



### SPLASH! milk science update FEBRUARY 2013 issue

We are pleased to announce new additions to our editorial staff: [Prof. Foteini Hassiotou](#), [Dr. Jeroen Heck](#), [Prof. Kevin Nicholas](#), [Dr. Lauren Milligan](#), and [Prof. Peter Williamson](#).

This month's issue features heart-healthy cheese, milk microRNAs, milk-induced temperament, and protective sugars. Enjoy!

## A Hearty Helping of Dairy

- **People with high dairy intakes are less likely to suffer a stroke.**
- **Inconsistent study results suggest that men and women probably derive similar benefits from dairy consumption.**
- **The cardiovascular benefits of dairy appear to be driven by cheese and yogurt intake.**

Most people know they hike the odds of developing cardiovascular disease by incessantly puffing on cigarettes and by eschewing the gym in favor of the TV. Stuffing saturated fats down one's gullet is another well-known risk factor, leading to an increase in low-density lipoprotein in the blood and thus to clogged arteries. On that basis, dairy products seem unlikely protectors of a healthy heart. But various studies suggest they might be just that, particularly—and bizarrely given its high fat content—cheese.

The evidence is not straightforward, nor, where it exists, very dramatic. Most studies that have looked for a statistically significant trend between dairy consumption and cardiovascular disease have found none. However, those that have formally claimed an association have usually concluded that eating milk-based foods is good for you. The theory goes that unlike most other foodstuffs with high levels of saturated fat, dairy products package “bad fats” with many other nutrients, which, in turn, seem to have positive effects sufficient to override the saturated fats' badness.

Meta-analyses, which statistically combine many different studies, attempt to provide overall answers to grand questions about health. And cohorts where study participants record what they eat from the outset (prospective studies)—rather than try to recall it afterwards—are less warped by the imperfections of human memory.

Combining those principles, a 2004 meta-analysis of 10 prospective cohort studies found that people with high dairy intakes are less likely to suffer a stroke than those with little dairy in their diets. But this analysis reported no significant trend for heart disease. So, in 2010, a follow-up meta-analysis was performed by some of the same researchers at the University Hospital of Wales in Cardiff, which included more studies in the analysis. It did find a small protective effect for dairy and underscored the previous results for stroke.

Those kind of general answers are helpful, but health guidelines would benefit more from specific information on how different dairy foods influence cardiovascular risk and how putting dairy into different bodies (namely those of men versus women) modifies that risk.

### A man's portion

Any sex differences appear muddled, and that may be because they aren't really there. In Japan, where people generally gobble little dairy, a study comparing men and women found a 14% cut in the risk of death by cardiovascular disease for every extra 100 g of dairy product that women consumed per day [Ref 1], but there was no effect for men. The academics behind the study offer various explanations for this, including Japanese men's much lower dairy intake next to



Japanese women's intake—which may have made any effect too hard to ascertain—and the possible masking effects of higher levels of workplace stress among the men.

The result is certainly strange next to another study that followed 2,500 middle-aged Welshmen into old age [Ref 2], and regularly noted their blood pressure and arterial wall stiffness. In this group, dairy intake did indeed lead to less stiff arteries later in life and also strongly predicted systolic (maximum resting) blood pressure 23 years later; impressively, the men in the quartile with the highest dairy consumption in the study recorded blood pressure scores 10.4 mm Hg lower, on average, than the men who didn't consume milk products.

Morbid stats demonstrate how noteworthy this is. The medical literature equates a 4 mm Hg decrease in systolic blood pressure to a 15.7% reduced chance of death by stroke, and to 9.9% lower odds of having a heart attack.

Studies of other northern Europeans offer yet more conflicting messages on sex differences. A massive cohort in the Netherlands—of more than 120,000 people—reported that the health of men was unaffected, whereas that of women was *negatively* impacted by dairy consumption, although only slightly [Ref 3]. This finding is again contradicted by a survey of over 33,000 Swedish women [Ref 4], who, as a group, tended to eat an awful lot of dairy. But the more dairy they ate, the lower their risk of heart attack.

