Characterisation of the miRNA content of human milk: Novel molecules with multifunctional significance for the mother and the infant

Mohammed Alsaweed (BSc, MBSc)

This thesis is presented for the degree of Doctor of Philosophy at The University of Western Australia, Faculty of Science, School of Chemistry and Biochemistry, and School of Women’s and Infant’s Health.
PREFACE

This thesis was supervised by Assistant Professor Foteini Kakulas (formerly Hassiotou), Associate Professor Donna T. Geddes, Emeritus Professor Peter E. Hartmann (School of Chemistry and Biochemistry, The University of Western Australia, UWA), and Professor John Newnham (School of Women's and Infants' Health, The University of Western Australia, UWA). My PhD candidature was financially supported by Majmaah University, Almajmaah, Riyadh, Saudi Arabia, and Medela AG (Switzerland).

The work presented in this thesis is my own work except when stated. It was primarily carried out at the School of Chemistry and Biochemistry, Faculty of Science, UWA. Some of the experimental work was conducted at the Genomics lab, Faculty of Medicine, UWA. The work presented in this thesis has not been presented for any other degree.

This thesis is presented as a series of scientific papers. Chapters 2 and 3 have been published, and Chapters 4, 5 and 6 are currently under review for publication. Permission has been granted by co-authors for inclusion of their work in this thesis, and their contribution is outlined in the Statement of Candidature Contribution (page 13). Each chapter is identical to the respective paper either published or submitted for publication. The work of this thesis has also been presented at scientific conferences in both oral and poster formats, as outlined in Publications (page 9).
ABSTRACT

Human milk (HM) is recognized as the optimal food for term infants, meeting their nutritional, protective and developmental needs especially in the first six months of life. In recent years, a plethora of novel bioactive components have been discovered in HM, with potential regulatory functions in both the mother and the infant during the breastfeeding period. Amongst these are microRNAs (miRNAs), non-coding RNA molecules that are abundant in HM and other mammals’ milks. Particularly in HM, miRNAs and their regulatory roles are still largely unexplored, and the effects of factors that may influence the content and composition of HM in these molecules, such as milk removal or the stage of lactation, are still unknown. Importantly, the origin of miRNAs in HM has not yet been investigated, whether it be the maternal bloodstream and/or the mammary epithelium.

Prior to investigating the miRNA content of HM, extraction of intact total RNAs and miRNAs from each HM fraction (cells, lipids and skim milk) needs to be optimized. In the first experimental chapter of this thesis, eight commercially available miRNA extraction kits were evaluated in each HM fraction, testing different extraction methods (filter column; phenol/guanidine; or a combination of filter column and phenol/guanidine). The efficacy and quality of each kit were tested in 472 samples of different HM fractions obtained from 29 lactating women. The extracted miRNAs were measured using a NanoDrop (for total quantity) and a Bioanalyzer (for small RNA to miRNA ratio). Two individual highly expressed miRNAs in HM were also quantified using qPCR. The column-based phenol-free method was found to be the most effective method for extraction of miRNA from all three HM fractions, with specific kits performing better than others depending on the HM fraction. Although skim milk has been mainly used previously for miRNA studies in HM, here the milk cell fraction yielded higher total RNA and miRNA quantities than the lipid and skim milk fractions, with the lowest quantities found in skim milk. These results suggested that careful consideration of sampling and the miRNA extraction method for each fraction of HM should be taken for HM miRNA studies, such as profiling and functional analyses.

Next, we sought to give insight into the origin of miRNAs in HM by profiling miRNAs in HM and maternal blood samples collected together from lactating women. The content of miRNA was compared between HM cells and lipids, and with maternal peripheral blood mononuclear cells (PBMCs) and blood plasma. The Taqman OpenArray technology was used to profile 681 human mature known miRNAs in 10 samples of HM
cells, HM lipids, PBMCs, and plasma (n=40 samples in total), and in two commercially available infant formulae (bovine milk- and soy milk-based). HM cells conserved more miRNA species than lipids, with 450 and 337 miRNAs profiled in cells and lipids, respectively. The miRNA species and expression patterns were very similar between HM cells and lipids, whilst PBMCs and plasma were largely different to HM. Infant formulae were extremely low in miRNAs compared to HM. These findings suggested that HM miRNAs primarily originate from the mammary epithelium, with a small contribution of the maternal circulation to the total miRNA content of HM. The processing of milk during manufacture of infant formulae is likely to deplete it in miRNA, since it discards the miRNA-rich cell and lipid fractions from animal or plant milk-based formulae.

To investigate factors that may influence the miRNA content of HM, such as infant feeding, a study was conducted that utilized Solexa small RNA sequencing to profile mature known and novel miRNAs in HM cell samples collected pre- and post-feeding from 10 exclusively breastfeeding mothers in month 2 of lactation. In both pre- and post-feed milk, 1,467 known mature miRNAs were identified, and 1,996 novel miRNAs were predicted, in which 89 novel miRNAs were considered as high-confidence. In accordance with previous studies, cell content was higher in post-feed milk than pre-feed milk, with higher cell content relating to greater miRNA concentration and number of miRNA species. Upregulation of 29 known and 2 novel miRNA species was observed post-feeding, whilst only 4 known and 1 novel miRNAs were upregulated in pre-feed milk, suggesting involvement of the upregulated miRNAs post-feeding in milk synthesis. Indeed, advanced functional prediction analysis highlighted specific functions of the top most highly expressed HM miRNAs in the synthesis of milk components. This study showed that HM cells are highly enriched in miRNAs, conserving numerous known and novel miRNA species, with milk removal by the infant influencing the cell content of HM, and through it, its miRNA composition. Some of the abundant HM miRNAs also display functions in the control of body fluid balance, thirst, appetite, immune response, and development, implicating their functional significance for the infant.

In addition to short-term effects of infant feeding, the stage of lactation was also examined as a potential factor that may influence the miRNA content of HM. Deep sequencing was used to profile miRNAs in post-feeding cell and lipid fractions of HM collected longitudinally from 10 lactating women in months 2, 4, and 6 postpartum. In both HM fractions, 1,195 mature known miRNAs were identified, in which cells contained higher number of known miRNAs compared to lipids (1,136 and 835 miRNAs,
respectively). These miRNAs were positively associated with the cell and lipid contents of HM. An additional 5,167 novel miRNA species were predicted using strict criteria, of which 235 were high-confidence miRNAs expressed in ≥3 samples with total reads of >20. The majority of 496 known mature miRNAs (63.9%) were similarly expressed between the two milk fractions, whilst 146 were upregulated in HM cells compared to lipids, and 133 in HM lipids compared to cells. During the first 6 months of lactation, the profiles of most known miRNA species did not change, yet approximately a third of known miRNAs were differentially expressed between these months postpartum. Great variation was seen in the composition of novel miRNA species both within and between mothers, suggesting that many of them are dyad-specific. These findings indicate that, overall, the total miRNA concentration and profile of most highly expressed known miRNAs in HM cells and lipids do not change in first 6 months of lactation, consistent with maintenance of milk production during this period. However, the composition of HM in certain miRNAs is altered, particularly in month 4 compared to months 2 and 6, potentially reflecting involvement of these miRNAs in the remodeling of the gland in response to infant feeding patterns, which usually change after exclusive breastfeeding, and thus adaptation to infant needs.

This thesis encompasses a comprehensive first characterization of HM miRNAs in healthy lactating women, providing a better understanding of the origin and regulation of these molecules in HM, and predicting their potential roles in the lactating mammary gland and in the breastfed infant as exogenous regulatory agents of immunity, growth and development. HM is extremely rich in miRNAs, which are abundantly conserved in its cellular and lipid fractions. Originating primarily from the lactating mammary gland, HM miRNA profiles appear to be unique to a mother-infant dyad, and may be used as indicators of lactation performance. They are influenced in the short-term during breastfeeding as well as during the first 6 months of lactation, but the most highly expressed and common HM miRNAs remain largely unchanged. These findings open new avenues to further investigate the role of miRNAs in milk synthesis and their physiological significance in the breastfed infant.
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“This thesis is dedicated to my mother who breastfed her 12 children”

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Mohammed Alsaweed
12 April 2016
The original articles and conference papers that have been achieved during my PhD period within the scope of this thesis are presented below:

**JOURNAL PAPERS**


CONFERENCE PAPERS


STATEMENT OF CANDIDATURE CONTRIBUTION

Mohammed Alsaweed completed most of the work presented in each chapter of this thesis. Acknowledgment for the contribution from other individuals in designing projects, data analysis or critical editing is stated below for each chapter.

Chapter 2
Mohammed Alsaweed and Dr Foteini Kakulas wrote the manuscript. Professor Peter Hartmann and Dr Donna Geddes critically reviewed the manuscript.

Chapter 3
Mohammed Alsaweed collected samples, conducted experiments and data analyses, interpreted results, and wrote the manuscript; Anna Hepworth conducted statistical analyses and critically reviewed the manuscript; Professor Peter Hartmann contributed to study design and critically reviewed the manuscript; Dr Donna Geddes designed the study, interpreted results and critically reviewed the manuscript; Dr Foteini Kakulas designed the study, conducted data analyses, interpreted results, wrote and critically reviewed the manuscript. All authors approved the final manuscript.

Chapter 4
Mohammed Alsaweed collected samples, conducted experiments and data analyses, interpreted results, and wrote the manuscript; Dr Ching Tat Lai conducted statistical analyses and critically reviewed the manuscript; Professor Peter Hartmann contributed to study design and critically reviewed the manuscript; Dr Donna Geddes designed the study, interpreted results and critically reviewed the manuscript; Dr Foteini Kakulas designed the study, collected samples, interpreted results, wrote and critically reviewed the manuscript. All authors approved the final manuscript.

Chapter 5
Mohammed Alsaweed collected samples, conducted experiments and data analyses, interpreted results, and wrote the manuscript; Dr Ching Tat Lai conducted statistical analyses; Professor Peter Hartmann contributed to study design and critically reviewed the manuscript; Dr Donna Geddes designed the study, interpreted results and critically reviewed the manuscript; Dr Foteini Kakulas designed the study, conducted data analyses, interpreted
results, wrote and critically reviewed the manuscript. All authors approved the final manuscript.

Chapter 6
Mohammed Alsaweed collected samples, conducted experiments and data analyses, interpreted results, and wrote the manuscript; Dr Ching Tat Lai conducted statistical analyses and critically reviewed the manuscript; Professor Peter Hartmann contributed to study design and critically reviewed the manuscript; Dr Donna Geddes designed the study, interpreted results and critically reviewed the manuscript; Dr Foteini Kakulas designed the study, conducted data analyses, interpreted results, wrote and critically reviewed the manuscript. All authors approved the final manuscript.

The candidate

The coordinating supervisor

Mohammed Alsaweed

Professor Peter E. Hartmann
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6.1 ABSTRACT
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>miRNA</td>
<td>Micro Ribonucleic Acid</td>
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<tr>
<td>Pri-miRNA</td>
<td>Primary Micro Ribonucleic Acid</td>
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<tr>
<td>Pre-miRNA</td>
<td>Precursor Micro Ribonucleic Acid</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
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<td>tRNA</td>
<td>Transfer Ribonucleic Acid</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal Ribonucleic Acid</td>
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<tr>
<td>piRNA</td>
<td>Piwi-interacting Ribonucleic Acid</td>
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<tr>
<td>RISC</td>
<td>The RNA-induced silencing complex</td>
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<tr>
<td>HM</td>
<td>Human milk</td>
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<tr>
<td>Pre-feed milk</td>
<td>Before feed human milk</td>
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<tr>
<td>Post-feed milk</td>
<td>After feed human milk</td>
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<tr>
<td>hMG</td>
<td>Human mammary gland</td>
</tr>
<tr>
<td>HuMEC</td>
<td>Human mammary epithelial cell</td>
</tr>
<tr>
<td>MFG</td>
<td>Milk fat globule</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>(q)RT-PCR</td>
<td>(quantitative) Real-Time Polymerase Chain Reaction</td>
</tr>
<tr>
<td>NGS</td>
<td>Next Generation Sequence</td>
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<tr>
<td>IPA</td>
<td>Ingenuity Pathway Analysis</td>
</tr>
<tr>
<td>GO</td>
<td>Gene Ontology</td>
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<tr>
<td>KEGG</td>
<td>Kyoto Encyclopedia of Genes and Genomes</td>
</tr>
<tr>
<td>UWA</td>
<td>The University of Western Australia</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organisation</td>
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CHAPTER 1

GENERAL INTRODUCTION
1.1 BACKGROUND

Human milk (HM) is unique for human infants, providing all the required elements to facilitate immune protection, growth and development early in life (Kramer, 2010). Because of this, the World Health Organization (WHO) recommends exclusive breastfeeding for the first six months, with continuation of breastfeeding for 2 years and beyond (Jeurink et al., 2013). HM contains various types of nutritional elements, including proteins, fat, carbohydrates, and vitamins (German et al., 2002) as well as live cellular components and molecules with bioactivity that support immunity and development (Hassiotou and Geddes, 2015, Hassiotou and Hartmann, 2014). The latter include recently discovered in HM small non-coding RNAs called microRNAs (miRNAs) that are ~22 nucleotides long and are known to regulate gene expression at the post-transcriptional level (Bartel, 2004). In addition to HM and a range of other body fluids such as plasma, miRNAs are present in all our tissues and cells (Kosaka et al., 2010, Weber et al., 2010, Wang et al., 2012a). They are involved in almost all biological processes in humans, such as cell differentiation, cell cycle, apoptosis and immunity (Krol et al., 2010). They are essential for growth and development in early life, especially in the nervous and immune systems (Amiel et al., 2012, Lindsay, 2008). To date, 2,588 mature miRNAs are known to be present in humans (miRBase version 21.0, release 2014) (Kozomara and Griffiths-Jones, 2014), although new miRNA species are being continuously discovered. Research in HM miRNAs is in its infancy, particularly the miRNA composition of the different fractions of HM (cells, lipids, and skim milk), with great scientific interest in their roles for the breastfed infant and in the lactating breast as well as in factors that may influence their content in HM.

Despite the recent advances on food-derived miRNAs, several barriers have hindered good understanding of the roles of exogenous miRNAs in our bodies (Yang et al., 2015). Since 2012, a number of studies have begun to investigate the fate and potential roles of exogenous food-derived miRNAs, including those obtained from both animal- and plant-based food sources (Zhang et al., 2012a, Baier et al., 2014, Wolf et al., 2015, Arntz et al., 2015). First, the survival of miRNAs in conditions of the gastrointestinal tract was studied, with conclusive findings demonstrating high stability of these molecules, in contrast to messenger RNA, under harsh conditions such as temperature, acidic pH and RNase-rich environments (Zhou et al., 2012, Kosaka et al., 2010, Hata et al., 2010, Modepalli et al., 2014). And although a couple of studies did not conclude that absorption of certain exogenous miRNAs in the adult is significant (Modepalli et al., 2014, Zhou et al., 2012), studies that utilized appropriate methodology and experimental models presented
convincing evidence supporting absorption of food- and bovine milk-derived miRNAs in the adult gastrointestinal tract (Wolf et al., 2015, Baier et al., 2014, Zhang et al., 2012a, Arntz et al., 2015). Particularly for HM miRNAs, the infant’s gastrointestinal tract enzymes have no major effects (Kosaka et al., 2010), the infant’s gut is less acidic than that of adults (Fallingborg, 1999), and is more permeable (van Elburg et al., 1992), suggesting a more efficient absorption. Moreover, paracellular diffusion in the gastrointestinal tract is enhanced in infancy (Wada and Lonnerdal, 2014). Finally, HM miRNAs are thought to be further protected within microvesicles, such as exosomes, and maternal cells, both of which have been suggested to facilitate their survival and absorption in the infant (Alsaweed et al., 2015a).

HM miRNAs are present in all three major milk fractions, including cells, lipids and skim milk (Alsaweed et al., 2015a), although most research to date has focused on skim milk and to a lesser extent on lipids and exosomes (Zhou et al., 2012, Kosaka et al., 2010, Weber et al., 2010, Munch et al., 2013). Although, approximately 700 mature miRNA species have been identified using qPCR and microarray technologies in skim milk (Weber et al., 2010), recent sequencing studies have revealed more miRNA species in HM lipids (Munch et al., 2013). Milk lipids, which mainly comprise fat globules, conserve more miRNAs than skim milk since they are derived from cells (the lactocytes), the site of miRNA synthesis in the mammary gland. The contribution of maternal cells of milk to its miRNA content has not been investigated yet in either HM or other species’ milks, potentially presenting a significant source of miRNAs for the infant compared to the other two milk fractions. Importantly, HM cells represent the cellular hierarchy of the lactating epithelium (Hassiotou et al., 2013a), providing a non-invasive and easily accessible source of miRNAs from the lactating breast.

In addition to characterising the miRNA composition of different HM fractions, research has started to focus on maternal and/or infant factors that may influence the miRNA content of HM, similar to its lipid, cellular and other components. For example, milk removal by the infant during breastfeeding is associated with an increased fat and cell content of HM post-feeding (Hassiotou et al., 2013ah). The stage of lactation may also influence milk composition in various components such as immune cells and other milk immunomodulatory factors, from colostrum to mature milk (Hassiotou and Geddes, 2015); yet other components, such as fat, lactose and protein, do not systematically change during lactation (Dewey et al., 1984). Immune-related miRNAs have been abundantly identified in HM, potentially contributing immunoprotective functions in the infant (Kosaka et al., 2010),
therefore delineating factors that may influence the miRNA content of HM is a research priority (Amiel et al., 2012).

1.2 RESEARCH OBJECTIVES

The aim of this thesis was first to optimise the extraction of miRNA from different fractions of HM, and then to characterise their miRNA composition, the origin of these molecules in HM, and factors that may influence them, such as milk removal by the infant and the stage of lactation. This was done using various technologies and tools, including evaluating and optimising different miRNA extraction methods, performing qPCR and deep sequencing for profiling of known and novel miRNAs, and functional analyses. The purpose of these studies was to standardize the methodology for isolating and analysing miRNAs from HM, and to provide a solid basis for further examinations of the role of these molecules for the breastfed infant, and their potential use as diagnostic biomarkers of lactation performance and the health status of the lactating breast.

The specific objectives of this thesis included:

1. Optimisation of miRNA extraction from different fractions of HM (Chapter 3).
2. Examination of the origin of HM miRNAs, and comparison of miRNA content between different HM fractions (Chapter 4).
3. Profiling of known miRNAs in HM cells, discovery of novel miRNA species, and effects of milk removal by the infant (Chapter 5).
4. Profiling of known and novel miRNA species in different HM fractions, and investigation of changes in the miRNA profile of HM during the first 6 months of lactation (Chapter 6).
CHAPTER 2

microRNAs IN BREASTMILK AND THE LACTATING BREAST: POTENTIAL IMMUNOPROTECTORS AND DEVELOPMENTAL REGULATORS FOR THE INFANT AND THE MOTHER

2.1 ABSTRACT

Human milk (HM) is the optimal source of nutrition, protection and developmental programming for infants. It is species-specific and consists of various bioactive components, including microRNAs, small non-coding RNAs regulating gene expression at the post-transcriptional level. microRNAs are both intra- and extra-cellular and are present in body fluids of humans and animals. Of these body fluids, HM 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. Potential effects of exogenous food-derived microRNAs on gene expression have been demonstrated, together with the stability of milk-derived microRNAs in the gastrointestinal tract. Taken together, these strongly support the notion that milk microRNAs enter the systemic circulation of the HM fed infant and exert tissue-specific immunoprotective and developmental functions. This has initiated intensive research on the origin, fate and functional significance of milk microRNAs. Importantly, recent studies have provided evidence of endogenous synthesis of HM microRNA within the human lactating mammary epithelium. These findings will now form the basis for investigations of the role of microRNA in the epigenetic control of normal and aberrant mammary development, and particularly lactation performance.

2.2 INTRODUCTION

Since their recent discovery in 1993, microRNAs (also known as miRNAs) have emerged as key regulators of gene expression at the post-transcriptional level in humans, animals and plants (Lee et al., 1993, Bartel, 2004). They act by binding to an mRNA target to either inhibit the translation of mRNA into protein and/or promote its degradation (He and Hannon, 2004, Pritchard et al., 2012, Fabian et al., 2010). The microRNA family includes extremely small non-coding RNA (~22 nucleotide in length) that have been isolated from cells, tissues and body fluids of various mammalian species (Krol et al., 2010, Winter et al., 2009). The biogenesis of microRNA comprises three main processes(Kim et al., 2009a): microRNAs are first transcribed into primary microRNA (pri-microRNA) from specific independent genes on DNA by RNA polymerase II, and are then converted into hairpin precursor microRNA (pre-microRNA) by the Drosha–DGCR8 complex. The enzyme Dicer then produces mature microRNA from pre-microRNA in the cytoplasm (Figure 1) (Winter et al., 2009, Du and Zamore, 2005). According
to miRBase version 21.0 (http://www.mirbase.org) released in June 2014, the number of pre-microRNAs in the human is estimated to be 1881. These correspond to 2588 known mature microRNAs, while the number of human that are considered to be targets of microRNAs is estimated to be approximately 20,000-25,000 (International Human Genome Sequencing, 2004). Therefore, a single mature microRNA can bind and regulate multiple mRNAs (genes) (Krol et al., 2010). Importantly, ongoing research is still discovering new microRNAs. In mammalian cells, various functional studies have demonstrated that microRNAs regulate up to 50% of protein synthesis (gene expression) (Pritchard et al., 2012). Several roles of different microRNAs were investigated experimentally and they are involved in regulating a range of biological processes in plants and mammals (including humans) (He and Hannon, 2004, Williams, 2008, O'Driscoll, 2006). In addition to controlling normal physiological processes, microRNAs have been implicated in pathologies such as cancer, autoimmune diseases, gastrointestinal diseases, and diseases of the reproductive system (He and Hannon, 2004, Pritchard et al., 2012). microRNAs have recently been reported to be important regulators of pluripotency-related genes and they have been used to reprogram somatic cells into induced pluripotent stem cells (iPSCs) (Houbaviy et al., 2003, Judson et al., 2009, Xu et al., 2009). This regulation could potentially be an important method for regenerative medicine and biomedical research, as it eliminates the need for viral vectors. Viral vectors are used to reprogram cells into iPSCs, however they have been shown to uncontrollably influence reprogramming via random insertion of exogenous sequences into the genome (Huangfu et al., 2008, Yoshida et al., 2009). Further, to their role in the epigenetic regulation of stem cell fate and function, microRNAs also regulate the mammalian immune system. Their functions include regulation of T and B cell development (Ventura et al., 2008, Xiao et al., 2008), release of inflammatory mediators (Jing et al., 2005), proliferation of neutrophils and monocytes (Landgraf et al., 2007), and differentiation of dendritic cells and macrophages (O'Connell et al., 2007). microRNAs are also thought to be involved in haematopoiesis (Monticelli et al., 2005), cardiac muscle development (Yang et al., 2007), insulin secretion (Poy et al., 2004), and neurogenesis (Giraldez et al., 2005). Given their role in numerous physiological processes, deregulation of microRNA function can lead to disease; therefore, increasing evidence supports their use as diagnostic biomarkers. Either upregulation or downregulation of microRNAs has been found to be associated with initiation and progression of some types of cancers (Mahn et al., 2011).
These include breast cancer (Gotte, 2010), where upregulation of oncogene miR-2 has been shown to be involved in both initiation and progression of the disease (Si et al., 2007). High levels of microRNAs have been detected in body fluids, such as plasma, urine, saliva, seminal fluid, tears, cerebrospinal fluid (CSF) and more recently, milk (Weber et al., 2010). Milk is a non-invasive source of numerous biomolecules that are either synthesized in the lactating breast or are transferred via the systemic circulation and provide important functions for both the lactating mother and the breastfed infant. To a large extent, milk microRNAs appear to be endogenous to the mammary gland (Silveri et al., 2006) 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. Further, food-derived microRNAs (e.g., exogenous miR-168a) (Zhang et al., 2012a) have been suggested to survive the mammalian gastrointestinal (GI) tract and regulate mammalian genes (Zhang et al., 2012a, Palmer et al., 2014, Jiang et al., 2012). As human milk (HM, breastmilk) is highly enriched in microRNAs, it would be of great interest to illuminate the fate and function of this breastmilk 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 HM (Alsaweed et al., 2015ab, Zhou et al., 2012), have been shown to survive the GI tract of adult humans and increase in their serum post-consumption (Baier et al., 2014). 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), further highlighting the important role of vehicle-mediated transfer of milk microRNA (Pieters et al., 2015).
Figure 1. The predicted biogenesis of microRNA. MicroRNA are first transcribed from specific genes on DNA as primary microRNA (pri-microRNA) by RNA polymerase II (RNAPII). In the nucleus, pri-microRNA are converted into ~70-nucleotide precursor hairpin microRNA (pre-microRNA) by the enzymatic Drosha–DGCR8 complex. Pre-microRNA are then transported from nucleus to the cytoplasm by Exportin 5. There, the Dicer–TRBP complex produces ~20 base pair 3′ microRNA and 5′ microRNA duplex. Dicer with assistance from argonaute 2 (AGO2) generates mature microRNA by cleaving the double strand of pre-microRNA. Only one strand of microRNA (3′ microRNA or 5′ microRNA) can be attached into the RNA-induced silencing complex (RISC). 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.
2.3 microRNAs ARE HIGHLY ENRICHED IN MILK

2.3.1 microRNAs in mammalian milk
HM is considered the optimal food for term infants in the first six months of life (Kramer, 2010), with the World Health Organization recommending exclusive breastfeeding for up to six months, with continuation of breastfeeding for at least the first two years (Kramer and Kakuma, 2004). In addition to providing nutrition, HM has long been known to protect the infant from infections and to play developmental functions integral to the infant, in which microRNAs are likely to be highly involved. microRNAs can be isolated and experimentally studied in the main three fractions of milk, the cells, lipids, and skim milk (Figure 2). Interestingly, HM is one of the richest microRNA source of all body fluids in the human, containing up to ~1400 mature microRNAs (Figure 3) (Alsaweed et al., 2015ab, Zhou et al., 2012, Wang et al., 2010a, Kosaka et al., 2010, Munch et al., 2013, Alsaweed et al., 2015bs). Cellular and lipid fractions of HM contain a greater amount of microRNAs compared to the skim milk fraction (Alsaweed et al., 2015ab, Munch et al., 2013), which is important to consider when analyzing milk microRNA. Not surprisingly, a wide variation in microRNA expression amongst lactating women has also been shown (Kosaka et al., 2010), with the factors that influence this variation having not been studied to date. Further, animal studies have shown that the type and expression levels of microRNA are distinctly different between the lactating and non-lactating mammary glands in the cow (Li et al., 2012). microRNAs were also found to be in involved in mammary gland development in murine models (Avril-Sassen et al., 2009). These animal studies have suggested a key role of microRNAs in the regulation of the development and performance of the lactating mammary gland, and they therefore have the potential to influence milk synthesis.
Figure 2. A workflow of microRNA identification in HM. Whole HM can be fractionated by centrifugation for 20 min at 800 g at 20°C to obtain three fractions including the cells, the lipid layer and skim milk. Total RNA and microRNA can be extracted from each fraction using the optimal kit (Alsaweed et al., 2015ab). Profiling of microRNA after quantification and measurement of its quality can be performed using three different methods (Alsaweed et al., 2015ab): phenol/guanidine, filter column, and a combination of the filter column and phenol/guanidine methods. Small RNA sequencing can determine novel microRNAs and identify all microRNAs in a sample. Microarray analysis and qPCR-based methods can on principle only measure specific microRNAs. Validation of presence and expression patterns of a microRNA of interest is done using qPCR as it is highly sensitive and specific.
Figure 3. Differences in the microRNA content between the different fractions of human milk. (A) Box plots showing the total RNA content (enriched in microRNA) measured using NanoDrop 2000 in HM cells (n=30 milk samples from 20 mothers), lipids (n=127 milk samples from 79 mothers) and skim milk (n=116 milk samples from 79 mothers) obtained from healthy breastfeeding mothers (Alsaweed et al., 2015bb, Alsaweed et al., 2015bs, Alsaweed et al., 2015ab). (B) Box plot showing the number of mature microRNA species that have been identified in HM cells, lipids and exosomes using deep small RNA sequencing. HM cell microRNAs were profiled in n=20 samples collected from 10 healthy exclusively breastfeeding mothers in month 2 of lactation (Alsaweed et al., 2015bb) using Illumina HiSeq2000, with total clean reads of 268,681,616 matched to miRBase version 20.0. This study identified 1,467 different mature known microRNAs in HM cells. HM lipid samples (n=7) were sequenced using an Illumina 1G Genome analyzer, with 124,110,646 clean reads mapped to miRBase version 16.0. This study identified 308 mature known microRNAs in HM lipids (Munch et al., 2013). HM exosome samples (n=4) were sequenced using an Illumina Genome analyzer II, with 83,520,000 clean reads matched to miRBase version 17.0. This study identified 602 mature known microRNAs in HM exosomes (Zhou et al., 2012).
2.3.2 microRNAs in different milk fractions

In 2010, Weber et al. isolated and profiled microRNA from 12 different human body fluids, including human colostrum and milk (Weber et al., 2010). In the same year, microRNAs were profiled in skimmed HM (Kosaka et al., 2010). Since then, a few studies have profiled microRNA in HM and in the milk of other mammalian species (Table 1) (Zhou et al., 2012, Munch et al., 2013, Kosaka et al., 2010). This was carried out using different platforms including qPCR, microarray analysis and small RNA sequencing (Weber et al., 2010, Zhou et al., 2012, Munch et al., 2013, Kosaka et al., 2010, Gu et al., 2012, Chen et al., 2010). Although qPCR is generally the method of choice providing high sensitivity and specificity, with low RNA input requirements, small RNA sequencing is often employed to screen for all microRNAs present. This allows identification of novel microRNAs and it is sensitive enough for microRNA quantification. Small RNA sequencing is done either using high throughput next generation sequencing (NGS) or small-scale NGS platforms (Pritchard et al., 2012).

Amongst the 12 human body fluids analysed, Weber et al. used qPCR to profile 429 known mature microRNAs in mature skimmed HM and 386 known mature microRNAs in skimmed human colostrum (the mammary secretion produced in the first few days postpartum) (Weber et al., 2010). This study demonstrated for the first time the high abundance of microRNA in HM, 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) (Weber et al., 2010). Microarrays were first used to profile microRNAs in HM by Kosaka et al., who examined 281 different microRNAs in skimmed HM obtained in the first six months of lactation (Kosaka et al., 2010). Although these two pioneering studies focused on microRNA analysis in skimmed HM, it has been argued that the other two main milk fractions (lipids and cells) are likely to harbor large quantities of microRNAs. Indeed, later Munch et al. analyzed the lipid fraction of HM using small RNA sequencing and found high quantities of microRNA, reporting 308 microRNA species that are predicted to target a total of 9074 genes (Munch et al., 2013). In addition, a subsequent study performed deep sequencing to profile microRNAs from HM exosomes (Zhou et al., 2012), which are small cell-derived vesicles carrying proteins and molecules present in all body fluids (Hata et al., 2010). This study found 639 exosomal mature microRNAs originating from 452 pre-microRNAs (Zhou et al., 2012).
More recently, we used the Taqman OpenArray Panel system (Applied Biosystems, Foster City, CA, USA) to screen 754 human mature microRNAs in the cellular and lipid fractions of HM. In addition, we performed comparisons with the microRNA content of maternal peripheral blood mononuclear cells (PBMCs) and plasma (Alsaweed et al., 2015bs). This analysis identified 293 and 233 microRNA species in the breastmilk cell and lipid fractions, receptively. Maternal PBMCs contained 345 different microRNAs, whereas only 169 were found in maternal plasma. Breastmilk cells and PBMCs had significantly higher microRNA content compared to breastmilk lipids and plasma, respectively ($p < 0.05$). Correlation and cluster analyses showed that breastmilk cells and lipids were highly related in terms of microRNA expression patterns and species, however PBMC microRNAs were not correlated with breastmilk microRNAs. In plasma, marked inter-individual variation in expression levels of single microRNA species was observed. This study together with our previously published optimization of microRNA extraction from HM (Alsaweed et al., 2015ab) demonstrated the presence of microRNAs in all three fractions of HM (cells, lipids, and skim milk), and revealed that HM conserves more microRNAs with different expression patterns compared to maternal plasma (Alsaweed et al., 2015bs), but also other human body fluids (Weber et al., 2010). Skim milk (known as the plasma phase of milk) is cell and fat globule free milk, and appeared to be extremely low in microRNAs and total RNAs compared to the cell and lipid milk fractions (Alsaweed et al., 2015ab).

In addition to HM, microRNA have been analyzed in other mammals’ milk, particularly the dairy cow, in various studies (Table 1) (Gu et al., 2012, Chen et al., 2010, Hata et al., 2010, Izumi et al., 2012). Mature bovine skim milk has been shown to contain 213 microRNA species using deep sequencing (Chen et al., 2010) and 53 using microarrays (Izumi et al., 2012). Some of these microRNA are enriched in either mature milk or colostrum (Chen et al., 2010). Specifically, bovine colostrum has been found to be richer in microRNA (both higher total content and species number) compared to mature bovine milk, where 230 and 100 microRNAs were identified in skimmed bovine colostrum in the above studies, respectively (Table 1) (Chen et al., 2010, Izumi et al., 2012). Similarly, bovine milk-derived microvesicle RNA and total milk RNA levels have been shown to be higher in colostrum compared to mature milk (Hata et al., 2010, Izumi et al., 2012). Unlikely bovine milk, skimmed rat colostrum was shown to conserve fewer microRNA species (128) than mature rat skim milk (144) (Table 1) (Izumi et
al., 2014). Izumi et al. (Izumi et al., 2012) isolated 53 mature microRNAs from mature bovine skim milk, and 100 from skimmed bovine colostrum (Table 1). Gu et al. examined the exosomes 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 HM (Table 1) (Gu et al., 2012). However, 39 pre-microRNA were identified that were not homologues of HM microRNA (Gu et al., 2012). This was one of the first efforts to quantify changes in milk microRNA content over time, in this case within the first month of lactation, which can provide insight into both their involvement in the maturation of the mammary gland into an organ that synthesizes copious amounts of milk and their role(s) in the development and protection of the offspring.

Another study in porcine milk exosomes identified 491 mature microRNAs by small RNA sequencing in the first 6 days postpartum (Table 1) (Chen et al., 2014). These pre-microRNA that are shared between different species potentially have similar physio-pathological mechanisms of action and functions in milk amongst mammals (Gu et al., 2012). In all animal milk microRNA studies to date, only a few microRNA were found to be highly expressed (Gu et al., 2012, Weber et al., 2010, Zhou et al., 2012, Munch et al., 2013, Kosaka et al., 2010, Chen et al., 2010). For example, the top 10 most highly expressed microRNAs in exosomal porcine milk were contributing approximately 87% of the total 234 microRNA (Gu et al., 2012).
Table 1. Number of mature known microRNA species reported for different mammalian species for the three milk fractions (cells, lipids and skim milk).

