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Milk derived Micro RNAs: Implications in health & diseases

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Abstract
Milk has been recognized as a functionally active nutrient system promoting neonatal growth of mammals. It is species-specific and consists of various bioactive components, including microRNAs, small non-coding RNAs that regulate gene expression at the post-transcriptional level and long-range cell-to-cell communication mediators. Transfer of these components through blood transfusion, diet, during breast feeding etc have sparked the interest on studies of miRNA.It can be both intra and extra cellular and are present in body fluids of humans and animals. Of these body fluids, milk appears to be one of the richest sources of microRNA, which are highly conserved in its different fractions, with milk cells containing more microRNAs than milk lipids, followed by skim milk. Milk microRNAs can enter the systemic circulation of the milk fed infant and exert tissue-specific immuno protective and developmental functions. Human breast milk is the ideal food for infants allowing appropriate postnatal growth and species-specific metabolic programming, but persistent high milk signaling during adolescence and adulthood by continued cow milk consumption may promote diseases of civilization. Key findings surrounding milk microRNAs in human, cow and goat milk among other species and their biological properties, use as disease biomarkers, consequences of transfer between individuals or species, and their putative or verified functions in health and disease of infants and adult consumers are discussed.

Keywords: diseases of civilization, microrna, immuno protective, neonatal growth, milk

1. Introduction
Milk is a highly specialized, complex nutrient system developed by mammalian evolution to promote postnatal growth. In contrast to feeding artificial infant formula, human milk allows appropriate metabolic programming and protects against diseases of civilization later in life. However, continued consumption of cow milk and dairy products during adolescence and adulthood is an evolutionarily novel behaviour that may have long-term adverse effects on human health (Wiley, 2012) [36]. Milk is an abundant source of microRNAs (miRNAs), which are evolutionarily conserved small non-coding RNAs, involved in post-transcriptional regulation of target mRNA in humans, animals and plants. MicroRNAs are responsible for regulating 40% to 60% of gene expression at the posttranscriptional level by binding to complimentary sites at the 3'-untranslated region of target mRNA. They have been identified as key regulators of diverse biological and developmental processes in eukaryotes, including cell proliferation and differentiation, apoptosis, immune system development and immune responses. Their enrichment in milk has generated the interest on microRNA transfer through diet, especially from mothers to infants during breastfeeding. The extension of such hypothesis led to the study of milk microRNAs in the case of cow or goat milk consumption in adults. To a large extent, milk microRNAs appear to be endogenous to the mammary gland and could therefore be employed as biomarkers for both the performance and health status of the gland during lactation, and its aberrant growth associated with breast cancer. As human milk is highly enriched in microRNAs, it would be of great interest to illuminate the fate and function of this breast milk component in the infant during breastfeeding and any long-term effects conferred during this period. Interestingly, bovine milk microRNAs miR-29b and miR-200c, which are also present in human milk, have been shown to survive the GI tract of adult humans and increase in their serum concentration post-consumption (Baier *et al.* 2014) [4]. More recently, bovine milk exosomal microRNA transfer was demonstrated in human intestinal colon cells and rat small intestinal cells by endocytosis *in vitro* (Wolf *et al.* 2015) [37], further highlighting the important role of vehicle-mediated transfer of milk microRNA.

2. Biogenesis of milk derived mi RNA

Mammary gland is the main source of milk microRNA, with the maternal circulation having a smaller contribution (Alsaweed *et al*. 2015) [1]. MicroRNA is first transcribed from specific genes on DNA as primary microRNA (pri-microRNA) by RNA polymerase II (RNAP II). In the nucleus, pri-microRNA is converted into ~70-nucleotide precursor hairpin microRNA (Pre-microRNA) by the enzymatic Drosha–DGCR8 complex. Pre-microRNA is then transported from nucleus to the cytoplasm by exportin 5. There, the Dicer-TRBP complex produces ~20 base pair microRNA duplex. Dicer with assistance from Argonaut 2 (AGO2) generates mature microRNA by cleaving the double strand of pre-micro RNA. The mi RNA strand with the most unstable base pairing at the 5′ end usually acts as the guide strand, while the strand with stable base pairing at the 5′ end (Known as the passenger or Mir* strand) is usually degraded. The guide strand directs the RNA-induced silencing complex (RISC) to the target mRNA through sequence complementarity. Finally, the microRNA/RISC complex binds into specific mRNA during protein translation, recognizing their target via a 6–8 nucleotides match-mir process (Seed region). This results in either repression of the mRNA translation into protein or mRNA degradation (Figure 1).

