings support an immunological connection between the mother and the breastfed baby, reinforcing the argument of “human milk for human babies”. They also raise important questions as to how the baby’s infection may stimulate an immune response in the lactating breast and whether this also provides local protection for the breast. We have proposed that oral infant pathogens may be transported into the breast via the retrograde ductal flow during the second half of milk ejection thereby stimulating a local response within the breast<sup>5, 8</sup>. Although this merits further investigation, the breast milk immune cell response to the infant’s infection highlights an underestimated component of the protective nature of breast milk. It also further reinforces the previously proposed functions of breast milk immune cells for the infant: conferring active immunity at a time when it is most needed and promoting the development of immunocompetence. Breast milk immune cells exert these functions not only in the gastrointestinal tract of the baby but also in distant sites where they are transported via the systemic circulation<sup>9, 10</sup>. A recent study reinforces these connections by showing a differential lymphocyte distribution between colostrum and maternal peripheral blood, with B lymphocytes, CD4+ T cells, effector cells, and memory cells being present at higher amounts in colostrum than in the mother’s blood<sup>11</sup>. This suggests a selective migration of these immune cell subsets from peripheral blood to colostrum, supporting the functional significance of these cells early on in the protection of the newborn and potentially reflecting the different status of the mammary environment during the early postnatal period.

### Diagnostic opportunities

Is the breast milk immune cell response to maternal and infant infections a mechanism of protection of not only the baby but also the lactating breast? Can it be used as a diagnostic tool for the assessment of the health status of the lactating breast? Surprisingly, the lactating breast is the only metabolically significant organ in the human body that does not have any medical test of normality. Somatic cell count is a parameter routinely used in the dairy industry to assess the quality of bovine milk and the presence of intramammary infection. The current data on human breast milk refine this test, specifying it for immune cells and extending it to human lactation<sup>5</sup>. Obviously, differences exist in the cell populations between human and bovine milk, with the latter containing large amounts of immune cells throughout lactation<sup>2, 12</sup>.

Despite these differences, intramammary infection, such as mastitis, causes significant increases in both total (somatic) cell and immune cell counts in both bovine and human milk, suggesting that these parameters may be a valuable tool for early diagnosis of breast conditions in women and their successful treatment. The importance of this cannot be overemphasized, as mastitis is a primary cause for early weaning and cessation of breastfeeding. Early diagnosis is likely to be instrumental in successful treatment, thus allowing for longer duration of breastfeeding and ensuring the healthiest start for the infant as well as multidimensional benefits to the mother. It is anticipated that as we learn more about the immune cells of human milk and factors influencing them, a deeper understanding and appreciation will be developed for the attributes and intricacies of this wonderful gift, breast milk.

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