<table>
<thead>
<tr>
<th>Species</th>
<th>Milk fraction</th>
<th>microRNAs*</th>
<th>Profiling method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Skim milk (mature)</td>
<td>429</td>
<td>qPCR</td>
<td>(Weber et al., 2010)</td>
</tr>
<tr>
<td>Human</td>
<td>Skim milk (colostrum)</td>
<td>386</td>
<td>qPCR</td>
<td>(Weber et al., 2010)</td>
</tr>
<tr>
<td>Human</td>
<td>Skim milk (mature)</td>
<td>281</td>
<td>Microarray</td>
<td>(Kosaka et al., 2010)</td>
</tr>
<tr>
<td>Human</td>
<td>Milk exosomes</td>
<td>639***</td>
<td>Solexa deep sequencing</td>
<td>(Zhou et al., 2012)</td>
</tr>
<tr>
<td>Human</td>
<td>Milk lipids</td>
<td>308</td>
<td>Solexa deep sequencing</td>
<td>(Munch et al., 2013)</td>
</tr>
<tr>
<td>Human</td>
<td>Milk cells</td>
<td>450***</td>
<td>TaqMan OpenArray</td>
<td>(Alsaweed et al., 2015bs)</td>
</tr>
<tr>
<td>Human</td>
<td>Milk cell pre-feed</td>
<td>337***</td>
<td>TaqMan OpenArray</td>
<td>(Alsaweed et al., 2015bs)</td>
</tr>
<tr>
<td>Human</td>
<td>Milk cell post-feed</td>
<td>1308</td>
<td>Solexa deep sequencing</td>
<td>(Alsaweed et al., 2015bb)</td>
</tr>
<tr>
<td>Bovine</td>
<td>Skim milk (colostrum)</td>
<td>230</td>
<td>Solexa deep sequencing</td>
<td>(Chen et al., 2010)</td>
</tr>
<tr>
<td>Bovine</td>
<td>Skim milk (mature)</td>
<td>213</td>
<td>Solexa deep sequencing</td>
<td>(Chen et al., 2010)</td>
</tr>
<tr>
<td>Bovine</td>
<td>Skim milk (colostrum)</td>
<td>100</td>
<td>Microarray</td>
<td>(Izumi et al., 2012)</td>
</tr>
<tr>
<td>Bovine</td>
<td>Skim milk (mature)</td>
<td>53</td>
<td>Microarray</td>
<td>(Izumi et al., 2012)</td>
</tr>
<tr>
<td>Porcine</td>
<td>Milk exosomes</td>
<td>180**</td>
<td>Solexa deep sequencing</td>
<td>(Gu et al., 2012)</td>
</tr>
<tr>
<td>Porcine</td>
<td>Milk exosomes (colostrum)</td>
<td>491</td>
<td>Solexa deep sequencing</td>
<td>(Chen et al., 2014)</td>
</tr>
<tr>
<td>Murine (rat)</td>
<td>Skim milk (colostrum)</td>
<td>128</td>
<td>Microarray</td>
<td>(Izumi et al., 2014)</td>
</tr>
<tr>
<td>Murine (rat)</td>
<td>Skim milk (mature)</td>
<td>144</td>
<td>Microarray</td>
<td>(Izumi et al., 2014)</td>
</tr>
</tbody>
</table>

* Number of detectable mature microRNAs; ** Precursor microRNAs (pre-microRNAs); *** Ct \(\geq 8\) and \(\leq 29\).

2.3.3 Origin of milk microRNAs

Our comparisons between breastmilk blood microRNA provided important insight into the origin of milk microRNA, with the mammary gland appearing to be the main source of milk microRNA, with the maternal circulation having a smaller contribution (Alsaweed et al., 2015bs). This was consistent with a recent microRNA analysis in tammar wallaby milk. In the tammar study, most microRNAs were differentially expressed between skim milk and blood serum, although the total number of microRNA species was similar in both milk and serum (86 and 82 microRNAs, respectively) (Modepalli et al., 2014). Prior to these findings, it was believed that because ribosomal RNA (18S and 28S) measured by the Bioanalyzer is usually absent or low during the isolation and quantification of microRNA, microRNA are less likely to
be secreted from milk cells (Gu et al., 2012, Izumi et al., 2012). Nevertheless, taken together, the similarities between milk cell and lipid microRNAs, the differences with PBMCs and plasma both in the human (Alsaweed et al., 2015bs) and dairy cow (Chen et al., 2010), and the known secretion of milk lipids from lactocytes, which are the most abundant cell type in HM under healthy conditions (Hassiotou et al., 2013a), strongly suggest that the milk cell and lipid microRNAs are primarily endogenously synthesized in the mammary gland (Alsaweed et al., 2015bs, Modepalli et al., 2014). Moving forward, it will be important to understand the factors controlling mammary microRNA synthesis during pregnancy and lactation, as this is likely to impact the health and development of both the mammary gland and the infant.

2.3.4 Milk microRNAs as diagnostic tools
Although the microRNA content and composition of different milk fractions is being intensively investigated, the understanding of factors influencing them as well as the roles and functions of these molecules in the lactating mammary gland and for the breastfed offspring is still very poor. A number of maternal and/or infant characteristics have previously been reported to influence the composition of HM, including infant feeding, preterm birth, the stage of lactation, parity, maternal body mass index (BMI), infant sex, and the health status of the mother and the infant (Molinari et al., 2013, Mitoulas et al., 2002, Hassiotou et al., 2013x, Hassiotou et al., 2013ah, Powe et al., 2010, Bachour et al., 2012, Bauer and Gerss, 2011). It is not unlikely that some of these factors may affect the microRNA content of HM, yet very few studies have examined these associations. Recently, the effects of infant feeding and milk removal on HM microRNA content and composition were investigated (Alsaweed et al., 2015ab, Alsaweed et al., 2015bs). It is well established that post-feed milk contains more fat and cells compared to pre-feed milk (Hassiotou et al., 2013ah, Mitoulas et al., 2002). Similarly, additional microRNA species were detected in post-feed milk, however the difference in the total number of microRNA species or the expression of the majority of microRNAs between pre- and post-feed milk was not statistically significant in a group of 10 lactating women examined in month 2 of lactation (n = 10, p > 0.05). Yet, a subgroup of 27 known and 1 novel microRNAs in this study were expressed more highly post-feeding (p < 0.05). From these findings, it can be concluded that milk removal may influence the content and/or expression of certain microRNAs in HM, but the overall microRNA composition appears to remain constant.
This is in agreement with Kosaka et al., who showed variable microRNA expression patterns between mothers, but claimed no significant intra-individual variation in microRNA expression (Kosaka et al., 2010). Yet, this study only collected 2–4 samples from each of eight lactating women at different stages of lactation without standardizing the sampling based on infant feeding/milk removal or time of the day. Therefore, further studies are required to shed light into factors that may influence HM microRNA content within a mother. Nevertheless, Kosaka et al. (Kosaka et al., 2010) as well as the more recent study by Alsaweed et al. (Alsaweed et al., 2015bb) showed great inter-individual variation in HM microRNA content, which could potentially be associated with parity, preterm birth, infant characteristics or environmental factors (e.g., maternal diet) (Baier et al., 2014, Zhang et al., 2012p). Interestingly, maternal diet has been shown to influence other HM components, such as fatty acids (Makrides et al., 1996) during lactation, but also fetal growth and health during pregnancy (Melnik et al., 2015, Jiang et al., 2014).

Similarly to numerous other immunological components of HM (immune cells, lysozyme, lactoferrin, immunoglobulins (Hassiotou et al., 2013x, Hassiotou and Geddes, 2015)), immune-related milk microRNA may be influenced by the health status of the mother and/or the infant. Although this has not been investigated in HM, a recent study in the dairy cow examined milk from healthy cows and those infected with S. uberis 0140J. It was found that 26 microRNAs isolated from milk cells described as monocytes were differentially expressed between the two cohorts (Lawless et al., 2014). The majority of the differentially expressed microRNAs are implicated in innate immunity, suggesting that infection of the lactating breast changes the milk microRNA profile to enhance immunoprotection and facilitate recovery. It is of note that in this study, milk monocytes were identified using the marker CD14, which is also expressed by milk epithelial cells (Hassiotou et al., 2013a, Hassiotou and Geddes, 2015). Although epithelial cells are not the dominant cell type in bovine milk, in contrast to HM (Hassiotou et al., 2013a, Hassiotou and Geddes, 2015), the microRNA profiles reported in this study are likely to represent more cell types than just monocytic immune cells of milk.

Although in HM the effects of infection on microRNA profiles are currently under investigation, evidence from animal studies supports the use of milk microRNA as a tool of assessing the health status of the lactating breast as well as the response to treatment, similarly to what has been previously shown for breastmilk immune cells (Hassiotou et al., 2013x,
Hassiotou and Geddes, 2015). The potential diagnostic value of milk microRNA has also been suggested by other animal studies. miR-148a-3p, which has been found to be the most highly expressed microRNA in exosomes of HM (Zhou et al., 2012), bovine (Chen et al., 2010) and porcine milk (Gu et al., 2012), has been proposed as a biomarker for raw milk quality control in the dairy industry, and also for artificial infant formulae (Chen et al., 2010). The use of microRNAs as biomarkers for milk quality control was first proposed by Chen et al. due to their high stability in milk, even under very harsh conditions including the sterilizing process during product manufacture and milk processing (Chen et al., 2010). However, Weber et al. (Weber et al., 2010) reported a lower concentration of miR-148a in skimmed HM than what was previously shown in bovine skimmed milk by Chen et al., in HM exosomes by Zhou et al., and in porcine milk exosomes by Gu et al. (Gu et al., 2012). In addition to miR-148-3p, controversies exist over miR-494, which has been identified to be present in high concentrations in both HM (Kosaka et al., 2010) and bovine milk by Izumi et al. (Izumi et al., 2012), but in very low concentrations in bovine milk by Chen et al. (Chen et al., 2010). It is not clear the degree to which inter- and intra-species variations and factors associated with them have contributed to these differences. Further, differing methodological approaches and lack of standardization of milk collection, storage, processing, milk fractionation (if any), and RNA extraction are also likely sources of potential variation in results. Due to the rapidly evolving techniques in this field, the need is arising for greater emphasis of studies to optimize and standardize the methodology employed in milk microRNA research. These procedures had already been optimized for microRNA extraction and analysis in blood and plasma (Sourvinou et al., 2013), and only recently this has been done for HM (Alsaweed et al., 2015ab).

The optimization of microRNA and total RNA extraction from HM was conducted in three main milk fractions (cell, lipids and skim milk) using different extraction methods and commercially available kits. The most efficient kits and methods were reported for each HM fraction (Alsaweed et al., 2015ab). In this study, microRNAs were found to be enriched in HM, with different milk fractions yielding different microRNA concentrations (Alsaweed et al., 2015bs). Therefore, it became clear that different fractions of HM require different processing for extraction, profiling and functional studies. Importantly, milk samples were fractionated and analysed fresh upon expression and not after storage, enabling extraction and analysis of microRNA specific to each milk fraction. Previous studies on skim milk or milk lipids have
typically analysed milk after freezing (Zhou et al., 2012, Kosaka et al., 2010, Gu et al., 2012), a process that is likely to result in cross-contamination between milk fractions due to membrane lysis (in milk cells and potentially fat globules) that is known to occur during freezing. These are important considerations for future investigations, with this optimization study now providing a standard protocol for HM microRNA analysis (Alsaweed et al., 2015ab).

Furthermore, the optimisation and standardization of the methodology for milk microRNA analyses (Alsaweed et al., 2015ab) opens new avenues for clinical exploitation of these molecules diagnostically, particularly given their non-invasive access via breastmilk. The suggested origin of many milk microRNA from the mammary gland (Alsaweed et al., 2015bs, Modepalli et al., 2014) makes them an attractive target as biomarkers of the health status and performance of the lactating breast as well as of breast aberrations such as cancer. Epigenetic modification has been suggested to be involved in the normal development of the mammary gland, although the specific mechanisms are still largely unexplored (Wang et al., 2012b). For example, miR-29s was found to regulate important lactation-related genes in mammary epithelial cells from the dairy cow, such as casein alpha S1 (CSN1S1), E74-like factor 5 (EIF5), and glucose transporter 1 (GLUT1) (Chen et al., 2010, Bian et al., 2015). Decreasing expression of miR-29s was associated with reduction of lactoprotein, triglycerides (TG) and lactose (Chen et al., 2010, Bian et al., 2015). These findings can form the basis for examination of potential avenues for enhancement and optimisation of milk quality in the dairy cow, as well as improvement of lactation performance in women with insufficient milk supply.

2.4 FUNCTIONS OF MILK microRNAs

2.4.1. Stability and uptake of food-derived microRNAs
Accumulating evidence confirms that microRNAs are present in all food sources. A number of studies have begun to investigate the fate of food-derived microRNAs, and whether they survive the GI tract and influence gene expression in mammals, including humans (Zhang et al., 2012a, Palmer et al., 2014, Baier et al., 2014, Wolf et al., 2015, Arntz et al., 2015). Food-specific microRNAs ingested orally have been found to be present in tissues and sera of different animals. Specifically, exogenous miR-168a, which is a rice-specific microRNA, was present in human sera in a Chinese cohort (Zhang et al., 2012a). miR-168a was found to bind
low-density lipoprotein receptor adapter protein 1 (LDLRAP1) in the human and mouse and to inhibit LDLRAP1 expression in the liver (Zhang et al., 2012a), demonstrating not only survival and uptake of this food-derived microRNA in humans, but also epigenetic regulation influencing tissue function. However, not all cross-microRNAs from food sources increase after consumption in mammals. For example, miR-167a and miR-824 are highly expressed in broccoli. Extensive consumption of broccoli sprouts by healthy humans did not change the expression pattern of either microRNA in the plasma (Baier et al., 2014). Moreover, Dickinson et al. investigated exogenous microRNA uptake in mice by feeding them rice-containing chow. This study failed to report gene-targeting functions of the plant-derived microRNA or change in expression levels in the liver or plasma of the animals (Dickinson et al., 2013). The above discrepancies between studies examining plant-derived microRNA transfer to mammals may reflect the lack and/or minimal contribution of exosomal transfer, since very limited evidence currently supports the production of exosome-like structures by plants that can be uptaken by mammalian cells (Mu et al., 2014). Indeed, the packaging of microRNA within “transporting vehicles” may play an important role for their transfer and function in the recipient.

In the case of HM microRNA, it has been suggested that their transfer to the infant’s bloodstream is further facilitated by the known packaging of milk microRNA in “vehicle” structures, such as somatic cells, exosomes and other microvesicles, 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 endosomal transfer via epithelial cells of the intestine (Wolf et al., 2015). Through these vehicles, milk-derived microRNA are thought to be uptaken by the infant and participate in the epigenetic regulation of various functions including immune protection and development (Figure 4) (Garcia-Segura et al., 2013, Jiang et al., 2012). In particular, recent studies have emphasized the importance of exosomal transfer of milk-derived microRNA. Extracellular vesicles including exosomes were shown to attach to different types of cells by endocytosis and to carry microRNA such as miR-21, which downregulated expression of TGFβRII and TPM1 in the recipient cells (Tian et al., 2010, Tian et al., 2013, Tian et al., 2014). Extracellular vesicles were further investigated in commercial bovine milk, and were found to carry immunoregulatory microRNAs. These milk-derived extracellular vesicles were resistant to harsh conditions such as low pH (Pieters et al., 2015). Moreover, uptake and functionality,
including therapeutic effects, of milk microRNAs has been recently demonstrated both in vitro and in vivo by Arntz et al. (Arntz et al., 2015). In this study, immune-related microRNAs (miR-30a, miR-223, miR-92a) were highly expressed in bovine milk-derived extracellular vesicles, which were uptaken in vitro by splenocytes and intestinal cells. When orally administered to BALB/c mice with experimental rheumatoid arthritis, these milk-derived extracellular vesicles were uptaken by RAW264.7 macrophages after 1–3 h as well as by the ileum tissue of the animals after 24 h. After 9 weeks of daily oral administration, arthritis was delayed, with reduction in cartilage depletion and joint inflammation (Arntz et al., 2015).

In addition to exosomal transfer, HM cells may significantly contribute to the transfer of milk-derived microRNA to the infant. It has been recently shown that stem cells and immune cells from milk are transferred to the bloodstream of suckling pups in mice, and from there to different tissues (Hassiotou et al., 2015, Hassiotou et al., 2014a). Given that the cells of milk are highly rich in microRNA (Alsaweed et al., 2015bs, Alsaweed et al., 2015bb), this is likely to be an important source of microRNA for neonates in addition to milk exosomes.

MicroRNAs contained in infant formulae may also, to a small extent, be transferred to the infant’s circulation. Baier et al. investigated bovine milk-derived miR-29b and miR-200c in human adults after consuming cow’s milk and found that both microRNAs were increased 2-fold in human PBMCs and could potentially alter gene expression (Baier et al., 2014). Both microRNAs were also highly expressed in the human plasma after few hours of consuming cow’s milk, and returned to the normal baseline expression level after 24 h of the initial milk consumption (Baier et al., 2014). 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, a process that was influenced by glycoproteins on the surface of host cells (Wolf et al., 2015). However, differences exist in the microRNA content between bovine milk and infant formula, with the latter 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 (Chen et al., 2010, Sun et al., 2013, Alsaweed et al., 2015bs)). 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.
One of the major requirements for confirmation of the functionality of food-derived microRNAs in mammals is to demonstrate their survival in the GI tract. Several studies have shown that microRNAs are extremely stable under various harsh conditions in vitro (Zhou et al., 2012, Kosaka et al., 2010, Ji and Chen, 2012). For breastmilk microRNA, the main considerations are resistance to RNase digestion and tolerance of low pH, and temperature and freeze/thaw cycles in the case of frozen HM (Zhou et al., 2012, Kosaka et al., 2010, Hata et al., 2010). Exosomal microRNA has been suggested to be protected (Zhou et al., 2012), but other microvesicles including fat globules are also considered to be involved in microRNA protection, such as apoptotic bodies (small vesicles derived from apoptotic cell death) (Kosaka et al., 2010), which have not been investigated yet. 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 to home in different organs (Zhang et al., 2012a, Modepalli et al., 2014).

With regard to free microRNA in milk (such as those present in skim milk), a few theories have been proposed. Ribonuclease (RNase), which has been found to exist in all body fluids (Chen et al., 2008), degrades RNA molecules into small fragments, and is thus a key enzyme in the RNA maturation process (Bai et al., 2013). Milk is known to have high RNase activities (Zhou et al., 2012, Kosaka et al., 2010). On the other hand, it is known that RNAs are unstable under harsh conditions (Mitchell et al., 2008, Gilad et al., 2008). Furthermore, HM and raw milk-derived microRNAs are found to be extremely stable even after RNase treatment in vitro (Kosaka et al., 2010, Gu et al., 2012, Izumi et al., 2012, Hata et al., 2010). The effects of low pH solution on microRNA integrity were examined using qPCR, showing that they are very stable (Kosaka et al., 2010, Gu et al., 2012). It is important to note that the GI tract of infants is less acidic than that of adults (Fallingborg, 1999), 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, Kosaka et al., 2010, Gu et al., 2012). As microRNA do not denature if subjected to different temperature cycles (at least those tested), microRNAs in stored HM fed to hospitalized infants are likely to be unaffected (Kosaka et al., 2010). The above observations strongly support the survival of the natural microRNA content of HM 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 HM.

A recent study utilized a mouse model of miR-375 and miR-200c knockout (KO) pups fed by wildtype (WT) foster mothers or KO mothers (Title et al., 2015). The study concluded that no evidence was found for intestinal uptake in KO or WT pups of miR-375 and miR-200c derived from foster mother milk (Title et al., 2015). However, a small increase in the plasma levels of both of these microRNAs was detected in KO pups after nursing, suggesting that some microRNA copies are actually transferred to the bloodstream. It is of note that the examined microRNAs were not highly expressed in the WT mother milk of the murine model used. Further, both miR-375 and miR-200c are known to be involved in the control of endocytosis and/or exocytosis and to modulate epithelial function, which may influence exosomal endocytosis and thus uptake of the examined microRNAs (Bar-Sagi et al., 1987, Salunkhe et al., 2015). Therefore, this KO mouse model and the chosen microRNAs may be inappropriate for investigating milk microRNA uptake by the nursed offspring. As it has been previously shown (Baier et al., 2014, Wolf et al., 2015), not all dietary microRNAs are ideal for exogenous microRNA uptake studies (Yang et al., 2015). Further studies are required to confirm the findings of Title et al. (Title et al., 2015) as well as investigate milk microRNA uptake in more appropriate models.

On the other hand, similar to human serum (Gilad et al., 2008), exogenous spiked-in synthesized (artificial) microRNAs in bovine milk were unstable and degraded compared to natural, endogenous microRNAs in bovine milk, which remained stable (Gu et al., 2012, Izumi et al., 2012). Interestingly, it is known that HM contains high quantities of very stable microRNAs, which are resistant to the pasteurization and milk bank storage procedures (Zhou et al., 2012, Kosaka et al., 2010). 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). In C. elegans, several microRNAs were stably expressed after UV-C treatment, such as miR-57-5p and miR-55-3p (Kagias et al., 2014). In a study of human primary keratinocytes exposed to UV-A and UV-B for 6 h, most microRNAs survived and no difference in expression was seen, except for few microRNAs such as miR-23b (upregulated) and miR-10a (downregulated) (Kraemer et al., 2013). Yet, other studies have reported upregulation of skin microRNA in response to UV irradiation (Syed et al., 2013), which may be involved in
cancer initiation in the skin (Grignol et al., 2011). In summary, although ultraviolet radiation may not affect the majority of microRNA in mammalian cells, current evidence suggests that some microRNA species may be affected, and therefore this requires further investigation. In general, as it is also emphasized by the food industry and the industry of processing raw milk products such as milk powder (Chen et al., 2010, Izumi et al., 2012), microRNAs in food and milk are very stable under and resistant to harsh conditions, and are therefore likely to be taken up by the HM fed infant, especially when protected within vehicle structures such as exosomes and live milk cells.

**Figure 4.** A potential scenario depicting the sources of exogenous microRNA for the infant (breastmilk and infant formulae) and uptake of them along with other macro/micronutrients (i.e., fatty acids and amino acids) in the infant’s gastrointestinal (GI) tract. Breastmilk microRNAs can be delivered to the infant either as free molecules in skim milk, or via uptake of breastmilk cells, exosomes and other milk microvesicles in the GI tract. There, absorption is thought to occur through intestinal epithelial cells, from which milk-derived microRNA may
reach various organs and tissues via the bloodstream to potentially perform functions, such as immunoprotection and developmental programming. It is of note that infant formulae are extremely poor in microRNA compared to HM, with potential differences also in the biological activity of these molecules in formula that merit further investigation.

2.4.2 microRNAs act as immune regulators

The tolerance of microRNAs of harsh conditions and the evidence that they migrate to the bloodstream and potentially different organs of the breastfed infant, suggest that they may play functional roles in the epigenetic regulation of development. Most of the microRNAs in HM are known for their immunocompetence (Izumi et al., 2012, Lindsay, 2008, Lu and Liston, 2009), and they are particularly abundant (Table 2) (Weber et al., 2010, Zhou et al., 2012, Munch et al., 2013, Kosaka et al., 2010). 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 (Lindsay, 2008, Lu and Liston, 2009). In addition, microRNA can play key roles in autoimmune conditions, such as inflammatory bowel disease (IBD), and regulate the development or prevention of these diseases (Pauley et al., 2009). Therefore, they could potentially be used as milk biomarkers to diagnose immune disorders such as allergic conditions (Lu et al., 2009, Oddy, 2009).

Kosaka et al. (Kosaka et al., 2010) reported high quantities of microRNAs in HM with functions associated with the immune system during the first 6 months of lactation, including, but not limited to miR-181a, miR-17, miR-155, miR-150, and miR-223. In particular, miR-181 and miR-155, which are known to regulate B cell differentiation (Kosaka et al., 2010, Chen et al., 2004, Vigorito et al., 2007), are present in high concentrations in HM (Kosaka et al., 2010, Munch et al., 2013), suggesting a function in the development of the infant’s immune system.

In addition, microRNA clusters miR-17 and miR-92 have been detected at high levels in HM, and given their function in regulating monocyte development as well as B and T cell differentiation and maturation (Ventura et al., 2008, Koralov et al., 2008), 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), is also found at high levels in HM (Kosaka et al., 2010). HM is rich in B cell-related microRNAs, such as miR-181 and miR-155, which potentially induce B cell differentiation (de Yebenes et al., 2008, Quinn et
al., 2014). On the other hand, miR-150, which is present in lower concentrations in HM, is known to act as a B cell suppressor (Zhou et al., 2007, Xiao et al., 2007). Interestingly, Zhou and colleagues identified a large number of microRNAs in HM exosomes (Zhou et al., 2012). 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 (Zhou et al., 2012). Specifically, miR-30b-5p is known to induce immunosuppression and reduce immune cell activation (Gaziel-Sovran et al., 2011). In contrast, miR-182-5p induces T cell-mediated immune responses (Stittrich et al., 2010). In the same study, 59 pre-microRNAs out of 87 (67.8%) that were detected in HM exosomes are considered to have immunological functions (Zhou et al., 2012), which is consistent with a previous study in human skim milk microRNA (Kosaka et al., 2010). The miR-17-92 cluster was also highly expressed in HM exosomes, with a speculated function as a developmental regulator of the immune system (Xiao et al., 2008).

Some microRNAs present in milk may have more than one function. Interestingly, miR-17-92, which is known to have immunological functions, has also been implicated in oncogenesis by promoting cell proliferation and inhibiting apoptosis (Mendell, 2008), although its role in the breastfed infant is poorly understood. Given that genes known to act as oncogenes have recently also been implicated in normal lactation (Hassiotou et al., 2013a, Hassiotou et al., 2012, Hassiotou et al., 2013v), it can be hypothesised that miRN-17-92, as well as other microRNAs with similar functions may participate in the milk-secretory function of the lactating breast rather than act as oncogenes in the context of lactation and breastfeeding. Some of these microRNAs therefore may be indicators of lactation performance.

In addition to HM, microRNAs with immunological functions have been identified in the milk of other mammalian species (Gu et al., 2012, Izumi et al., 2012). Profiling of bovine milk showed a high similarity of microRNA content to HM in respect to immune-related microRNAs (Kosaka et al., 2010, Chen et al., 2010, Hata et al., 2010), although this does not directly translate to the human infant. miR-181a and miR-155, which play important roles in immune system regulation and inflammation (Munch et al., 2013, Sonkoly et al., 2008), were profiled in both bovine colostrum and mature milk, and were detected in high quantities, more so in colostrum (Chen et al., 2010, Hata et al., 2010). More specifically, bovine immune-related microRNAs are present at higher concentrations in colostrum compared to mature milk (Chen et al., 2010), although this is yet to be investigated in HM. This appears to be one of the factors
involved in providing greater immunological support required early in life, and is consistent with the higher numbers of immune cells and concentrations of various humoral immunological factors such as lactoferrin and secretory IgA in colostrum compared to mature HM (Hassiotou et al., 2013a, Hassiotou et al., 2013x, Keller et al., 1988, Goldman et al., 1982). Bovine milk 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 (Izumi et al., 2012). Additionally, the expression levels of a selected bovine microRNA group, including miR-223, miR-106b, miR-15b, miR-155, and miR-34a, have been analyzed using qPCR and compared between colostrum and mature milk, where they were found to be present at significantly different levels (Izumi et al., 2012). In contrast to bovine milk (Chen et al., 2010, Izumi et al., 2012), rat skim milk (Izumi et al., 2014) and porcine milk exosomes (Gu et al., 2012), a study showed that the levels of microRNA concentration and expression in HM are lower in colostrum compared to mature milk, where 429 different microRNAs were identified in human mature milk vs. 386 different microRNAs in human colostrum (Weber et al., 2010). This warrants validation, together with investigation of differences in immune-related microRNAs between human colostrum and mature HM, since these molecules are likely to contribute in the immunoprotection of the neonate in the first days postpartum when it is most susceptible, as well as in the development of infant’s immune system and long-term protection against infections.

Kosaka et al. (Kosaka et al., 2010) and Gu et al. (Gu et al., 2012) first showed that human skim milk and porcine milk exosomes, respectively, contain microRNAs related to immune responses (Table 2). Gu et al. found that 58 out of 84 immune-related microRNAs listed in the Pathway Central Database (Qiagen, Valencia, CA, USA) were enriched in porcine milk exosomes (Gu et al., 2012), consistent with another recent study showing that HM exosomes were enriched with immune-related microRNA (Zhou et al., 2012). This study identified 12 out of 13 high abundance microRNAs in porcine milk exosomes to be expressed at higher levels in the first 3 days postpartum compared to later in month 1 postpartum (days 7, 14, 21 and 28) (Gu et al., 2012). These 12 microRNAs (let-7a-5p, miR-182-5p, miR-191-5p, miR-200c-3p, miR-21-5p, miR-23b-3p, miR27b-3p, miR-30a-5p, miR-30c-2-5p & -1-5p, miR-30d-5p, miR-375-3p, and miR-574-3p) (Gu et al., 2012) are all immune-related and they regulate immune response genes and proteins (Xiao and Rajewsky, 2009). More specifically, miR-30c-2-5p and

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miR-1-5p are immunosuppression regulators (Gaziel-Sovran et al., 2011), whereas let-7a-1-5p regulates inflammation-associated cytokine IL-6 (interleukin-6) that induces STAT3 (signal transducers and activators of transcription 3) signaling (Meng et al., 2007b). The innate immune receptors can be activated and regulated by porcine milk miR-21-5p, toll-like receptor 4 (TLR4), and a key cytokine receptor via targeting programmed cell death protein 4 (PDCD4) and interleukin 12 (IL-12), respectively (Sheedy et al., 2010). Also, IL-12 that is negatively controlled by miR-21-5p, is also responsible for regulating T cells and natural killer cells (Manetti et al., 1993). Further, the abundant porcine milk miR-27b was found to induce lipopolysaccharide (LPS), which inhibits and de-stabilizes the peroxisome proliferator-activated receptor c (PPARc), which is important in dampening inflammation via macrophage immune response (Jennewein et al., 2010).

Several bovine immune-related microRNAs (Lindsay, 2008, Lu and Liston, 2009) have been isolated from milk-derived microvesicles (Hata et al., 2010), and were also found to be expressed in the mammary gland using small RNA sequencing (Table 2) (Gu et al., 2007). These microRNAs include miR101 and miR150, which are known regulators of T cells (Yu et al., 2007, Cobb et al., 2006), and also miR-223 that has been reported to modulate innate immune cell (granulocytes and neutrophils) differentiation and activation (Lindsay, 2008, Johnnidis et al., 2008, Fazi et al., 2005). miR-155 and miR-223 have been detected in bovine milk and are both involved in many immune functions, and potentially have anti-inflammatory effects especially in bovine colostrum. In addition to this function, miR-155 regulates T and B cell differentiation, and is a known modulator of T helper cells (Th1/Th2 balance) (Thai et al., 2007). In contrast, miR-223 negatively regulates neutrophil proliferation and activation (Lindsay, 2008). miR-25-3p targets KLF4, which is a potent mediator of inflammation (Kuhn et al., 2010), and has a crucial role in the development of the immune system (Gu et al., 2012). miR-30a-5p targets GalNActransferase 7 (GALNT7) to promote cellular invasion and immunosuppression (Gaziel-Sovran et al., 2011). miR-182-5p promotes T cell-mediated immune responses by inhibiting forkhead box protein O1 (FOXO1) (Stittrich et al., 2010), a gene that is also targeted by miR-21 in cancer (Lei et al., 2014, Song et al., 2015). miR-200c-3p has been identified to regulate T cell differentiation by targeting zinc finger E-box-binding homeobox 1 (ZEB1) (Gregory et al., 2008), and also to regulate CD4 differentiation (Brabletz et al., 1999). Moreover, as mentioned earlier, bovine milk microRNA expression patterns have
been recently found to be altered during severe inflammation of mammary gland such as mastitis (Lawless et al., 2014). Gene target analysis of the up- and down-regulated milk microRNAs such as miR-223 and miR-15b, respectively, revealed several roles of these microRNAs in response to mastitis (Naeem et al., 2012). Also, most milk microRNAs were downregulated during mastitis, suggesting that they actively control the mammary immune response to *S. uberis*, which causes mastitis in the dairy cow (Naeem et al., 2012).

Collectively, the current data highlight that breastmilk is a complex system of different microRNA molecules with synergistic and antagonistic relationships, controlling specific immune responses in the infant and the lactating breast (Zhou et al., 2012, Wang et al., 2010a, Kosaka et al., 2010, Munch et al., 2013). 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 both the infant and the lactating breast, further supporting the potential use of these molecules diagnostically.
<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Regulatory Function(s)</th>
<th>References</th>
<th>Presence in HM</th>
<th>References</th>
<th>Presence in animal milk</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-181a</td>
<td>Cell signaling. Development of B cells.</td>
<td>(Chen et al., 2010)</td>
<td>Skim milk. Milk lipids.</td>
<td>(Li et al., 2007, Chen et al., 2004)</td>
<td>Bovine skim milk (colostrum and mature milk). Rat milk whey. Porcine milk exosomes.</td>
<td>(Kosaka et al., 2010, Weber et al., 2010, Munch et al., 2013)</td>
</tr>
<tr>
<td>miR-181b</td>
<td>Switch recombination in activated B cells. Increased activity of NF-kB.</td>
<td>(Chen et al., 2010, Izumi et al., 2014, Chen et al., 2014)</td>
<td>Skim milk (colostrum and mature milk). Milk lipids.</td>
<td>(Chen et al., 2004, de Yebenes et al., 2008, Iliopoulos et al., 2010)</td>
<td>Bovine skim milk (colostrum and mature milk). Rat milk whey. Porcine milk exosomes.</td>
<td>(Kosaka et al., 2010, Weber et al., 2010, Munch et al., 2013)</td>
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<tr>
<td>miR-17</td>
<td>B and T cells. Monocyte development.</td>
<td>(Chen et al., 2010, Izumi et al., 2012)</td>
<td>Skim milk. Milk lipids. Milk exosomes.</td>
<td>(Fontana et al., 2007, Admyre et al., 2007)</td>
<td>Bovine skim milk (colostrum and mature milk). Rat milk whey. Porcine milk exosomes.</td>
<td>(Kosaka et al., 2010, Zhou et al., 2012, Munch et al., 2013)</td>
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<tr>
<td>miR-125b</td>
<td>Tumor necrosis factor-a production. Innate immune response TLR signaling.</td>
<td>(Chen et al., 2010, Izumi et al., 2012, Izumi et al., 2014, Chen et al., 2014)</td>
<td>Skim milk (colostrum and mature milk). Milk lipids.</td>
<td>(Tili et al., 2007)</td>
<td>Bovine skim milk (colostrum and mature milk). Rat milk whey. Porcine milk exosomes.</td>
<td>(Kosaka et al., 2010, Weber et al., 2010, Munch et al., 2013)</td>
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</table>

Table 2. Immune-related microRNAs expressed in different fractions of milk from different mammalian species that have been highlighted in previous studies.
<table>
<thead>
<tr>
<th>miR</th>
<th>Function</th>
<th>Source</th>
<th>Source</th>
<th>Source</th>
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</table>

Note: The table entries include functions and source references for microRNAs (miR) involved in immune responses and their associated sources.
<table>
<thead>
<tr>
<th>miR</th>
<th>Function</th>
<th>Source/Details</th>
</tr>
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<tbody>
<tr>
<td>miR-15a</td>
<td>Downregulated in chronic lymphocytic leukemia.</td>
<td>(Chen et al., 2010, Izumi et al., 2012, Izumi et al., 2014, Chen et al., 2014)</td>
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<td></td>
<td></td>
<td>Skim milk (colostrum and mature milk). Milk lipid. Milk exosomes.</td>
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<td></td>
<td></td>
<td>(Fulci et al., 2007, Calin et al., 2002)</td>
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<td>Bovine skim milk (colostrum and mature milk). Porcine milk exosomes.</td>
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<td></td>
<td></td>
<td>(Zhou et al., 2012, Munch et al., 2013, Weber et al., 2010)</td>
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<tr>
<td>miR-16</td>
<td>Induces TNFa mRNA degradation. Upregulated in rheumatoid arthritis.</td>
<td>(Chen et al., 2010, Izumi et al., 2012, Chen et al., 2014)</td>
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<td></td>
<td></td>
<td>Skim milk. Milk lipids.</td>
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<td></td>
<td></td>
<td>(Jing et al., 2005, Pauley et al., 2008)</td>
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<td>Bovine skim milk (colostrum and mature milk). Porcine milk exosomes.</td>
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<td></td>
<td></td>
<td>(Munch et al., 2013, Weber et al., 2010)</td>
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<tr>
<td>miR-21</td>
<td>Up-regulated in B-cell lymphoma and chronic lymphocytic leukemia.</td>
<td>(Chen et al., 2010, Izumi et al., 2014, Chen et al., 2014)</td>
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<td></td>
<td></td>
<td>(Lawrie et al., 2007)</td>
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<td>Bovine skim milk (colostrum and mature milk). Porcine milk exosomes.</td>
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<td>(Weber et al., 2010, Munch et al., 2013, Zhou et al., 2012)</td>
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<tr>
<td>miR-20a</td>
<td>Inhibits monocyte proliferation, differentiation and maturation.</td>
<td>(Chen et al., 2010, Izumi et al., 2014, Gu et al., 2012, Chen et al., 2014)</td>
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<td></td>
<td></td>
<td>(Fontana et al., 2007)</td>
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<td>Bovine skim milk (colostrum and mature milk). Porcine milk exosomes.</td>
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<td>(Weber et al., 2010, Munch et al., 2013, Zhou et al., 2012)</td>
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<tr>
<td>miR-106a</td>
<td>Inhibits monocyte proliferation, differentiation and maturation.</td>
<td>(Chen et al., 2010, Izumi et al., 2012, Izumi et al., 2014)</td>
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<td></td>
<td></td>
<td>Milk lipids. Milk exosomes.</td>
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<tr>
<td></td>
<td></td>
<td>(Fontana et al., 2007)</td>
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<td>Bovine skim milk (colostrum and mature milk). Porcine milk exosomes.</td>
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<td>(Munch et al., 2013, Zhou et al., 2012)</td>
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2.4.3 microRNAs are key regulators of milk lipid metabolism

MicroRNAs have been isolated from HM lipid vesicles including fat globules in large numbers (Munch et al., 2013), as well as from milk cells and skim milk (Munch et al., 2013, Alsaweed et al., 2015bs, Vickers and Remaley, 2012). This has formed the basis for the potential use of both extracellular and intra-vesicle milk microRNAs as biomarkers in molecular diagnostics for a range of diseases (Rayner and Hennessy, 2013). Although lipid metabolism is usually regulated extracellularly, microRNAs have recently been identified to regulate genes associated with lipid metabolism at the post-transcriptional level (Vickers and Remaley, 2012). These genes control functions related to cholesterol homeostasis, fatty acid oxidation, and lipogenesis, offering new opportunities for the treatment of various diseases such as dyslipidemias (Fernandez-Hernando et al., 2011).