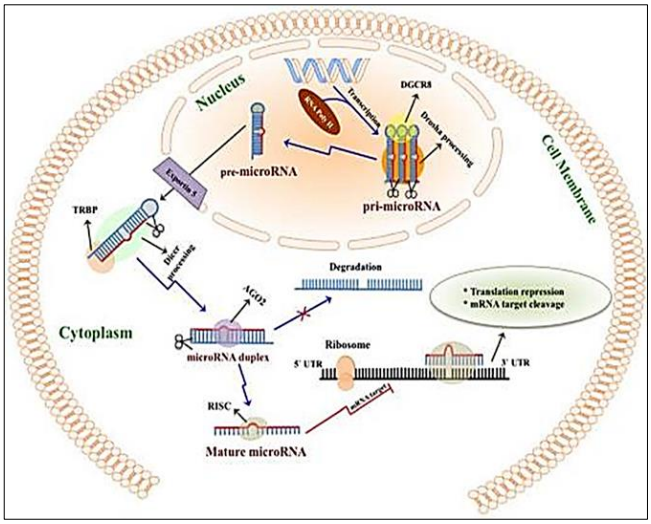

Fig 1: Biogenesis of mi RNA

3. Micro RNAs in Milk

Milk is one of the most important biological fluids, not only in terms of volumes produced by various mammals but also because it is the only food for newborns that ensures infant development and health. Maternal milk is one of the very rich source of nutrients indispensable for the infant and also a functional food shaped by evolution (Alsaweed *et al*., 2015) [1] with various bioactive compounds like antimicrobial molecules, growth factors, casein fragments and lipids, immune cells, antibodies, and catalytically active antibodies. It even contains maternal cells that integrate into the infant's gut walls creating localized micro chimerism ensuring proper development of immunity for the infant. MicroRNAs can be isolated and experimentally studied in the main three fractions of milk, the cells, lipids, and skim milk. MicroRNAs present in milk can occur in extracellular vehicles (EVs), which are nano sized membrane vesicles released, by many cell types as a means of intercellular communication. The membrane of EVs protects enclosed miRNAs from degradation and harbours molecules that allow specific targeting to recipient cells. MicroRNAs are abundant in human milk, bovine milk and milk derived from other livestock, and they have functional roles in infants and in the secretion process of mammary glands.

Extracellular vesicles are cell-derived lipid bilayer-enclosed vesicles containing selectively incorporated proteins, lipids, and nucleic acids (mainly small RNAs), which are in turn selectively delivered to recipient cells to modulate their functions. Hence, the transfer of maternal milk EVs to the new born allows for cross-organism communication. They have been identified and characterized in human milk, as well as in milk from other species, such as cow, buffalo, pig, wallaby, horse, camel, rat and panda (Alsaweed *et al*. 2015) [1]. Most interestingly, the major microRNAs found in these milk often overlap with human milk microRNAs, suggesting a conserved evolution process that lead to the release of specific milk microRNAs (Melnik,, 2015) [23]. Considering domestic animal derived milk that are part of human consumption (cow and goat), it is interesting to note that the top 10 microRNAs found in these two species, is quite close to human milk microRNA profiles.These abundantly present mi RNA species might be involved in the specific targeting of signalling pathways in the new born and can be delivered to various tissues in humans. Importantly, it has recently been shown that food derived miRNAs are very stable in the gastrointestinal tract and that they can be transferred to the blood circulation in adults, thereby influencing gene expression in different tissues.

4. Milk Derived microRNAs in different species

In 2010, Weber *et al.* isolated and profiled microRNA from 12 different human body fluids, including human colostrum and milk. They used qPCR to profile 429 known mature microRNAs in mature skimmed human milk and 386 known mature microRNAs in skimmed human colostrum. This study demonstrated for the first time, the high abundance of microRNA in human milk, which is in accordance with its high total RNA content compared to other body fluids (47,240 µg/L *vs*. 308 µg/L in plasma and 94 µg/L in urine). MicroRNA hsa-miR-148a-3p is consistently the most abundant microRNA in breast milk, most likely because of its importance for lactation and its enrichment in mammary gland cells.

Mature bovine skim milk has been shown to contain 213 microRNA species using deep sequencing (Hata *et al*. 2010) [12] and 53 using microarrays (Izumi *et al.* 2012) [15]. Some of these microRNAs were enriched in either mature milk or colostrum. Specifically, bovine colostrum has been found to be richer in microRNA compared to mature bovine milk. Again, miR-148a the most expressed milk microRNA, and it is consistently reported in all milk small RNA sequencing, supporting its evolutionary importance in lactation and possibly for the newborn's development and health (Friedrich *et al.*, 2017) [7]. Moreover, the sequences of human and bovine miR-148a are identical. As this microRNA is a regulator of the DNA methyl-trans ferase 1 (Dnmt1), this fact raised concerns over the impact of recurrent milk consumption on epigenetic regulation of the human genome (Melnik & Schmitz, 2017) [24]. Gu *et al.* (2012) [9] examined the exospores of porcine milk using small RNA sequencing at six time points of lactation within the first month postpartum (0, 3, 7, 14, 21, and 28 days after birth) and found 180 pre-microRNAs encoding 237 mature microRNAs, which are also

found in human milk. In all animal milk microRNA studies to date, only a few microRNA were found to be highly expressed. For example, the top 10 most highly expressed microRNAs in exosomal porcine milk were contributing approximately 87% of the total 234 microRNA.