### **If you're gonna have a burger, add cheese**

A logical explanation behind this messy thicket of data probably lies with the kinds of dairy products the people in question were consuming. Some of the aforementioned studies did not subdivide dairy in much detail. But those that did suggest that butter increases some of the risk factors for poor cardiovascular health, meanwhile cheese and yogurt do the opposite.

The dairy-loving Swedish women, for example, were international anomalies in their fondness for cheese. When the study authors from the Karolinska Institute, in Stockholm, statistically reorganized their analysis to examine this oddity, the first thing they noted was an extra-strong protective effect: the group of women who ate the most cheese (a whopping six servings per day!) were 26% less likely to have a heart attack than those that ate a single serving on about two out of three days.

Cheese, being laced with saturated fat, is unlikely to cause such an effect without invoking some other mechanism. So these researchers then probed all of their dairy consumption and health data for correlations that might link the ingestion of individual minerals to cardiovascular robustness. This exercise revealed that the cheesy goodness can be explained by the packaging of saturated fat with calcium. Inside the intestines of cheese-loving Swedish ladies, dietary calcium probably binds fats that could otherwise have contributed to ill-health into insoluble soapy substances. And these would have found their way into the ladies' feces, not their bloodstreams.

The health-giving effects of yogurts seem to derive from the details, too. In this case, the strain of starter culture employed in the fermentation process appears to be all-important. Yogurts made with conventional strains, such as *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, don't do much to alter lipoprotein levels in blood. But products manufactured with strains known as probiotics, which encourage the growth of healthy bacteria in the guts of yogurt eaters (and others), appear more useful. The reasons why are merely postulated. Prominent among them is the idea that certain bacteria do a better job of breaking down complex carbohydrates inside the intestine, leading to an increase in short-chain fatty acids in the blood and consequently to an inhibition of cholesterol synthesis by the liver.

Whatever is going on in the body, these mechanisms are unlikely to capture the full picture of how dairy influences cardiovascular health. Little is known, for example, about how bioactive peptides derived from casein proteins shift the regulation of blood pressure through their inhibition of angiotensin I-converting enzyme—nor about the likely vasodilatory consequences of several minerals present in milk.

Although the combination of all of these effects appears to skew health outcomes only slightly over time, this is surely important enough for health systems to take note. In the United States, a country hardly famed for a population in fine cardiovascular fettle, dairy products account for 21% of all saturated fat consumed. So maybe, rather than just nagging people to eat less saturated fat, health professionals could place more emphasis on which fatty foods aren't so bad for you. They could start by offering around the cheese plate.

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## MicroRNAs in Milk

- **MicroRNAs control levels of protein produced by milk-making cells.**
- **MicroRNAs are a biological component of milk.**
- **MicroRNAs survive strong acidic conditions and milk processing.**
- **The biological activity of milk microRNAs may potentially be transferred to gut cells.**



Small things remain invisible unless one knows to look for them. Case in point: tiny RNAs. What are they? Why are they in milk? Are they good for the consumer?

Proteins are a fundamental nutritional and bioactive component of milk. These proteins are primarily produced by cells in the mammary gland. Gene activation dictates the amount of protein produced, and critical to this process is something called messenger RNA. Messenger RNA, or mRNA, is a molecule that serves as an intermediary between gene and protein. In other words, gene activation leads to the production of mRNA, which then leads to the synthesis of proteins, including those in milk. Genes beget mRNA; mRNA begets proteins.

Although we've known about mRNA for decades, just twenty years ago, scientists discovered a new class of RNA molecules that have turned out to play a crucial role in affecting the rate of protein synthesis. Prior to this groundbreaking finding, we believed the speed at which proteins were produced was controlled by the amount of mRNA present that coded for the protein or perhaps the stability of that mRNA. Now we know that these new RNA molecules, called microRNA, or miRNA (discovered in worms, no less!), function as the brake pedal in protein synthesis. That is, their natural function is to retard the rate of production of proteins in cells by disrupting the molecular machinery required for translating those mRNA strands to proteins. In short, microRNAs slow protein production.