The known lipid regulatory microRNAs are few and include amongst others miR-335, miR-33, miR-122, miR-370, miR-378-3p, and miR-125a-5p (Fernandez-Hernando et al., 2011). Interestingly, these microRNAs have been identified in abundance in the HM lipid fraction (Munch et al., 2013), human skim milk (Weber et al., 2010), HM cells (Alsaweed et al., 2015bb), human colostrum (Weber et al., 2010), HM exosomes (Zhou et al., 2012), as well as in bovine skim milk and colostrum (Chen et al., 2010), suggesting that they play critical roles in the lipid metabolism and/or synthesis in the lactating breast. For example, miR-33 has been shown to regulate cholesterol homeostasis at the cellular level (Rayner et al., 2010, Najafi-Shoushtari et al., 2010). One of the most significant predicted gene targets of miR-33 is ABCA1, which produces cholesterol efflux regulatory protein (CERP). CERP is responsible for regulating cellular cholesterol and phosphate homeostasis, and also transporting cholesterol outside of the cell (Schmitz and Langmann, 2001). miR-33 also targets ABCG1, which reduces the efflux of cholesterol to high-density lipoprotein (HDL) and serum in macrophages (Rayner and Hennessy, 2013, Yvan-Charvet et al., 2010).

miR-125a-5p is another microRNA found abundantly in human and other species milk, regulating oxysterol binding protein-related Protein 9 (ORP9) (Chen et al., 2009), which is involved in various processes of lipid metabolism (Olkkonen and Levine, 2004, Raychaudhuri et al., 2006) including induction of lipid uptake by macrophages (Chen et al., 2009). Furthermore, HM miR-103 (Munch et al., 2013, Weber et al., 2010) is known to regulate milk fat synthesis, promoting fat globule synthesis and accumulation of triglyceride and unsaturated
fatty acids (Lin et al., 2013). Overexpression of miR-103 has been identified as a crucial regulator of milk fat synthesis and composition as well as milk nutrient levels (Lin et al., 2013). Interestingly, downregulation of miR-103 was not shown to affect fat accumulation in caprine milk lactocytes, suggesting that there may be alternative and/or compensatory mechanisms controlling mammary fat metabolism (Lin et al., 2013). Further, miR-193b and miR-365, also present in milk, were shown to control lipid synthesis, upregulating brown fat differentiation via enhancing expression of Runt-related transcription factor 1 translocated to 1 (Runx1t1) (Sun et al., 2011).

Interestingly, in addition to fat globule-related microRNA, many microRNA have been found to be packaged into other lipid-based carriers, such as exosomes, microvesicles and apoptotic bodies, which are secreted by various cell types, such as immune cells (Thery et al., 2002), and many of which are found in HM (Zhou et al., 2012). These are known as lipid particle carriers (Subra et al., 2007, Huber et al., 2002), packaging not only microRNA, but also lipoproteins (Vickers and Remaley, 2012), and have the important function of delivering extracellular microRNA to recipient cells. For example, miR-150 is transported via microvesicles from macrophage-like cells to human microvascular endothelial cells, where it is thought to target c-Myb and regulate cell migration (Kopecki et al., 2007, Zhang et al., 2010, Xiao et al., 2007). Moreover, adipocyte-derived microRNA such as miR-27a, miR-146b and miR-16, are transported to other cell recipients via microvesicles (Kopecki et al., 2007). Therefore, it can be postulated that microRNAs contained in milk microvesicles/exosomes as well as fat globules are transferred to recipient cells in the GI tract of infants, and a proportion may transfer to the blood circulation from where they are transported to the infant’s tissues, playing regulatory functions.

### 2.4.4 Various potential benefits of human milk microRNAs
The function of extracellular microRNA is still poorly understood (Kosaka et al., 2010). Current evidence supports the notion that extracellular microRNAs play crucial roles in cell-cell communication (Wang et al., 2010a, Valadi et al., 2007, Liang et al., 2007). microRNAs have been shown to be exported by cells in culture (Wang et al., 2010a). Moreover, proteins and mRNA can be taken up by neurons through exosomes from adjacent cells, suggesting that the same is possible for microRNA (Wang et al., 2010a). The existence of microRNA in
exosomes and their potential function as extracellular regulators have opened up a new field of possibilities for use of microRNAs as biomarkers in health and disease (Chen et al., 2008, Mitchell et al., 2008) as well as in therapeutic modeling (Wang and Wu, 2009, Bader et al., 2010).

HM microRNA are potentially involved in many physiopathological functions, including regulating cell growth and differentiation (Kosaka et al., 2010) as well as influencing development in the infant (Chen et al., 2010). For example, one of the most highly expressed microRNA in HM, miR-148a-3p (Munch et al., 2013, Zhou et al., 2012), which is also found in other species’ milk (Chen et al., 2010, Gu et al., 2012), targets DNA methyltransferase 3b (DNMT3B) and suppresses its expression, potentially to facilitate DNA methylation during development (Duursma et al., 2008). At the same time, given that the majority of cells in mature HM under healthy conditions are lactocytes (Hassiotou et al., 2013a), HM microRNA are reflective of the microRNA composition and function of the lactating mammary epithelium, and this can form the basis for further explorations of their use as non-invasive, easily accessible biomarker of the functionality of the lactating breast.

Moreover, some tissue-related microRNAs have been found in HM (Kosaka et al., 2010), but less abundantly than in tissue and organs (Zhou et al., 2012). For example, miR-142-5p and miR-142-3p (hematopoietic system), miR-122 (liver), and miR-216 and miR-217 (pancreas) were highly expressed in these organs and less abundantly in HM (Kosaka et al., 2010, Gao et al., 2011), suggesting that these HM microRNAs may originate from the maternal bloodstream to specifically target the development, growth and function of the corresponding organs in the HM fed infant. At the same time, they may have specialized functions in the breast during lactation. Similarly in bovine milk, microRNA have been identified as tissue-specific microRNA present in low quantities in milk and with lower expression in both bovine colostrum and mature milk (Chen et al., 2010). These include for example muscle miR-1 and miR-133 (Liang et al., 2007), brain miR-9 and miR-124a (Sempere et al., 2004), pancreatic miR-216 and miR-217 (Szafranska et al., 2007), liver miR-122 (Liang et al., 2007, Landgraf et al., 2007), blood cell miR-451 (Masaki et al., 2007), and endothelial cell miR-126 (Wang et al., 2008).

microRNAs isolated from HM fat globules have been shown to be regulated by a maternal high-fat diet, and this may modify metabolic pathways in HM fed infants (Munch et
This is in agreement with the putative roles of circulating microRNAs, which when altered in either composition or concentration can be associated with cardiovascular morbidity and mortality (Zhu and Fan, 2011, Wang et al., 2009). Munch et al. found that gene targets of 308 microRNAs in HM lipids have a wide range of functions, particularly in the regulation of gene expression and metabolism, and immune responses (Munch et al., 2013), suggesting the potential importance of these microRNAs for HM fed infants (Munch et al., 2013, Neville et al., 2012). Further to these functions, some HM microRNA are thought to participate in the regulation of the central nervous system (CNS). For example, Munch et al (Munch et al., 2013) showed that HM miR-118.2 targets Teneurin Transmembrane Protein 2 (TENM2), the encoding protein of which is found at high levels in the CNS (Baumgartner et al., 1994), suggesting a regulatory function in the infant’s neural development and promotion of connection formation within the nervous system (Oohashi et al., 1999, Zheng et al., 2011). Adipogenesis may also be targeted in the infant via milk-derived microRNAs. Overexpression of miR-155 (Izumi et al., 2012) has been speculated to decrease brown adipose tissue mass by targeting the adipogenic transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) (Chen et al., 2013). Milk-derived miR-29a inactivates the INSIG-1 gene (Chen et al., 2010, Munch et al., 2013, Weber et al., 2010), which is likely to regulate adipogenesis (He et al., 2007), and this was positively associated with body mass index (BMI) (Dehwah et al., 2012). In addition to direct effects on fat deposition, milk microRNA may be involved in the short- and/or long-term appetite control conferred to the infant via breastfeeding, together with the numerous appetite regulatory hormones of breastmilk, such as leptin, adiponectin, ghrelin, insulin and others (Hassiotou and Geddes, 2014).

In addition to their involvement in normal metabolism and tissue function, many microRNAs have been shown to target genes related to cancer (van Kouwenhove et al., 2011, Calin and Croce, 2006), 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) and some of them are present in milk, and more specifically in HM. Although epidemiological evidence has previously associated bovine milk consumption with increased risk of certain cancers in adults (Gaard et al., 1995, Duarte-Salles et al., 2014), and this could be related to the content of bovine milk in oncogenic microRNA (Melnik, 2015b), the microRNAs in HM appear to have normal lactation-specific functions for the lactating...
mammary gland and the infant (Alsaweed et al., 2015). Interestingly, HM microRNA have been proposed to protect the infant against cancer through to adulthood (Munch et al., 2013). For example, miR-21, which is present in both HM and bovine milk (Chen et al., 2010, Zhou et al., 2012), is also known to be overexpressed in human hepatocellular cancer (HCC). Therefore, any deregulation of miR-21 can be associated with HCC growth by modulating mTORC1 signaling, i.e., PTEN expression (Meng et al., 2007a). miR-21 is an abundant microRNA in bovine milk (Chen et al., 2010) and has been isolated from both colostrum and mature HM (Weber et al., 2010). It is also abundant in human plasma (Olivieri et al., 2012), and in infants it is thought to be involved in promoting postnatal growth (Melnik et al., 2013). In addition, miR-21 has other normal tissue functions, including regulation of adipogenic differentiation in mesenchymal stem cells (MSCs) of human adipose tissue (Kim et al., 2009b). Further, HM microRNAs may directly regulate tumor suppressor genes (Hammond, 2007), such as the which is involved in decreasing lung tumor growth by directly targeting the RAS oncogene (Johnson et al., 2005). The specific normal functions of HM microRNA for the infant and in the lactating breast warrant further investigation.

2.5 Infant formula is poor in microRNAs compared to human milk

HM is much more than nutrition for the infant, containing fat, carbohydrates, proteins, vitamins and minerals, but also immunoprotective and regulatory biomolecules as well as viable cells that provide essential signals for the infant’s optimal growth, development and protection (Hassiotou and Geddes, 2015, Hassiotou and Geddes, 2014, Miller et al., 2013, Melnik, 2015a). However, a rapid worldwide population growth over the last 100 years, and the high demand to provide artificial milk for infants has led scientists and industrial companies to successfully produce infant formula from bovine milk as an alternative or complementary food for infants initially without access to HM (Greer et al., 2008). And although many have expressed the view that infant formula should only be made available to the infant if mother’s own milk is not sufficient, infant formula has recently become controversially more popular within some communities for non-medical reasons (Fein and Falci, 1999).

HM is a complex biofluid containing maternal somatic cells, beneficial microbiota, and molecules including microRNAs with functional roles (Weber et al., 2010, Hassiotou et al., 2012, Cregan et al., 2007, Bode et al., 2014). Most infant formulae are bovine milk-based
(Munch et al., 2013), and similar to HM, bovine milk microRNAs are most likely to be highly conserved in both fat globules (lipid fraction) and cells. Due to their stability, microRNAs may largely survive the industrial milk preparation procedures, however the milk cell and lipid fractions are usually discarded from formula (Food and Drug Administration, 2014, Gigli and Maizon, 2013), so the microRNA presence in formulae is significantly reduced. This has been confirmed by studies showing that the expression level of microRNAs in formulae is much lower than that of raw bovine milk (Table 3) (Izumi et al., 2012, Chen et al., 2010, Melnik et al., 2014). Chen et al. selected and surveyed seven microRNAs as quality control markers for raw milk and different brands of infant formula (Chen et al., 2010). The expression level of these seven microRNAs was significantly lower in formulae compared to raw milk (Chen et al., 2010). Izumi et al. found that the total RNA concentration of three types of infant formulae (standard formula, follow-on formula, and extensively hydrolyzed formula) was significantly lower than in raw bovine milk (Izumi et al., 2012). Also, two highly expressed microRNAs (miR-148a and miR-200c) in bovine milk were differentially expressed among the three different types of infant formula (Izumi et al., 2012). In a recent study, we have compared the human microRNA content and expression levels of two infant formulae from the Australian market, a bovine-milk based and a soy-based formula (Alsaweed et al., 2015bs). Out of 754 human mature microRNAs tested using Taqman Openarray (Applied Biosystems), only 45 microRNAs were identified in the bovine milk formula, and only 22 microRNAs in the soy formula (Table 3) (Alsaweed et al., 2015bs). Moreover, the biological activity of the remaining few formula microRNAs may be altered by the formula processing procedures, something that requires further investigation. Although the functional effects of non-human milk microRNAs on infants have not been investigated, it is possible that some microRNAs that are shared between bovine and human milk may play similar beneficial functions for the offspring across mammals, emphasizing the potential detrimental effects for infants of the low microRNA content of artificial formulae. This, together with the near absence in formulae of other immunoprotective factors of HM are likely to at least partially explain the reduction in protection from disease in infants fed artificial formulae (Melnik et al., 2014). Donor milk is the preferred alternative to formula, and even if pasteurized, donor milk is likely to retain many HM microRNA, potentially conferring more benefits to the infant than formula.
2.6 CONCLUSIONS AND OUTLOOK

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 (Xiao et al., 2011). Deregulation of microRNAs has also been shown to be associated with disease, for which they are useful diagnostic biomarkers (Blenkiron and Miska, 2007, van Kouwenhove et al., 2011). The recent discovery and identification of microRNA in HM requires further study to elucidate the biology of microRNA and their normal functions in the development and protection of the human breast and the infant. Although the use of circulating microRNA as biomarkers is still in its infancy (Rayner and Hennessy, 2013), microRNA have been proposed as biomarkers for various abnormalities (Chen et al., 2008), including breast cancer (Heneghan et al., 2009, Heneghan et al., 2010) and more recently, milk microRNA as biomarkers for lactation performance (Chen et al., 2010) and mastitis (Lawless et al., 2014). This is of particular interest since milk can be accessed easily and non-invasively and is plentiful. Importantly, microRNAs are extremely stable, and are transferred to humans via food, and also to infants via HM. 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. The investigation of the roles of HM microRNA for the infant and the mother will not only reveal novel attributes of breastfeeding, but it may also open new diagnostic and therapeutic avenues.
Table 3. Comparison of selected microRNAs and their abundance in infant formulae, bovine milk, and human milk (HM).

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Mature sequence (miRBase 20.0)</th>
<th>Existence</th>
<th>Expression Level</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>bta-miR-26a</td>
<td>uccaagauaauccaggaaggccu</td>
<td>Bovine milk</td>
<td>Low in formulae compared to raw bovine milk. Highly expressed in HM lipid fraction.</td>
<td>(Izumi et al., 2012, Chen et al., 2014, Chen et al., 2010)</td>
</tr>
<tr>
<td>hsa-miR-26a-5p</td>
<td>uccaagauaauccaggaaggccu</td>
<td>Infant formula HM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bta-miR-26b</td>
<td>uccaagauaauccaggaaggcug</td>
<td>Bovine milk</td>
<td>Low in formulae compared to raw bovine milk. Highly expressed in HM lipids and exosomes.</td>
<td>(Chen et al., 2010, Munch et al., 2013)</td>
</tr>
<tr>
<td>hsa-miR-26b-5p</td>
<td>uccaagauaauccaggaaggcugg</td>
<td>Infant formula HM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bta-miR-200c</td>
<td>uaaauacugcccggguauagugga</td>
<td>Bovine milk</td>
<td>Low in formulae compared to raw bovine milk. Highly expressed in HM lipid fraction.</td>
<td>(Chen et al., 2010, Munch et al., 2013, Zhou et al., 2012)</td>
</tr>
<tr>
<td>hsa-miR-200c-3p</td>
<td>uaaauacugcccggguauagugga</td>
<td>Infant formula HM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bta-miR-21-5p</td>
<td>uagcuauacgagauaguugacu</td>
<td>Bovine milk</td>
<td>Low in formulae compared to raw bovine milk. Highly expressed in HM lipids, skim milk and exosomes.</td>
<td>(Chen et al., 2010, Munch et al., 2013, Izumi et al., 2012)</td>
</tr>
<tr>
<td>hsa-miR-21-5p</td>
<td>uagcuauacgagauaguugagina</td>
<td>Infant formula HM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bta-miR-30d</td>
<td>uguauacacucccgcaggaacggcug</td>
<td>Bovine milk</td>
<td>Low in formulae compared to raw bovine milk. Highly expressed in HM lipids and skim milk.</td>
<td>(Chen et al., 2010, Munch et al., 2013, Weber et al., 2010, Kosaka et al., 2010, Zhou et al., 2012)</td>
</tr>
<tr>
<td>hsa-miR-30d-5p</td>
<td>uguauacacucccgcaggaacggcugag</td>
<td>Infant formula HM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bta-miR-99a-5p</td>
<td>aacccguagacagauuccugug</td>
<td>Bovine milk</td>
<td>Low in formulae compared to raw bovine milk. Highly expressed in HM lipids and skim milk.</td>
<td>(Chen et al., 2010, Munch et al., 2013, Weber et al., 2010)</td>
</tr>
<tr>
<td>hsa-miR-99a-5p</td>
<td>aacccguagacagauuccugug</td>
<td>Infant formula HM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bta-miR-148</td>
<td>uccagucucuacagacucuuccuug</td>
<td>Bovine milk</td>
<td>Low in formulae compared to raw bovine milk. Highly expressed in HM lipid, skim milk and exosomes.</td>
<td>(Chen et al., 2010, Munch et al., 2013, Weber et al., 2010)</td>
</tr>
<tr>
<td>hsa-miR-148a-3p</td>
<td>uccagucucuacagacucuuccuugg</td>
<td>Infant formula HM</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

hsa refers to human, bta refers to bos taurus (bovine) species.
HUMAN MILK microRNA AND TOTAL RNA DIFFER DEPENDING ON MILK FRACTIONATION

3.1 ABSTRACT
MicroRNA have been recently discovered in human milk signifying potentially important functions for both the lactating breast and the infant. Whilst human milk microRNA have started to be explored, little data exist on the evaluation of sample processing, and analysis to ensure that a full spectrum of microRNA can be obtained. Human milk comprises three main fractions: cells, skim milk, and lipids. Typically, the skim milk fraction has been measured in isolation despite evidence that the lipid fraction may contain more microRNA. This study aimed to standardize isolation of microRNA and total RNA from all three fractions of human milk to determine the most appropriate sampling and analysis procedure for future studies. Three different methods from eight commercially available kits were tested for their efficacy in extracting total RNA and microRNA from the lipid, skim, and cell fractions of human milk. Each fraction yielded different concentrations of RNA and microRNA, with the highest quantities found in the cell and lipid fractions, and the lowest in skim milk. The column-based phenol-free method was the most efficient extraction method for all three milk fractions. Two microRNAs were expressed and validated in the three milk fractions by qPCR using the three recommended extraction kits for each fraction. High expression levels were identified in the skim and lipid milk factions for these microRNAs. These results suggest that careful consideration of both the human milk sample preparation and extraction protocols should be made prior to embarking upon research in this area.

3.2 INTRODUCTION
Human milk (breastmilk) is a complex secretion of the mammary gland that is the main source of nutrition, immune protection, and developmental programming for the infant (Hassiotou and Geddes, 2013). In addition to being a balanced food for infants containing water, minerals, vitamins, proteins, carbohydrates, and lipids, human milk is a potent source of immunomodulatory factors (Hanson et al., 1997, Kramer, 2010). These include bioactive molecules, such as immunoglobulins and lactoferrin (Lonnerdal, 2003, Hassiotou et al., 2013a), and immune cells amongst stem cells, progenitor cells, and epithelial cells that constitute a cellular hierarchy in human milk (Hassiotou et al., 2012). Further to these components, milk is a rich source of RNAs, and microRNAs (Lemay et al., 2013). The latter have been recently discovered in human milk (Kosaka et al., 2010, Weber et al., 2010, Zhou et al., 2012, Munch et
and in the milk of other mammalian species (Chen et al., 2010, Gu et al., 2012, Bai et al., 2013, Izumi et al., 2014), suggesting that they may play crucial roles both in the lactating mammary gland and for the breastfed infant (Munch et al., 2013, Zhou et al., 2012).

MicroRNAs are small non-coding RNA molecules found in plants and animals (Ambros, 2004). First discovered in 1993 in Caenorhabditis elegans (Lee et al., 1993, Wightman et al., 1993), they are considered to be crucial regulators of gene expression at the post-transcriptional level by attaching to messenger RNA (mRNA) to either inhibit protein translation and/or induce mRNA degradation (He and Hannon, 2004, Pritchard et al., 2012). They are thus involved in a number of developmental and physiological processes, including cellular differentiation, apoptosis, proliferation, immune response, and maintenance of cell and tissue identity (Bartel, 2004). Deregulation of microRNAs is associated with aberrant cell functions leading to cancers (Calin and Croce, 2006, Esquela-Kerscher and Slack, 2006) and other diseases (Lu et al., 2008). MicroRNA exhibit diversified expression patterns, with some of them being specific to certain organs, such as miR-122, which is primarily found in the liver (Lagos-Quintana et al., 2002), or miR-1, which predominantly exists in the mammalian heart (Lee and Ambros, 2001). The number of new microRNA molecules discovered is increasing, with over 2,000 having been identified in humans thus far (http://www.mirbase.org). In addition to cells and tissues, microRNA are present in body fluids, such as plasma (Sourvinou et al., 2013) urine, saliva, and tears (Cortez et al., 2011) as well as abundantly in milk (Weber et al., 2010, Munch et al., 2013). The few studies that have examined microRNA in human milk have largely focused on its skimmed fraction. In 2010, Kosaka et al. reported 281 novel microRNA in skim milk (Kosaka et al., 2010), while Weber et al. found 429 microRNA in mature skim milk and 368 in skimmed colostrum (Weber et al., 2010). In addition to skim milk microRNAs, human milk contains microRNA packaged in vesicles. These have been examined more recently, with 452 pre-microRNA detected in human milk exosomes (Zhou et al., 2012). Exosomes are small membrane vesicles secreted from mammalian cells that protect molecules and proteins, which are then transported into the extracellular environment participating in cell-cell communication (Admyre et al., 2007). Human milk exosomes are rich in microRNA and immune-associated proteins, particularly these that have been isolated from skim milk (Admyre et al., 2007). So far, 59 immune-related microRNA have been described within human milk exosomes (Zhou et al., 2012). Further to those, other particles in human milk also contain
microRNA. Recently, Munch et al. showed that the milk fat globule encompasses novel microRNAs (Munch et al., 2013). Similar to adults (Baier et al., 2014), most likely human milk carries these microRNAs to the infant. Although some hypotheses were raised that oral microRNAs do not survive in the human gastrointestinal tract (Dickinson et al., 2013, Witwer and Hirschi, 2014), milk microRNAs are protected within fat globules, exosomes, or cells, and therefore are likely to be transferred intact across to the infant's blood. Moreover, human milk is known to contain maternal cells (Hassiotou et al., 2013a), which are transferred to the infant. These cells are rich in microRNA (Alsaweed et al., 2015dc). Exogenous microRNAs have been previously described to play functional roles in adults (Baier et al., 2014, Zhang et al., 2012a), and therefore it is likely that cellular microRNAs from human milk contribute regulatory functions in the infant.

The properties and regulation of microRNA in the different cell types present in human milk as well as in the milk fat globule, skim milk, and exosomes remain unknown. Early evidence suggests that certain human milk microRNA support the immune system of the infant, especially in the first six months of life, such as the highly abundant in milk miR-155, which regulates T and B cells and has a role in the innate immune response (Kosaka et al., 2010). Prior to investigating the properties and roles of human milk microRNA in either the mammary gland or the infant, it is critical to establish appropriate methodology that allows consistent isolation and quantification of these molecules, similar to that of plasma (Sourvinou et al., 2013), serum (Fariina et al., 2014), and blood cells (Hammerle-Fickinger et al., 2010, Monleau et al., 2014). Given that only the skim milk fraction of human milk has been widely investigated, it is not known what contribution the lipid or cell fractions make to the total microRNA population of human milk.

Considering that handling and isolation protocols play critical roles in the reliable quantification of microRNA in plasma (Sourvinou et al., 2013), it is logical to expect that special protocols, and handling procedures may apply to human milk. In addition, different methods have been utilized, contributing to the wide variation of total RNA, and microRNA published in human milk studies. In this study, we determined whether the microRNA content differed between the skim, lipid, and cell fractions of human milk and investigated the efficacy (yield and quality) of microRNA extraction for eight commercially available kits in each milk fraction. This provided valuable insight into the abundance and content of microRNA in
different human milk fractions, setting the basis for profiling, and functional microRNA studies in human milk.

3.3 MATERIALS AND METHODS

3.3.1 Human milk collection
The study was approved by the Human Research Ethics Committee of The University of Western Australia. All participants provided informed written consent. Fresh human milk samples (n=49) were collected from n=29 breastfeeding mothers on 1-4 occasions under sterile conditions. Symphony pumps (Medela AG, Switzerland) were used for Human milk expression and sample volumes ranged 14-135 mL. All participants and their infants were healthy at the time of milk collection, with current smoking, and medication use being exclusion criteria for participation. Lactation stages at sample collection ranged from 3 to 158 weeks.

![Diagram](image)

**Figure 1.** Brief workflow showing the steps to obtain the three main fractions (cells, lipid, and skim milk) of human milk using multiple centrifugation steps depending on the fraction, and quantity, quality and expression level of RNA/ microRNA.
3.3.2 Human milk fractionation

All human milk samples were fractionated immediately after expression by centrifugation at 720g for 20min. Three fractions were obtained from each sample (cells, lipids, and skim milk), and were transferred to 15-mL RNase free tubes (Fig. 1). Purification of the milk fractions involved an additional centrifugation step in the lipid and skim milk fractions, whilst cells were washed twice in sterile phosphate-buffered saline (PBS, Gibco).

3.3.3 Total RNA/microRNA extraction

All RNA/microRNA extractions were done on ice immediately after separation and purification of the three milk fractions (Fig. 1) using eight commercially available kits (Table 1). Each fraction of each milk sample was separated into 2-5 identical aliquots depending on the original sample volume and the size of the cell pellet. Each aliquot was used for extraction by a different kit such that the number of aliquots obtained per sample reflected the number of kits used for this sample. Table II shows the number of milk samples tested with (small RNA) phases and precipitation of the former was done with either chloroform or alcohol. After that, isopropanol or column separation was used for RNA precipitation. Then, different washing steps depending on the kit were required to obtain pure small RNA, which was eluted either in elution buffer or suspended in RNase free water.

3.3.4 MicroRNA analysis and quantification

Concentration and purity (260:280 ratio) of the extracted total RNA was measured using a spectrophotometer (NanoDrop™ 1000, Wilmington, DE). The microRNA concentration and microRNA/ small RNA ratios were quantified by capillary electrophoresis using the small RNA Chip kit (Agilent, CA) in an Agilent Bioanalyzer 2100 instrument. The amount of small RNA was normalised to 100, and the amount of microRNA was presented as a percentage of this value. Using this kit, we were able to quantify the small RNA in a sample including microRNA, which ranged in size between 6 and 150 nucleotides for small RNAs, and 10–40 nucleotides for microRNAs.
3.3.5 Reverse transcription and quantitative RT-PCR

Two whole human milk samples from two different mothers were fractionated as described above. RNA including microRNA was extracted using recommended kits for each fraction (Table 3). RT-PCR was used to validate the presence of two mature microRNAs using the TaqMan miRNA assay (Life Technologies, Foster City, CA). The expression level of hsa-miR-148a-3p and hsa-miR-30a-5p was done in two steps according to the manufacturer’s protocol. The reverse transcription was performed using 600ng of input RNA using the TaqMan miRNA reverse transcription kit and pooled both microRNA primers (5x primers) and endogenous control (RNU48). The RT reaction was processed using BioRad C-1000 thermo cycler (Hercules, CA) as follows: 16°C for 30 min; 42°C for 30 min; 85°C for 5 min, then the sample was held at 4°C. The PCR reaction was performed using Fast advanced master mix in triplicates and TaqMan microRNA probes for both examined microRNAs and the endogenous control (20x) using 7500 Fast Real Time-PCR system as follows: 50°C for 2 min; 95°C for 20 s followed by 40 cycles at 95°C for 3 s; and finally 60°C for 30 s. Comparative Ct (RQ) analysis was performed using 7500 software V2.0.6 by normalizing all samples to milk cell A sample.

3.3.6 Statistical analysis

Statistical analysis was done using R 2.9.0 for MacOSX (Team Development Core R, 2009) using the base packages, and the libraries nlme, multcomp, and lattice for linear mixed effects models, general linear hypothesis tests, and graphical presentation of data, respectively. Extraction kits were compared in terms of: (a) their efficiency in extracting total RNA, (b) the purity of the extracted RNA (260/280 ratio, whereby values of 1.80-2.19 were considered good; values of 1.51-1.79 were considered moderate; and values >2.20 or <1.50 were considered poor), (c) their efficiency in extracting microRNA, and (d) the ratio of microRNA to small RNA. This was done separately for each human milk fraction (cells, lipids, and skim milk). Comparison of the kits was done in two sets reflecting the kits tested in aliquots of the same milk samples (Table 1). In the first set (Set 1), three kits were compared: miRNeasy micro Kit (Qiagen, Hilden, Germany), mirVana microRNA Isolation Kit (Ambion, Austin, TX), and RNAzol-RT Reagent (Molecular Research Center, Inc.). In the second set (Set 2), five kits were compared: miRNeasy mini Kit (Qiagen, Hilden, Germany), TRIzol-LS Reagent (Invitrogen, CA), miRCURY RNA Isolation-Cell&Plant Kit, miRCURY RNA Isolation-Biofluids Kit.
(Exiqon, Vedbaek, Denmark) and mirPremier microRNA Isolation Kit (Sigma–Aldrich, St. Louis, MO).

To determine whether the four measures differed among the kits, linear regression and linear mixed effects models were used, with the measure of interest as the response and the kit (factor with either 3 or 5 levels) as the predictor. Two models were created for each combination of measure, kit set, and milk fraction. Ordinary least squares (OLS) regression, and a linear mixed effects (LME) model with random effects of different baseline levels per sample. Models were compared using analysis of variance. LME models are reported where found to be more appropriate, and regression otherwise. Overall P-values for differences among kits were tested using ANOVA, and where this was significantly different Tukey’s HSD was used to identify which kits differed. Significance values reported in tables are from the ANOVA, with significances among kits being from Tukey’s HSD. Differences were considered to be significant if P < 0.05.

Table 1. RNA/microRNA extraction kits, suppliers, and the extraction based method that used for evaluation and comparison of extraction efficacy in all the three breastmilk fractions in the two comparison sets.

<table>
<thead>
<tr>
<th>Kit</th>
<th>Company</th>
<th>Extraction method</th>
</tr>
</thead>
<tbody>
<tr>
<td>miRNeasy micro Kit</td>
<td>Qiagen</td>
<td>Filter column</td>
</tr>
<tr>
<td>mirVana microRNA Isolation Kit</td>
<td>Ambion</td>
<td>Filter column &amp; phenol/guanidine</td>
</tr>
<tr>
<td>RNAzol-RT Reagent</td>
<td>Molecular Res. Center</td>
<td>Phenol/guanidine</td>
</tr>
<tr>
<td>miRNeasy mini Kit</td>
<td>Qiagen</td>
<td>Filter column</td>
</tr>
<tr>
<td>TRIzol-LS Reagent</td>
<td>Invitrogen</td>
<td>Phenol/guanidine</td>
</tr>
<tr>
<td>miRCURY RNA Isolation-Cell/Plant Kit</td>
<td>Exiqon</td>
<td>Filter column</td>
</tr>
<tr>
<td>miRCURY RNA Isolation-Biofluids Kit</td>
<td>Exiqon</td>
<td>Filter column</td>
</tr>
<tr>
<td>mirPremier microRNA Isolation Kit</td>
<td>Sigma–Aldrich</td>
<td>Filter column</td>
</tr>
</tbody>
</table>

3.4 RESULTS

3.4.1 Purity of extracted RNA

Patterns of RNA purity (Table 4; Supplementary 1, Table S1) were found to be significantly different among the RNA extraction kits examined (P≤0.001), with the column-based/phenol-
free kits being the best performers and the phenol/guanidine-based kits being the worst performers. Moreover, each kit performed differently for different human milk fractions. Notably, the mirPremier microRNA Isolation Kit yielded good 260/280 ratios for the lipid and cellular fractions, but low ratios for the skim milk fraction. In general, all tested kits performed well in the cellular and lipid fractions, with more variation and lower RNA purity seen for the skim milk fraction.

In the milk lipid fraction and comparison Set 1, 260/280 ratios of mirVana and miRNeasy micro kits were significantly higher than those of RNAzol-RT (P<0.001 for both), which had a mean 260/280 ratio that was outside the acceptable range (1.65). Similarly, differences were seen among the TRIzol-LS, miRNeasy mini and miRCURY-Cell&Plant kits, where 260/280 ratios for TRIzol-LS were lower than in the other two kits (P=0.016 and P=0.019, respectively). However, the mean 260/280 ratio for TRIzol-LS was within the acceptable range (1.84), but lower than in the other two kits. Given the 260/280 cutoffs used, RNAzol-RT, and Trizol-LS were outside the acceptable range for lipids, indicating that these are not optimal choices for extracting total RNA or microRNA from milk lipids.

In skim milk and comparison Set 1, 260/280 ratio for mirVana was significantly higher than those of miRNeasy micro (P<0.001) and RNAzol-RT (P=0.041), both of which were outside the acceptable range. In comparison Set 2, although a wide range of average values was obtained among miRNeasy mini, TRIzol-LS, miRCURY-Cell&Plant, miRCURY-Biofluids, and mirPremier, no significant differences were found between the five kits tested (P=0.631). Notably, the mean 260/280 values of TRIzol-LS and mirPremier were outside the acceptable range (1.60 and 1.41, respectively).