5. Stability and uptake of milk -derived microRNAs

a. Stability of Milk -Derived microRNAs

MicroRNAs are extremely stable under various harsh conditions *in vitro*. For breast milk microRNA, the main considerations are resistance to RNase digestion and tolerance of low pH, temperature and freeze/thaw cycles in the case of frozen human milk (Zhou *et al.* 2012) [41]. Eexosomal microRNA has been suggested to be protected, but other micro vesicles including fat globules are also considered to be involved in micro RNA protection, such as apoptotic bodies (small vesicles derived from apoptotic cell death). Moreover as stated above, milk cellular microRNA may be transferred intact as it is protected within cells, which have been shown to survive the GI tract of the offspring and in different organs. Ribonuclease (RNase), which has been found to exist in all body fluids, degrades RNA molecules into small fragments, and is thus a key enzyme in the RNA maturation process. Milk is known to have high RNase activities (Kosaka *et al.* 2010) [19]. Human milk and raw milk-derived microRNAs are found to be extremely stable even after RNase treatment *in vitro* (Hata *et al.* 2010) [12].

The effects of low pH on microRNA integrity examined using qPCR (Kosaka *et al.* 2010) [19], also showed that miRNAs are very stable. It is important to note that the GI tract of infants is less acidic than that of adults and have low enzymatic activity, allowing even immune cells and other cells of milk to survive and settle within the infant digestive tract wall which further supports increased survival of milk microRNA activity. Moreover, milk microRNAs are resistant to milk storage under different temperatures, such as incubation at 100 °C for 10 min, and freeze-thaw cycles (Zhou *et al.* 2012) [41]. As microRNA do not denature if subjected to different temperature cycles, microRNAs in stored human milk fed to hospitalized infants are likely to be unaffected. The above observations strongly support the survival of the natural microRNA content of human milk in the infant's GI tract, either as free molecules or packaged in vesicles/cells, and thus suggest a potential function of these transferable and stable molecules in the breastfed infant, including the hospitalized infant receiving stored human milk. Milk contains high quantities of highly stable microRNAs, which are resistant to the pasteurization and milk bank storage procedures (Kosaka *et al.* 2010) [19]. Additionally, microRNAs were found to be active and still regulate their target genes after subjection to ultraviolet radiation (UV-A, UV-B, and UV-C) (Pothof *et al.* 2009) [26]. Howard *et al.* (2015) [14] reported high mi RNA content in unprocessed milk compared to pasteurized and stored milk. They also observed miRNAs loss during homogenization and processing of milk into dairy products.

b. Uptake of milk -derived microRNAs

In the case of human milk microRNA, their transfer to the infant's bloodstream is facilitated by the packaging of milk microRNA in "vehicle" structures, such as somatic cells, exosomes and other micro vesicles, which may be essential for the long-distance transport of microRNA, given that they are surrounded by a lipid bi-layered membrane and are equipped with adherence molecules, both of which facilitate their ordered endosome transfer via epithelial cells of the intestine (Wolf *et al.* 2015) [37]. Through these vehicles, milk-derived microRNA are thought to be up taken by the infant and participate in the epigenetic regulation of various functions including immune protection and development. Extracellular vesicles including exosomes were shown to attach to different types of cells by endocytosis and carry microRNA to the recipient cells. Uptake and functionality, including therapeutic effects, of milk microRNAs has been recently demonstrated both *in vitro* and *in vivo* by Arntz *et al.* (2015) [3] In this study, immune-related microRNAs (miR-30a, miR-223, miR-92a) were highly expressed in bovine milk-derived extracellular vesicles, were taken up *in vitro* by Splenocytes and intestinal cells.

MicroRNAs contained in infant formulae may also, to a small extent, be transferred to the infant's circulation. Baier *et al.* (2014) [4] investigated bovine milk-derived miR-29b and miR-200c in human adults after consuming cow milk and found that both microRNAs were increased 2-fold in human PBMCs and could potentially alter gene expression. Both microRNAs were also highly expressed in the human plasma after few hours of consuming cow milk and returned to the normal baseline expression level after 24 hour of the initial milk consumption. Furthermore, bovine milk exosomes isolated from commercial milk products were shown to be transported into human intestinal colon carcinoma Caco-2 cells and rat primary small intestinal IEC-6 cells by endocytosis *in vitro* (Wolf *et al.* 2015) [37]. However, differences exist in the microRNA content between bovine milk and infant formula, with the later lacking exosomes and viable cells, and thus containing much lower microRNA concentrations (approximately 100-fold lower in bovine milk-based formula compared to raw bovine milk and colostrum (Sun *et al*.2013) [32]. Moreover, the non-human origin of formula microRNA and/or the procedures of formula preparation may be associated with altered biological activity of any remaining microRNA in infant formula.

The major organs which are responsible for milk EV uptake are the liver, spleen, lung and the small intestine, through immune cells (Zempleni, 2017) [40] with a potential uptake of milk EVs by the gut micro biota (Yu *et al*., 2019) [39]. Notably, milk EVs reached the brain of mice after oral administration (Manca *et al.*, 2018) [22].