### Is miRNA found in human milk?

In 2010, the results of a fundamental collaborative research project between the Nutritional Science Laboratory of the Morinaga Milk Industry in Kanagawa, Japan, and the National Cancer Center Research Institute in Tokyo were published, demonstrating the presence of miRNA in breast milk (2). The study focused on miRNA species that were previously implicated in downregulating levels of immune-related proteins. The study also reported that these miRNAs were relatively resistant to low acid pH levels similar to those found in the human stomach.

A study just published online in *BMC Genomics* (December 2012)(1) is the latest in a series of articles dealing with miRNA and lactation. Because miRNAs are molecules that help regulate the levels of proteins that are involved in biological processes, their presence in milk could mean they contribute to the regulation of developmental and physiological functions. This latest study shows that miRNA differs in the lactating and non-lactating mammary gland. This is similar to the difference in patterns of gene expression that are observed when the mammary gland begins to make milk.

## What about bovine milk and commercial milk products?

Now that scientists are discovering miRNAs in human milk, it's important to know if the same, different, or any miRNAs are found in other milk sources, such as bovine milk and commercial milk products. A few studies have shown that miRNA is found in raw and processed bovine milk (3-5). The miRNA found by individual investigators varied between the different reports, although this was undoubtedly influenced by study design. Colostrum and milk show differences in the absolute number of each miRNA found, and there is a greater variety in colostrum. Not surprisingly, different stages of milk storage and processing show considerable variation in the levels of most miRNA molecules in milk. Additionally, in infant formula, some miRNA molecules are found at one-tenth the level at which they exist in unprocessed milk. Seven miRNA molecules were identified as relatively stable in raw milk, and these may be useful as indicators of milk processing effects or spoilage. Although miRNA is relatively resistant to milk processing, they seem to undergo a considerable loss in concentration during the process, perhaps due to disruption of small bubble-like compartments that contain the majority of miRNA molecules. Whether this is of any significance to the consumer remains to be seen.

## Does milk-derived miRNA have a biological function?

The evidence strongly supports that miRNA are delivered to the infant via milk, and because of their remarkable resilience to degradation, they would transit through the stomach. The resistance to degradation may be attributed to the fact that miRNA is predominantly found in exosomes.

Exosomes are very small bubble-like compartments that form inside cells and are secreted into the fluid surrounding these cells. Milk-producing cells secrete exosomes into milk. Exosomes contain not only miRNA, but also other biomolecules, including proteins. There is convincing evidence that exosomes can fuse into the cell membrane of target cells and release their contents, including miRNA. When exosomes are put together with cells in the laboratory, they have been shown to modify the cellular responses of their target cells, including the release of immune modulatory molecules (4, 6). However, clear evidence that miRNA is delivered in this way and affects receiving cells is still lacking. Furthermore, extrapolating from the laboratory into an entire person or animal has drawbacks, and so it is unknown whether ingested miRNAs are functional in the recipient.

However, one intriguing finding published recently showed the presence of plant-derived miRNAs in the bloodstream of adults who had eaten rice (7). The researchers showed that a miRNA from rice slows the production of a liver protein in a mouse! This study demonstrated for the first time that a plant food component potentially regulates mammalian cells. It should be even easier for a mammalian food (milk) to regulate mammalian cells.

There are still a lot of questions to be answered, but we do know that miRNA are highly conserved between species, so if these molecules do have biological activity, it is likely that bovine-derived miRNA will have similar effects as human miRNA. Do miRNAs in milk affect the health of the consumer? In a word: maybe.

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## Hormones in Mother's Milk Influence Baby's Behavior

- **Hormones in mother's milk may program offspring behavioral development.**
- **Cortisol in mother's milk influences offspring temperament.**
- **Cortisol ingested via milk has different consequences for sons and daughters.**



Mammalian young are not just passive creatures allocating mother's milk solely to survival and growth. The calories young need to be behaviorally active, from the hesitant romping of the young foal to the arm-waving, ear-splitting tantrum of a newborn baby, come from mother's milk (Hinde and Capitanio, 2010). But other bioactive constituents in mother's milk, namely hormones, may also influence **HOW** the infant behaves.