In the cellular fraction and comparison Set 1, 260/280 ratios for miRNeasy micro and mirVana were significantly higher than those for RNAzol-RT (P<0.001 and P<0.003, respectively), where RNAzol-RT ratio averaged at 1.61, which is considered to be a low ratio. In comparison Set 2, only the 260/280 ratios for TRIzol-LS were significantly lower than all other kits (P<0.001), and they were also outside the acceptable 260/280 range (1.70).
Table 2. Number of the three-breastmilk fraction samples used for RNA/microRNA extraction in evaluating their performance using the eight kits.

<table>
<thead>
<tr>
<th>Kit/Fraction</th>
<th>Cells</th>
<th>Lipid</th>
<th>Skim</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>miRNeasy micro Kit</td>
<td>14</td>
<td>27</td>
<td>29</td>
<td>70</td>
</tr>
<tr>
<td>mirVana microRNA Isolation Kit</td>
<td>11</td>
<td>23</td>
<td>26</td>
<td>60</td>
</tr>
<tr>
<td>RNAzol-RT Reagent</td>
<td>11</td>
<td>22</td>
<td>25</td>
<td>58</td>
</tr>
<tr>
<td>miRNeasy mini Kit</td>
<td>14</td>
<td>22</td>
<td>22</td>
<td>58</td>
</tr>
<tr>
<td>TRIzol-LS Reagent</td>
<td>12</td>
<td>21</td>
<td>17</td>
<td>50</td>
</tr>
<tr>
<td>miRCURY RNA Isolation-Cell &amp; Plant</td>
<td>15</td>
<td>19</td>
<td>23</td>
<td>57</td>
</tr>
<tr>
<td>miRCURY RNA Isolation-Biofluids Kit</td>
<td>15</td>
<td>24</td>
<td>23</td>
<td>62</td>
</tr>
<tr>
<td>mirPremier microRNA Isolation Kit</td>
<td>14</td>
<td>24</td>
<td>19</td>
<td>57</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>106</strong></td>
<td><strong>182</strong></td>
<td><strong>184</strong></td>
<td><strong>472</strong></td>
</tr>
</tbody>
</table>

### 3.4.2 Total RNA concentration

Total RNA concentration differed both among kits and human milk fractions for the same kit (Table 4, Fig. 2). The lipid fraction contained significantly higher concentration of RNA compared to skim milk (P<0.05) (Fig. 3). In the lipid fraction, the mean ± S.D. of total RNA concentration ranged from 7.02 ± 3.99 ng/mL in the mirPremier kit to 49.90 ± 61.06 ng/mL in the miRCURY-Cell&Plant kit. In comparison Set 1, no significant difference was found between miRNeasy micro, mirVana, and RNAzol-RT (P=0.600). In comparison Set 2, mirPremier yielded significantly lower total RNA values than TRIzol-LS (P=0.05), miRNeasy micro (P=0.007) and miRCURY-Cell&Plant kits (P=0.005). No significant differences were found among the remaining four kits (P>0.05). TRIzol-LS, miRNeasy mini kit and miRCURY-Cell&Plant kit yielded high amount of total RNA from the human milk lipid fraction.

In the skim milk fraction, the mean ± S.D. of RNA concentration ranged 0.60 ± 0.46 ng/mL (mirPremier) to 9.26 ± 18.23 ng/mL (TRIzol-LS). In comparison Set 1, RNAzol-RT was clearly a better performer than miRNeasy micro and mirVana in terms of amount of extracted RNA (P<0.001). In comparison Set 2, TRIzol-LS was the best performer (mean=9.26 ng/mL), although this was only significantly different from mirPremier (P=0.064), which had the lowest performance amongst those tested (mean = 0.60 ng/mL). In human milk cells, the mean ± S.D. of total RNA amount extracted from equal cell aliquots ranged from 1,907 ± 3,321 ng in the mirPremier kit to 11,083 ± 15,106 ng in the miRNeasy mini kit. In comparison Set 1, total RNA
extracted with miRNeasy micro kit was significantly higher than that obtained with RNAzol-RT (P=0.017). The highest levels of extracted RNA were seen for the miRNeasy micro kit, followed by mirVana and RNAzol-RT. While the differences between mirVana and the other two kits were not significant, the results were closer to those of the miRNeasy micro kit than to RNAzol-RT. In comparison Set 2, miRNeasy mini yielded significantly higher total RNA amount than mirPremier (P=0.003), with the former kit yielding the highest average amount of RNA among all the other 7 kits (11,083).

3.4.3 MicroRNA concentration

MicroRNA and small RNA were identified on electropherograms in all three human milk fractions, with varying profiles both within and among milk fractions and for different microRNA extraction kits (Fig. 4). Similar to total RNA concentration, microRNA concentration was higher in the lipid fraction compared to skim milk (P<0.05) (Fig. 3). The tested kits performed differently in different human milk fractions (P=0.110 to P=0.720) (Table 4, Fig. 2). In the lipid fraction, the highest mean microRNA concentration was obtained by miRCURY-Biofluids (12.74 ± 24.44 ng/mL), followed by mirPremier (9.43 ± 8.06 ng/mL), while the lowest by mirVana (0.80 ± 1.70 ng/mL). In comparison Set 1, no overall difference was seen among the three kits (P=0.169). Tukey’s HSD comparison showed a borderline difference between miRNeasy micro kit and mirVana, with a tendency for higher levels in the former (P=0.092). In comparison Set 2, there was no overall difference among the kits (P=0.216). Further, multiple comparisons of means/Tukey’s HSD showed no significant differences, with the smallest P-value being 0.210. Thus, all the tested kits performed relatively well in extracting high levels of microRNA from the lipid fraction of human milk.

In skim milk, all kits yielded relatively low quantities of microRNA. Specifically, miRCURY-Cell&Plant kit yielded the highest mean concentration of extracted microRNAs (2.17 ± 5.66 ng/mL), whereas the lowest mean concentration was obtained with mirVana (0.10 ± 0.15 ng/mL) and miRNeasy micro kits (0.10 ± 0.9 ng/mL). In comparison Set 1, there was no evidence of significant difference among either the three kits overall (P=0.342) or using multiple comparisons of means (P≥0.386). In comparison Set 2, no evidence of difference was found among the five kits either overall (P=0.363) or using multiple comparisons of means (all P=0.449). Therefore, similar to the lipid fraction, in skim milk all eight tested kits performed
similarly in extracting microRNA, although in almost all cases the extracted quantities of microRNA were very low in this fraction compared to the other two human milk fractions.

In human milk cells, TRIzol-LS showed the largest mean amount of microRNA (1,443 ± 3,448 ng), while mirVana yielded the smallest mean amount of microRNA (65.67 ± 84.38 ng). In comparison Set 1, there was no evidence of difference among the miRNeasy micro kit, mirVana, and RNAzol-RT either overall (P=0.308) or for multiple comparisons of means (all P>0.241). Similarly, in comparison Set 2, no significant difference was found among the five kits in terms of levels of extracted microRNA either overall (P=0.722) or for multiple comparisons of means (P>0.614).

### 3.4.4 MicroRNA/small RNA ratio (%)

To further assess the efficiency of the examined kits to extract microRNA from human milk fractions, we compared the percentage ratio of microRNA to small RNA obtained with each kit (Table 4, Fig. 2). In the lipid fraction, the highest mean microRNA/small RNA ratio was seen with the miRNeasy micro kit (54.4%) and RNAzol-RT (50%). In comparison Set 1, there were significant differences among kits (P=0.041), while in comparison Set 2 there was no overall evidence of significant differences among the five kits compared (P=0.217). Thus, although all the examined kits performed similarly in terms of microRNA/small RNA ratios in the human milk lipid fraction, the highest mean was obtained by the miRNeasy micro kit.

In the skim milk fraction, the highest microRNA/small RNA ratio was obtained by mirPremier (57.8%), followed by RNAzol-RT (48.5%). In comparison Set 1, significant differences were seen among kits (P<0.001). The highest ratios were observed with RNAzol-RT, which were significantly higher than both miRNeasy micro and mirVana (P<0.001), which did not significantly differ from one another (P=0.732). Similarly, comparison Set 2 yielded significant differences among kits (P<0.001). mirPremier kit was higher compared to all other kits (57.8%), and yielded significantly higher microRNA/ small RNA ratios than either miRCURY-Cell&Plant or miRNeasy mini kit (P<0.001). Moreover, ratios obtained by miRCURY-Biofluids and TRIzol-LS were significantly higher than those obtained by miRCURY-Cell&Plant (P=0.049 and P=0.063, respectively), although the significance of the difference between TRIzol-LS and miRCURY-Cell&Plant was much weaker, suggesting that TRIzol-LS yields more variable microRNA/small RNA ratios than miRCURY-Biofluids.
Finally, no significant differences were seen between miRCURY-Biofluids and mirPremier (P=0.196). Overall, all examined kits performed similarly in terms of microRNA/small RNA ratios in the skim milk fraction of human milk, with the preference to three kits being mirPremier, RNAzol-RT, and TRIzol-LS.

In human milk cells, TRIzol-LS and miRNeasy mini kit showed the highest mean microRNA/small RNA ratio with 51.1% and 36.1%, respectively. In comparison Set 1, no significant difference was seen among the kits overall (P=0.152). In comparison Set 2, the kits differed significantly (P<0.001). TRIzol-LS yielded higher ratios than miRCURY-Biofluids, miRCURY-Cell&Plant, and mirPremier (P<0.001), while ratios for miRNeasy mini were significantly higher than those of miRCURY-Cell&Plant (P=0.034).
Table 3. Mean and standard deviation of quantity and quality of RNA and microRNA extracted from different fractions of breastmilk (cells, lipids, and skim milk) by the eight kits in two separate comparison sets. Total RNA and 260/280 ratio were obtained by Nanodrop, while total microRNA, and microRNA/small RNA ratio were measured by Bioanalyzer using small RNA kit.

<table>
<thead>
<tr>
<th></th>
<th>Comparison Set 1</th>
<th></th>
<th>Comparison Set 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>miRNeasy</td>
<td>mirVana</td>
<td>RNAzol-RT</td>
<td>p-value</td>
</tr>
<tr>
<td><strong>Total RNA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells (ng)</td>
<td>6,598±6,275</td>
<td>4,816±4,660</td>
<td>3,389±3,320</td>
<td>0.055*</td>
</tr>
<tr>
<td>Lipids (ng/µL)</td>
<td>37.5±32.6</td>
<td>26.0±26.4</td>
<td>29.0±20.0</td>
<td>0.600*</td>
</tr>
<tr>
<td>Skim Milk (ng/µL)</td>
<td>1.92±2.10</td>
<td>2.04±2.00</td>
<td>6.40±4.30</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>260/280 ratio</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>2.01±0.17</td>
<td>1.98±0.16</td>
<td>1.61±0.18</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lipids</td>
<td>2.03±0.07</td>
<td>2.03±0.19</td>
<td>1.65±0.15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Skim Milk</td>
<td>1.41±0.30</td>
<td>1.78±0.27</td>
<td>1.54±0.19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Total MicroRNA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells (ng)</td>
<td>110.6±95.0</td>
<td>65.7±84.0</td>
<td>139.1±166.0</td>
<td>0.508</td>
</tr>
<tr>
<td>Lipids (ng/µL)</td>
<td>3.40±6.30</td>
<td>0.80±1.70</td>
<td>1.10±1.40</td>
<td>0.169</td>
</tr>
<tr>
<td>Skim Milk (ng/µL)</td>
<td>0.10±0.09</td>
<td>0.10±0.15</td>
<td>0.19±0.29</td>
<td>0.342</td>
</tr>
<tr>
<td><strong>MicroRNA to Small RNA (%)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>24.4±13.7</td>
<td>19.5±8.7</td>
<td>33.5±16.0</td>
<td>0.152</td>
</tr>
<tr>
<td>Lipids</td>
<td>53.25±31.4</td>
<td>28.6±22.7</td>
<td>46.3±23.9</td>
<td>0.041</td>
</tr>
<tr>
<td>Skim Milk</td>
<td>20.5±8.5</td>
<td>24.0±12.4</td>
<td>48.5±20.6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* Results from linear mixed effects models, indicating that there are significant correlations between measures within a sample.
Table 4. The three most effective kits for each breastmilk fraction in each of the four evaluation criteria (total RNA extracted, quality of extracted RNA, total microRNA extracted, and microRNA/small RNA ratio). Total RNA and microRNA are presented in ng for the cellular fraction and in ng/mL for the lipid and skim milk fractions. All values represented are means ± standard deviations.

<table>
<thead>
<tr>
<th>Milk fraction</th>
<th>microRNA</th>
<th>260/280 ratio</th>
<th>RNA</th>
<th>microRNA Small RNA Ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TRIzol LS</td>
<td>miRNeasy mini (100%); (2.04±0.03)</td>
<td>miRNeasy mini (11082.7±151.063 ng)</td>
<td>TRIzol LS (51.1%±24.5)</td>
</tr>
<tr>
<td></td>
<td>miRCURY RNA Isolation kit-Cell &amp; Plant (806.9±1660.8 ng)</td>
<td>miRNeasy mini (100%); (2.10±0.04)</td>
<td>miRNeasy micro (6598.5±6275.2 ng)</td>
<td>miRNeasy mini (36.1%±11.8)</td>
</tr>
<tr>
<td></td>
<td>miRNeasy mini (770.6±2005.5 ng)</td>
<td>miRNeasy mini (100%); (1.98±0.16)</td>
<td>miRNeasy mini (6089.1±2853.2 ng)</td>
<td>RNAzol RT (33.5%±16.0)</td>
</tr>
<tr>
<td><strong>Lipids</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>miRCURY RNA Isolation Biofluids (12.7±24.4 ng/μL)</td>
<td>miRNeasy mini (100%); (2.03±0.05)</td>
<td>miRNeasy mini (49.9±61.1 ng/μL)</td>
<td>miRNeasy micro (53.2%±31.4)</td>
</tr>
<tr>
<td></td>
<td>mirPremier microRNA Isolation (9.4±8.5 ng/μL)</td>
<td>miRNeasy mini (100%); (2.04±0.079)</td>
<td>miRNeasy mini (46.9±34.1 ng/μL)</td>
<td>RNAzol RT (46.3%±23.9)</td>
</tr>
<tr>
<td></td>
<td>miRNeasy mini (6.5±1.1 ng/μL)</td>
<td>miRNeasy micro (100%); (2.03±0.07)</td>
<td>TRIzol LS (44.1±26.9 ng/μL)</td>
<td>TRIzol LS (44.4%±20.4)</td>
</tr>
<tr>
<td><strong>Skim milk</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>miRCURY RNA Isolation Cell &amp; Plant (2.17±5.66 ng/μL)</td>
<td>miRNeasy mini (80% good, 13.33 moderate, 13.33 poor); (1.99±0.17)</td>
<td>TRIzol LS (9.26±18.23 ng/μL)</td>
<td>mirPremier microRNA Isolation (5.7%±13.7)</td>
</tr>
<tr>
<td></td>
<td>miRCURY RNA Isolation Biofluids (1.0±1.09 ng/μL)</td>
<td>mirVana microRNA Isolation (53.3% good, 26.66 moderate, 20% poor); (1.78±0.27)</td>
<td>RNAzol RT (6.4±6.29 ng/μL)</td>
<td>RNAzol RT (48.5%±26.0)</td>
</tr>
<tr>
<td></td>
<td>miRNeasy mini (0.3±0.29 ng/μL)</td>
<td>miRNeasy mini (80% good, 40% moderate, 26.66% poor); (2.13±2.26)</td>
<td>miRNeasy mini (4.03±6.18 ng/μL)</td>
<td>TRIzol LS (47.7%±14.0)</td>
</tr>
</tbody>
</table>
3.4.5 Validation of microRNA expression in different human milk fractions

RT-PCR was used to validate the efficiency of the recommended kits (Table 3) in extracting microRNA using two different samples of each fraction (milk cells, lipids, and skim milk). Using comparative Ct (RQ) analysis, it was found that hsa-miR-148a-3p and hsa-miR-30a-5p were expressed in all three fractions for both milk samples tested (Fig. 5). These two microRNAs showed a similar relative quantification (RQ) value in two different milk lipid samples (A and B samples) (Fig. 5). Despite the low total RNA and microRNA concentration of skim milk compared to the other two human milk fractions, hsa-miR-30a-5p was highly expressed in one of the skim milk samples tested (B); however, the other skim milk sample showed much lower expression for both microRNAs. In the milk cell fraction, both microRNAs were expressed at relatively low levels compared to lipids and skim milk (Fig. 5). In the milk lipid fraction, both microRNAs were expressed more consistently in the two mothers compared to the cellular and skim milk fractions.

Figure 2. Total RNA (A), microRNA (B) and microRNA to small RNA ratio (%) (C) obtained by the eight kits tested. Different shades of grey represent different individuals (human milk samples). RNA and microRNA (A and B, respectively) are presented in ng for
the cellular fraction and in ng/mL for the lipid and skim milk fractions. All values represented are means ± standard deviations.

3.5 DISCUSSION
Recent advances in human milk compositional studies have revealed the presence of RNA (Lemay et al., 2013) and microRNA molecules (Weber et al., 2010, Munch et al., 2013), similar to those previously found in other biological fluids (Hassiotou et al., 2012). With breakthrough studies demonstrating an active role of food-derived microRNAs in regulating gene expression in adults (Baier et al., 2014, Zhang et al., 2012a), the discovery of these molecules in human milk highlights their potential significance for the breastfed infant. In addition, these milk molecules may provide novel diagnostic opportunities in relation to disease (Chen et al., 2008, Turchinovich et al., 2011). Extensive profiling and quantification of microRNA is therefore essential to the understanding and exploration of these molecules and their functions in human milk. The complexity of human milk composition, including lipid, cellular, and skim milk fractions, also suggests that each fraction potentially requires a different handling procedure and extraction kits for optimal RNA and microRNA isolation. In previous studies of human milk microRNA, the expression and type of microRNAs differed between milk lipids and skim milk, with the latter showing lower levels of microRNA expression than the lipid fraction (Weber et al., 2010, Munch et al., 2013, Kosaka et al., 2010). This suggests that microRNA content may also be different in human milk cells, as we showed in this study. MicroRNAs in the three human milk fractions could be used for different diagnostic and prognostic purposes, especially in monitoring the performance and related pathologies of the lactating mammary gland.

Studies investigating human milk microRNA content have focused mainly on the skim milk fraction and no comparisons have been made with the milk lipid fraction within the same sample. In this study, the human milk lipid and cell fractions clearly contained higher quantities of both total RNA and microRNA (P < 0.001) (Fig. 2) compared to skim milk. Specifically, the lipid fraction was on average 10- and 8-fold richer in microRNA and total RNA, respectively, than skim milk (Fig. 3).

With respect to total RNA in milk lipids, all methods performed similarly in our hands (Table 4), except one of the filter column kits (mirPremier), which yielded significantly lower total RNA than the TRizol-LS (P=0.010), miRNeasy mini (P=0.007), and miRCURY-Cell&Plant (P=0.005) kits. Moreover, RNAzol-RT gave significantly higher total RNA yields than mirVana (P=0.002) and the miRNeasy micro kits (P=0.0002). The
above suggest that the phenol/ guanidine-based method (RNAzol-RT and TRIzol-LS) yields higher total RNA concentrations in the milk lipid fraction.

**Figure 3.** Comparison of overall concentration of total RNA using NanoDrop 1000, and microRNA using Bioanalyzer 2100, respectively, between the lipid and skim milk fractions obtained for all samples using the eight extraction kits.

Sample RNA purity in milk lipids varied according to the method used. The optimal range considered was between 1.8 and 2.2 (Supplementary 1, Table S1). We found that the 260/280 ratios for TRIzol-LS (Table 4) were significantly lower to those of miRNeasy mini (P=0.019) and miRCURY-Cell&Plant (P=0.016). Generally, the phenol/guanidine method (TRIzol-LS) yielded less pure RNA than the other methods examined. However, when phenol/guanidine was combined with filter column (e.g., mirVana), higher microRNA to small RNA ratios were obtained (P=0.018), suggesting it as an appropriate method for extracting high quantities of microRNA from milk lipids.

With respect to microRNA content in the milk lipid fraction, significantly higher microRNA/small RNA ratios were obtained with the miRNeasy micro kit compared to mirVana (P<0.035). No differences were seen between RNAzol-RT and miRNeasy micro kit (P < 0.190). TRIzol-LS (phenol/guanidine) was not different to other filter column kits.
These findings suggest that both the phenol/guanidine and the filter column methods extract more micro-RNA than the filter column combined with phenol/guanidine method.

For the cellular portion of the human milk, we present our results as amounts as it was not possible to determine the concentration in this fraction without cell counts of the total sample (whole milk). This limits the comparison to the lipid and skim milk fractions. Nevertheless, RNA/microRNA amounts of the human milk cell fraction were relatively high and comparable to other cells such as mast cells (Eldh et al., 2012). In the first set of samples, we found that RNAzol-RT yielded less total RNA compared to miRNeasy micro (P=0.017). In the second sample set, mirPremier yielded less total RNA than miRNeasy mini (P=0.003). It is prudent to note that the mirPremier kit yielded the lowest amounts of RNA of all kits. However, no significant difference was seen between kits in the microRNA content. In terms of purity of RNA, the phenol/guanidine method yielded significantly lower values compared to the other methods tested (RNAzol-RT: P<0.001; TRIzol-LS: P<0.001). In terms of microRNA/small RNA ratio, TRIzol-LS yielded significantly more small RNA (P < 0.001). In summary, it appears that the filter column-based kits yield similar amounts of microRNA and total RNA with good purity in the milk cell fraction.

In the skim milk fraction, significantly purer RNA was obtained using mirVana than either miRNeasy micro or RNAzol-RT kits (P<0.001). Nevertheless, RNAzol-RT yielded

**Figure 4.** Examples of electropherograms for different human milk fractions (cells, lipids and skim milk) for two human milk samples obtained using the Agilent 2100 Bioanalyzer for small RNA.
significantly higher total RNA than mirVana (P=0.001). No differences were seen in extracted microRNA levels amongst all kits tested. However, significant differences were observed between kits in the microRNA/small RNA ratios (P<0.001). RNAzol-RT had significantly higher microRNA/small RNA ratio than either miRNeasy micro or mirVana (P<0.001). Further, microRNA/small RNA ratio of mirPremier was higher than of miRCURY-Cell&Plant or miRNeasy mini kits (P<0.001). Interestingly, although the phenol/guanidine method (RNAzol-RT and TRIzol-LS) was very efficient in extracting high amounts of total RNA and microRNA from skim milk, the purity of the extracted RNA was rather poor.

By using RT-PCR, the validation of microRNA presence in biological samples has been conducted (Chen et al., 2005, Shi and Chiang, 2005, Doleshal et al., 2008). MicroRNA expression patterns do not correlate with total RNA concentration that is usually measured by Bioanalyzer or Nanodrop (Doleshal et al., 2008, Moret et al., 2013). We confirmed this in this study by examining hsa-miR-148a-3p and hsa-miR-30a-5p expression in different human milk fractions. These microRNA were detected in all human milk fractions using the most effective extraction kits (Table 3). As expected, both microRNAs were expressed at high levels consistently in two different milk lipid samples; in particular, hsa-miR-30a-5p was expressed at higher levels than hsa-miR-148a-3p in the lipid fraction. In contrast, one skim milk sample had low expression of these microRNAs, whereas the other skim milk sample showed high expression. Moreover, these microRNAs were not expressed at high levels in both milk cell fraction samples compared to the other milk fractions. These findings suggest an enrichment for these microRNAs in the fat globules secreted by lactocytes as well as secretion in the skim milk. Expression levels may change rapidly in cells, whereas the same is not expected for either the fat globule or the skim milk. The above merit further investigation.
Figure 5. RT-PCR data for two milk cells, lipids and skim milk samples extracted using three different extraction kits (miRNeasy mini, miRCURY Biofluids, and miRCURY-Cell&Plant kits respectively) and analyzed using TaqMan miRNA assay for has-miR-148a-3p (black boxes) and has-miR-30a-5p (grey boxes). RNU48 was used as a housekeeping gene and all samples were normalized to milk cell sample A.

Our findings indicate that microRNA in human milk are conserved and protected either within cells or fat globules/other vesicles such as exosomes, and very few can be isolated from the skim milk fraction, which has also been called the plasma phase of milk. Importantly, most previous studies examined skim milk and not the cellular or the lipid fraction (Kosaka et al., 2010, Weber et al., 2010), and they have therefore excluded the fractions of human milk that appear to be richer in microRNA. Most recently, Munch et al. (2013) stated that human milk lipids are richer in microRNAs than skim milk (Munch et al., 2013). Our findings are in agreement with this and strongly suggest that it is necessary to examine microRNA in all three fractions of human milk and not just in one, to allow complete analysis of this component of human milk, its origin, properties and functions. Also, the microRNA content of the human milk cell fraction has not been profiled as done in human milk lipids, although it potentially conserves more novel microRNAs (Munch et al., 2013). Finally, our results support the rigorous investigation and standardization of sampling, processing, extraction and storage criteria for the investigation of microRNA in different biofluids. The recommended kits for each human milk fraction based on the quantity and quality of RNA/microRNA were listed in Table 3, and could be applied for
highly efficient extraction of RNA/microRNA from exosomes, fat globules, and human fluid cells in addition to human milk fractions.

A potential explanation for the differences between the kits in extraction performance is that the differences in the lysis solution between kits, which is an important step to release intact RNA/ microRNA from cells and fat globules. Therefore, the composition and efficiency of the lysis solution must be carefully selected based on the requirements of RNA/microRNA for subsequent studies, such as profiling using qPCR or Microarrays. Importantly, although most of the kits were designed for cellular fractions, we show that they can be used for body fluid samples such as skim milk, with good performance. However, using higher amounts (than those recommended by the manufacturer for fluids) of skim milk for extraction may help to increase the concentration of RNA/microRNA. It is also suggested to use smaller amounts of lysis solution in extracting RNA from skim milk because they are already free in skim milk, and the lysis solution may influence the integrity of RNA transcripts. Another consideration is that microRNAs may be fragmented into smaller pieces during the washing steps, and may not be subsequently conserved in the filter columns. On the other hand, in the phenol/ guanidine-based kits, the main issue in our findings was the poor RNA quality, suggesting that RNAs may be influenced during precipitation due to the long term exposure to ethanol and phenol.

This will now generate new avenues for examination of the types, properties and functions of these human milk molecules. Further, the variability amongst and within lactating women and factors that may influence them, such as the stage of lactation or milk removal, can be now robustly and consistently investigated. Opportunities arise for the use of these molecules as diagnostic markers of disease during lactation. Given the recently postulated function of human milk microRNA in providing immunological support to the infant (Kosaka et al., 2010, Zhou et al., 2012). Although the benefits of oral microRNAs have been recently challenged (Dickinson et al., 2013, Witwer and Hirschi, 2014), our study sets the basis for further examination using sound methodology of the potential significance of microRNAs in the lactating mammary gland and/or in the infant (Heneghan et al., 2009, Gotte, 2010). Future studies should consider the methodology developed herein to address important questions of immunological as well as developmental benefits conferred to the infant by human milk microRNA, and the potential to use them as diagnostic markers for the human mammary gland.
3.6 CONCLUSIONS
We demonstrated the presence of RNA and specifically microRNA in all three fractions of human milk, including the cells, lipids and skim milk, with the highest levels of both RNA and microRNA obtained in the lipid and cellular fractions. We presented a comparison analysis in a comprehensive dataset of 472 human milk samples, assessing three different extraction methods in eight commercially available kits. These results allow researchers to choose the most appropriate method for measurement of microRNA for their sample composition and fraction of human milk.
CHAPTER 4

HUMAN MILK miRNAs PRIMARILY ORIGINATE FROM THE MAMMARY GLAND RESULTING IN UNIQUE MIRNA PROFILES OF FRACTIONATED MILK

4.1 ABSTRACT
Human milk (HM) contains regulatory biomolecules including miRNAs, the origin and functional significance of which are still undetermined. We used TaqMan OpenArrays to profile 681 mature miRNAs in HM cells and fat, and compared them with maternal peripheral blood mononuclear cells (PBMCs) and plasma, and bovine and soy infant formulae. HM cells and PBMCs (292 and 345 miRNAs, respectively) had higher miRNA content than HM fat and plasma (242 and 219 miRNAs, respectively) (p < 0.05). A strong association in miRNA profiles was found between HM cells and fat, whilst PBMCs and plasma were distinctly different to HM, displaying marked inter-individual variation. Considering the dominance of epithelial cells in mature milk of healthy women, these results suggest that HM miRNAs primarily originate from the mammary epithelium, whilst the maternal circulation may have a smaller contribution. Our findings demonstrate that unlike infant formulae, which contained very few human miRNA, HM is a rich source of lactation-specific miRNA, which could be used as biomarkers of the performance and health status of the lactating mammary gland. Given the recently identified stability, uptake and functionality of food- and milk-derived miRNA in vivo, HM miRNA are likely to contribute to infant protection and development.

4.2 INTRODUCTION
Human milk (HM) is the optimal nutrition for term infants (Kramer, 2010). In addition to being a food source, HM confers developmental programming to the infant and protection against infections, resulting in decreased risk of sudden infant death syndrome and reduced mortality and morbidity both in the short- and long-term (Ip et al., 2007, Hassiotou and Geddes, 2014, Dewey et al., 1995, Newburg and Walker, 2007). These effects are mediated by HM-specific regulatory factors including both cellular and biochemical components (Hassiotou and Geddes, 2015, Hassiotou et al., 2013a, Hassiotou and Hartmann, 2014, Hanson and Winberg, 1972). In contrast, artificial infant formula cannot confer such protective and developmental functions as it lacks important HM components with bioactivity (Eudeknab and Schanler, 2012, Alsaweed et al., 2015a). An additional unique bioactive component of HM that has been recently discovered is miRNAs (Kosaka et al., 2010, Weber et al., 2010).
miRNAs are small non-coding RNAs, which regulate gene expression, thus control protein synthesis at the post-transcriptional level in eukaryotic cells (Bartel, 2004). They have been identified as key regulators of diverse biological and developmental processes in eukaryotes (cell proliferation and differentiation, apoptosis, immune system development and immune response) (Williams, 2008, O'Driscoll, 2006) by targeting messenger RNA (mRNA) during its translation into protein, either degrading the mRNA or inhibiting the translation process (Krol et al., 2010). Aberrant miRNA expression has been found to be associated with pathologies, including different types of cancer, inflammation and diabetes (Lu et al., 2008). Importantly, food-derived miRNA have been recently shown to be very stable in the gastrointestinal tract and be transferred to the blood circulation of adults, influencing gene expression in different tissues (Zhang et al., 2012a). In addition to tissues and cells, miRNAs have been isolated from body fluids, such as plasma, urine, saliva and tears (Weber et al., 2010). Further, exosomes, small cell-derived vesicles present in body fluids and carrying proteins and molecules, have been shown to take up miRNAs mediating their protection against digestion and facilitating their regulatory functions in different tissues and organs (Valadi et al., 2007).

Most recently, miRNAs have been isolated in high quantities from both animal and HM, and were shown to be present both as free molecules in skim milk (Kosaka et al., 2010, Weber et al., 2010, Chen et al., 2010, Modepalli et al., 2014, Izumi et al., 2014) and packaged in vesicles such as milk exosomes and the fat globule (Zhou et al., 2012, Munch et al., 2013, Chen et al., 2014, Gu et al., 2012). Bovine milk exosomes can be transported by intestinal cells via endocytosis in the human and rat colon (Wolf et al., 2015). Moreover, studies of bovine milk consumed by adult humans showed that at least some bovine milk miRNA can be transferred to the bloodstream (Baier et al., 2014). This was further reinforced by Arntz et al. (Arntz et al., 2015), who demonstrated in vitro uptake by splenocytes and intestinal cells of miRNA derived from bovine milk extracellular vesicles, and their therapeutic role in delaying the onset of experimental arthritis when delivered orally to mice. Collectively, these findings strongly suggest that HM miRNA survive the gastrointestinal tract of the infant to exert regulatory functions during breastfeeding (Alsaweed et al., 2015a), similar to what has been recently shown for maternal milk-derived stem cells (Hassiotou et al., 2015). It is therefore important to
elucidate the origin, properties, distribution and functional significance of HM miRNA as a novel regulatory component of milk.

Most previous research in milk miRNA has focused on animal milk, including the bovine (Chen et al., 2010), porcine (Gu et al., 2012) and murine (Izumi et al., 2014). In these animal studies, next generation sequencing (NGS) has been mainly employed as a miRNA profiling method. Studies using NGS and other global miRNA profiling methods in HM are scarce, with many previous investigations mainly using qPCR-based technology for a limited number of miRNAs. Further, skim milk and milk lipids have been the milk fraction of choice in previous milk miRNA studies (Kosaka et al., 2010, Weber et al., 2010, Munch et al., 2013, Zhou et al., 2012), whilst the milk cellular fraction has been largely neglected despite being a rich source of RNA (Hassiotou et al., 2012). Although Munch et al. (2013) stated that HM lipids were the richest milk fraction in miRNA (Munch et al., 2013), this was not compared with HM cells, which potentially conserve high quantities of miRNAs. Recently, we have shown that skim milk has the lowest miRNA content amongst the three HM fractions (cells, lipids and skim milk) (Alsaweed et al., 2015ab). Considering that breastfed infants consume whole HM, it is imperative that the miRNA content of all three fractions is examined to illuminate the contribution of these molecules to infant health. At the same time, investigation of all milk fractions and maternal blood may shed light into the origin of milk miRNA, which is still unexplored.

In this study, we profiled 681 HM mature miRNAs using the TaqMan miRNA OpenArray system (Applied Biosystems), with the aim to determine the miRNA composition of the cell and fat fractions of HM and compare it with maternal peripheral blood mononuclear cells (PBMCs) and plasma collected from exclusively breastfeeding women as well as two commercial infant formulae. In addition, gene ontology for miRNA targets and pathway analyses were conducted for the miRNAs that were differentially expressed between HM and maternal blood. Our study elucidates the origin of HM miRNAs and reveals the contributions of the cells and fat fractions to the total content of miRNAs in HM.
4.3 MATERIALS AND METHODS

4.3.1 Ethics, sample collection and processing
This study was approved by the Human Research Ethics Committee of The University of Western Australia. Informed written consent was provided by all participants, which included 10 exclusively breastfeeding dyads in month 2 postpartum (week 4 to 8) to ensure established lactation. All participating dyads and their infants were healthy at the time of collection. The workflow from sample collection to analysis is shown in Supplementary 2, Figure S6. Fresh HM samples (24-78 mL) were collected early in the morning. Aseptic collection of the samples was carried out using a breast pump, sterile bottles and other accessories (Medela AG, Switzerland). Maternal blood samples were collected at the time of milk collection by an accredited phlebotomist. All the blood samples were collected into EDTA tubes (Becton Dickinson, Mountain View, CA, USA). Samples were transferred immediately to the laboratory in the dark for processing. HM samples were processed for miRNA analyses as previously described (Alsaweed et al., 2015ab, Hassiotou et al., 2012). Briefly, fresh milk samples were diluted 1:1 with phosphate buffered saline (PBS; Gibco, Life Technologies, Foster, CA), and were then centrifuged at 800 g for 20 min at 20°C for fractionation. HM cells and lipids were transferred separately into new RNAse free tubes. Cells were then washed three times with PBS, stained with Trypan blue (ProSciTech, Queensland, Australia) and counted using a haemocytometer as previously described (Hassiotou et al., 2012). Lipid samples were centrifuged twice at 450 g for 20 min at 20°C to obtain a pure lipid fraction. For blood fractionation, the whole blood samples were collected in EDTA-coated centrifuge tubes and were centrifuged at 800 g at 20°C for 10 min to separate the plasma from cells. The plasma was then transferred to a new tube and centrifuged further at 3,500 g for 20 min at 4°C to remove all residual cells and other debris. Blood peripheral mononuclear cells (PBMCs) were isolated from blood cell samples based on Secoll separation (Serana, Australia). Briefly, after transferring the plasma into a new tube, the buffy coat in the top layer of the whole blood was transferred to a new tube with PBS. PBMCs in PBS were gently overlaid onto 4 mL of Secoll in a new tube and centrifuged at 800 g for 15 min at 20°C. The middle layer containing PBMCs was collected and was washed three times in PBS, then counted as described for HM cells.
above. Plasma samples were centrifuged for 15 min at 4°C at 14,000 g to further purify plasma. miRNAs were extracted from all samples immediately without cryopreservation.