6. Implications of Milk Derived microRNAs in Health

a. MicroRNAs Act as Immune Regulators

The tolerance of microRNAs to harsh conditions and the evidence that they migrate to the blood stream and potentially to different organs of the breastfed infant, suggest that they may play functional roles in the epigenetic regulation of development. Most of the microRNAs in human milk are known for their immunocompetence (Izumi, *et al.* 2012) [15] and they are particularly abundant in milk. They are thought to be involved in several mechanisms of the immune system, such as regulation of B and T cell differentiation and development, and innate/adaptive immune responses. In addition, micro RNAs can play key roles in autoimmune conditions, such as inflammatory bowel disease (IBD), and regulate the development or prevention of these diseases. Therefore, they could potentially be used as milk biomarkers to diagnose immune disorders such as allergic conditions.

In addition, microRNA clusters miR-17 and miR-92 have been detected at high levels in human milk and given their function in regulating monocyte development as well as B

and T cell differentiation and maturation, they are also thought to contribute to the maturation of the infant's immune system early in life. MiR-223, which is predicted to activate proliferation of granulocytes (Johnnidis *et al.* 2008) [17], is also found at high levels in human milk. It is rich in B cell-related microRNAs, such as miR-181 and miR-155, which potentially induce B cell differentiation. On the other hand, miR-150, which is present in lower concentrations in human milk, is known to act as a B cell suppressor. They also identified a large number of microRNAs in human milk exosomes. Of the 10 most abundant, 4 microRNAs were associated with immune functions, including miR-148a-3p, miR-30b-5p, miR-182-5p and miR-200a-3p. Specifically, miR-30b-5p is known to induce immunosuppression and reduce immune cell activation. In contrast, miR-182-5p induces T cell-mediated immune responses (Stittrich *et al.* 2010) [31].

Bovine milk mi RNA; miR-15b, miR-27b, miR-34a, miR-106b, miR-130a, miR-155 and miR-223 which are all considered as immune and development related microRNAs, have been found in higher levels in colostrum than in mature milk. Kosaka *et al.* (2010) [19] and Gu *et al.* (2012) [9] first showed human skim milk and porcine milk exosomes, respectively, and identified many microRNAs related to immune responses.

They are also found to be altered during mastitis and most milk microRNAs were downregulated during mastitis, suggesting that they actively control the mammary immune response. Collectively, the current data highlight that milk is a complex system of different microRNA molecules with synergistic and antagonistic relationships, controlling specific immune responses in the infant and the lactating mammary gland. Factors such as the stage of lactation (Colostrum *vs.* mature milk) and infection/inflammation have been shown to influence the microRNA-mediated epigenetic regulation of immune responses and development in the infant (Lawless *et al.* 2014) [20].

b. Various potential benefits of human milk microRNAs

The existence of microRNA in exosomes and their potential function as extracellular regulators have opened a new field of possibilities for use of microRNAs as biomarkers in health and disease as well as in therapeutic modeling. MicroRNAs are potentially involved in many physio pathological functions, including regulating cell growth and differentiation as well as influencing development of the infant.

- **Epigenetic regulation on growth**

Lactation describes the secretion of milk from the mammary glands and the period of time that a mother lactates to feed and epigenetically program her young. During lactation MECs dramatically enhance milk protein and milk lipid synthesis. A network of genes participates in coordinating bovine milk fat synthesis and secretion. One of the most highly expressed microRNA in human milk, miR-148a-3p, which is also found in other species milk targets DNA methi ltrans ferase 3b (DNMT3B) and suppresses its expression, potentially to facilitate DNA methylation during development (Duursma *et al*. 2008) [6]. In accordance, abundant lactation-specific miRNAs that target DNA methi ltrans ferases (DNMTs) are involved in the activation of lactation-related biosynthetic pathways.

- **Appetite control and feeding reward**

The newborn infant needs continuous access to calories and milk-derived signal transduction for appropriate postnatal growth. Epigenetic mechanisms of milk may thus regulate the magnitude of appetite and reward signals in order to guarantee adequate and continuous calorie intake during the postnatal growth phase.

Milk-mediated epigenetic activation of fat mass and obesity-associated protein (FTO) expression modifies the epitranscriptome. Milk-derived DNMT-targeting miRNAs reduce methylation critical DNA CpG islets thereby increasing FTO gene expression. The RNA m6A deme thy lase FTO erases m6A marks on mRNAs, thereby enhancing FTO-dependent mRNA transcription and mRNA splice variant production such as radiogenic short form of RNX1T1. The mRNAs of ghrelin and dopamine receptor 3 (DRD3) are targets of FTO-mediated upregulation. Resulting hyperplasia and feeding rewards support milk intake for infant growth requirements.