A growing body of evidence has demonstrated that hormones from the mother, ingested through milk, bind to receptors within the young. Once these "maternal-origin" hormones bind, they seemingly trigger hormonal signaling cascades as would the young's own hormones. Recently, Skip Bartol and colleagues coined the term "lactocrine programming" to describe the process by which hormones present in mother's milk permanently shape physiological processes within the young, their main target of interest being the reproductive system (Bartol et al., 2009, Miller et al., 2013). [A previous column](#) described how hormones present in milk--specifically leptin and adiponectin--are associated with infant growth rates, although the mechanisms within the infant remain unknown.

Some hormonal pathways, though, underlie our behavioral tendencies--our individual predilection toward impulsivity, aggression, shyness, extroversion, etc. The phenomenon of individual behavioral tendencies is variably known as temperament, personality, or behavioral syndrome, depending to some extent on one's professional field (Hinde, 2013). The endocrine pathway of particular interest to behaviorists is the hypothalamic-pituitary-adrenal (HPA) axis. The HPA axis is colloquially referred to as the "stress axis" because it is activated when individuals are in danger, social conflict, or perceive that they are under stress (such as having to meet the deadline for my SPLASH! contribution this month). The adrenal glands at the end of this pathway produce glucocorticoids (cortisol in primates and corticosterone in rodents) that serve important metabolic, immunological, and neurobiological functions. For example, glucocorticoids regulate the use and storage of glucose and fats to meet energy needs during fight or flight.

What might this mean for infants? Well, glucocorticoids present in the mother's blood circulation end up in milk, are absorbed intact across the infant's intestinal tract (the epithelium), and then bind to glucocorticoid receptors there (Angelucci et al., 1983, Pácha, 2000). For this reason, cortisol in mother's milk likely reflects the mother's environment and degree of danger or social conflict she encounters—it's an "honest" signal of her own HPA axis activation. Most interestingly, the density of glucocorticoid receptors in the intestinal tract of mammalian young decreases after weaning, suggesting that those receptors are present specifically to bind to hormones ingested via mother's milk (Pácha, 2000). Importantly glucocorticoids ingested via mother's milk are shaping the HPA axis and behavioral tendencies of offspring.

A series of elegant experimental studies in rats by Angelucci, Catalani, and Casolini's research group in the Department of Human Physiology and Pharmacology, University of Rome, represents the most comprehensive investigation of ingested glucocorticoids on behavioral, cognitive, and physiological outcomes for any mammal. Their studies indicated that ingestion of glucocorticoids via mother's milk has beneficial programming effects in offspring that persist into adulthood. In their study design, rat dams consumed glucocorticoids in their water, increasing glucocorticoids in their blood and their milk. As juveniles, individuals who ingested elevated glucocorticoids through their mother's milk had better spatial memory (Catalani et al., 1993, Casolini, 1997). In adulthood, these individuals demonstrated better coping behavior during stress challenges; they showed less anxiety and more exploration (Catalani et al., 2000, Catalani et al., 2002, Meerlo et al., 2001). The elevated glucocorticoids in mother's milk altered areas of the brain that regulate the stress response and regulation of the HPA axis (Catalani, 2000). Interestingly these neurobiological effects were only seen in males, not females, even though the behavioral effects were similar in both sexes. These rodent studies demonstrate, using the gold standard of experimental manipulation, that hormones in mother's milk shape behavioral outcomes in offspring, but that the neurobiological pathways may differ for [sons and daughters](#).

These effects have not just been documented in rodents, but also in monkeys and humans. In a sample of 44 rhesus monkey mothers, we found substantial individual variation in cortisol concentrations in milk. Infants whose mothers produced higher concentrations of cortisol in milk were characterized as more "Confident," a factor score derived from systematic ratings of confident, bold, active, curious, and playful trait adjectives. However, this effect was driven by sons--milk cortisol concentrations did not predict 'Confidence' factor scores in daughters (Sullivan et al., 2011). The results from

monkeys are partially consistent with the results from rodents--glucocorticoids ingested through mother's milk are associated with less fearful, more exploratory tendencies, but only for sons.