4.3.2 Infant milk formulae

Equal amounts (2 g) of two different types of infant formula powder were dissolved in 4 mL of Trizol LS Reagent (Invitrogen, CA, USA). Standard, whey dominant, infant formula known as bovine milk-based formula (S-26 Gold) and soy-based infant formula (S-26 Gold Soy) manufactured by Aspen Nutritional Australia, were used. As per the manufacturer’s bottle instructions, both are considered to be suitable for infants from birth to one year old. The semi-dissolved powder in Trizol was incubated for 30 min at 37°C for complete dissolution.

4.3.3 Milk lipid content

Lipid content of whole fresh HM samples was measured using Creamatocrit Plus (Medela, Inc, McHenry, Illinois) as previously described (Lucas et al., 1978). Briefly, whole HM was taken up by a capillary tube, then centrifuged for 10 min at 11,731 g in a microcentrifuge (BHG Hermle, Germany) to separate the milk lipids from skim milk and cells.

4.3.4 Extraction and quantification of miRNA

miRNA were isolated from different fractions of HM according to our previous study (Alsaweed et al., 2015ab), where the miRNeasy mini kit (Qiagen, Hilden, Germany) was used to extract miRNAs for HM cells and maternal PBMCs, the miRCURY RNA Isolation-Biofluids Kit (Exiqon, Vedbaek, Denmark) for HM lipids and both infant formulae, and the mirVana PARIS Kit (Ambion, Austin, TX, USA) for maternal plasma. These three miRNA extraction kits all use filter column-based methods. All the extractions were done according to the manufacturer’s protocol and as described previously ensuring complete lysis of cells and membranous components via (Alsaweed et al., 2015ab). The volume of milk lipids and lysis reagent for the miRCURY RNA Isolation-Biofluids Kit were increased to 400 µL and 120 µL, respectively. The concentration and purity of the extracted miRNA were measured using a NanoDrop 2000 Spectrophotometer (Wilmington, DE, USA) and Agilent Bioanalyzer 2100 (Agilent, CA, USA) with the RNA 6000 NanoChip kit. All miRNA samples were then stored at -80°C until further analyses incubated for 30 min at 37°C for complete dissolution.
4.3.5 Reverse transcription (RT), preamplification and TaqMan OpenArray analysis

The expression levels of 681 human mature miRNAs that have been functionally validated with miRNA artificial templates were profiled in the milk, formulae and maternal PBMCs and plasma samples using the TaqMan miRNA OpenArray panel system (Life Technologies, CA, USA). The panel originally profiled 758 human mature miRNAs, however since its release, recent updates of miRNA integrity and function confirmed 681 out of the 758 targets as true human miRNAs (Supplementary 2, Table S22). Human ath-miR159a was used as a negative control for the human samples, and RNU48, RNU44 and U6 rRNA were used as housekeeping controls. According to the manufacturer’s instructions, total extracted miRNA (>50 ng of total RNA) was combined with reverse transcription primers using the Megaplex RT Primers (Life Technologies) in a MicroAmp Fast Optical 96 well plate (Life Technologies) to synthesize single strand cDNA. This was followed by preamplification, which employed the Megaplex PreAmp Primers (Life Technologies) to increase the quantity of desired cDNA. All preamplification samples were diluted (1 in 40) using 0.1X TE pH 8.0 (Promega, WI, USA). TaqMan OpenArray Real Time PCR Master Mix was added to the diluted samples to amplify the preamplified cDNA using 681 unique human mature miRNA primers on the OpenArray 384 well plate. The samples were loaded from a 384-well plate into the TaqMan OpenArray Panel using the OpenArray AccuFill System, then the panel was analysed on the TaqMan OpenArray Cycler platform (Life Technologies). Cycle threshold (Ct) values for targeted mature miRNAs, including the controls, were automatically measured and calculated using the supplied OpenArray software. Infant formulae made from soy milk expectedly contained ath-miR159a, which is a plant-specific miRNA. Therefore, ath-miR159a was used as a negative control for HM and blood samples, and as an endogenous target miRNA for both infant formulae samples. This miRNA (5′UUUGGAUUGAAGGGAGCUCUA3′; miRBase accession MIPF0000010) was screened for 16 replicates in each formula, and was considered a miRNA with high reliability.

4.3.6 Gene Ontology and gene target analysis

Pathway enrichment analysis of the reliable (8≤Ct≤29 and detected in at least 4 samples per group) and significantly differentially expressed miRNAs for each comparison was done using
the MetaCore pathway analysis tool by GeneGO (Thomson Reuters). The software only recognized and analyzed miR-200a; 205; 200c; 141; 429; 200b; 106b; 20a; 17; 34a; 34c; 340-5p; 137-3p; 195. \( p \) values were calculated using the hypergeometric test (Adams and Skopek, 1987) and adjusted for multiple testing using the False Discovery Rate “FDR” method (Benjamini et al., 2001). GeneGO categories were found to be significant (adjusted \( p \) value<0.05) for each list of differentially expressed miRNAs, and pathways were generated. Due to the limited number of miRNA that are recognized and can be analyzed using MetaCore, Ingenuity Pathway Analysis was also employed to map all the reliable miRNAs to IPA database to identify associated biological or pathological functions.

4.3.7 Statistical analyses
The qRT-PCR quality characteristics, the differential expression analysis, and comparative analysis for each of the samples were investigated using the R Studio Version 0.98.1103 package (RStudio Team, 2015) and the HTqPCR software, which provides biostatistical tools for the analysis of Ct values obtained from the TaqMan OpenArray assays (Dvinge and Bertone, 2009), including data loading, quality assessment, normalisation, visualisation and parametric or non-parametric testing for statistical significance in Ct values between features (the individual miRNAs). Linear mixed effects (LME) models, general linear hypothesis tests, and graphical exploration of the data were used to examine associations between cell and total RNA enriched in miRNA content. Tukey’s HSD test was employed to identify differences in miRNA species between different sample groups (HM cells and lipids, and maternal PBMCs and plasma). \( P<0.05 \) was considered statistically significant. Reliable miRNA were \( 8 \leq \text{Ct} \leq 29 \) and detected in at least 4 samples per group, whilst unreliable miRNA correspond to markedly low Ct value (<5) or showing excessive variation between replicates. Undetermined miRNA correspond to a failed assay or a rare miRNA species (Ct>29). The DataAssist software (Life Technologies, Foster, CA) was used to calculate the delta Ct value and fold change of each identified miRNA and to perform the differential expression analyses to confirm the results obtained by the HTqPCR.
4.4 RESULTS

4.4.1 Concentration and quality of RNA enriched in miRNA in maternal milk and blood

Total RNA was extracted using the miRNeasy mini kit for milk cells and PBMCs, miRCURY RNA Isolation-Biofluids Kit for milk lipids, and mirVana PARIS Kit for plasma, which have been shown to enrich for miRNA; therefore, the total RNA concentration in the text below refers to total RNA enriched in miRNA. A high correlation between RNA Integrity Number (RIN) using the Bioanalyzer and 260/280 ratio using the NanoDrop was obtained ($p<0.001$).

The total RNA concentration of PBMCs (ng/106 cells) was not different to that of milk cells using either the Bioanalyzer ($p=0.960$) or the NanoDrop ($p=0.800$) (Table 1, Figure 1A). However, both the total RNA concentration and RNA quality of plasma were significantly lower than that of milk lipids using both instruments ($p<0.001$) (Figures 1B-1D). The quality of RNA obtained from milk lipids was also lower than that of milk cells and PBMCs when using Bioanalyzer ($p<0.001$), although still within the acceptable range when using NanoDrop (Figures 1C, 1D).

High HM cell content was associated with high total RNA amounts extracted from milk cells ($p=0.004$ and $p=0.005$, using Bioanalyzer and NanoDrop, respectively), although the same was not observed for either maternal PBMCs and their total RNA content ($p=0.502$ and $p=0.762$, using Bioanalyzer and NanoDrop, respectively) or HM lipid content and total RNA/µL lipids extracted ($p=0.664$ and $p=0.825$ using the Bioanalyser and NanoDrop, respectively) (Figures 1E-1G). A trend for a positive association was seen between the volume of milk expressed and the total RNA extracted from milk cells as well as the maternal blood volume collected and the total RNA extracted from PBMCs, although these were not statistically significant ($n=10$) ($p=0.172$, 0.712 and $p=0.482$, 0.117 using Bioanalyzer and NanoDrop for HM and blood, respectively) (Figures 1H, 1I). Further, no relationship existed between the total number of milk cells and PBMCs ($p=0.459$) (Figure 1J).
Table 1. Sample characteristics for HM cells and lipids, and maternal PBMCs and plasma (n=10 for each sample group), including quantity and quality of total RNA enriched in miRNA extracted from using NanoDrop 2000 and Bioanalyzer 2100, total number of miRNA species detected, and top 10 most highly expressed miRNAs. (SD: standard deviation).

<table>
<thead>
<tr>
<th>Extraction kit used</th>
<th>Milk cells</th>
<th>Milk lipids</th>
<th>PBMCs</th>
<th>Plasma</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>miRNeasy mini</td>
<td>miRCURY Biofluids</td>
<td>miRNeasy mini</td>
<td>mirVana</td>
</tr>
<tr>
<td><strong>Cell content/mL milk or blood (mean±SD) (cell viability %)</strong></td>
<td>435,787±311,96 7 (92.5)</td>
<td>-</td>
<td>724,073±309,44 9 (85.1)</td>
<td>-</td>
</tr>
<tr>
<td><strong>Lipid content of milk (%) to (mean±SD)</strong></td>
<td>-</td>
<td>8.4±3.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Total RNA (mean±SD) using NanoDrop 2000</strong></td>
<td>2,525 ng/10⁶ cells±1048</td>
<td>29 ng/µl of fat±17.5</td>
<td>2,648 ng/10⁶ cells±1821</td>
<td>4.62 ng/µl of plasma±3.0</td>
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<tr>
<td><strong>RNA purity (OD 260/280) (mean±SD) using NanoDrop 2000</strong></td>
<td>2.04±0.02</td>
<td>2.00±0.04</td>
<td>2.07±0.01</td>
<td>1.26±0.46</td>
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<tr>
<td><strong>Total RNA (mean±SD) using Bioanalyser 2100</strong></td>
<td>2,674 ng/10⁶ cells±1173</td>
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<td>2,709 ng/10⁶ cells±1821</td>
<td>0.09 ng/µl of plasma±0.08</td>
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<tr>
<td><strong>RNA Integrity Number (RIN) (mean±SD) using Bioanalyser 2100</strong></td>
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<td><strong>Number of detectable miRNA species</strong></td>
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<td>345</td>
<td>219</td>
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<td>hsa-miR-106a</td>
<td>hsa-miR-30c</td>
<td>hsa-miR-199a-3p</td>
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¹Total RNA (ng) per 1×10⁶ of milk cells or PBMCs, or ng/µL of milk lipids or blood plasma.

²The number of reliable miRNAs species based on our criterion of presence in ≥4 samples per group with 8≤Ct.
4.4.2 Human milk has a diverse miRNA composition that differs from maternal blood

To investigate the miRNA composition of HM and maternal blood, TaqMan OpenArray was used to measure Ct values for 681 human known mature miRNAs. Using a first criterion of $8 \leq \text{Ct} \leq 29$, HM cells and lipids, and PBMCs and plasma were found to conserve 450, 337, 488, and 319 miRNAs, respectively. To determine the most reliably measured miRNAs, we used a novelty criterion stating $8 \leq \text{Ct} \leq 29$ and miRNA presence in $\geq 4$ out of the 10 samples tested for each of milk cells, milk lipids, PBMCs and plasma. Unreliable miRNAs were determined based on Ct<5 or excessive variation between replicates. Undermined miRNAs (miRNAs that were tested in the OpenArray panel but were not detected in any of the samples examined) did not match the above criteria (Figure 2A). Using this novelty criterion, 292 miRNA species were determined in HM cells, 242 miRNAs in HM lipids, 345 miRNAs in PBMCs, and 219 miRNAs in blood plasma (Table 1; Supplementary 2, Table S1A). Plasma was diverse in miRNA composition, with great variation between participants, and contained fewer miRNA species than PBMCs and HM cells ($p<0.001$) or HM lipids ($p=0.015$). HM cells contained more miRNA species than HM lipids ($p=0.038$). PBMCs conserved a high number of miRNAs in most samples, which was significantly higher than in HM lipids ($p=0.001$), but not statistically different to HM cells ($p=0.084$) (Figure 2B). Of the miRNA identified, the top 10 and 20 most highly expressed in all four sample groups (HM cells and lipids, and maternal PBMCs and plasma) are listed in Table 1 and Supplementary 2, Table S2, respectively. All the miRNA species detected in each sample are listed in Supplementary 2, Table S1B.

Heat map analysis and plotting of miRNA profiles showed that most miRNAs in HM cell and lipid samples are clustered together, whilst PBMC and plasma miRNAs do not significantly relate to any of HM cells or lipid samples (Figure 2C). The relationship between sample groups was further examined using Principal Component Analysis (PCA), which demonstrated the distribution and clustering of the individual group samples (Figure 3A). PBMCs formed a tight separate cluster, whilst plasma miRNA showed a broader distribution suggestive of biological variation. Most of the HM cells and lipid samples were grouped together.
Differential miRNA expression analysis for the reliable miRNAs identified was performed in R (HTqPCR package) using Linear Models for Microarray Analysis (limma) (Figure 3C). The full set of miRNAs and their differential expression p values are shown in Supplementary 2, Tables S3-S13. HM cell and lipid miRNA expression was highly correlated, with the exception of hsa-miR-564, which was higher in lipids than in milk cells. Most of the detected miRNAs in the other sample groups were differentially expressed amongst them. These findings were confirmed using correlation analysis in DataAssist, which showed a high correlation in miRNA expression between HM cells and lipids (r=0.90), whilst the correlation between PBMC miRNAs and miRNAs in HM cells or lipids was poorer (r=0.62; r=0.57, respectively). Plasma miRNAs were expressed at distinctly different levels than all other three sample groups (r=-0.08, -0.001, -0.27 for HM cell, lipids, and PBMCs comparisons respectively).

Further, a comparative analysis of the miRNA species present in each group was performed using the most reliable miRNA identified. This also confirmed the high correlation in miRNA content between HM cells and lipids (Figure 3B). PBMC miRNAs were weakly related to HM miRNAs and also to plasma miRNAs (Figure 3B). Euler diagrammatic analysis revealed the overlapping (most reliable) miRNAs between the different sample groups examined, showing 43 miRNAs that were specific to HM and were not found in maternal blood (Figure 4A; Supplementary 2, Table S14A). Of these, 14 miRNA were specific to HM cells, 6 were specific to HM lipids, and 23 were commonly specific between HM cells and lipids. Similarly, 84 miRNA species were specific to maternal PBMCs, and 11 to plasma (Figure 4A; Supplementary 2, Table S14A). In total, 221 miRNA species were shared between HM cells and lipids (Figure 4A; Supplementary 2, Table S14A). Of the 681 miRNAs assayed, 114 were detected in all four sample groups, 34 were specific to HM cells and PBMCs, 2 were specific to HM lipids and PBMCs, and 12 were specific to PBMCs and plasma (Figure 4A; Supplementary 2, Table S14A). Interestingly, no miRNAs were identified that were specific to plasma and HM only. These data provide evidence supporting the endogenous synthesis of the majority of HM miRNA in the lactating mammary epithelium, with a small contribution of the maternal circulation. A proportion of HM miRNA appear to be unique to lactation, as they were not detected in maternal blood.
Figure 1. RNA enriched in miRNA in HM cells and lipids, and maternal PBMCs and plasma, and associations with HM components. (A, B) RNA concentration of HM cells, PBMCs, HM lipids, and plasma, obtained with NanoDrop 2000 (N) and the Bioanalyzer 2100 (B). (C, D) RNA integrity measured by the Bioanalyzer 2100, and RNA purity (260/280 ratio) using NanoDrop 2000 in all four sample groups. (E, F) Associations between total RNA enriched in miRNA and HM cell content or maternal blood PBMC content using Bioanalyzer 2100 and Nanodrop 2000. (G) HM lipid content (%) and RNA concentration of HM lipids (ng). (H, I) Associations between HM volume or maternal blood volume with the total RNA enriched in miRNA. (J) Association between PBMC content of blood and HM cell content.

4.4.3 Infant formulae are low in human miRNA compared to human milk

Both infant formulae examined (bovine milk-based and soy-based) contained very few human mature miRNA species, and those were present at very low expression levels compared to HM fractions (Figure 2A). Although the RNA input of the soy-based formula was higher than that of the bovine milk-based formula (Table 2), the latter contained 45 mature human miRNAs compared to 22 in the soy-based formula with \(8 \leq \text{Ct} \leq 35\), whilst only 26 and 19 respectively with the more reliable \(8 \leq \text{Ct} \leq 29\) (Supplementary 2, Table S15). These differences between the two formulae potentially reflect differences between animal and plant miRNA. The miRNA content and expression patterns of the bovine milk-based infant formula were more similar to that of HM than the maternal PBMCs or plasma, and less so for the plant-based formula (Figure 3B). Of the miRNA detected in the formulae, 33 were common between the bovine milk-based formula and HM cells and lipids, whilst only 8 miRNAs were common between the soy-based formula and HM fractions (Figure 4B; Supplementary 2, Table S14B). Interestingly, miR159a, which is known to be plant-specific and was not expressed in any of the HM samples, was detected in not only the soy-based formula, but also in the bovine milk-based formula, at 16 replicates and high expression levels compared to the other identified miRNAs in this formula (Figure 4C; Supplementary 2, Table S16).
Table 2. Characteristics of extracted miRNA from bovine milk- and soy-based infant formulae. Although the criterion of miRNA presence in ≥4 samples with 8≤Ct≤29 cannot be applied in the formula samples tested due to examination of only one sample for each formula, here we report the top 10 most highly expressed miRNAs in each formula, which had 8≤Ct≤35. The miRCURY Biofluids kit was used to extract miRNA from both formulae.

<table>
<thead>
<tr>
<th></th>
<th>Bovine milk-based formula</th>
<th>Soy-based formula</th>
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<tr>
<td><strong>Total RNA using NanoDrop 2000 (ng/µL of formula)</strong>*</td>
<td>1.37 ng/µl</td>
<td>116.4 ng/µl</td>
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<tr>
<td><strong>RNA purity (OD 260/280) using NanoDrop 2000</strong></td>
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<td>2.12</td>
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<tr>
<td><strong>Total RNA using Bioanalyser 2100 (ng/µL of formula)</strong>*</td>
<td>0.1 ng/µl</td>
<td>69.9 ng/µl</td>
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<tr>
<td><strong>Number of detectable miRNAs</strong></td>
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<tr>
<td>ath-miR159a</td>
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<td>has-miR-520c-3p</td>
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* 2 grams of formula powder dissolved in 4 mL of Trizol LS reagent.
Figure 2. (A) Ranking for the classified Ct values across each sample run on the OpenArray platform. Reliable miRNAs had 8≤Ct≤29 and were detected in at least 4 samples per group, whilst unreliable miRNAs had markedly low Ct values (<5) or showed excessive variation between replicates. Undetermined miRNAs correspond to a failed assay or a rare miRNA species (Ct>29). (B) Boxplot showing the number of miRNA species in all mothers and each sample group (n=10 per sample group). (C) Heat map showing an overview of the Ct quality categories that have been assigned to the different miRNAs. The categories correspond to “Reliable”, ”Undetermined” and “Unreliable” with the colour codes “green”, ”red” and “yellow”, respectively.
4.4.4 Pathway enrichment and ingenuity pathway analyses reveal a plethora of biological functions associated with human milk miRNAs

Pathway enrichment analysis of some of the significantly differentially expressed miRNAs in HM cells and lipids (miR-200a; 205; 200c; 141; 429; 200b; 106b; 20a; 17; 34a; 34c; 340-5p; 137-3p; 195) was done using the MetaCore pathway analysis tool pipelines by GeneGO (GO) (Gomez et al., 2015). Several of these miRNAs were found to interact in the molecular pathways regulating the inflammation action of Endothelin-1, cardiovascular disease, and sickle cell disease. They are also known to be involved in microphthalmia-associated transcription factor (MITF) in melanoma, epithelial-mesenchymal transition (EMT), lung epithelial progenitor cell differentiation, and tumor protein p53 signaling in prostate cancer (Figure 5; Supplementary 2, Figures S2-S5).

Due to the limited number of miRNA that are recognized and can be analyzed using MetaCore, we also employed ingenuity pathway analysis to further explore functional and disease pathways associated with the reliably expressed miRNAs (8≤Ct≤29) across all samples through 6 mir seed regions, which is the mRNA-miRNA binding site. All the identified miRNAs were mapped to biological processes to determine their contribution in a variety of normal and abnormal conditions (Supplementary 2, Tables S17-S21). Due to the overrepresentation of knowledge associated with some diseases including cancer and inflammation, compared to normal biological functions, many miRNAs in this study have been identified to be associated with abnormal conditions. However, numerous miRNAs also have important roles in normal conditions. As shown by the differential expression analysis (Supplementary 2, Table S6), miR-564 was differentially expressed between HM cells and lipids, where it was upregulated in HM lipids. This miRNA is known to regulate the differentiation of adipose tissue-derived stem cells (ADSCs) (Cho et al., 2011), and in the case of HM, it may participate in milk or lipid synthesis in the lactocyte. A plethora of biological functions across all sample groups examined were regulated by the reliably identified miRNAs. These functions included cellular development and movement, cell cycle, growth and proliferation, and immune responses (Supplementary 2, Tables S17-S21).
Figure 3. (A) Principal Component Analysis (PCA) showing the distribution and clustering of the individual sample groups. The miRNA content in PBMCs form a tight cluster mainly in the top right of the graph, whilst the plasma samples show a broader distribution suggestive of high inter-individual biological variation. The HM cell and lipid miRNAs are clustered together and share a similar space on the graph. (B) Correlation plot demonstrating the relationship between different sample groups. The regions of the graph that are shaded in hotter colours correspond to more similar profiles of miRNA presence and relative abundance. The HM cell and lipids samples are all well correlated with each other, whilst a slight correlation between maternal PBMCs and plasma samples could be seen. Two plasma samples were markedly different to all other samples. (C) Differential expression analysis for the reliable miRNAs identified using Linear Models for Microarray Analysis (limma) in the R HTqPCR package. Results are presented in volcano plots, where the log-fold change is plotted on the x-axis and the -log10 (adjusted p value) on the y-axis. The six volcano plots demonstrate a comparison between the four sample groups (HM cells and lipids, and maternal PBMCs and plasma) by graphic fold change versus significant (p<0.05) to exhibit differences in miRNA expression between sample groups.
4.5 DISCUSSION

The discovery of novel bioactive components in HM, such as stem cells and miRNAs, re-emphasizes its importance as a nutritional, developmental and protective agent for infants. Increasing evidence suggests the involvement of miRNAs in both normal mammary development and function and infant health via HM (Alsaweed et al., 2015a, Zhou et al., 2012, Li et al., 2012). The abundance of miRNA in milk and their stability in the GI tract in adults further support their survival and functional significance in the breastfed infant (Alsaweed et al., 2015a, Kosaka et al., 2010, Munch et al., 2013, Zhou et al., 2012). Over the past few years, HM miRNAs have been investigated in either skim milk (Kosaka et al., 2010, Weber et al., 2010) or milk lipids (Munch et al., 2013) and exosomes (Zhou et al., 2012), whilst the cellular component of milk has been largely ignored despite its high RNA content (Hassiotou et al., 2012). We used the OpenArray Taqman technology to profile 681 human mature miRNAs in the cell and lipid fractions of HM and compare it with maternal blood (PBMCs and plasma) as well as with two commercially available bovine milk- and plant-based formulae. HM cells were found to conserve higher quantities of miRNA, both in total content and miRNA species composition, compared to previously studied HM lipids and skim milk (Kosaka et al., 2010, Weber et al., 2010, Munch et al., 2013). The miRNA composition of the cell and lipid fractions of HM was similar, whilst maternal PBMCs and plasma displayed distinctly different miRNA profiles to HM. This finding, together with the previously shown dominance of lactocytes in mature HM of healthy mothers and infants (Hassiotou and Geddes, 2015, Hassiotou et al., 2013a, Hassiotou et al., 2013x), suggest endogenous synthesis of the majority of HM miRNA in the mammary epithelium, with a small contribution of the maternal circulation.

The total RNA content enriched in miRNA was not significantly different between PBMCs and HM cells (Figure 1E, 1F), indicating similar transcription activity in both milk-derived mammary epithelial cells and maternal blood-derived PBMCs. In contrast, HM lipids conserved higher RNA concentration compared to maternal plasma (Figure 1B), with similar miRNA content to that of HM cells. This is in accordance with the origin of milk fat globule from the lactocyte (Hassiotou et al., 2013a) and with previous milk lipid miRNA studies (Munch et al., 2013). Thus, HM fat, in addition to being an essential nutritive component for the infant (Hale and Hartmann, 2007), is also an important carrier of bioactive miRNA molecules to infants. No relationship was seen between HM lipid content and the total RNA of HM lipids, which is in agreement with the fact that total HM fat content does not necessarily reflect the number of fat globules (Hamosh et al., 1999). This
suggests that the lipid miRNAs are primarily packaged within the fat globule or other microvesicles such as exosomes contained within the milk fat. A positive relationship between HM cells or PBMCs and total RNA content was seen as expected. However, this was stronger for PBMCs, which represent a specific cell type in contrast to HM cells, which are a heterogeneous population of cells including primarily lactocytes (in mature milk from healthy mothers and infants), but also smaller populations of stem cells, progenitor cells, and immune cells (Hassiotou and Geddes, 2015, Hassiotou et al., 2013a, Hassiotou and Hartmann, 2014, Hassiotou et al., 2013x). The weaker association between HM cells and total RNA content may reflect differing RNA transcription between these different cell populations of HM.

Although both HM cells and PBMCs were rich in different miRNA species (292 and 345 miRNAs, respectively) and not significantly different to each other in our study cohort \((p=0.084)\), HM lipids had a lower miRNA species number (242 miRNAs) \((p=0.038\) for the comparison with HM cells; \(p<0.001\) for the comparison with PBMCs), followed by maternal plasma (219 miRNAs) \((p<0.001\) for the comparison with HM cells and PBMCs; \(p=0.015\) for the comparison with HM lipids). The poor miRNA species content of maternal plasma has also been seen in other body fluids, such as human urine (Weber et al., 2010), and also in skim milk (Kosaka et al., 2010). Therefore, the HM cellular component conserves more miRNA species than the HM lipid and skim milk fractions, emphasizing the need to include it in milk miRNA investigations, particularly since it better represents what the infant receives. However, caution needs to be exercised when analyzing whole milk samples to ensure complete lysis of HM cells and membranous microvesicles for miRNA extraction. This is better performed when fractionating the HM into cells, skim milk, and lipids immediately upon expression and prior to freezing, and then extracting miRNA after rigorous cell lysing, as has been previously described (Twigger et al., 2015, Alsaweed et al., 2015a).
**Figure 4.** Shared reliable miRNAs \( (8 \leq \text{Ct} \leq 29 \text{ and present in at least 4 samples per group}) \) between the four sample groups examined. The bovine milk- and soy-based formulae are the results of a single assay and the observations are only illustrative \( (8 \leq \text{Ct} \leq 35) \). (A) Euler diagram showing overlapping reliable miRNA species between sample groups. (B) Euler diagram showing the number of reliable miRNA species in the HM cell and lipid samples and their overlap with infant formulae. (C) Box plot showing high expression of plant-based miR-159a (16 replicates in each infant formula) in the two formulae tested.

Differential miRNA expression analysis together with heat mapping and comparative analysis of miRNA species showed strong similarities in miRNA composition and profiles, including expression levels, between HM cell and lipid fractions. At the same time, maternal blood, both its PBMCs and plasma, had distinctly different miRNA profiles to HM cells and lipids (Figures 3A-3C). It has been previously shown that in mature HM of healthy mother-infant dyads, which is what was analysed in our study, the dominant cell type is the lactocyte (secretory mammary epithelial cell), which also secretes the HM lipids (Hassiotou and Geddes, 2015, Hassiotou et al., 2013a, Hassiotou et al., 2013x). Therefore, our findings strongly support the origin of HM cell and lipid miRNAs primarily from the mammary
epithelium via endogenous synthesis in the lactocytes. The maternal circulation may still likely have a small contribution to milk miRNA. This is in agreement with a recent study in the tammar wallaby reporting a weak correlation between maternal serum and skim milk miRNAs, suggesting that milk miRNAs are primarily synthesized in the mammary gland (Modepalli et al., 2014).

The origin of HM miRNA primarily from the mammary gland emphasizes their potential use as biomarkers of both lactation performance and the health of the gland. Indeed, HM immune cells have been shown to rapidly respond to maternal infections, with the most pronounced responses seen in abnormal conditions of the lactating breast such as mastitis (Hassiotou and Geddes, 2015, Hassiotou et al., 2013x), which if left untreated or are managed late, can result in early cessation of breastfeeding, with detrimental effects to both the infant and the mother (Fetherston, 1998). Recent studies have shown distinct miRNA responses in the milk of the dairy cow during mastitis, consistent with the immune cell response (Lawless et al., 2014). Moreover, family members of miR-29 (miR-29a/b/c), an abundant miRNA in mammalian milk, which was also found to be highly expressed in HM cell and lipid fractions in our study, was shown to epigenetically regulate lactation performance in the dairy cow (Bian et al., 2015). The distinct changes of milk miRNA in response to the status of the mammary gland together with our data supporting the mammary origin of milk miRNA highlight their potential diagnostic value as non-invasive and easily accessible biomarkers of mammary gland function and health to facilitate timely management of lactation difficulties and maintenance of breastfeeding for longer periods. In a similar context, circulating miRNAs in plasma have been successfully used as early biomarkers (Chen et al., 2008, Etheridge et al., 2011) of aberrant growth in breast cancer (Mattie et al., 2006) and of other diseases such as type 2 diabetes (Zampetaki et al., 2010).

To give insight into the content of artificial infant formulae in miRNA, we compared the miRNA profiles of a bovine milk-based and a soy-based formulae that are in high demand in the Australian market. We found very few human mature miRNAs in both of these formulae and a poor miRNA representation compared to HM (Figure 2A). The bovine milk-based formula clustered more closely with HM cell and lipid miRNAs than the soy-based formula (Figure 2A, 2B), likely due to the mammalian milk basis of the former versus the plant basis of the latter, yet still very poorly correlated with HM. Skim bovine milk has been shown to harbor 245 miRNAs (Chen et al., 2010), some of which are similar to those in HM, but most of these miRNAs were not found to be present in the bovine milk-based formula analysed in our study. Although miRNAs have been shown to be highly stable in
infant formula (Chen et al., 2010, Izumi et al., 2012), the first step of the formula manufacturing process (for both animal milk-based and plant-based formulae) discards the milk fat layer as well as cell debris by extremely high speed centrifugation followed by pasteurization (Food and Drug Administration, 2014, Gigli and Maizon, 2013), and therefore likely excludes the sources that are rich in miRNA.

Interestingly, miR-159a was the most highly expressed miRNA in both the bovine milk-based and the soy-based formulae (Figure 4C). This is a plant-specific miRNA not detected in any of the HM samples analyzed. It is possible that it originated from the nutrition of the animals from which milk was sourced for the bovine formula. Moreover, it may come from vegetable fats, such as soybean oil, that are added to the bovine formula during manufacturing as per its ingredients, though intentional addition of this miRNA or contamination during preparation and processing cannot be excluded either. This requires validation with further samples of this formula. It is not known whether this miRNA survives in and can be absorbed by the GI tract of the infant to exert gene regulatory functions, and this warrants further investigation. This is likely given that other plant food-derived miRNAs, such as miR-168a, have been shown to influence gene expression in adult humans (Zhang et al., 2012a).
Figure 5. Example of molecular pathway (epithelial-to-mesenchymal transition, EMT) controlled by some of the most highly expressed reliable miRNAs detected in HM (miR-200a/b/c, miR-429, miR-141, and 205), and how they interact with other genes to regulate EMT.

Recently, exogenous miRNAs have been experimentally proven to regulate gene expression in mammalian cells (Zhang et al., 2012a, Baier et al., 2014, Arntz et al., 2015). These miRNAs are transferred to humans via consumption of food, therefore it is highly likely that the same transfer of HM miRNA to the breastfed infant occurs, especially since the neonatal stomach is less acidic (Fallingborg, 1999) and the gut highly leaky early in life.
(Gareau, 2011). A recent study in adult humans demonstrated that after 4 to 8 hours of consuming bovine milk, miR-29b and miR-200c increased in the plasma, returning to baseline levels after 24 hours of the initial consumption (Baier et al., 2014). Further, after bovine milk consumption, the expression of runt-related transcription factor 2 (RUNX2), targeted by miR-29b, was elevated in PBMCs (Baier et al., 2014). A recent study by Arntz et al. further demonstrated uptake of milk-derived miRNA by mammalian cells and a therapeutic function in ameliorating experimental arthritis in mice (Arntz et al., 2015). Therefore, the high presence of miRNAs in HM further supports its function as a biofluid initiating epigenetic signals in infants that could potentially influence infant development and health. In our study, GeneGo analysis and Ingenuity Pathway Analysis (IPA) revealed a number of biological functions and pathways that are controlled by miRNA enriched in HM, including immunity, growth and development, cell proliferation and apoptosis, lung epithelial progenitor cell differentiation, and epithelial-to-mesenchymal transition (EMT) (Figures 5). EMT has been found to be a key player in cell differentiation, motility and migration in multiple tissues and organs, particularly during embryogenesis and in cancer (Shackleton et al., 2006). It has been shown to be normally present in both the human (Hassiotou et al., 2012) and the murine mammary gland (May et al., 2011), with potential important functions in normal breast remodeling required for milk synthesis. Our results suggest that the EMT process in the normal lactating breast may be mediated by pregnancy- and lactation-specific miRNAs, which requires further investigation.

Consistent with previous studies in HM, some of the most abundant milk miRNAs detected here are known to contribute to metabolic processes, decrease the cancer risk of infants (Munch et al., 2013), participate in the development of the infant’s immune system, and protect infants from infections (Kosaka et al., 2010, Zhou et al., 2012, Munch et al., 2013). Moreover, miRpath (Vlachos et al., 2012) and KEGG (Kanehisa et al., 2014) analyses showed that the majority of the top 10 highly expressed miRNAs in both HM fractions examined here (cells and lipids) are involved in the regulation of the cell cycle and the RNA transportation process during development (Supplementary 2, Figure S1A). Normal cell cycle and RNA transport in the lactating breast is required for normal cell differentiation and proliferation of the lactocyte (Hassiotou et al., 2013a), and also to prevent breast cancer initiation by controlling cell proliferation (Fernandez et al., 1998). In contrast, the most highly expressed miRNAs in PBMCs were found to be involved in different molecular pathways to those of HM, such as the maintenance of normal gene activity of K-Ras, HER2 and CDK4, which are known oncogenes (Supplementary 2, Figure
S1B) (Calin and Croce, 2006). Further, miRNAs found to be highly expressed in the maternal plasma are known to be involved in the control of oncogenes in melanoma, pancreatic and colorectal cancers (Etheridge et al., 2011).