Persistent uptake of bovine milk exosomal miRNAs may epigenetically enhance long-term orexigenic signaling promoting overgrowth and obesity of the human consumer of cow's milk. In fact, epidemiological studies confirmed enhanced BMI and linear growth in relation to cow's milk consumption in children and adolescents (Hoppe *et al.* 2006) [13]. Activation of dopaminergic signaling plays an important role in the midbrain and frontal cortex functioning during postnatal development and regulates extrapyramidal movement and important cognitive functions, including motivation, habit learning, and reward associations. Persistently over activated FTO expression by continued cow milk consumption may maintain a state of hyperplasia promoting obesity (Melnik and Schmitz, 2017) [24].

- **Intestinal Growth**

Human milk contains and transfers milk stem cells with multi lineage differentiation potential to the newborn infant (Hassiotou *et al.* 2014) [11]. Notably, DNMT inhibition promoted the differentiation of human induced pluripotent stem cells into functional enterocytes. It is thus conceivable that milk-derived DNMT-targeting miRNAs support intestinal epithelial cells maturation as well as milk stem cell differentiation into enterocytes, potential contributions for appropriate growth, maturation and function of the infant's gut. Intestinal epithelial cells are also able to take up bovine milk exosomes (Arntz *et al.* 2015) [3].

- **Myogenesis**

Muscle mass acquisition in the adult human is primarily dependent on mechanical stimuli and active muscle contraction, which activates mTORC1 signaling (Goodman, 2014) [8]. Repetitive active muscle contractions significantly up regulate the metabolic transcription factor NR4A3. The newborn infant, however, with a still undeveloped neuromuscular system may depend on other stimuli for muscle cell differentiation and growth. In fact, NR4A3 expression is involved in postnatal development and its expression critically depends on nutritional status. Milk-derived exosomal miRNAs apparently provide the required epigenetic signals for muscle cell differentiation and appropriate muscle protein acquisition. NR4A3 expression is epigenetically induced by NR4A3 promoter demethylation (Yeh *et al.* 2016) [38]. Milk-mi RNA-mediated suppression of DNMT1 expression may thus augment myogenesis via

epigenetic activation of myogenic transcription factors, which closely interact with mTORC1 signaling.

- **Osteogenesis**

Milk exosomal mi RNA-mediated suppression of DNMTs may promote NRF2 (nuclear factor-E2-related factor2) driven postnatal osteogenesis. Importantly, miRNA-29b promotes osteogenesis by directly down-regulating histone deacetylase (HDAC4) and transforming growth factor beta 3 (TGFβ3), the negative regulators of osteoblast differentiation by binding to target 3'-UTR sequences. Thus, miRNA-29b is a key regulator of development of the osteoblast phenotype by targeting anti-osteogenic factors. Notably, bovine milk-derived miRNA-29b, which shares the identical seed sequence as human miRNA-29b, has been shown to increase dose-dependently in the serum of healthy human adults after consumption of pasteurized cow's milk and increased Runt-related transcription factor 2 (RUNX2) expressions in PBMCs of the milk consumers. It is thus conceivable that milk exosomes-derived miRNAs control the epigenetic status of RUNX2 and NRF2 promoting osteogenesis.

- **Epidermal Differentiation**

After birth, milk-derived miRNAs targeting DNMTs may support epigenetic NRF2-mediated expression of cornified envelope proteins crucial for skin barrier function. The epidermal growth factor receptor (EGFR) plays a key role for the regulation of epidermal proliferation. Milk-derived exosomal miRNA-148a may promote epidermal proliferation as well as proliferation of other EGFR-dependent cells (Kim *et al.* 2014) [18].

7. Implications of Milk Derived miRNAs in Diseases

Role of mi RNA signaling during the perinatal period for life-long epigenetic programming is very important. The great majority of clinical and epidemiological studies demonstrated that cow milk consumption during pregnancy increased foetal growth and birth weight of the newborn infant (Melnik, 2015) [23]. High birth weight and accelerated postnatal weight gain are associated with an increased risk of obesity. Accelerated infant and childhood weight gain are associated with increased energy intake and diminished satiety response at the age of 5 years. Perinatal programming of energy intake and eating behavior provide a potential mechanism linking early life influences with later obesity, type 2 diabetes and cardiovascular disease.

Continued uptake of milk-derived exosomes that carry DNMT-targeting miRNAs may modify early programming of the human epigenome promoting FTO-driven food intake and the development of diseases of civilization such as diabetes, cardio vascular diseases, obesity, allergy, neurodegenerative diseases and cancer. Continued consumption of cow's milk during childhood and adolescence accelerates growth trajectories associated with increased BMI, linear growth and early onset of menarche.

a. Obesity

In humans, new adipocyte formation occurs throughout childhood and adolescence, with fat cell numbers plateauing around the age of 20 years. Approximately 10% of fat cells are renewed annually at all adult ages (Spalding *et al.* 2008) [30]. MiRNA-21, another abundant exosomal mi RNA of human, bovine and porcine milk, has recently been shown to enhance adipogenic differentiation from porcine bone marrow-derived mesenchymal stem cells (An *et al.* 2016). Milk mi rna-mediated dnmt suppression may thus activate fto-mediated activation of adipogenic transcription factors such as runx1t1, pparγ, cebpα, srebp1, and pgc1α. Milk mediated exosomal transfer of dnmt-targeting mirnas might exert inhibitory posttranscriptional activity on adipose tissue dnmt1 expression promoting adipocyte differentiation and adipogenesis.