In 2007, a study of 253 human infants provided suggestive evidence that cortisol in breast milk may also contribute to behavioral tendencies in humans (Glynn et al., 2007). Breastfeeding mothers with higher plasma cortisol concentrations, used as a proxy for milk cortisol concentrations, rated their infants as significantly more fearful than did breastfeeding mothers with lower plasma cortisol concentrations. Importantly, maternal cortisol concentrations were not associated with infant fearfulness among mothers who were formula-feeding their infants. These data suggested that the cortisol ingested via milk directly contributed to infant fearful temperament, rather than maternal cortisol influencing behavioral care of the infant or the mother's rating of her infant's temperament (Glynn et al., 2007).

Recently the same research team directly investigated cortisol concentrations in milk among 52 breastfeeding mothers (Grey et al., 2012). Grey and colleagues discovered that higher concentrations of cortisol in breastmilk were associated with greater "Negative Affectivity." This is a composite score of the infant's tendency toward fear, sadness, discomfort, anger/frustration, and reduced soothability as reported by the infant's mother. These results were not explained by other factors such as maternal depression or perceived stress. And most interestingly, the effect was driven by only daughters--the relationship between milk cortisol concentrations and negative affectivity was not present in sons!

These foundational studies demonstrate effectively that ingested glucocorticoids are predictive of infant behavior. Additionally, sons and daughters may have different sensitivities to maternal hormones for reasons that remain unclear. Interestingly the human results differ markedly from findings in both monkeys and rats; in humans, ingested glucocorticoids seemingly make infants more fearful and the effect is present in daughters but not sons. The reverse was found in monkeys; milk cortisol was associated with sons being more exploratory (seemingly less fearful), but there was no effect on the behavioral tendencies of daughters. In rodents the behavioral outcomes are present in both sons and daughters, but the neurobiological pathways underlying the outcomes differ between sons and daughters. Clearly, much, much, MUCH more research is needed to better understand the relationships among maternal psychological state, milk hormones, infant behavior, and sex-specific sensitivities.

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## To Secrete or Not to Secrete

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- **A mother's "secretor status" dictates amount and type of oligosaccharides (sugars) in her milk.**
- **Compared with term milk, oligosaccharides in premature milk are more variable.**
- **Milk oligosaccharide supplements may be beneficial for premature babies.**

On a cruise ship with a Norovirus outbreak, the chance of becoming infected is largely determined by a single gene: FUT2. Likewise, a mother's "secretor status"—whether or not she secretes certain protective sugars in milk—is determined by this same gene.

In 2004, Dr. Ardythe Morrow and others made the important discovery that breastfed infants of mothers who produced a certain milk sugar were protected against diarrhea<sup>1</sup>. (For those who must know, it is 2'-FL, a specific 2-linked fucosyloligosaccharide.) Why don't all moms produce this sugar? It comes back to that gene, FUT2.

### How "secretor status" changes milk sugars

Moms whose milk-making cells turn "on" the FUT2 gene produce a different set of milk sugars that are more protective than those produced in the absence of a functioning FUT2 gene. Moms with a functional FUT2 gene are called "secretors". "Nonsecretors" have a disabling mutation in the FUT2 ( $\alpha$ -1,2-fucosyltransferase) gene and therefore produce sugars with different linkages in their milk.

To understand the difference between sugars produced by secretors and nonsecretors, let's imagine a complex sugar made up of a lactose molecule attached to a fucose molecule. Both secretors and nonsecretors produce the lactose and fucose molecule, but an important difference is how the fucose attaches to the lactose. If the fucose attaches via an  $\alpha$  1-2 linkage, the sugar is called 2'-FL and has a variety of protective actions; if the fucose links via an  $\alpha$  1-3 linkage, the sugar is called 3'-FL and loses some of its magic powers, rendering it less protective for the infants.

Perhaps a simpler way to visualize these differing linkages is to use Stick People Chemistry. The difference in sugar structures created by a nonsecretor or secretor is like attaching a Shoe to the left hand or the left foot of a Stick Man. Even though the Stick Man and the Shoe are individually the same in both cases, their ability to function as a whole is different depending on whether the Shoe is on Stick Man's hand or foot.