Collectively, our data together with previous studies demonstrate that miRNAs are abundant in HM and likely play significant roles in the development and normal function of the lactating mammary gland, and in the HM fed infant. We have provided evidence that HM miRNA are primarily synthesized in the mammary epithelium, and may therefore be used as novel diagnostic biomarkers of lactation performance and breast infection. Further research is required to identify the functions of these miRNAs and examine potential novel miRNAs that may be present in the milk and the breast. Moreover, factors that may influence the miRNA content and expression levels of HM should be investigated in an effort to standardize milk miRNA studies and elucidate the maternal-infant interaction in the regulation of these molecules during breastfeeding.
CHAPTER 5

HUMAN MILK CELLS CONTAIN NUMEROUS KNOWN AND NOVEL miRNAs THAT REGULATE MULTIPLE PHYSIOLOGICAL PROCESSES AND MAY CHANGE IN RESPONSE TO MILK REMOVAL

5.1 ABSTRACT

Human milk (HM) is a complex biofluid conferring nutritional, protective and developmental elements required for optimal infant growth. Amongst these are maternal eukaryotic cells, which change in response to feeding and were recently shown to be a rich source of miRNAs. We used next generation sequencing to characterise the cellular miRNA profile of HM collected before and after feeding from exclusively breastfeeding mothers in month 2 of lactation. HM cells conserved higher miRNA content than previously published lipid and skim HM fractions or other body fluids. In both pre- and post-feed milk, 1,467 known mature and 1,996 novel miRNAs were identified, with 89 high-confidence novel miRNAs. HM cell content was higher post-feeding (p<0.05), and was positively associated with total miRNA content (p=0.014) and species number (p<0.001). This coincided with upregulation of 29 known and 2 novel miRNAs, and downregulation of 4 known and 1 novel miRNAs post-feeding (p<0.05), but no statistically significant change in expression was detected for the remaining miRNAs (p>0.05). These findings suggest that feeding may influence the miRNA content of HM cells, with the most highly and differentially expressed miRNAs being key regulators of milk components, indicating their diagnostic value in lactation performance. They are also involved in the control of body fluid balance, thirst, appetite, immune response, and development, implicating their functional significance for the infant.

5.2 INTRODUCTION

Human milk (HM) is a complex system of nutritional and bioactive components that together offer the essential building blocks for the optimal growth, development and protection of the human infant (Hassiotou and Geddes, 2013, Stevens et al., 2009). Latest research has shifted focus from the nutritional components of HM, which have been well studied, to its bioactive elements, including maternal cells and the molecules they secrete, such as microRNAs (miRNAs) (Alsaweed et al., 2015u, Hassiotou and Geddes, 2015, Hassiotou and Hartmann, 2014). The maternal cells of HM are primarily of mammary epithelial origin when the mother and infant are healthy, but they are dominated by immune cells originating from the maternal circulation in the first days postpartum (colostrum) and during periods of infection of either the mother or the infant (Hassiotou et al., 2013af, Hassiotou and Geddes, 2015, Riskin et al., 2012). miRNAs are small non-coding RNA molecules (~22 nucleotides long) (Kosaka et al., 2010, Weber et al., 2010, Zhou et al., 2012, Munch et al., 2013) that are present in all three fractions of HM (cells, lipids and skim milk)
(Weber et al., 2010, Kosaka et al., 2010, Munch et al., 2013, Alsaweed et al., 2015bs), and have been shown to originate mainly from mammary epithelial cells (Alsaweed et al., 2015bs). They are potent regulators of gene expression at the post-transcriptional level (Bartel, 2004), and are involved in several biological processes including apoptosis, cell differentiation, development and growth of various tissues and organs (He and Hannon, 2004). miRNAs are abundant in HM compared to other body fluids, such as plasma and peripheral blood mononucleated cells (Weber et al., 2010, Kosaka et al., 2010, Munch et al., 2013, Alsaweed et al., 2015ab, Alsaweed et al., 2015bs), and are likely to be transferred to the infant via the exosomal and cellular HM components (Lasser et al., 2011, Admyre et al., 2007, Alsaweed et al., 2015u). The exosomes release miRNAs by three main mechanisms. These are fuse with the recipient cells, internalized into the recipient cells and directly interact with recipient cell’s receptors (Zhang et al., 2015). Also, cells may release their miRNAs using one of the above mechanisms, especially fusing mechanisms.

The roles of HM miRNA in the infant have not yet been explored. However, most recently, food-derived miRNAs, including those derived from commercially available bovine milk, were demonstrated to integrate into human and animal cells \textit{in vitro}, and also survive the gastrointestinal tract \textit{in vivo}, transferring to the systemic circulation and various organs, where they exert gene regulatory functions at the cellular level (Zhang et al., 2012a, Baier et al., 2014, Jiang et al., 2012, Zhang et al., 2012q, Arntz et al., 2015, Wolf et al., 2015, Vaucheret and Chupeau, 2012, Zhou et al., 2015). Some of these miRNAs were further shown to have therapeutic effects \textit{in vivo}, including amelioration of experimental rheumatoid arthritis in a mouse model (Arntz et al., 2015). And due to the lack of supporting evidence for transfer of certain plant-derived miRNAs into mammalian cells (Dickinson et al., 2013, Yang et al., 2015), it has been proposed that the packaging of miRNA in transporting ‘vehicles’, including exosomes and somatic cells, which is typical of milk miRNA, plays an essential role in their survival and integration in mammals (Alsaweed et al., 2015u). This, together with the high stability of miRNAs under harsh conditions (Zhou et al., 2012, Kosaka et al., 2010), and given that the infant’s stomach is less acidic and the gut more permeable than adults (Fallingborg, 1999), have provided strong support for the survival, absorption and integration of HM miRNA in the infant, and their potential functional significance in early growth, protection, and development. It is therefore important to characterise the miRNA content of HM and examine factors that may influence it.
Milk miRNA have mostly been studied in the skim milk fraction (Weber et al., 2010, Kosaka et al., 2010, Chen et al., 2010, Izumi et al., 2014, Modepalli et al., 2014), with recent studies in milk lipids (Munch et al., 2013, Alsaweed et al., 2015ab) and cells (Alsaweed et al., 2015bs) showing that skim milk (also called milk plasma) is not as rich in miRNA content and composition as the other two milk fractions, and that milk cells conserve more miRNA species than milk lipids. Indeed, this is also the case for blood plasma compared to blood cells (Alsaweed et al., 2015bs). Yet, studies on the miRNA content of milk cells are scarce (Alsaweed et al., 2015bs), with no study to our knowledge, examining the milk cellular miRNA profile using deep sequencing for discovery of novel miRNA. In 2013, Munch et al. used next generation sequencing in the HM lipid fraction and detected 21 novel miRNAs (Munch et al., 2013). It is likely that HM cells, being richer in miRNA, conserve more novel miRNA than HM lipids, which may be influenced by milk removal. Cell content is known to increase post-feeding in HM (Hassiotou et al., 2013ah), similar to fat content (Hassiotou et al., 2013ah, Khan et al., 2013), suggesting that changes in the milk miRNA content and composition may also occur with feeding. To further examine the effects of milk removal and characterise the miRNA profile of the cells of HM, we purified and quantified miRNAs from the cell fraction of HM collected before and after feeding from exclusively breastfeeding mothers in month 2 of lactation, and performed deep sequencing using the Illumina (Solexa) platform.

5.3 MATERIALS AND METHODS

5.3.1 Ethics statement and sample collection
This study was approved by the Human Research Ethics Committee of The University of Western Australia, and all methods were conducted in accordance with the approved guidelines (Supplementary 3, Figure S10). All participants provided informed written consent. Exclusively breastfeeding mothers in month 2 of lactation (week 4 to 8) were recruited in this study (n=16), and were all healthy, including their infants, at the time of sample collection. A volume of 5 mL of milk was obtained before a morning breastfeeding session (pre-feed sample) from the fuller breast, and then a second 5-mL milk sample was collected from the same breast immediately after feeding (post-feed sample). The feeding sessions lasted for at least 5 minutes and the amount of milk taken by the infant was measured by weighing the infant before and after feeding, as described previously (Hassiotou et al., 2013ah). Samples were collected aseptically using an electric breast pump.
(Medela AG, Switzerland), and were immediately transported to the laboratory for milk fractionation and miRNA extraction.

5.3.2 Cell isolation and miRNA extraction from human milk
HM fat content was measured as previously described (Alsaweed et al., 2015bs). HM was fractionated into cells, skim milk and lipids as described previously (Hassiotou et al., 2012, Alsaweed et al., 2015bs). Briefly, freshly expressed HM was diluted 1:1 with PBS (Gibco, Life Technology, Foster, CA) and centrifuged at 800 g for 20 min at 20°C. Purified milk cells were washed in PBS at 800 g for 5 min at 20°C, and were then counted using a haemocytometer as previously described(Hassiotou et al., 2012). miRNA were extracted from all samples immediately without cryopreservation using the miRNeasy mini Kit (Qiagen, Hilden, Germany) according to previous optimization studies(Alsaweed et al., 2015ab). The concentration and purity of miRNAs were measured using a NanoDrop 2000 Spectrophotometer (Wilmington, DE, USA) and an Agilent Bioanalyzer 2100 instrument (Agilent, CA, USA) with the RNA 6000 Nano Chip kit (Agilent, CA, USA). All extracted miRNA samples were stored at -80°C, and pre- and post-feed samples from a subgroup of 10 mothers were used for small RNA sequencing and qPCR validation (Supplementary 3, Figure S10).

5.3.3 Statistical analysis of human milk miRNA content
All analyses were performed using R Studio Version 0.98.1103 package (RStudio Team, 2015). The additional packages nlme(Pinheiro et al., 2013) and lattice (Sarkar, 2015) were used for linear mixed effects modeling (LME) and graphical exploration of the data, respectively. Differences were considered to be significant if p<0.05. General linear hypothesis tests and LME were used to determine the differences between pre- and post-feed samples in total cell content, fat content, miRNA content and species number, as well as account for the effect of milk intake and other demographic characteristics of individual mother/infant dyads.
Table 1. A list of all known and novel miRNA species number in each sample with the total number of reads that were generated from small RNA sequencing using Solexa.

<table>
<thead>
<tr>
<th>Sample ID*</th>
<th>Number of known miRNAs</th>
<th>Number of known miRNA reads</th>
<th>Number of novel miRNAs</th>
<th>Number of novel miRNA reads</th>
</tr>
</thead>
<tbody>
<tr>
<td>1_B</td>
<td>790</td>
<td>10,037,707</td>
<td>107</td>
<td>1,507</td>
</tr>
<tr>
<td>1_A</td>
<td>859</td>
<td>9,203,046</td>
<td>74</td>
<td>1,374</td>
</tr>
<tr>
<td>2_B</td>
<td>563</td>
<td>6,483,425</td>
<td>209</td>
<td>2,267</td>
</tr>
<tr>
<td>2_A</td>
<td>532</td>
<td>8,344,307</td>
<td>249</td>
<td>3,667</td>
</tr>
<tr>
<td>3_B</td>
<td>840</td>
<td>10,137,502</td>
<td>108</td>
<td>1,182</td>
</tr>
<tr>
<td>3_A</td>
<td>876</td>
<td>9,424,413</td>
<td>103</td>
<td>1,045</td>
</tr>
<tr>
<td>4_B</td>
<td>820</td>
<td>9,539,244</td>
<td>90</td>
<td>1,138</td>
</tr>
<tr>
<td>4_A</td>
<td>814</td>
<td>9,694,427</td>
<td>90</td>
<td>1,242</td>
</tr>
<tr>
<td>5_B</td>
<td>750</td>
<td>5,658,703</td>
<td>35</td>
<td>366</td>
</tr>
<tr>
<td>5_A</td>
<td>823</td>
<td>8,164,895</td>
<td>53</td>
<td>709</td>
</tr>
<tr>
<td>6_B</td>
<td>833</td>
<td>9,378,155</td>
<td>140</td>
<td>1,410</td>
</tr>
<tr>
<td>6_A</td>
<td>899</td>
<td>8,811,523</td>
<td>131</td>
<td>1,224</td>
</tr>
<tr>
<td>7_B</td>
<td>468</td>
<td>6,534,047</td>
<td>184</td>
<td>1,918</td>
</tr>
<tr>
<td>7_A</td>
<td>662</td>
<td>8,058,217</td>
<td>157</td>
<td>1,268</td>
</tr>
<tr>
<td>8_B</td>
<td>759</td>
<td>9,732,524</td>
<td>115</td>
<td>1,240</td>
</tr>
<tr>
<td>8_A</td>
<td>707</td>
<td>7,599,182</td>
<td>118</td>
<td>1,178</td>
</tr>
<tr>
<td>9_B</td>
<td>750</td>
<td>7,744,119</td>
<td>143</td>
<td>1,310</td>
</tr>
<tr>
<td>9_A</td>
<td>586</td>
<td>7897409</td>
<td>222</td>
<td>2,009</td>
</tr>
<tr>
<td>10_B</td>
<td>789</td>
<td>10,327,701</td>
<td>179</td>
<td>1,864</td>
</tr>
<tr>
<td>10_A</td>
<td>780</td>
<td>11,415,988</td>
<td>330</td>
<td>3,405</td>
</tr>
</tbody>
</table>

Total: 1,467 174,186,534 Total: 1,996 31,323

* B refers to milk before feed (pre-feed milk); A refers to milk after feed (post-feed milk).
Table 2. Number of known and novel miRNAs in each sample group (pre- and post-feed milk) with the total reads (expression level).

<table>
<thead>
<tr>
<th>Sample group</th>
<th>Pre-feed milk (n=10)</th>
<th>Post-feed milk (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of known miRNAs</td>
<td>1,287</td>
<td>1,308</td>
</tr>
<tr>
<td>Total reads (known)</td>
<td>85,573,127</td>
<td>88,613,407</td>
</tr>
<tr>
<td>Number of novel miRNAs</td>
<td>961 (35**)</td>
<td>1,215 (41**)</td>
</tr>
<tr>
<td>Total reads (novel)</td>
<td>14,202 (5,282**)</td>
<td>17,121 (6,950**)</td>
</tr>
<tr>
<td>Number of specific miRNAs</td>
<td>159</td>
<td>180</td>
</tr>
</tbody>
</table>

*Novel miRNAs with > 20 total reads and identified in ≥4 samples
**High confidence novel miRNAs.

5.3.4 Library construction, small RNA sequencing, and bioinformatics analysis

All human milk cell miRNA samples to be analysed using small RNA sequencing (n=20) were standardized to 500 ng/µL. Libraries were created for each sample individually as previously described (Yi et al., 2013, Liu et al., 2014). Briefly, by using size fractionation, 18-30 nt in length of small RNAs (sRNAs) were obtained and ligated to 5’-RNA and 3’-RNA adapters. cDNA was created using small RNA primers. All cDNA were sequenced into two SE50 lanes, where ~10M reads were generated for each sample using Illumina HiSeq 2000 platform (Supplementary 3, Figure S10). Cleanup reads were done on raw reads to trim low or contaminate reads such as 5’ primer contaminants, no insert tags, oversized insertion, low quality reads, poly A reads, etc. Clean reads were distributed by length, where sRNAs were considered between 18-30 nucleotides in length. This step was utilised to identify miRNAs within different sRNAs (for example, miRNA is usually between 21 and 22 nt in length, siRNA is 24 nt, and piRNA is 30 nt). Further, the clean RNA reads were annotated by BLAST into different categories against Rfam (ftp://sanger.ac.uk/pub/databases/Rfam/) and GenBank (http://blast.ncbi.nlm.nih.gov/) to determine miRNA, siRNA, piRNA, rRNA, tRNA, snRNA, snoRNA, and repeat associated sRNA. Degraded fragments of mRNA within sRNAs were detected by alignment to exons and introns of mRNAs. Reads were then mapped to the human genome by bowtie to analyze their expression and distribution on the human genome. Any mapped sRNAs to exons, introns or intergenic regions of the human genome, which did not match any other RNAs, were predicted as novel miRNAs. Further, miRNAs were mapped to miRBase 21.0
(released June 2014) (http://www.mirbase.org/) using BLAST to identify human known mature miRNAs and their precursors. Unmatched miRNAs to known mature miRNAs in miRBase were mapped again to the human genome using the SOAP software to predict potential miRNAs. Usually, the characteristic precursor structure of miRNAs (pre-miRNAs) must be designed to predict potential novel miRNAs. The Mireap software (http://sourceforge.net/projects/mireap/) was used to predict the stem loop (pre-miRNAs) for novel mature miRNAs by exploring the secondary structure, the dicer cleavage site, and the minimum free energy of the unannotated small RNA reads. Then, potential miRNAs were further assessed by identification of base bias on the first position and the nucleotide length on each position.

5.3.5 Differential expression analysis

A comparison of the expression of both known and novel mature miRNAs between pre- and post-feed milk samples was done to investigate differentially expressed miRNAs. Expression between pre- and post-feed milk samples was normalised to obtain expression levels of transcript per million (TPM) using the following normalisation formula:

\[ \text{normalised expression} = \frac{\text{actual miRNA count}}{\text{total count of clean reads}} \times 1,000,000 \]

Fold change was calculated using the following formula:

\[ \text{Fold change} = \log_2(\text{normalised expressed miRNA from post-feed milk} / \text{normalised expressed miRNAs from pre-feed milk}) \]

DEGseq (R package) (Wang et al., 2010f) was used to determine p-values for fold expression change between pre- and post-feed samples, and to generate scatter plots. miRNA with p<0.05 was considered to be differentially expressed miRNA between pre- and post-feed milk.

5.3.6 qPCR validation

The top 5 most highly expressed known miRNAs and the top 4 novel miRNAs across all samples tested were used for qPCR validation of their presence and expression levels in pre- and post-feed HM samples from n=7 mothers. Milk samples were standardized equally to 500 ng of total miRNA. The known miRNAs examined were: hsa-let-7f-5p, hsa-miR-181a-5p, hsa-miR-148a-3p, hsa-miR-22-3p, and hsa-miR-182-5p. The novel miRNA sequences examined are listed below, with all primers and probes synthesized by Life Technologies and assayed using custom TaqMan small RNA (Life Technologies, CA): novel_mir_7-p5 UCCAUUAUCCCAACCUCUCAGAGU, novel_mir_299-5p ACUAGGAUUGUCUUCCCUGG, novel_mir_367-3p
UGCAGCGACCAUAGAGCCU, novel_mir_39-5p UCUGGCAUGGCCUUGGGCACU.

Reverse transcription and qPCR reaction were performed as previously described (Alsaweed et al., 2015ab). Relative quantitation (RQ) was obtained using 7500 software V2.0.6, and was compared between samples in the R Studio Version 0.98.1103 package (RStudio Team, 2015) using linear mixed effects (LME) models, where $p \leq 0.05$ was considered statistically significant.

5.3.7 Target prediction and functional analysis

Three different databases/algorithms were used to predict gene targets of all identified miRNAs and of the top 10 most highly expressed known and novel miRNAs. These are targetscan (http://www.targetscan.org/), RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid), and miRanda (http://www.microrna.org/microrna/home.do). Determined target genes (identified in two or three of the above databases) were used for the functional analysis, where the predicted target gene candidates of known and novel miRNAs were annotated to predict the number of genes involved in different cellular and signaling functions using the Gene Ontology (GO) database (http://www.geneontology.org/) (Ashburner et al., 2000). These target genes were classified into three enriched GO terms (cellular component, molecular function and biological process). This was conducted by mapping target gene candidates to GO terms using its database (http://www.geneontology.org/). The hypergeometric test was used to predict the significant GO terms in target gene candidates. Furthermore, enriched metabolic pathways were determined for target gene candidates using Kyoto Encyclopaedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) (Kanehisa et al., 2014).

5.3.8 Analysis of pathways, networks, and miRNA gene targets

The top 700 most highly expressed known mature miRNAs in HM cells (total count $\geq 40$ reads) in all sequenced samples ($n=20$) were uploaded and analysed in QIAGEN’s Ingenuity Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). This computer simulation software was used to determine the possible interactions and relationships of the identified highly expressed known miRNAs in different signalling and metabolic pathways. Further, it was used to build molecular networks between the target genes and the identified highly expressed mature known miRNAs through the 6-8 seed region between mRNA and miRNA. Target genes of the uploaded miRNAs (100K reads or above = top 23 miRNAs) were identified using different databases including TarBase,
miRecords, Targetscan (IPA tool “MicroRNA Target Filter”), and the IPA findings. All identified target genes were either experimentally verified or were strongly predicted to interact with mature miRNA sequences. Functional analyses included biological roles and related diseases that were associated with more miRNA species (100K reads or above = top 23 miRNAs). p-value was calculated using Fisher’s exact test to determine pathways (biological function and/or related disease) that significantly associate with the uploaded unique miRNAs.

5.3.9 Availability of supporting data

All raw small RNA sequences are available in the NCBI Gene Expression Omnibus database under accession number GSE71098. Additional information is also included in Supplementary 3.

![Figure 1](image)

**Figure 1.** Filtering and annotation of the small RNA reads. (A) The total number of small RNA raw reads including miRNAs, and the total number of clean reads that were generated after filter analysis. (B) Percentage of the nucleotide length distribution of clean small RNAs after filter analysis. (C) Total clean reads were annotated to one or more RNA categories (i.e. rRNA, tRNA, scRNA etc.). (D) Small RNAs were mapped to only one RNA
category using the following priority rule: rRNA etc (in which Genbank > Rfam) > known miRNA > piRNA > repeat > exon > intron. rRNAs were used as a marker of sample quality, with the criterion of high quality when <40% in each sample.

### 5.4 RESULTS

#### 5.4.1 Human milk cells conserve numerous known and novel miRNAs

Species and expression levels of known and novel miRNAs were examined using Illumina HiSeq 2000 in pre- and post-feed HM samples collected from a subgroup of 10 mother/infant dyads. By using two sequencing lanes, 293,932,547 reads were generated from all samples, in which 268,681,616 (91.40%) were determined as clean reads of small RNAs (Figure 1A) after filtering analysis. Length distribution analysis was done on the clean reads where a highly percentage (61.42%) of the reads were distributed between 21-23 nucleotides in length, which is considered as the ideal transcript length for miRNAs (Figure 1B). The total and unique clean reads were matched to Genebank and RFam to identify and remove tRNA, rRNA, snRNA, snoRNA, and repeat small RNAs (Figure 1C-1D). Thereafter, 208,829,362 reads were retained for miRNA analysis, consisting of 2,612,363 unique sequences. Retained small RNA reads were aligned to human genome using SOAP to analyze expression and distribution of these small RNAs on the genome (Supplementary 3, Figure S1). All retained reads (208,829,362) were matched to miRBase 21.0 ([http://www.mirbase.org/](http://www.mirbase.org/)) using BLAST to identify known miRNAs. Matched miRNA sequences were analysed to determine the base bias on the first position with certain length and on each position (Supplementary 3, Figure S2A-S2B). Unannotated reads (unmapped reads) to the human genome that did not map to any other RNA classes, including miRNAs that were identified as known in miRBase, were used for novel miRNA prediction analysis (Figure 1C-1D). To predict hairpin structure of miRNA precursors for unannotated mature miRNA reads, the mireap software ([http://sourceforge.net/projects/mireap/](http://sourceforge.net/projects/mireap/)) was used to explore the secondary structure, the Dicer cleavage site and the minimum free energy of the unannotated small RNA reads.

With no mismatched allowed between the profiled sequences and miRBase, 1,467 known miRNAs were identified, consisting of 174,186,534 reads (Table 1; Supplementary 3, Table S1). Of these, 1,287 known miRNAs (total reads=85,573,127) were detected in pre-feed HM, whilst 1,308 known miRNAs (total reads=88,613,407) in post-feed HM (Table 2). Moreover, a total of 1,996 miRNA species was discovered as novel miRNAs in
all samples (Table 1 and Supplementary 3, Table S2). Of these, 961 novel miRNAs (14,202 reads) were identified in pre-feed HM, whilst 1,215 novel miRNAs (17,121 reads) in post-feed HM (Table 2). To narrow down the novel miRNA species number to those of high confidence, we selected those predicted novel miRNAs with ≥20 reads and which were identified in ≥4 out of 20 pre- and post-feed milk samples. Using this criterion, 89 novel miRNAs were identified in all samples tested, with a total of 15,337 reads (Supplementary 3, Table S3).

The hairpin structure of novel miRNA precursors were predicted for all identified novel mature miRNAs, and the structures of the high confidence novel miRNAs are listed in Supplementary 3, Table S4. qPCR was used to validate the presence of the top 4 most highly expressed novel miRNAs across samples. Except novel-miR-299-5p, which was not expressed in 2 samples (pre-feed milk sample 6, and post-feed milk sample 3), all other 3 novel miRNAs tested were expressed at high levels in all samples (Figure 2F-2I).

Higher cell content in either pre- or post-feed milk was associated with greater miRNA content measured using the Bioanalyzer (p=0.014, n=20 samples). In turn, greater miRNA content was associated with higher number of known and total (known+novel) miRNA species (p=0.003 and p<0.001, respectively, n=20 samples) (Figure 3A-3B); however the same was not observed for novel miRNAs (p=0.679, n=20 samples). Similarly, greater cell content was associated with more known and total (known+novel) miRNAs (p=0.004 and p<0.001, respectively, n=20 samples), but not novel miRNAs (p=0.644, n=20 samples) (Figure 3C). The volume taken by the infant was not related to known (p=0.50, n=10) or novel (p=0.90, n=10) miRNA species number. Interestingly, an inverse relationship was found between the number of identified known and novel miRNAs, where samples with high number of novel miRNA species had low number of known miRNA species, and vice versa (p=0.02, n=10).
Figure 2. Bar plots showing the expression patterns of the top 5 most highly expressed known miRNAs, and the top 4 most highly expressed novel miRNAs using qPCR. Y axes indicate the expression of each miRNA relative to the endogenous control used (RNU48), whilst X axes indicate Participant ID. A subgroup of 7 mothers was used for qPCR validation. Linear mixed effects modeling showed no significant differences (p>0.05) in expression of these known and novel miRNAs between pre- and post-feed milk samples for each mother. novel-miR-299-5p was not detected in sample ID 6 (pre-feed milk) and in sample ID 3 (post-feed milk).

5.4.2 Effects of feeding on the miRNA content of human milk cells

Overall, the total number of cells per mL of HM was higher post-feeding compared to pre-feeding (p=0.028, n=16) (Figure 3D; Table 3), and this increase was not related to the milk volume taken by the infant (p=0.207, n=16). It is of note that in the smaller subgroup of n=10 mothers, the change in cell content post-feeding did not reach significance (p=0.668, n=10). HM fat content was significantly higher in post-feed milk (p<0.001, n=16) than pre-feed milk, and this change was related to the volume taken by the infant (p=0.025, n=16). Further, a positive association was found between fat content and cell content of HM (p=0.006, n=16) (Supplementary 3, Figure S3A-S3B). Within a dyad, the total miRNA
content per 10⁶ cells did not differ between pre- and post-feed milk (p=0.658 with NanoDrop, n=16; p=0.971 with Bioanalyzer, n=10) (Figure 3E; Table 3), even after accounting for the milk volume taken by the infant. In 7 out of 10 participants, 9-167 additional total (known+novel) miRNA species (76 ± 60 mean ± standard deviation) were detected in post-feed milk. In 3 out of 10 participants, the number of miRNA species was slightly lower in pre-feed than post-feed milk. Interestingly, in those participants, the differences in cell content pre- and post-feeding were also minimal compared to the other participants. In these 10 mothers, no statistically significant difference in the known or novel or total (known+novel) miRNA species number was found between pre- and post-feed milk within a dyad (p=0.756, p=0.509, and p=0.412, respectively, n=10), and this was not influenced by the milk volume taken by the infant (p>0.05). However, when comparisons were made between mother/infant dyads in post-feed samples, the total cell content per mL milk was positively associated with the number of known and total (known+novel) miRNA species (p=0.035 and p<0.001, respectively, n=10), but not with the number of novel miRNAs (p=0.689, n=10). This was in agreement with the associations observed when all pre- and post-feed milk samples from all dyads were considered (see above).

The number of reads was used to identify the most highly expressed miRNAs. The top 19 most highly expressed known miRNAs were consistently present in pre- and post-feed milk, and represented 86.2% of all expressed miRNAs (Figure 3F-3G). The top 5 most highly expressed known and top 4 novel miRNAs were validated using qPCR. Expression patterns of the top 5 known miRNAs did not differ between pre- and post-feed milk (p>0.05, n=7) (Figure 2A-2I), similar to the sequencing analysis. After normalizing the 5 selected known miRNAs to RNU48, mother with ID 1 showed greater expression of all the 5 miRNAs compared to the other 6 mothers including the reference sample (mother with ID 4). Expression patterns were very similar amongst mothers (Figure 2A-2E). LME modeling showed no differences in expression of the 4 novel miRNAs examined between pre- and post-feed milk (p>0.05, n=7) (Figure 2F-I).

Known miRNAs specific to either pre- or post-feed milk were also seen, with 159 miRNAs specific to pre-feed milk that were not identified in any post-feed milk samples, and 180 miRNAs specific to post-feed milk that were not identified in any pre-feed milk samples. These pre- or post-feed milk-specific miRNAs were expressed at low levels (<14 total reads in 10 post-feed milk samples, and <12 total reads in 10 pre-feed milk samples). However, the majority of known miRNAs were identified in both pre- and post-feed milk,
with 1,128 out of 1,467 known mature miRNAs commonly determined in pre- and post-feed milk samples (Supplementary 3, Table S5). In contrast, most of the novel miRNAs were discovered in either pre- or post-feed milk, and only 180 novel miRNAs were seen in both pre- and post-feed milk. More novel miRNAs were found to be specific to post-feed milk than pre-feed milk samples, with 1,035 novel miRNAs only seen in post-feed milk, and 781 novel miRNAs only seen in pre-feed milk (Supplementary 3, Table S6). The effects of feeding on the expression of known and novel miRNAs in both pre- and post-feed milk samples are shown in scatter plots (Figure 4A-4B). Of the miRNAs commonly found in pre- and post-feed milk, 33 known miRNAs were differentially expressed between pre- and post-feed milk (p<0.05, n=10), of which 29 were upregulated in post-feed milk (Supplementary 3, Table S7). Of the novel miRNAs universally present in both pre- and post-feed milk samples, 3 were differentially expressed between pre- and post-feed milk, of which 2 were upregulated post-feeding (Supplementary 3, Table S8).

Interestingly, few of the differentially expressed miRNAs were highly expressed, such as hsa-miR-191-5p, which was abundant in both pre- and post-feed milk (total reads=1,140,649) and was upregulated post-feeding (p=0.002). Most of the top 10 most highly expressed known miRNAs were similarly expressed within a mother/infant dyad (pre- and post-feeding). However in one dyad, has-miR-141-3p was downregulated in post-feed milk compared to pre-feed milk, whereas in another dyad has-miR-375 was upregulated in post-feed milk. The top 10 most highly expressed miRNAs were clustered together, with a strong correlation in expression in pre- and post-feed milk seen between has-miR-30d-5p and has-miR-22-3p, and between has-let-7f-5p and has-let-7a-5p (Figure 4C).
Table 3. The mean±standard deviation of HM cell content, and miRNA content and quality using NanoDrop 2000 and Bioanalyzer 2100 respectively, in pre-feed and post-feed milk samples (n=10 each).

<table>
<thead>
<tr>
<th></th>
<th>Mean ± S.D. of the number of total milk cells/mL milk (cell viability %)</th>
<th>Mean ± S.D. of total miRNA content (ng/10⁶ cells)</th>
<th>Mean ± S.D. of miRNA quality (OD 260/280 &amp; RIN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All samples (n=20)</td>
<td>1,222,860 ± 767,091 (92.7%)</td>
<td>1,414 ± 519</td>
<td>2.05 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>NanoDrop 2000</td>
<td>1,000 ± 438</td>
<td>8.67 ± 1.22</td>
</tr>
<tr>
<td></td>
<td>Bioanalyzer 2100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-feed milk (n=10)</td>
<td>1,146,364 ± 843,594 (91.3%)</td>
<td>1,391 ± 571</td>
<td>2.04 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>NanoDrop 2000</td>
<td>996 ± 481</td>
<td>8.57 ± 1.69</td>
</tr>
<tr>
<td></td>
<td>Bioanalyzer 2100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Post-feed milk (n=10)</td>
<td>1,299,356 ± 719,432 (93.3%)</td>
<td>1,438 ± 490</td>
<td>2.05 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>NanoDrop 2000</td>
<td>1,004 ± 417</td>
<td>8.77 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>Bioanalyzer 2100</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Top 20 most highly expressed known and novel miRNAs across all 20 pre- and post-feed milk samples with the total reads, and the number of samples that each miRNA was detected in.

<table>
<thead>
<tr>
<th>Known miRNA</th>
<th>Number of reads</th>
<th>Number of samples detected in (out of 20 samples)</th>
<th>Novel miRNA</th>
<th>Number of reads</th>
<th>Number of samples detected in (out of 20 samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. hsa-let-7f-5p</td>
<td>25,479,884</td>
<td>20</td>
<td>novel_mir_7</td>
<td>3890</td>
<td>13</td>
</tr>
<tr>
<td>2. hsa-miR-181a-5p</td>
<td>21,601,482</td>
<td>20</td>
<td>novel_mir_299</td>
<td>942</td>
<td>19</td>
</tr>
<tr>
<td>3. hsa-miR-148a-3p</td>
<td>14,925,495</td>
<td>20</td>
<td>novel_mir_367</td>
<td>804</td>
<td>17</td>
</tr>
<tr>
<td>4. hsa-miR-22-3p</td>
<td>12,999,002</td>
<td>20</td>
<td>novel_mir_39</td>
<td>789</td>
<td>20</td>
</tr>
<tr>
<td>5. hsa-miR-182-5p</td>
<td>11,288,364</td>
<td>20</td>
<td>novel_mir_115</td>
<td>760</td>
<td>17</td>
</tr>
<tr>
<td>6. hsa-let-7a-5p</td>
<td>10,343,580</td>
<td>20</td>
<td>novel_mir_476</td>
<td>658</td>
<td>4</td>
</tr>
<tr>
<td>7. hsa-miR-375</td>
<td>9,145,308</td>
<td>20</td>
<td>novel_mir_90</td>
<td>586</td>
<td>17</td>
</tr>
<tr>
<td>8. hsa-miR-141-3p</td>
<td>8,577,962</td>
<td>20</td>
<td>novel_mir_41</td>
<td>341</td>
<td>12</td>
</tr>
<tr>
<td>9. hsa-miR-30a-5p</td>
<td>7,844,936</td>
<td>20</td>
<td>novel_mir_269</td>
<td>322</td>
<td>15</td>
</tr>
<tr>
<td>10. hsa-miR-30d-5p</td>
<td>6,173,556</td>
<td>20</td>
<td>novel_mir_161</td>
<td>295</td>
<td>9</td>
</tr>
<tr>
<td>11. hsa-miR-146b-5p</td>
<td>4,140,259</td>
<td>20</td>
<td>novel_mir_278</td>
<td>247</td>
<td>14</td>
</tr>
<tr>
<td>12. hsa-miR-26a-5p</td>
<td>3,876,162</td>
<td>20</td>
<td>novel_mir_76</td>
<td>222</td>
<td>13</td>
</tr>
<tr>
<td>13. hsa-miR-21-5p</td>
<td>3,457,523</td>
<td>20</td>
<td>novel_mir_430</td>
<td>194</td>
<td>14</td>
</tr>
<tr>
<td>14. hsa-let-7i-5p</td>
<td>2,264,279</td>
<td>20</td>
<td>novel_mir_144</td>
<td>189</td>
<td>3</td>
</tr>
<tr>
<td>15. hsa-miR-92a-3p</td>
<td>1,918,918</td>
<td>20</td>
<td>novel_mir_456</td>
<td>178</td>
<td>9</td>
</tr>
<tr>
<td>16. hsa-miR-27b-3p</td>
<td>1,849,454</td>
<td>20</td>
<td>novel_mir_202</td>
<td>174</td>
<td>12</td>
</tr>
<tr>
<td>17. hsa-miR-181b-5p</td>
<td>1,594,211</td>
<td>20</td>
<td>novel_mir_159</td>
<td>173</td>
<td>12</td>
</tr>
<tr>
<td>18. hsa-miR-423-5p</td>
<td>1,374,443</td>
<td>20</td>
<td>novel_mir_411</td>
<td>169</td>
<td>11</td>
</tr>
<tr>
<td>19. hsa-miR-125a-5p</td>
<td>1,260,164</td>
<td>20</td>
<td>novel_mir_251</td>
<td>156</td>
<td>8</td>
</tr>
<tr>
<td>20. hsa-miR-10a-5p</td>
<td>1,145,612</td>
<td>20</td>
<td>novel_mir_425</td>
<td>154</td>
<td>7</td>
</tr>
</tbody>
</table>
5.4.3 Human milk cellular miRNAs are regulatory agents in the mammary gland

Due to the large number of the identified known and novel miRNAs in this study, only the top 10 known and novel miRNAs were used for gene target, GO and KEGG analyses (Table 4). A range of computational approaches was used to predict the target genes of the top 10 miRNAs, including targetscan, RNAhybrid and miRanda. A total of 26,200 targets were predicted for the top 10 known miRNAs (17,586 unique targets), whilst 16,453 targets were predicted for the top 10 novel miRNAs (13,066 unique targets). All identified targets that were regulated by the top 10 most highly expressed known and novel miRNAs were classified using GO and KEGG databases to determine gene functions and metabolic pathways, respectively (Supplementary 3, Table S9-S12; Supplementary 3, Figure S4A-S4B). Similar to the above target prediction analysis, IPA was used to predict the targets (experimentally confirmed or highly predicted) of the most highly expressed miRNAs (100K reads or above = top 23 miRNAs). 8,925 unique targets were determined for the top 23 most highly expressed miRNA (Supplementary 3, Table S13), with functions in immune response, development, growth, metabolism, and cell cycle (Supplementary 3, Table S14).