Milk-derived mirna-148a and mirna-21 are critically involved in adipogenesis. Persistent intake of both adipogenic miRNAs apparently promotes obesity. Mirna-148a directly targets the pivotal genes regulating triglyceride synthesis (Fas), cholesterol homeostasis (ldlr), cholesterol efflux (abca1) and β-oxidation (ctpa1) (Wagschal *et al.* 2015). Mirna-148a via targeting dnmt1 and subsequent promoter hypo methylation enhances adipogenic gene expression including insulin (ins), insulin-like growth factor-1 (igf1), caveolin-1 (cav1), leptin (Lep), ppar-γ2 (pparg2), fatty acid-binding protein 4 (fabp4) and lipoprotein lipase (Lpl). Additionally, milk mirna-148a-mediated fto promoter demethylation may further enhance rna transcription of the key adipogenic transcription factors runx1t1, pparγ, cebpα, and pgc1α via erasing m6a marks on their target Mrnas.

Milk-mediated exosomal transfer of dnmt-targeting mirnas may thus exert inhibitory posttranscriptional activity on adipose tissue DNMT1 expression promoting adipocyte differentiation and adipogenesis.

Cow milk is a rich source of exosomal miRNA-21 and it has been demonstrated that miRNA-21 acts as a bidirectional switch in the formation of insulin-producing cells by regulating the expression of target and downstream genes (SOX6, RPBJ and HES1). Deletion of miRNA-21 in hepatocytes increased insulin sensitivity and modulated the expression of multiple key metabolic transcription factors involved in fatty acid uptake, de novo-lipogenesis, gluconeogenesis and glucose output. Furthermore, long-term inhibition of miRNA-21 reduced body weight and adipocyte size in db/db mice (Seeger *et al.* 2014) [28]. Thus, persistent uptake of exosomal miRNA-21 via persistent cow milk consumption may enhance the risk for obesity and diabetes. Taken together, cow milk transfers obesogenic and orexigenic miRNAs, predominantly miRNA-148a and miRNA-21, that maintain an epigenetic status that is intimately involved in the pathogenesis of diabesity. Remarkably, cow milk contains substantial amounts of miR-155. The target of miR-155 is the adipogenic transcription factor CCAAT/enhancer-binding protein β (C/EBPβ). Overexpression of miR-155 in mice has been shown to reduce brown adipose tissue (BAT) mass. Thus, milk miR-155 intake may attenuate thermogenesis of BAT, an unfavourable condition promoting lipid and energy storage in WAT promoting obesity.

b. Type 2 Diabetes Mellitus (T2DM)

Early onset of menarche, which is related to milk consumption in early childhood, is associated with increased risk of T2DM (Janghorbani *et al.* 2014) [16]. Intake of cow's milk in contrast to fermented milk products has been associated with incident T2DM (Sluijs *et al.* 2012) [29]. Cow's milk consumption increases miRNA-29b levels in PBMCs in a dose-dependent manner (Baier *et al.* 2014) [4]. The miRNA-29 family is among the most abundantly expressed miRNAs in pancreas and liver and is regarded as a diabetogenic risk marker.

In the liver both miRNA-29a and miRNA-29c were important negative regulators of insulin signaling via PI3K regulation.

Hepatic insufficiency of miRNA-29 potently inhibited obesity and prevented the onset of diet induced insulin resistance. These results confirmed strong regulatory functions for the miRNA-29 family in diabesity. Persistent transfer of milk exosomal miRNA-29 via cow's milk consumption may thus represent a critical epigenetic factor in the pathogenesis type 2 diabetes.

Hypo methylation of specific CpG sites of FTO have been reported to enhance FTO expression. In fact, decreased FTO methylation has been demonstrated in pancreatic islets of T2DM patients compared to non-diabetic controls. Milk contains substantial amounts of let7a, let7b, let7c and let7f. There is accumulating evidence that the Lin28/let-7 axis regulates glucose metabolism.

c. Cancer

In addition to the involvement in normal metabolism and tissue function, many microRNAs have been shown to target genes related to cancer, with some of these gene targets known to increase or decrease cancer risk. These microRNAs could be used as cancer biomarkers for both prognosis and diagnosis (Kosaka *et al.* 2010) [19] and some of them are present in milk and more specifically in human milk. Although epidemiological evidence has previously associated bovine milk consumption with increased risk of certain cancers in adults, this could be related to the content of bovine milk in oncogenic microRNA (Melnik, 2015) [23].The microRNAs in human milk appear to have normal lactation-specific functions for the lactating mammary gland and the infant (Alsaweed *et al.* 2015) [1]. Interestingly, human milk microRNA has been proposed to protect the infant against cancer through to adulthood. For example, miR-21, which is present in both human and bovine milk, is also known to be overexpressed in human hepatocellular cancer (HCC). Therefore, any dysregulation of miR-21 can be associated with HCC growth by modulating mTORC1 signaling. MiR-21 is an abundant microRNA in bovine milk and has been isolated from both colostrum and mature human milk. It is also abundant in human plasma and in infants it is thought to be involved in promoting postnatal growth.