### Why is the milk of secretors more protective?

All moms produce milk oligosaccharides (sugars). These sugars are not there to feed the infant, despite the fact they are the third most abundant component in milk. Milk oligosaccharides have at least two key functions. The first is to feed beneficial bacteria in the infant's gut. Some milk sugars selectively feed beneficial bacteria because only these bacteria can sever the link between the sugars to consume it. In our Stick People Chemistry analogy, while some bacteria can remove the Shoe from the Foot, even fewer can remove the Shoe from the Hand.

The second superpower of human milk oligosaccharides (sugars) is to sequester unfavorable bacteria. This sounds complicated, but actually the sugars act as a simple decoy. Cells along our intestines produce a variety of sugars that stick out like tree branches. Bacteria attach to these sugars, enabling them to hang out in the intestines like monkeys in a tree. Some of the sugars in milk, like the 2'FL, are made to mimic these intestinal cell sugars so that the bacteria bind instead to the milk sugars and get flushed out into the baby's diaper. If a monkey grabs a decoy tree branch that is not connected to a tree, the monkey will fall to the ground.

So why is secretor milk more protective? While it is not known with certainty, the milk sugars from secretor milk are almost certainly better at either feeding beneficial bacteria or binding/flushing bad bacteria.

## Preemies: Not just tiny babies

Premature babies (“preemies”) could certainly benefit from some extra secretor-milk-like protection. Preemies are infants born too soon, before the end of gestation, i.e., before 37 weeks of pregnancy. Many preemies survive birth but face a multiplicity of health complications and prolonged stays in a neonatal intensive care unit (NICU). They are vulnerable to gastrointestinal diseases and infections—in particular, necrotizing enterocolitis (NEC)—due to the delayed establishment of protective intestinal microbiota. Secretor phenotype influences these risks. In a cohort of preemies born at less than 32 weeks gestation, 15% of preemies with low secretor phenotype died, compared with only 2% of those with high secretor phenotype<sup>2</sup>.

While exclusive breastfeeding provides the ideal nutrition for infants, the optimum for preemies is less clear. Premature milk is not nutritionally complete, nor does it recover in the postpartum period. Perhaps mammary gland development is prematurely halted with the baby’s birth. From an evolutionary perspective, young preemies did not survive, so there would have been no selective pressure for early maturation of mammary glands. Therefore, breast milk needs to be supplemented, particularly for those whose neonates were born at less than 32 weeks gestation.

### Milk sugars for preemies

Human milk contains a few hundred different types of oligosaccharides (sugars), the most of any mammal. Does the mom of a preemie produce all of these sugars? In a recent paper from the *Journal of Proteome Research*, De Leoz and colleagues present the first comprehensive profile of the milk oligosaccharides in milk from women delivering preterm<sup>3</sup>. They studied variation in the sugars over time and between individuals as well as certain chemical characteristics, such as fucosylation and sialylation, which are important in the prevention of disease.

The results of this study by De Leoz et al. suggest that the composition of sugars in preterm milk is not consistent (has a higher degree of variability) compared to term milk. The milk from three subjects did not even obey the law of “milk secretor status”: their milk sometimes contained the fucosylated milk sugars and sometimes did not. Given the postulated importance of these sugars in pathogen binding, their fluctuation in mothers’ milk may further compromise the gut health of their premature offspring.

A pool of milk from many donor moms is one possible solution. Donor milk from full term secretor moms is enriched with protective milk oligosaccharides. Fortification of mom’s milk or formula with these protective sugars may be especially beneficial for nonsecretor infants. In *Breastfeeding Medicine*, Stellwagen et al. propose something low tech: pool milk from the birth mom’s separate pumping sessions<sup>4</sup>. While they suggest this method to remedy known inconsistencies in macronutrient content, it may help the problem with milk sugar variability as well.

### Life is not all bad for nonsecretors

If being a secretor or consuming the milk from a secretor mom is so wonderful, one might ask why nonsecretors haven’t all died off. It turns out there are some advantages to being a nonsecretor, such as increased resistance to HIV and Norovirus. For preemies in the NICU, milk from a secretor mom is probably best. For those babies born to HIV+ moms on a Norovirus cruise ship, nonsecretor status should improve their otherwise incredibly bad luck.

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