More specifically, KEGG pathway analysis revealed involvement of the abundant HM miRNAs in many complex metabolic networks, such as glycerophospholipid metabolism, porphyrin and chlorophyll metabolism, and nitrogen metabolism (Supplementary 3, Table S11-S12), with the top one targeted pathway being the renin-angiotensin system (RAS) (Supplementary 3, Figure S5), which controls body fluid balance and blood pressure. Some of the highly expressed and abundant HM cell miRNAs (miR-181a-5p/101-3p/148a-3p/30a-5p/16-5p/141-3p/22-3p/182-5p and let-7f-5p) control ATPase expression and triacylglycerol synthesis (Figure 5A), with the latter forming the basis of HM lipids (Figure 5B), as well as regulate GLUT1 expression, which is associated with lactose synthesis (Figures 6). Furthermore, some of the most highly expressed HM cell miRNAs (miR-181a-5p/375-3p/148a-3p/30a-5p/16-5p/141-3p/22-3p/182-5p/125b-5p and let-7f-5p) control mammary signaling via direct effects on numerous receptors, including the growth hormone receptor (GHR) and its phosphorylation by JAK2 (Supplementary 3, Figures S6); the insulin-like growth factor-I receptor (IGF-IR); the insulin receptor (INSR) (Supplementary 3, Figures S7), and estrogen receptor genes (ERα and ERβ) (Supplementary 3, Figures S8). miRNAs associated with anti-cancer effects in the breast and other organs (miR-181a-5p/148a-3p/30a-5p/141-3p/22-3p/182-5p and let-7f-5p) as well as with immune responses to disease (miR-148a-3p/ miR-181a-5p/182-5p/16-5p/99b/5p and let-7f-5p) were also identified at high expression levels in HM cells.
Figure 3. Comparison of HM cell content, total miRNA content, and profiled miRNA species between pre- and post-feed milk. (A, B) A positive association was found between the total miRNA content and the number of known miRNAs, using either NanoDrop or Bioanalyzer. (C) Positive relationship between total cell content per mL of HM and the number of known miRNA species. (D, E) Comparisons of total cell content per mL of HM and miRNA content (ng/10^6 cells) between pre- and post-feed milk. The top 19 most highly expressed miRNA species were identical in pre-feed milk (F) and post-feed milk (G), and accounted for 86.2% of the total miRNAs in all samples (n=20).
5.5 DISCUSSION

Human milk is one of the richest sources of miRNA known to date, with the majority of milk miRNA being protected within milk cells, fat globules and exosomes, and primarily originating from the lactating epithelium, potentially exerting lactation-specific functions (Alsaweed et al., 2015u, Alsaweed et al., 2015ab, Alsaweed et al., 2015bs, Zhou et al., 2012, Munch et al., 2013). Their high stability to harsh conditions (Kosaka et al., 2010, Zhou et al., 2012) and their vehicle-mediated transporting mechanism (Alsaweed et al., 2015u) further increase their likelihood of survival in the gastrointestinal tract of the infant, to subsequently be absorbed into the bloodstream for tissue-specific functions (Alsaweed et al., 2015u). Indeed, miRNA derived from bovine milk consumed by adults or animals have been shown to transfer into the plasma and perform regulatory and therapeutic functions (Alsaweed et al., 2015u, Baier et al., 2014, Wolf et al., 2015). HM cells are known to survive in the infant’s gut, diapedese through the intestinal mucosa and enter the bloodstream as well as various tissues, where they integrate and differentiate into functional cells (Hassiotou et al., 2015). HM cells including epithelial cells are therefore important carriers of miRNA that provide regulatory signals to the infant, via realising their miRNAs in the infant’s gut. Here, we characterised the miRNA content of HM cells using next generation sequencing, which has only previously been carried out in the lipid and skim milk fractions (Munch et al., 2013, Zhou et al., 2012). Numerous known and novel miRNA species were identified, which positively correlated with the number of HM cells (Figure 3C). Although some of the most highly expressed known miRNAs in HM cells were conserved amongst lactating mothers (Figure 3F-3G), the variation seen in individual dyads (Supplementary 3, Table S7), particularly in respect to the novel miRNA species, suggests that a component of miRNA-mediated regulation is dyad-specific. Whilst milk removal by the infant does not consistently influence the content and/or expression of miRNAs in HM cells (Figure 4A-4B), specific miRNAs did increase from pre- to post-feed milk, similar to HM cell and fat content (Hassiotou et al., 2013ah).
Table 5. Number of highly expressed experimentally validated known miRNAs (reads>100K) involved in different molecular, cellular and developmental functions, or disease.

<table>
<thead>
<tr>
<th>Top function or disease involvement</th>
<th>P value</th>
<th>Number of miRNAs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molecular and Cellular Functions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell development</td>
<td>5.00E-02 - 6.09E-10</td>
<td>24</td>
</tr>
<tr>
<td>Cell growth and proliferation</td>
<td>4.88E-02 - 6.09E-10</td>
<td>26</td>
</tr>
<tr>
<td>Cell movement</td>
<td>4.21E-02 - 1.17E-06</td>
<td>15</td>
</tr>
<tr>
<td>Cell death and survival</td>
<td>4.41E-02 - 2.45E-05</td>
<td>17</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>3.82E-02 - 3.80E-05</td>
<td>9</td>
</tr>
<tr>
<td><strong>Physiological System Development and Function</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organismal development</td>
<td>4.39E-06 - 9.21E-10</td>
<td>5</td>
</tr>
<tr>
<td>Digestive system development and function</td>
<td>2.54E-06 - 2.54E-06</td>
<td>4</td>
</tr>
<tr>
<td>Hepatic system development and function</td>
<td>2.54E-06 - 2.54E-06</td>
<td>4</td>
</tr>
<tr>
<td>Organ development</td>
<td>1.63E-02 - 2.54E-06</td>
<td>5</td>
</tr>
<tr>
<td>Connective tissue development and function</td>
<td>6.13E-03 - 3.80E-05</td>
<td>4</td>
</tr>
<tr>
<td><strong>Diseases and Disorders</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cancer</td>
<td>4.41E-02 - 1.21E-34</td>
<td>41</td>
</tr>
<tr>
<td>Hematological diseases</td>
<td>3.01E-02 - 1.21E-34</td>
<td>26</td>
</tr>
<tr>
<td>Immunological diseases</td>
<td>3.01E-02 - 1.21E-34</td>
<td>24</td>
</tr>
<tr>
<td>Organismal injury and abnormalities</td>
<td>4.41E-02 - 1.21E-34</td>
<td>41</td>
</tr>
<tr>
<td>Reproductive system diseases</td>
<td>2.60E-02 - 2.10E-28</td>
<td>31</td>
</tr>
</tbody>
</table>

Similar to previous reports (Hassiotou et al., 2013ah), the total cell and fat content of HM increased post-feeding (Figure 3D; Supplementary 3, Figures S3A), suggesting an association with milk synthesis, cell turnover during breastfeeding, and/or potentially active migration of epithelial cells into the alveolar and ductal lumen (Hassiotou et al., 2013ah, Hassiotou et al., 2013a). When pre- and post-feeding milk samples were considered together (n=20), HM cell content was positively associated with the number of known and total (known+novel) miRNA species, as well as the total miRNA content, and the latter was further related to more known and total miRNA (known+novel) species. However, within mother/infant dyads comparisons examining the effects of feeding on HM cell miRNA (n=10) showed no significant differences in total number of miRNA species and total miRNA content pre- and post-feeding (Figure 3D), something that may be due to the lack of significance in change in HM cell content in this small group (p=0.668 for n=10, whilst p=0.028 in the larger group of n=16 mothers), which is one limitation of the study.
Similarly, expression levels of the majority of miRNAs were not different pre- and post-feeding, however a subgroup of 33 known and 3 novel miRNAs were differentially expressed in post-feed milk, of which 29 and 2 were upregulated, respectively (Figure 4A-4B). The top 3 included the known hsa-miR-191-5p (total reads 1,140,649) and hsa-miR-30e-3p (total reads 91,722) miRNAs and the novel_mir_39 (total reads 789), which were the most significantly upregulated post-feeding compared to the other differentially expressed known and novel miRNAs (Supplementary 3, Table S7-S8). hsa-miR-191-5p and hsa-miR-30e-3p are known to be involved in cell proliferation and different types of cancer (Calin and Croce, 2006). In particular, hsa-miR-191-5p is upregulated in breast cancer (Mar-Aguilar et al., 2013, Nagpal et al., 2013), and has been suggested as a prognostic marker for breast cancer progression (Hu et al., 2012). On the other hand, hsa-miR-30e-3p is a biomarker for inflammatory (autoimmune) disorders (Marchand et al., 2012). However, in the context of the normal lactating mammary gland, their upregulation post-feeding may be associated with activation of cell division to facilitate the generation of more milk-secretory lactocytes. Functional analysis using GO and KEGG showed that 146 genes involved in fatty acid synthesis are controlled by miRNAs found to be upregulated post-feeding. Moreover, some of these upregulated miRNAs are involved in the production of immunoglobulin A (IgA), which is known as the first line of defence in the human’s gastrointestinal tract against various infectious diseases (Isolauri et al., 2001).

Collectively, these data indicate that towards the end of a feed HM is richer in cells, which in turn contain greater amounts of miRNA, thus the total cellular miRNA content of HM is likely to be higher in emptier breasts in proportion to the increase in HM cell content with feeding. Considering the potential functional roles of HM miRNA for the infant, feeding on demand likely facilitates exposure of the infant to the full spectrum of HM miRNAs and not only to miRNAs characteristic of milk from fuller breasts. This is further supported by the fact that some miRNAs were detected only in pre- or post-feed milk (Supplementary 3, Table S5-S6) and were therefore specific to either fuller or emptier breasts, suggesting endogenous mammary synthesis of certain miRNA as the infant removes milk from the breast. These miRNA may be involved in the regulation of milk synthesis, which is increased as milk is removed from the breast, and/or of the infant’s appetite, and merit further investigation. Indeed, a study has previously shown that increased milk removal from the breast during pumping resulted in upregulation of gene expression of the milk component α-lactalbumin (Maningat et al., 2007), confirming that milk synthesis can be altered by different milk removal regimes.
Munch et al. (2013) stated that human milk lipids conserved the highest number of miRNAs (308 mature miRNAs) amongst human milk fractions (Munch et al., 2013), although skim milk profiled in 2010 using qPCR-based methods showed greater number of miRNAs (429 mature miRNAs) than the Munch et al. study on milk lipids (Weber et al., 2010). Further, exosomes isolated from skim HM were profiled using Solexa sequencing in 2012, where 602 mature miRNAs were determined (Zhou et al., 2012). However, the cell fraction of HM was not examined in any of these studies. Here, we provide evidence demonstrating that HM cells are richer in miRNA species than all other human milk fractions, containing a total of 1,467 known miRNA species and an additional 1,996 novel miRNAs. We used 208,829,362 reads to match the available 2,590 known miRNAs to the latest version of miRBase 21.0 (released June 2014), in contrast to Munch et al., who used 124,110,646 reads and an older version of miRBase (14.0) (Munch et al., 2013), and Zhou et al., who used ~83,520,000 reads matched to miRBase version 17.0 (Zhou et al., 2012). This difference may have also contributed to the higher number of miRNAs identified here. Importantly, 10M reads were used for each individual sample, which allowed reading low abundance miRNAs. Moreover, an optimized protocol for HM preparation and miRNA extraction was also used to achieve high efficiency of miRNA isolation (Alsaweed et al., 2015ab), in which the analysis of fresh HM and not frozen played an important role.
Figure 4. The two differential expression scatter plots show all identified known (A) and novel (B) miRNAs that were expressed in all pre- and post-feed milk samples (n=10 samples in each group). Each red and green dot represents an individual miRNA that is either up- or down-regulated between pre- and post-feed milk, respectively, whilst blue represents no difference in expression level. (C) Heat-map demonstrating the relationship and expression patterns of the top 10 most highly expressed miRNAs between pre-(B) and post-(A) feed milk of individual participants. The expression patterns were analysed hierarchically by clustering these 10 miRNAs, where green refers to low expression and red refers to high expression level.

Target genes were predicted for the top 10 most highly expressed known and novel miRNAs using different computational approaches (Targetscan, RNAhybrid and miRanda). Candidate targets were applied to GO and KEGG to identify their functions in the lactating mammary gland and for the infant. Most of the top 10 known miRNAs are involved in immune responses, development, growth, metabolic processes, reproduction, and exert enzyme regulatory activity. KEGG pathway analysis revealed involvement in many complex metabolic networks, such as glycerophospholipid metabolism, porphyrin and chlorophyll metabolism, and nitrogen metabolism. The top one targeted pathway was the renin-angiotensin system (RAS), which is a hormone system controlling body fluid balance and blood pressure. These highly abundant HM miRNAs reaching the infant’s gastrointestinal tract potentially control breastfeeding behaviour and appetite via RAS modulation. Further, some of the highly expressed HM cell miRNAs (let-7f-5p, miR-181a-5p/101-3p/148a-3p/30a-5p/16-3p) are known to control ATPase expression including ATP2C1, ATP2B3, ATP2A2, ATP2B4, ATP2B2, and ATP2B1 (Supplementary 3, Figure S9), and may thus facilitate calcium absorption in the infant since ATPase acts as a ion pump transporting ions including calcium (Ca^{2+}) to extracellular space (Brini and Carafoli, 2009). Indeed, HM calcium is more bioavailable to the infant than that of infant formula (Hallberg et al., 1992).

Notably, highly expressed HM cell miRNAs were significantly associated with disease pathways, such as influenza A and respiratory diseases. These miRNAs are likely to control immunity response to influenza A virus in the infant, enhancing the immunological protection provided via HM. This is in accordance with the numerous previous studies emphasising the protective effects of breastfeeding against infections (Walker, 2004, Dewey et al., 1995, Hanson and Winberg, 1972, Howie et al., 1990). For example, exclusive
breastfeeding in the first 4-6 months postpartum has been associated with a reduction of upper and lower respiratory infections in infants (Duijts et al., 2010, Bahl et al., 2005). Further, bioactive components of breastmilk were found to protect against pneumonia, mainly caused by viral infections, during infancy (Ford and Labbok, 1993, Cesar et al., 1999).

As expected, the miRNA profile of HM cells described in this study reflects the miRNA content and endogenous synthesis in the lactocytes, since that is the dominant cell type in mature HM when both the mother and infant are healthy (Hassiotou and Geddes, 2015). In addition to regulatory functions in the infant, highly expressed miRNAs known to regulate triacylglycerol synthesis were identified in HM cells and may be involved in the synthesis of milk lipids in the mammary gland (Figure 5A). Triacylglycerol forms the core of milk fat globules, which mainly contain fatty acids (Innis, 1992), and is the basis of human milk lipids (~98%) (Morera Pons et al., 1998). HM Fatty acids are derived from de novo synthesis in the lactocyte and from blood lipids (Hale and Hartmann, 2007). Specifically, AGPAT6 (1-acylglycerol-3-phosphate O-acyltransferase 6) is known to be regulated by the some of the top most highly expressed HM cell miRNAs (let-7f-5p, miR-182-5p, miR-148a-3p, and miR-22-3p), and has a direct effect on the synthesis of triacylglycerol and long chain acyl-CoA (fatty acids) (Takeuchi and Reue, 2009). Further to triacylglycerol synthesis, highly expressed HM cell miRNAs are involved in fatty acid biosynthesis including oleic, stearic, and palmitic acid (Figure 5B). FADS2 (fatty acid desaturase 2) that is modulated by let-7f-5p (Selbach et al., 2008), is involved in oleate biosynthesis. Moreover, THEM4 (thioesterase superfamily member 4) is controlled by miR-30a-5p, which is also essential for the phosphorylation and synthesis of fatty acids (Selbach et al., 2008) (Figure 5B).

HM lactose is specifically synthesized in the mammary gland, and is the primary sugar in HM contributing ~40% to the energy intake of the infant (Hambraeus, 1996). Lactose consists of two different monosaccharides, glucose and galactose that are joined by 1,4 β-glycosidic linkage (Hale and Hartmann, 2007). In the lactocyte, cytosolic glucose is converted to UDP-galactose by galactose-1-phosphate uridylyltransferase, and is then transported by a glucose transporter (GLUT1) to the lumen of Golgi vesicles (Kunz et al., 2000). GLUT1 expression is regulated by miR-148a-3p, miR-181a-5p, and miR-182-5p, which were highly expressed in HM cells. UDP-galactose interacts with glucose to synthesise lactose after linking to two different protein complexes in the Golgi membrane, β1-4 galactosyltransferase (β4GalT1) and α-lactalbumin (α-LA) (Kunz et al., 2000).
β4GalT1 is regulated by HM cell miR-181a-5p, whereas α-LA by HM cell miR-148a-3p (Figure 6). Therefore, abundant HM cell miRNA appear to regulate fat and lactose synthesis in the lactocyte, and could potentially be used as indicators of the level of milk synthesis in the mammary gland.

Furthermore, some of highly expressed HM cell miRNAs (let-7f-5p, miR-151-3p and miR-16-5p) control the growth hormone receptor (GHR). Human growth hormone (hGH) is critical to milk production in healthy women (Kulski and Hartmann, 1983) and variable levels have been reported in human and bovine milk (Jensen, 1995). It is required to maintain lactation performance (Hale and Hartmann, 2007), and has important roles in alveolar and ductal development in the mammary gland (Kelly et al., 2002). hGH binding to its receptor (GHR) activates Janus Kinase 2 (JAK2), which is mainly responsible for phosphorylation of GH receptor (Herrington and Carter-Su, 2001). The HM cell highly expressed miR-375-3p is a regulator of JAK2. GH-activated JAK2 also phosphorylates/activates signal transducers and activators of transcription (STAT) family including STAT1, STAT3 and STAT5 (Wang and Wood, 1995). STATs are involved in many molecular functions including nuclear localization and activation of transcription of target genes (Wang and Wood, 1995). All members of the STAT family are controlled by highly expressed HM cell miRNAs, including miR-181A-5P, miR-30a/d-5p, and miR-141-3p. Suppresser of cytokine signalling (SOCS) protein family is also responsible for termination of GH-activated STAT signalling (Herrington and Carter-Su, 2001), where the expression of SOCS1-7 proteins is regulated by HM cell miR-182-5p, let-7f-5p, miR-148a-3p, miR-22-3p, miR-16-5p, miR-181a-5p, miR-141-3p (Supplementary 3, Figure S6). These miRNAs involved in the control of GH-mediated mammary gland development and signalling could potentially be used as indicators of lactation performance.

Importantly, most of the highly expressed HM cell miRNA (let-7f-5p, miR-16-5p, miR141-3p, miR30a/d-5p, miR182-5p, andmiR375-3p) regulate the insulin-like growth factor-I receptor (IGF-IR) (Supplementary 3, Figure S6). HM contains insulin-like growth factor-I (IGF-I), which is higher in colostrum than mature milk and higher in HM than bovine milk (Nagashima et al., 1990). IGF-I is thought to be efficiently absorbed in the infant’s gastrointestinal tract and to increase in the serum post-feeding (Buyukkayhan et al., 2003). This growth factor is known to be involved in the development and growth of the infant via direct effects on cell differentiation and proliferation (Donovan et al., 1991).

Some of the highly expressed HM cell miRNAs were identified to have critical roles in insulin receptor (INSR) signalling, including miR-181-5p/182-5p/22-3p/141-3p/148a-
3p/30a-5p. Also INSR itself is regulated by the most highly expressed miRNA in HM, let-7f-5p, and also by miR-182-5p (Supplementary 3, Figure S7). Insulin via its binding to INSR plays a vital role in regulating different milk components, in particular, glucose and fat homeostasis (Strack et al., 1995). Different milk formulae, including standard formula, insulin formula (20 ng/ml) and insulin with trypsin inhibitor (1 U/ml) formula were administrated to rat pups, resulting in increase in plasma insulin, with a direct positive effect on pancreatic amylase activity required in early life (Kinouchi et al., 2000). Further, Insulin receptor (INSR) was detected in the epithelial cell of piglet intestine, suggesting that milk insulin plays a crucial role in the development of the newborn intestinal mucosa. Also, dentists of INSR found to increase the growth of epithelial cells of intestine (Georgiev et al., 2003). Indeed, insulin in HM is up to 30-fold higher than in infant formula (Shehadeh et al., 2001). INSR expression increases in response to nutrients to reduce glucose and synthesis of glycogen (Strack et al., 1995). Controlling the INSR via miRNAs in blood has been previously investigated, where some miRNAs were shown to inhibit INSR (Karolina et al., 2011). Human milk miRNAs potentially control the signalling of insulin receptor based on the needs to decrease glucose.

**Figure 5.** Some of the most highly expressed miRNAs in HM cells are involved in milk synthesis, regulating various pathways within complex molecular networks. (A) 8 highly expressed miRNAs (shown in light blue color) were identified to control the production and maintain the level of triacylglycerol in milk fat. (B) 6 highly expressed HM cell miRNAs (shown in orange color) that regulated different genes associated with fatty acid biosynthesis, including oleic acid, stearic acid, and palmitic acid.
Highly expressed miRNAs in HM cells are also associated with regulation of estrogen receptor genes (ERα and ERβ) (Supplementary 3, Figure S8), which may play important functions in maintaining a normal milk supply. High levels of estrogen inhibits milk production, thus it is present in very low levels to allow prolactin to maintain milk synthesis (Neville et al., 2002). Abundant in HM cells miR-181A-5P, miR-22-3p and miR-21-5p repress ERα (ESR1), whilst the milk most highly expressed miRNA, let-7f-5p, negatively regulates ERβ (ESR2) (Supplementary 3, Figure S8). Estrogen receptors (ERs) are overexpressed in ER+ breast cancer, which is the most common type of breast cancer (Zwart et al., 2011). The known treatment of this cancer is based on blocking the estrogen hormone to bind the excessive ERs on cancer cells, preventing cancer cell proliferation (Zwart et al., 2011). Highly expressed HM miRNAs synthesised in the mammary gland, which target ERs, could be used as a novel therapeutic approach in this cancer (O’Day and Lal, 2010).

Given the high metabolic rate of the lactating mammary gland, it was not surprising that miRNAs known to act as oncogenes, such as miR-21-5p (Si et al., 2007), were found to be highly expressed in HM. In the context of lactation, these HM miRNAs are likely to play important functions in the normal remodeling of the lactating mammary gland, and/or the development of the breastfed infant. They may also be useful biomarkers of lactation performance and the health status of the mammary gland (Heneghan et al., 2009), and require further investigation.

In summary, our study demonstrates that HM cells are rich in miRNA species and content compared to other HM fractions, such as lipids and skim milk, and all other human body fluids. Robust miRNA prediction analysis revealed numerous novel miRNAs, some of which may be specific to lactation. Milk removal by the infant during breastfeeding influences a small subset of HM miRNA species and their expression levels, and results in richer miRNA content in post-feed milk due to the higher number of cells and their associated miRNAs. The identified miRNA targets reveal critical potential roles of miRNAs in the infant and the lactating gland, and suggest using them as diagnostic biomarkers for both lactation performance and breast health.
**Figure 6.** HM lactose synthesis in the mammary gland during lactation is regulated by 4 highly expressed miRNAs in HM (shown in white color). Importantly, miR-182-5p and let-7f-5p regulate SLC2A3 (UDP-glucose transporter) and SL35A2 (UDP-galactose transporter).
CHAPTER 6

HUMAN MILK CELLS AND LIPIDS CONSERVE NUMEROUS KNOWN AND NOVEL miRNAs, SOME OF WHICH ARE DIFFERENTIALLY EXPRESSED DURING LACTATION

6.1 ABSTRACT

Human milk (HM) is rich in miRNAs, which are thought to contribute to infant protection and development. We used deep sequencing to profile miRNAs in the cell and lipid fractions of HM obtained post-feeding from 10 lactating women in months 2, 4, and 6 postpartum. In both HM fractions, 1,195 mature known miRNAs were identified, which were positively associated with the cell (p=0.048) and lipid (p=0.010) content of HM. An additional 5,167 novel miRNA species were predicted, of which 235 were high-confidence miRNAs. HM cells contained more known miRNAs than HM lipids (1,136 and 835 respectively, p<0.001). Although the profile of the novel miRNAs was very different between cells and lipids, with the majority conserved in the cell fraction and being mother-specific, 2/3 of the known miRNAs common between cells and lipids were similarly expressed (p>0.05). Great similarities between the two HM fractions were also found in the profile of the top 20 known miRNAs. These were largely similar also between the three lactation stages examined, as were the total miRNA concentration, and the number and expression of the known miRNAs common between cells and lipids (p>0.05). Yet, approximately a third of all known miRNAs were differentially expressed during the first 6 months of lactation (p<0.05), with more pronounced miRNA upregulation seen in month 4. These findings indicate that although the total miRNA concentration of HM cells and lipids provided to the infant does not change in first 6 months of lactation, the miRNA composition is altered, particularly in month 4 compared to months 2 and 6. This may reflect the remodeling of the gland in response to infant feeding patterns, which usually change after exclusive breastfeeding, suggesting adaptation to the infant’s needs.
6.2 INTRODUCTION

miRNAs (or microRNAs) are small non-coding RNA molecules, typically ~22 nucleotide long, that have emerged as crucial regulators of gene expression at the post-transcriptional level (Krol et al., 2010). They perform this function by binding on the mRNA during translation to either repress it or cause mRNA degradation (Ha and Kim, 2014). Via their action on the mRNA, they are involved in a wide range of biological processes in mammals, including normal development and disease, targeting cell functions such as cell cycle, proliferation, differentiation, apoptosis, and immune response (He and Hannon, 2004, Winter et al., 2009). These processes are all continuous in the mammary gland during its development, remodelling and maintenance of milk-secretory characteristics in both pregnancy and lactation (Hassiotou and Geddes, 2013), in which miRNAs are therefore likely to play key roles (Zhang et al., 2014, Alsaweed et al., 2015a, Tordonato et al., 2015, Tanaka et al., 2009b).

In addition to the lactating gland itself, miRNAs present in human milk (HM) are thought to have important functions for the infant (Alsaweed et al., 2015a). HM contains the required nutritional elements for the infant, including carbohydrates, proteins, lipids, and minerals (Hassiotou et al., 2013a), as well as bioactive factors with immunocompetence and developmental functions (Ballard and Morrow, 2013). At the same time, it contains prokaryotic cells that contribute to healthy gut colonisation in the infant (Bode et al., 2014), together with different types of maternal eukaryotic cells such as epithelial cells from the lactating mammary tissue, stem cells and immune cells (Hassiotou et al., 2013a, Hassiotou and Hartmann, 2014, Hassiotou and Geddes, 2015), some of which survive the infant’s gastrointestinal tract, potentially conferring immunoprotective and developmental functions (Hassiotou et al., 2014b, Zhou et al., 2000, Jain et al., 1989). It has been postulated that miRNAs, which are abundant in HM, also participate in these functions (Alsaweed et al., 2015a, Munch et al., 2013, Zhou et al., 2012). Accumulating evidence is suggesting that, similar to plant food-derived miRNA (Zhang et al., 2012a, Yang et al., 2015), bovine milk miRNA survive the gastrointestinal tract, enter the bloodstream, and exert tissue-specific regulatory functions in the adult (Baier et al., 2014, Chen et al., 2010, Jain et al., 1989, Wolf et al., 2015). These effects are thought to be mediated by the packaging of milk miRNA in exosomes as well as in milk cells and other microvesicles, which protect them from degradation and facilitate their cellular absorption (Adam et al., 2015, Arntz et al., 2015, Alsaweed et al., 2015a, Melnik et al., 2014, Pieters et al., 2015, Tian et al., 2014). Particularly in the case of HM consumption by the infant, these effects may be extenuated
by the fact that the infant gut is more permeable (Catassi et al., 1995) and less acidic than that of the adult (Fallingborg, 1999), further facilitating the survival, absorption and functionality of HM miRNA early in life. Yet, miRNAs as a bioactive component of HM are still largely unexplored.

Recently, it has been shown that HM miRNAs are primarily produced endogenously in the mammary gland, with small contributions from the maternal blood circulation (Alsaweed et al., 2015ct). Moreover, HM is one of the richest sources of miRNA amongst body fluids (Alsaweed et al., 2015bb). Together, these findings suggest lactation-specific regulation and functions of miRNAs for both the mother and the infant. Further, the different fractions of HM have been examined for their miRNA content, with the cell fraction containing more miRNA species than the lipid fraction from the same individuals (Alsaweed et al., 2015ct), and than previously profiled skim milk, lipids and milk exosomes (Zhou et al., 2012, Kosaka et al., 2010). Factors that can influence the miRNA content of HM are poorly understood, with a recent study demonstrating potential effects of milk removal during breastfeeding due to the increase in the cell content of HM post-feeding (Alsaweed et al., 2015bb). Other factors such as the stage of lactation are yet unexplored. Although major macronutrient components of HM, such as fat, protein and lactose, do not systematically change over the course of lactation (Mitoulas et al., 2002), immunological components such as secretory IgA, lactoferrin and activated leukocytes decrease from colostrum to mature milk, whereas humoral protection increases again later in lactation (Hassiotou et al., 2013af, Hassiotou and Geddes, 2015, Prentice et al., 1987, Rai et al., 2014). Further, the status of the lactating epithelium, as reflected by its gene expression, is altered during lactation (Twigger et al., 2015, Hassiotou et al., 2013v), potentially reflecting progressive differentiation of the gland. Therefore, it is not unlikely that the miRNA expression and content of HM may be subject to changes associated with lactation progression. In this connection, Kosaka et al. (2010) identified a few miRNAs in skimmed human milk, such as miR-181a and miR-155, which were expressed at lower levels after month 6 of lactation (Kosaka et al., 2010). In this study, we sought to determine whether the profile of mature miRNAs in HM cell and lipid fractions changed temporally during the first 6 months of lactation. We used deep sequencing (Solexa) to analyse miRNAs from milk samples collected from lactating women at months 2, 4 and 6 of lactation. The cellular and lipid miRNA composition was compared over the three stages of lactation, and novel miRNA species were predicted using mirdeep.
6.3 METHODS

6.3.1 Ethics statement and sampling
The study was approved by the Human Research Ethics Committee of The University of Western Australia, and all methods were conducted in accordance with the approved guidelines. All participants provided informed written consent. Healthy breastfeeding mothers (n=10) were recruited and donated 5 mL of post-feeding milk (at the end of a morning breastfeeding session) longitudinally in months 2 (M2), 4 (M4) and 6 (M6) postpartum (Table 1). Samples were collected aseptically using an electric breast pump (Medela AG, Switzerland), and were immediately transported to the laboratory for analyses.

6.3.2 Human milk processing and miRNA extraction
Fat content was measured in fresh whole HM using the Creamatocrit method, as previously described (Hassiotou et al., 2013ah, Alsaweed et al., 2015ct). Subsequently, whole milk was fractionated to obtain purified cell and lipid fractions, as previously described (Alsaweed et al., 2015ct). Briefly, freshly expressed HM was diluted 1:1 with PBS (Gibco, Life Technology, Foster, CA) and centrifuged at 800 g for 20 min at 20°C. Purified milk cell and lipid fractions were washed twice separately in PBS at 800 g for 5 min at 20°C, and cells were then counted using a haemocytometer. Small RNA sequencing was performed in all n=30 milk cell samples from the 10 study participants (3 per participant for each of the three lactation stages examined), and in a subgroup of n=15 lipid samples from 5 study participants (3 per participant for each of the three lactation stages examined). miRNA were extracted from total milk cells and total milk lipids immediately without cryopreservation using the miRNasy mini Kit (Qiagen, Hilden, Germany) and the miRCURY RNA Isolation-Biofluids Kit (Exiqon, Vedbaek, Denmark) respectively, as previously described (Alsaweed et al., 2015ab). An Agilent Bioanalyzer 2100 instrument (Agilent, CA, USA) with an RNA 6000 Nano Chip kit, and NanoDrop 2000 Spectrophotometer (Wilmington, DE, USA) were used to measure the concentration and purity of the extracted miRNA (Table 1). All miRNA samples were immediately stored at -80°C for small RNA sequencing.
Table 1. Demographic and HM sample characteristics of study participants (n=10). All values are presented as a range and as mean±standard deviation in brackets, for cell and lipid HM fractions, and in months M2, M4, and M6 of lactation.