Further, human milk microRNAs may directly regulate tumor suppressor genes, such as the let-7 family, which is involved in decreasing lung tumor growth by directly targeting the RAS oncogene (Hammond, 2007) [10]. There is also a strong association of whole milk consumption and prostate cancer (PCa), the most common cancer in men of civilized societies. A recent meta-analysis considering 11 population-based cohort studies involving 7, 78, 929 individuals demonstrated the existence of a linear dose-response relationship between whole milk intake and increase of PCA mortality risk. Intriguingly, recent evidence links miRNA-148a to the promotion of prostate cell growth. The addition of cow milk as an exogenous source of miRNA-148a to prostate cancer cells in vitro stimulated PCA cell growth producing an average increase in growth rate of over 30% (Tate *et al.* 2011) [33].

In contrast to PCA, a meta-analysis involving over 900,000 subjects and over 5200 colorectal cancer (CRC) cases supports an inverse association between non-fermented milk consumption and risk of CRC in men (Ralston *et al.* 2014) [27]. It should be noticed that in contrast to fermented milk, non-fermented milk contains higher amounts of bioactive miRNAs including miRNA-148a, the most abundant mi RNA of cow's milk. Downregulation of miRNA-148a expression plays a critical role in CRC carcinogenesis and progression. Thus, milk-derived miRNA-148a loaded exosomes may substitute miRNA-148a deficiency in colorectal adenoma cells thereby preventing their further progression to CRC. Hepatocellular carcinoma has recently been related to increased consumption of cow's milk (Salles *et al*. 2014) [5]. Exosomal milk-derived miRNA-148a and miRNA-21 may thus provide oncogenic signals inducing an epigenetic landscape for tumorigenesis maintained by the consumption of cow's milk in the majority of cancers except CRC. Milk exosomes via transfer of miR-148a and miR-148a-mediated suppression of DNA methyl transférase 1 (DNMT1) enhances the expression of estrogen receptor-α (ERα). ERα promotes des expression of miR-21, which targets critical genes involved in PI3K-AKT signaling and cell cycle control. Exosome-derived transforming growth factor-β (TGF-β) induces the expression of miR-155. MiR-155 enhances the expression of FoxP3, a critical inhibitor of the tumor suppressor breast cancer 1 gene (BRCA1). Downregulation of BRCA1 further enhances the expression of miR-155, which is a pivotal inhibitor of suppressor of cytokine signaling 1 (SOCS1) finally promoting epithelial–mesenchymal transition (EMT). Dairy milk exosomes thus contribute to BC tumor genesis via enhancing key oncogenic components involved in the pathogenesis of breast cancer.

d. Neurodegenerative Diseases

Continued consumption of cow's milk and persistent uptake of bovine exosomal miRNA-148a, which is identical with human miRNA-148a, may represent an epigenetic mechanism suppressing DNMT1, which via syncline alpha (SNCA) demethylation may increase the expression of α syncline, a key aggregating protein in Parkinson's disease. Similarly mTORC1 induces abnormally hyper phosphorylated tau proteins, which aggregate resulting in compromised microtubule stability. Abnormally hyper phosphorylated tau aggregates form paired helical filaments in neurofibrillary tangles, a key hallmark of Alzheimer's disease and other tauopathies. Mtorc1 is involved in regulating tau distribution in subcellular organelles and in the initiation of tau secretion from cells to extracellular space. Mtorc1 was activated in the AD brains and the activation level of Motor signaling correlates with cognitive severity of AD patients. As outlined above, FTO plays a pivotal role for mTORC1 activation and milk mi RNA has been identified as a critical activator of mTORC1-dependent translation.

e. Bone Diseases

Milk derived mi RNA play a key role in the regulation of bone remodeling executed by bone-resorbing osteoclasts and bone-forming osteoblasts. Blood monocytes are a primary source of osteoclast precursor cells. Upregulation of receptor activator of nuclear factor B ligand (RANKL), V-Fos FBJ murine osteosarcoma viral oncogene homolog (c-Fos) and transforming growth factor-β (TGF-β) promote osteoclast genesis. MiR-148a via targeting V-maf musculoaponeurotic fibro sarcoma oncogene homolog B (MAFB) increases RANKL expression. MiR-21 via targeting programmed cell death 4 (PDCD4) increases c-Fos activity. Notably, miR-148a, miR-21 and TGF-β are provided by dairy milk exosomes. Addition of commercial milk-derived exosomes to bone marrow-derived osteoclast precursor cells increased osteoclast formation. Overexpression of miR-148a triggers mesenchymal stem cells (MCS) to differentiate into adipocytes and attenuates osteoblast differentiation. Persistent

intake of dairy milk exosomes may thus disturb the delicate balance of bone remodeling favoring osteoclast genesis over osteoblast genesis, a critical mechanism promoting osteoporosis and fracture risk (Melnik & Schmitz, 2019) [24].

f. Cardiovascular Diseases

Milk exosomes are taken up by monocytes and macrophages. MiR-148a stimulates the differentiation of monocytes to macrophages, especially of macrophages of the pro-inflammatory M1 type. MiR-148a mediated suppression of low density-lipoprotein (LDL) receptor (LDLR) expression increases circulating LDLs that after ageing-dependent chemical modifications are scavenged by macrophages. MiR-148a-mediated suppression of ATP binding cassette transporter 1 (ABCA1) attenuates reverse cholesterol transport and thus further promotes lipid accumulation in macrophages. MiR-148a-mediated suppression of DNA methyl transférase 1 (DNMT1) enhances the expression of adipose differentiation-related protein (ADRP) further promoting foam cell formation.

g. Hyperplasia

Milk, a feeding and signaling system promoting postnatal anabolism and growth, most likely interferes with satiety control in the hypothalamus, which is possible as milk exosomes accumulate in the brain (Melnik & Schmitz, 2019) [24]. Cholecystokinin (CCK) is released by duodenal I-cell during intestinal nutrient abundance. CKK is an important hormone that induces satiety signals in the hypothalamus via binding to CCK receptor 2 (CCKR2). CCKR2 is a direct target of miR-148a. It is thus conceivable that milk exosomes maintain a "hungry brain" to increase milk intake during the breastfeeding period. Persistent milk exosomes intake by consumption of pasteurized cow's milk may maintain this hyperplasic state, a further mechanism promoting obesity.

h. Foetal Macrosomia

Milk exosomes-derived miR-21 may increase placental miR-21 content promoting mTORC1 signaling via inhibition of phosphatase and tensin homolog (PTEN) and other regulatory checkpoints. Increased mTORC1-mediated placental growth enhances the nutrient transfer to the fetus. In the trophoblastic, up regulated mTORC1 increases the expression of L-type amino acid transporters (LAT) and glucose transporter 1 (GLUT1), thus over stimulating the implacental flux of branched-chain amino acids (BCAAs) and glucose to the fetus promoting fetal overgrow (macrosomia). MiR-21 also targets CDKN1C, a critical checkpoint for fetal growth mutated in Beckwith-Weidman syndrome.

i. Allergic Diseases

Human breast milk and dairy milk exosomes transfer miR-148a and miR-29b, which both suppress DNA methyl transférase 1 (DNMT1). DNMT1 controls the methylation status of the Treg-specific demethylation region (TSDR) on the FOXP3 promoter. DNMT1 suppression (TSDR hypo methylation) increases FoxP3 expression. Milk exosomes-derived miR-155 inhibits suppressor of cytokine signaling 1 (SOCS1), a negative regulator of the JAK-STAT pathway that increases the expression of signal transducer and activator of transcription 5 (STAT5) promoting FoxP3 expression. Milk exosome-derived transforming growth factor-β (TGF-β) enhances SMAD5 signaling that further increases FoxP3 expression, especially in the thymus. Milk exosomes thus promote the induction of FoxP3, the master transcription factor of regulatory T cells (Tregs), the potential mechanism preventing allergy development by breast feeding or raw farm milk consumption during early infancy (Melnik & Schmitz, 2019) [24].

8. Conclusion

MicroRNAs play beneficial functions in humans and are actively involved in many normal developmental and physiological processes. They are crucial modulators of many normal functions, such as cardiac function and other cardiovascular processes, immune protection, and tissue function. Infant formula not only contains insufficient amounts of biologically active microRNAs, but it also has a completely different microRNA profile to human milk, with potential detrimental effects on the growth, development and protection of the infant. Routine milk consumption, which has been boosted by the introduction of refrigeration technology in the early 1950´s, is an evolutionarily novel dietary behaviour, which may have adverse long-term biological consequences. Milk is not just food but appears to represent a most sophisticated endocrine signalling system activating mTORC1 via special maternal milk-derived dietary messengers controlled by the mammalian lactation genome: Persistently increased mTORC1 signalling has been recognized as the fundamental driving force for the development of mTORC1-driven diseases of civilization. Therefore, future research in nutrition science should pay special attention to the function of milk-derived miRNAs and should clarify the potential role of milk´s exosomal miR-transfer on metabolic regulation in the milk recipient.

9. Future Prospects

Milk EVs, the major milk microRNA carriers, are bioavailable and bioactive compounds that ensure milk microRNA bio accessibility and uptake. The participation of microRNAs to milk EV functionality is yet to be validated and may represent great opportunities for disease management. Moreover, if such mechanisms were to be uncovered, concern would be raised on the detrimental chronic exposure to milk microRNAs and also the potential transfer of microRNAs form other species to human consumers. The study of the different stability of microRNAs between the species would also be a matter of interest for future investigations.

10. References

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