<table>
<thead>
<tr>
<th></th>
<th>Month 2-Cells (n=10 samples)</th>
<th>Month 2-Lipids (n=5 samples)</th>
<th>Month 4-Cells (n=10 samples)</th>
<th>Month 4-Lipids (n=5 samples)</th>
<th>Month 6-Cells (n=10 samples)</th>
<th>Month 6-Lipids (n=5 samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
<td>36-24 (32.4 ± 3.86)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parity (number of children)</td>
<td>1-3 (1.6 ± 0.69)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infant weight (g)</td>
<td>4,038-6,940 (5,494 ± 831.1)</td>
<td>5,940-8,945 (7,147 ± 900.3)</td>
<td>7,044-9,800 (8,186 ± 945.8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HM fat content (%)</td>
<td></td>
<td></td>
<td>8.1-15.6 (11.1 ± 2.9)</td>
<td></td>
<td>7.3-13.0 (10.7 ± 2.3)</td>
<td></td>
</tr>
<tr>
<td>HM cell content (/mL milk)</td>
<td>400,000-2,300,000 (1,299,356 ± 719,432)</td>
<td>142,222-4,360,000 (1,234,797 ± 1,246,005)</td>
<td>128,889-1,572,000 (666,146 ± 406,928)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cell Viability (%)</td>
<td>89-98 (93.28 ± 2.8)</td>
<td></td>
<td>95.95 ± 3.1</td>
<td></td>
<td>94.7 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>Total miRNA</td>
<td>(NANO Drop)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NanoDrop)*</td>
<td>(1.42 ± 0.49)</td>
<td>(0.052 ± 0.03)</td>
<td>(1.19 ± 0.47)</td>
<td>(0.052 ± 0.05)</td>
<td>(1.14 ± 0.57)</td>
<td>(0.031 ± 0.02)</td>
</tr>
<tr>
<td>Total miRNA</td>
<td>(Bioanalyzer)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Bioanalyzer)*</td>
<td>(1.24 ± 0.35)</td>
<td>(0.026 ± 0.01)</td>
<td>(0.90 ± 0.38)</td>
<td>(0.027 ± 0.01)</td>
<td>(1.16 ± 0.50)</td>
<td>(0.023 ± 0.01)</td>
</tr>
<tr>
<td>260/280 ratio (NanoDrop)</td>
<td>(2.03 ± 0.04)</td>
<td>(2.07 ± 0.05)</td>
<td>(2.02 ± 0.06)</td>
<td>(2.07 ± 0.01)</td>
<td>(2.01 ± 0.05)</td>
<td>(2.04 ± 0.04)</td>
</tr>
<tr>
<td>RIN (Bioanalyzer)</td>
<td>(8.39 ± 0.35)</td>
<td>(5.56 ± 2.07)</td>
<td>(8.22 ± 0.83)</td>
<td>(5.72 ± 1.01)</td>
<td>(8.28 ± 0.48)</td>
<td>(4.36 ± 1.56)</td>
</tr>
</tbody>
</table>

* Total miRNA concentration measured in 1 million cells (µg) or in 1 microliter (µg) of lipids
6.3.3 Small RNA sequencing and bioinformatics analysis

Sequencing libraries were prepared from HM cells and lipids miRNA samples using the Solexa small RNAs protocol as previously described (Yi et al., 2013, Alsaweed et al., 2015bb, Liu et al., 2014). By using polyacrylamide denture gels for size fractionation, small RNAs ranging 18-30 nucleotides (nt) long were obtained and ligated to 5′-RNA and 3′-RNA adapters, which were transcribed into cDNA. Small RNA primers (Illumina) were added to the cDNA for PCR amplification. After purification of the cDNA products, Illumina HiSeq 2000 platform was used with SE49 lanes to sequence all small RNAs. During data analysis, raw sequences were cleaned of all contaminated and low reads, such as 5′ primer contaminants, oversized insertions, and reads shorter than 18 nt (Supplementary 4, Table A in S1 File), and the quality of reads was also checked in individual samples (n=45) (Supplementary 4, S1 Fig). The clean reads of small RNAs were distributed based on the nucleotide size. The vast majority of miRNAs are between 21 and 22 nt, whilst piRNAs are 30 nt, and siRNAs are 24 nt. All clean reads were mapped to the human genome using the SOAP software to analyse their expression and distribution. Thereafter, all of these mapped and cleaned reads were annotated using BLAST to identify different small RNA classes. Therefore, reads were to aligned to Rfam (ftp://sanger.ac.uk/pub/databases/Rfam/) and GenBank (http://blast.ncbi.nlm.nih.gov/) to identify and remove rRNA, tRNA, snRNA, scRNA, snoRNA, and other ncRNAs. Also degraded fragments of mRNAs were removed after the alignment to exons and introns. The remaining reads were mapped to miRBase 21.0 (release August 2014) (http://www.mirbase.org/) using BLAST to determine the human known mature miRNA species. The clean reads that did not map to miRBase and other RNAs but mapped to the human genome, were used for novel miRNA prediction analysis. In this analysis, all unmatched clean reads were uploaded into mirdeep (http://sourceforge.net/projects/mirdeepstar/) by SOAP to explore the stem loop (secondary) structure, the Dicer cleavage site and the minimum free energy of the unannotated small RNAs. Then, base bias on the first position and the nucleotide length on each position were determined for each read for accurate novel miRNA prediction.

6.3.4 Differential expression analysis

All identified known and novel miRNAs were used for differential expression analysis between different time points (month 2, 4 and 6 of lactation), and between milk cell and lipid fractions. The expression levels of transcript per million (TPM) were obtained using the following normalisation formula: normalised expression = actual miRNA count / total
count of clean reads * 1,000,000. Then, fold change was obtained using the following formula: 

\[
\text{Fold change} = \log_2(\text{normalised expressed miRNAs from a milk fraction or lactation stage}) / \text{normalised expressed miRNAs from another milk fraction or lactation stage}
\]

DEGseq, an R package for identifying differentially expressed genes from RNA-seq data, was used to generate scatter plots and determine p-values based on the fold change (Wang et al., 2010f). miRNAs with p<0.05 were considered to be differentially expressed between different stages of lactation and between HM cells and lipids samples.

**6.3.5 miRNA target prediction, signaling and metabolic functional analysis**

Prediction of target genes of the top 20 most highly expressed known and novel miRNAs was done using three different databases/algorithms: targetscan (http://www.targetscan.org/), miRanda (http://www.microrna.org/microrna/home.do), and RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid). These three databases predict the binding region (miRNA seed region) between the mature miRNA sequence and the mRNA. Furthermore, these targets were classified into different signaling and cellular function ontologies (cellular component, molecular function and biological process) using Gene Ontology (GO) (http://www.geneontology.org/) (Ashburner et al., 2000) to investigate the functions of the miRNA target genes. Metabolic and cellular pathways of the identified target genes were also predicted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/) (Kanehisa et al., 2014).

**6.3.6 Statistical analysis**

Microsoft Excel and R Studio Version 0.98.1103 package were used to perform graphical exploration of the data and statistical analyses with linear mixed effects modeling (LME) (RStudio Team, 2015) via nlme (Pinheiro et al., 2013) and lattice (Sarkar, 2015) packages. Differences were considered to be significant if p<0.05. Differences in total cell content, fat content and total miRNA content between individual participants, HM cells and lipids, and during the three stages of lactation (M2, M4, and M6) were tested using general linear hypothesis tests and LME.

**6.3.7 Availability of supporting data**

All raw small RNA sequences are under preparation for submission to the NCBI Gene Expression Omnibus database. Additional information is also included in Supplementary 4.
Table 2. Top 20 most highly expressed known miRNAs identified in 10 mothers in each lactation stage (month 2, M2; month 4, M4; and month 6, M6) and in the cell and lipid fractions of HM, with the total reads. All the presented miRNAs were identified in all samples in each lactation stage and milk fraction.

<table>
<thead>
<tr>
<th>miRNA species</th>
<th>M2 (n=10)</th>
<th>M4 (n=10)</th>
<th>M6 (n=10)</th>
<th>Cells (n=30)</th>
<th>Lipids (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>let-7f-5p</td>
<td>12,401,040</td>
<td>18,703,845</td>
<td>24,056,451</td>
<td>41,882,696</td>
<td>13,278,640</td>
</tr>
<tr>
<td>miR-181a-5p</td>
<td>9,010,343</td>
<td>8,854,468</td>
<td>8,280,949</td>
<td>17,505,458</td>
<td>8,640,302</td>
</tr>
<tr>
<td>miR-182-5p</td>
<td>6,920,662</td>
<td>10,029,944</td>
<td>10,727,735</td>
<td>18,396,918</td>
<td>9,281,423</td>
</tr>
<tr>
<td>miR-148a-3p</td>
<td>6,113,951</td>
<td>6,211,182</td>
<td>6,018,492</td>
<td>13,491,305</td>
<td>4,852,320</td>
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<tr>
<td>let-7a-5p</td>
<td>4,390,350</td>
<td>6,082,456</td>
<td>7,290,866</td>
<td>13,054,286</td>
<td>4,709,386</td>
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<tr>
<td>miR-375</td>
<td>4,345,215</td>
<td>5,195,961</td>
<td>5,194,769</td>
<td>9,992,213</td>
<td>4,743,732</td>
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<tr>
<td>miR-22-3p</td>
<td>3,863,461</td>
<td>4,670,692</td>
<td>4,943,342</td>
<td>8,617,402</td>
<td>4,860,093</td>
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<td>miR-30a-5p</td>
<td>3,822,156</td>
<td>3,402,934</td>
<td>3,607,107</td>
<td>7,347,279</td>
<td>3,484,918</td>
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<tr>
<td>miR-141-3p</td>
<td>2,785,391</td>
<td>2,938,731</td>
<td>3,102,357</td>
<td>6,663,599</td>
<td>2,162,880</td>
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<tr>
<td>miR-30d-5p</td>
<td>2,679,770</td>
<td>4,339,844</td>
<td>4,995,894</td>
<td>7,859,146</td>
<td>4,156,362</td>
</tr>
<tr>
<td>miR-146b-5p</td>
<td>3,899,267</td>
<td>3,910,664</td>
<td>3,667,413</td>
<td>8,119,202.0</td>
<td>3,358,142</td>
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<tr>
<td>miR-99b-5p</td>
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<td>2,680,089</td>
<td>1,400,374</td>
<td>5,756,657.0</td>
<td>1,963,643</td>
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<tr>
<td>miR-125a-5p</td>
<td>2,033,776</td>
<td>1,596,856</td>
<td>1,166,779</td>
<td>3,549,138.0</td>
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<td>miR-10a-5p</td>
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<td>1,151,709</td>
<td>3,403,563.0</td>
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</tr>
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<td>1,460,349</td>
<td>1,009,074</td>
<td>2,737,027.0</td>
<td>860,967</td>
</tr>
<tr>
<td>miR-21-5p</td>
<td>1,297,811</td>
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<td>641,092</td>
<td>2,524,499.0</td>
<td>491,391</td>
</tr>
<tr>
<td>miR-191-5p</td>
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<td>1,590,406</td>
<td>817,349</td>
<td>2,402,640.0</td>
<td>1,002,760</td>
</tr>
<tr>
<td>miR-423-5p</td>
<td>816,934</td>
<td>750,918</td>
<td>665,056</td>
<td>1,789,507.0</td>
<td>443,401</td>
</tr>
<tr>
<td>let-7i-5p</td>
<td>879,471</td>
<td>800,140</td>
<td>800,269</td>
<td>1,787,511.0</td>
<td>692,369</td>
</tr>
<tr>
<td>miR-143-3p</td>
<td>162771</td>
<td>1,521,802</td>
<td>80,033</td>
<td>1,739,025.0</td>
<td>25,581</td>
</tr>
</tbody>
</table>
Figure 1. Annotation and filtering analysis of all clean small reads. (A) After the filtering analysis of the total reads, the total number of raw generated clean reads compared to the filtered clean reads is presented. (B) Total clean reads of small RNAs distributed based on their nucleotide length (12-30 nt). (C) Total raw and clean reads for each lactation stage (months 2, 4 and 6). (D) Small RNA unique reads categorised into only one RNA type, including miRNAs, using the following priority rule: rRNA etc (in which Genbank > Rfam) > known miRNA > piRNA > repeat > exon > intron3.

6.4 RESULTS

6.4.1 Cells are richer in miRNA species than lipids in human milk

Small RNAs (sRNA) including mature miRNAs were sequenced using Illumina HiSeq 2000 (50SE) in HM cell and lipids samples obtained longitudinally from 10 lactating mothers. Filtering analysis was used to determine clean reads, where 545,655,541 clean reads (91.93%; average=12,125,678.7 reads per sample) were obtained from the total generated 596,988,897 raw reads (Fig 1A). All clean reads were distributed based on their nucleotide (nt) length, in which reads of 22 nt length were the most common (29.13%) (Fig 1B). By using SOAP, 543,933,429 reads were mapped to the human genome to determine the
distribution and expression of small RNAs (Fig 1C). All the mapped and clean reads were annotated to the different RNA types in the to Genebank and RFam to identify and remove rRNA, tRNA, snoRNA, snRNA, scRNA and repeat small RNAs (Fig 1D). miRNAs and unannotated total reads were used for known and novel miRNA identification, respectively (Fig 1D). The total reads of 282,555,158 (unique reads 80,620) were matched using BLAST to the miRBase 21.0 to determine human known mature miRNAs. Specifically, higher matched reads were obtained from the cell sample group (n=30) compared to the lipid sample group (n=15), with 6,737,566.6 and 5,403,643 average clean reads, respectively (Supplementary 4, Table B in S1 File).
1,195 known mature miRNAs were identified in all HM cell and lipid samples (n=45), of which 1,136 known miRNA species (total matched reads= 202,126,997) and 835 known miRNA species (total matched reads=81,054,651) were determined in HM cell (n=30) and lipid (n=15) samples, respectively (Fig 2A; Table 1; Supplementary 4, Table C in S1 File), with the HM cell samples conserving significantly more known miRNAs than the lipids samples (p<0.001). The let-7 miRNA family was of the most highly expressed miRNAs in HM, with four let-7 mature miRNAs (let-7f-5p/7a-5p/7i-5p/7e-5p) ranking in the top 20, and other let-7 members (let-7g-5p/7b-5p/7c-5p/7d-5p) expressed at high levels (Table 2; Supplementary 4, Table C in S1 File). Amongst the identified known miRNAs, most species (776 miRNAs) were commonly observed in both cells and lipids, whereas 360 and 59 known miRNAs were specific to cells and lipids, respectively (Supplementary 4, Table D in S1 File).
Figure 3. (A, B) Scatter plots showing the differentially expressed (A) known and (B) novel miRNAs in cell and lipid HM samples. (C, D, E) Differentially expressed known miRNAs between months M2 and M4 (C), M2 and M6 (D), and M4 and M6 (E). (F, G, H) Differentially expressed novel miRNAs between months M2 and M4 (F), M2 and M6 (G), and M4 and M6 (H). Each dot represents an individual miRNA, where red and blue refer to up- and down-regulated miRNAs respectively, and grey dots refer to no change of expression of a given miRNA.
Unannotated reads (total 69,827,843; unique 3,629,263) mapped to sRNAs which corresponded to antisense exons, antisense introns, or intergenic regions on the human genome, and did not map to any other RNAs including known miRNAs, were used to predict novel miRNAs. First, mirdeep was used to predict the secondary structure (stem loop), the Dicer cleavage site and the minimum free energy of the unannotated small RNAs that could be mapped to the genome. Second, all unannotated small RNAs were further assessed to determine the predicted novel miRNA nucleotide base bias on the first position with certain length and on each position. In all HM cell and lipid samples (n=45), 5,167 novel miRNAs (total reads=225,937) were predicted using the above criteria. Similar to the known miRNA species, significantly more novel miRNA species were identified in the HM cell samples (n=30) (3,404 novel miRNAs, with total reads of 461,417) compared to the HM lipid samples (n=15) (2,072 novel miRNAs, with total reads of 454,673) (p=0.002) (Fig 2B; Supplementary 4, Table E in S1 File). Moreover, the number of total miRNA species (known+novel miRNAs) was significantly higher in the HM cell samples (p=0.005). High-confidence novel miRNAs were determined based on more strict criteria, whereby a novel miRNA identified in ≥3 samples and with total reads >20 was considered as high-confidence novel miRNA. This resulted in 235 high-confidence novel miRNAs (total reads=814,351) in all HM cell and lipid samples, of which 233 were found in HM cells and 187 in HM lipids (Supplementary 4, Table F in S1 File). Due to the large number of novel miRNAs predicted in this study, only the top 20 most highly expressed novel miRNAs (Table 3) are shown in S2 Fig (Supplementary 4), with their secondary structure (hairpin structure of the precursor miRNAs), the Dicer cleavage site, and the minimum free energy. Most of the novel miRNA species found in ≥2 out of the n=45 samples (276 miRNAs) were specific to HM cells, whilst only 72 novel miRNAs were specific to lipids, and only 81 were commonly seen between cells and lipids (Supplementary 4, Table G in S1 File). These findings demonstrate that the cell fraction of HM contains more known and novel miRNA species than the lipid fraction.

6.4.2 Human milk miRNAs are positively associated with milk cell and lipid contents

The total cell content of HM was not related to the cellular miRNA concentration (NanoDrop p=0.309, Bioanalyzer p=0.705; n=30). However, a significant positive association between the fat content of HM and the miRNA concentration of the lipid fraction was found (NanoDrop p=0.019, Bioanalyzer p=0.003; n=15). Further, samples with greater fat content contained more known miRNA species in the lipid fraction of HM.
(p=0.010, n=15) (Fig 2C), but the same was not seen for novel miRNAs (p=0.731). A significant positive association was also seen between the HM cell content (cell number per mL milk) and the number of known and novel miRNA species in HM cells (p=0.048 and p=0.021 respectively, n=30) (Fig 2D). Although in the HM cell samples, total miRNA concentration did not relate to the number of known (NanoDrop p=0.199, Bioanalyzer p=0.979; n=30) or novel miRNA species (NanoDrop p=0.249, Bioanalyzer p=0.890; n=30), in the HM lipid samples there was a positive association with the number of known miRNA species (NanoDrop p<0.001, Bioanalyzer p=0.052; n=15), but not the novel miRNAs (NanoDrop p=0.734, Bioanalyzer p=0.459; n=15) (Supplementary 4, Fig 2E-2H). When the total miRNA concentration in HM cells and lipids was considered together, the positive association between total miRNA concentration and the number of known miRNA species persisted (NanoDrop p<0.001, Bioanalyzer p=0.005; n=45). These data indicate that HM samples with high cell and lipid contents contain more miRNAs.

6.4.3 Cells and lipids of human milk share similar known miRNA profiles, but contain largely different novel miRNAs

Differential expression analysis was done for the 776 known miRNAs commonly detected between the HM cell and lipid fractions. Of these, 496 miRNAs (63.9%) were expressed at similar levels between cells and lipids (p>0.05) (Fig 3A). The remaining 280 known miRNAs were differentially expressed between HM cells and lipids (p<0.05), with 147 miRNAs found to be upregulated in cells and 133 in lipids (Supplementary 4, Table H in S1 File). Only miR-21-5p was in the top 20 most highly expressed known miRNAs in cells and lipids, and at the same time upregulated (p<0.001) in HM cells (total reads 41,882,696) compared to lipids (total reads 13,278,640), whilst all other top 19 known miRNAs were similarly expressed between the two milk fractions (Supplementary 4, S3 Fig A). For the novel miRNAs predicted, 309 species were commonly detected in both cell and lipid HM fractions and were thus used for differential expression analysis. Of these, 209 novel miRNAs (67.6%) were expressed at similar levels between the two milk fractions (p>0.05) (Fig 3B), whilst 100 were differentially expressed, with 59 species upregulated in cells and 41 in lipids (p<0.05) (Supplementary 4, Tables I-J in S1 File). Amongst the top 20 most highly expressed novel miRNAs (present in ≥4 samples), 13 novel miRNAs were upregulated in cells compared to lipids, whilst only 1 miRNA (novel_mir_8) was upregulated in lipids compared to cells (Supplementary 4, S3 Fig B). These results demonstrate that the cell fraction of HM is richer and more variable in novel miRNA
species than the lipid fraction, with the majority of novel miRNAs in HM cells being mother-specific. However, approximately 2/3 of all the known and the high-confidence novel miRNAs common between cells and lipids were similarly expressed in the two HM fractions.

6.4.4 Human milk miRNA expression profiles change with lactation

The overall HM cell and fat contents were not significantly different between the three stages of lactation examined (M2, M4, and M6) (p=0.109; p=0.691, respectively). In keeping with this, the total miRNA concentration extracted from HM cells or lipids and measured by NanoDrop and Bioanalyzer did not differ between the stages of lactation (p=0.226 and p=0.683 respectively for HM cells; p=0.452 and p=0.661 respectively for HM lipids). Overall, the total number of known and novel miRNA species was not different between the three stages of lactation amongst the participants (p=0.285 and p=0.355, respectively) (Fig 2I-2J). Specifically, in M2 951 known (total reads 83,832,174) and 1,601 novel (total reads 260,148) miRNAs in both milk cells and lipids were identified, whilst 996 known (total reads 99,378,288) and 2,181 novel (total reads 75,008) miRNAs were determined in M4, and 867 known (total reads 99,971,186) and 1,950 novel (total reads 311,005) miRNAs in M6. Although the number of novel miRNAs was largely different between the three lactation stages, this was not statistically significant (p>0.05) due to the great variability seen in the novel miRNA composition of each HM sample and the presence of many of these novel miRNAs in single samples.

The mean total contribution of the top 20 most highly expressed known miRNAs in the cell and lipid HM fractions together was 88.8 ± 1.4% mean ± S.D. (range 87.9-90.3; specifically for each month: M2 87.9%, M4 88.0%, M6 90.3%). Of this, 19.2 ± 4.6% mean ± S.D. (range 14.8-24.1; specifically for each month M2 14.8%, M4 24.1%, M6 18.8%) of copies were related to the most abundant miRNA in HM, let-7f-5p. In the HM cell and lipid fractions separately, the top 20 known miRNAs contributed 88.19 ± 3.2% and 89.4 ± 6.2% mean ± S.D., respectively, of all identified miRNAs in each milk fraction. In particular, the highest expressed miRNA let-7f-5p contributed 23.1 ± 5.7% mean ± S.D. in HM cells, and 17.9 ± 3.6% mean ± S.D. in HM lipids (Fig 4).
Table 3. Top 20 most highly expressed novel miRNAs identified in the 10 mothers studied in both cell and lipid HM fractions and across the three lactation stages examined (months 2, 4 and 6), with the total reads, and the number of samples that each miRNA was detected in (total samples n=45).

<table>
<thead>
<tr>
<th>miRNA species</th>
<th>Mature sequence</th>
<th>Total reads</th>
<th>Nucleotide length (nt)</th>
<th>Number of samples determined in (out of n=45)</th>
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</thead>
<tbody>
<tr>
<td>novel_mir_189</td>
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<td>751,632</td>
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<tr>
<td>novel_mir_472</td>
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<tr>
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<td>578</td>
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</table>
Differential expression analysis between the three stages of lactation for HM cells and lipids together included most of the expressed known miRNAs, since they were identified in all stages of lactation (808 known miRNAs were common between M2 and M4; 746 known miRNAs were common between M2 and M6; and 764 known miRNAs were common between M4 and M6) (Fig 3C-3E). When the cell and lipid HM fractions were considered together, most known miRNAs were similarly expressed between the three stages of lactation amongst participants, whereby 75.5% (610/808) and 71.6% (534/746) of miRNAs did not differ in M2 compared to either M4 or M6 respectively (p>0.05), and 66.6% (509/764) miRNAs were expressed similarly between M4 and M6 (p>0.05) (Supplementary 4, Table H in S1 File). Of the differentially expressed miRNAs, more known miRNAs were upregulated in M4 compared to M2 and M6 (112/198, 56.6%, and 158/174, 90.8% respectively). 12 of the top 20 known miRNAs in HM cells and lipids together were similarly expressed, based on total raw reads, between the three stages of lactation (p>0.05) (Figs 4 and 5; Supplementary 4, Table K in S1 File). The majority of upregulated known miRNAs out of the top 20 (4 miRNAs) were seen in M2 compared to M6. Interestingly, let-7f-5p was the most highly expressed miRNA in HM and dramatically increased from M2 to M6 (p<0.001) (Fig 6A; Supplementary 4, S3 Fig C).

In contrast to the known miRNA species, the vast majority of novel miRNAs (89.3%) were identified in few samples at low expression levels. Therefore, the novel miRNA species predicted appeared to be highly variable both within and between individuals, in both the cell and lipid HM fractions and throughout the first 6 months of lactation. When the cells and lipid HM fractions were considered together, most novel miRNAs common in all three lactation stages were similarly expressed between the three stages of lactation (p>0.05) (66.9%, 67.3% and 74.4% between M2 and M4, M2 and M6, and M4 and M6, respectively) (p<0.05) (Fig 3F-3H; Supplementary 4, Table I in S1 File). When the two milk fractions were considered separately, from the top 20 most highly expressed novel miRNAs only 9 were commonly determined in M2, M4 and M6 in HM cells. Amongst these 9 miRNAs, novel_mir_189 contributed 92.0 ± 1.95% mean ± standard deviation (range 93.8-98.9%). Similarly, only 7 novel miRNAs were commonly identified in M2, M4, M6 in the HM lipid fraction, with the same novel miRNA (novel_mir_189) contributing 98.1 ± 1.5% mean ± standard deviation (range 96.4-99.1%) (Supplementary 4, Table K in S1 File).

Out of the 235 high-confidence novel miRNAs, 36.9%, 39.2% and 33.1% were differentially expressed between M2 and M4, M2 and M6, and M4 and M6, respectively
Most of the differentially expressed miRNAs were upregulated between the three lactation stages (64.4% M2 to M4; 53.5% M2 to M6; and 67.9% M4 to M6). The expression patterns of the top 20 most highly expressed novel miRNAs (present in ≥4 samples) in HM cell and lipid samples together were analysed, with most changes occurring in the first 4 months of lactation and the fewest differentially expressed novel miRNAs out of the top 20 observed from M4 to M6 (6 versus 10 in M2 to M4, and M2 to M6). 7 and 3 novel miRNAs were up- and down-regulated respectively in M2 compared to M4; 4 and 6 novel miRNAs were up- and down-regulated respectively in M2 compared to M6; and 5 and 1 novel miRNAs were up- and down-regulated respectively in M4 compared to M6 (p<0.05) (Supplementary 4, Table K in S1 File). Interestingly, the most highly expressed novel miRNA (novel_mir_189) was enriched in M4 compared to M2 and M6 (Fig 6B; Supplementary 4, S3 Fig D).

6.4.5 Gene targets and functional analysis of miRNAs in human milk cells and lipids
To determine the main functions of the top 20 most highly expressed miRNAs in HM, target genes were first predicted using different databases (TargetScan RNAhybrid and miRanda). These were effectively the same between the cell and lipid fractions of HM because the top 20 most highly expressed known miRNAs were very similar between the two fractions (Fig 5D-5E). Differences were found for the top 20 novel miRNAs, which differed both between and within participants. The target genes of known and novel miRNAs were predicted using as the 2-7 binding site between the miRNA and mRNA, which is commonly known as the miRNA seed region. The total common number of the gene targets identified in these databases for the top 20 most highly expressed known miRNAs during the first 6 months of lactation was almost identical, where 50,308 gene targets were determined in HM cells and 50,301 in HM lipids (Supplementary 4, Table L in S1 File). Similarly, the total common number of the gene targets identified in these databases for the top 20 most highly expressed novel miRNAs that were common amongst all participants during the first 6 months of lactation was 50,313 gene targets in HM cells and 50,311 in HM lipids (Supplementary 4, Table M in S1 File). These gene targets were used to investigate the predicted roles of these miRNAs using GO and KEGG. Most of identified gene targets were involved in numerous essential biological processes including programmed cell death, cell-to-cell communication, cell adhesion, peptide transport, nervous and immune system development, and metabolic processes (Supplementary 4, Table N in S1 File). More than 600 gene targets were involved in the immune response to various infectious diseases, in particular of bacterial origin.
Further, ~500 genes participated in the development of the immune system (Supplementary 4, Table N in S1 File). Transporting molecules, such as proteins, into and out of cells was one of the most common functions of the genes targeted and regulated by the highly expressed miRNAs in HM. Numerous miRNA gene targets were also involved in growth factor receptor synthesis. Different metabolic processes were also targeted, including fat digestion and absorption and gluconeogenesis pathways (Supplementary 4, Table O in S1 File).

6.5 DISCUSSION

Human milk (HM) has been well characterised in the past few centuries as the optimal food source for infants, especially in the first 6 months postpartum (Stevens et al., 2009). It contains all the required nutritional elements to nourish the infant, including fat, carbohydrates, proteins, and vitamins (Kramer, 2010), with recent research advances revealing protective and developmental functions of HM conferred by an array of molecular and cellular components, which include miRNA (Alsaweed et al., 2015a, Hassiotou et al., 2013a, Hassiotou and Geddes, 2013, Hassiotou and Hartmann, 2014). These small non-coding RNA molecules have been profiled in the milk of different mammals, with HM found to be one of the richest sources of miRNAs in humans (Alsaweed et al., 2015ct, Alsaweed et al., 2015bb, Weber et al., 2010). HM miRNAs are protected within cells and exosomes (Zhou et al., 2012, Pieters et al., 2015, Tian et al., 2014), and are likely transferred to different infant tissues exerting regulatory functions (Hassiotou et al., 2015, Alsaweed et al., 2015a, Baier et al., 2014, Wolf et al., 2015). Further to their roles in the breastfed infant, we have recently identified several HM cell miRNAs that are endogenously synthesized in the breast and are involved in the synthesis and regulation of milk components such as triacylglycerol, fatty acids, lactose, and others (Alsaweed et al., 2015bb, Alsaweed et al., 2015ct), supporting the involvement of miRNA in the normal function of the lactating mammary gland (Alsaweed et al., 2015a). Here, we performed a first comparison of the miRNA profiles of the cell and lipid fractions of HM using Solexa sequencing, and examined how they vary temporally in the first 6 months postpartum. The high number of clean small RNA reads (~546M), the high quality and purification levels of the extracted miRNAs (91.9%) (Alsaweed et al., 2015ab), and the use of the most updated miRBase (21.0) resulted in the discovery of numerous putative miRNAs that exceeded those reported in previous investigations (Munch et al., 2013, Yi et al., 2013). Of these, 1,195
were known mature miRNAs, and 5,167 were novel predicted miRNAs, of which 235 were high-confidence miRNAs. This is in agreement with previous reports on porcine milk exosomes, which contain more novel predicted miRNAs than known miRNAs (Chen et al., 2014). The cell fraction of HM contained more known miRNAs than the lipid fraction, and had very different novel miRNA profiles, which varied greatly amongst mothers. 36.1% and 80% of known and novel miRNAs, respectively, were differentially expressed between the two milk fractions. However, most of the highly expressed known miRNAs were commonly found in both cell and lipid HM fractions, suggesting that these miRNAs originate from the lactocyte (Alsaweed et al., 2015ct), and they are protected within the cells and fat globules to resist digestion in the infant’s gastrointestinal tract (Wolf et al., 2015). These findings confirm previous postulations (Alsaweed et al., 2015ct, Alsaweed et al., 2015bb), and emphasize that cells are the richest in miRNA fraction of HM. Although the total miRNA concentration of HM cells and lipids did not systematically change in the first 6 months of lactation, changes in miRNA composition and expression levels were observed for some miRNAs, particularly in month 4 compared to months 2 and 6 postpartum, potentially reflecting adaptation to infant needs, which change after exclusive breastfeeding. Increasing the number of reads and using optimised miRNA extraction protocols (Alsaweed et al., 2015ab) contributed to the discovery of a significantly higher number of novel miRNAs (5,167, of which 235 were of high-confidence), compared to previous studies, which predicted 21 and 622 novel miRNAs in HM and tammar wallaby milk, respectively (Munch et al., 2013, Modepalli et al., 2014). Similar to known miRNAs, milk cells were richer in novel miRNAs (3,404) compared to lipids (2,072). These novel miRNAs were subjected to multiple strict criteria to be considered true novel miRNAs (Supplementary 4, Table F in S1 File). As expected, all of these miRNAs were present at very low expression levels, suggesting that potentially a large number of miRNAs remain to be discovered as detection methods improve.

The total miRNA concentration of the HM cells and lipids was positively associated with the number of both known and novel miRNA species (Fig 2C-2D). Moreover, HM fat content was positively related to the number of known miRNA species, and HM cell content was positively related to the number of both known and novel miRNA species (Fig 2G-2H). Therefore, the total fat and cell contents of HM of a given mother can be indicative of the miRNA concentration and number of species of these milk fractions, as they are also indicative of breast fullness (Hassiotou et al., 2013a, Hassiotou et al., 2013ah, Mitoulas et al., 2003). Indeed, emptier breasts (post-feeding) contain milk that is richer in both fat and
cells than fuller breasts (pre-feeding) (Mitoulas et al., 2003, Hassiotou et al., 2013a, Hassiotou et al., 2013ah), and appear to also contain more miRNAs associated with the cell and lipid fractions (Alsaweed et al., 2015bb). This supports ‘feeding on demand’ practices, which ensure provision to the infant of not only variable amounts of fat and cells in response to the specific feeding patterns of each infant, but also of the full spectrum of miRNAs of HM.

Of the top 20 most highly expressed known miRNAs identified in this study, very few were differentially expressed between the cell and lipid HM fractions and the three lactation stages (Figs 3A and 5A-5C; Supplementary 4, Table K in S1 File), indicating similar profiles both between and within individuals and suggesting potentially important functions for these highly conserved miRNAs. This, together with previous reports supporting that most milk miRNAs primarily originate from the lactating mammary gland (Alsaweed et al., 2015ct), suggests that some of these highly expressed and conserved during lactation HM miRNAs could be related to the continuous remodelling of the lactating breast associated with cell turnover and milk production, which is known to remain consistent during established lactation. Thus, they could potentially be used as indicators of lactation performance and any pathologies of the gland during the breastfeeding period. Further, it can be postulated that some of these miRNAs, which are known to be involved in numerous fundamental events of tissue development, play important regulatory functions in the rapid development of different tissues and organs of the infant that occurs early in life. Milk exosomes have been found to protect milk miRNAs from harsh digestive conditions, such RNase and high PH (Zhou et al., 2012, Kosaka et al., 2010), and facilitate transfer of milk miRNAs into the bloodstream and host cells via endocytosis (Wolf et al., 2015). Recently, Hassiotou et al. (2015) provided evidence that native milk stem cells migrate and integrate into the neonate’s stomach, thymus, liver, pancreas, spleen, and brain (Hassiotou et al., 2015), whereas previous reports have shown this for milk immune cells (Jain et al., 1989, Zhou et al., 2000, Weiler et al., 1983). It has been suggested that in addition to exosomes, milk cells and fat globules may provide a similar protection to miRNAs during breastfeeding to further facilitate their transport into the bloodstream and their functionality in the infant (Alsaweed et al., 2015a).
Figure 4. Top 20 known miRNAs in HM cells (A) and lipids (B) in each lactation stage (months 2, 4 and 6 postpartum). The contribution of the top 20 known miRNAs in the cell and lipid fractions was 88.4% and 88.2%, respectively, compared to all identified miRNAs in each fraction. *: p<0.05, **: p<0.01.

Consistent with previous studies (Alsaweed et al., 2015bb), 496 known miRNAs (63.9% of the total known miRNAs), were similarly expressed between the two HM fractions (Supplementary 4, Table I in S1 File). Apart from miR-21-5p, the top 19 highly expressed known miRNAs did not differ between the two HM fractions. These similarities in the profiles of the top 20 most highly expressed known miRNAs between HM cells and lipids suggest that the known miRNAs of the two fractions are mainly reflective of the lactocyte, from which HM fat originates (Lemay et al., 2013). Still, approximately a third of known miRNAs were differentially expressed between the two fractions (Supplementary 4, Table I in S1 File). Moreover, the novel miRNAs were mother-specific, with very different profiles between the two HM fractions (Supplementary 4, Table G in S1 File). This suggests
that the cell fraction of milk is more appropriate for novel miRNA discovery studies. Of the top 20 most highly expressed novel miRNAs, 13 were upregulated in cells and 1 in lipids. These differentially expressed known and novel miRNAs between HM cells and lipids need to be further investigated for any specific roles in the lactating breast and/or the infant, and they likely reflect the fact that the miRNA concentration of the lipid fraction is representative of the lactocytes, whilst that of the cell fraction of not only the lactocytes, but also the whole cellular hierarchy of the lactating epithelium. This is also reflected in the absence of a relationship between the miRNA concentration of the cells and the HM cell content or the number of known and novel miRNA species (p>0.05), contrary to the positive association between the miRNA concentration of the lipids and the HM fat content or the number of known miRNA species (p<0.05). The above further indicate that the miRNA content and composition of the HM fat represents specifically the lactocyte, whereas that of the HM cells represents a range of different cell types, with different transcription and miRNA synthesis rates, and is thus more variable.

The HM fat and cell contents and the miRNA concentration of cells and lipids did not change in the first 6 months of lactation. Yet, approximately a third of all known miRNAs were differentially expressed across this period. Although the abundant HM miRNAs that are conserved at similar expression levels may play important functions in the breast and/or infant that are consistent during the first 6 months postpartum, those miRNAs that are differentially regulated may reflect the remodelling of the mammary gland in response to changing infant feeding patterns, which usually occur in the transition from exclusive to non-exclusive breastfeeding. Similar changes in expression of certain milk miRNAs with the stage of lactation were previously reported in other mammals such as the bovine (Chen et al., 2010) and porcine (Gu et al., 2012).
Figure 5. Distribution percentage of the top 20 most highly expressed known miRNA species in each stage of lactation examined (months 2, 4 and 6; n=10 in each month) (A-C), and in HM cells (n=30) (D) and lipids (E) (n=15). The top 20 most highly expressed known miRNAs contributed 88.8±1.4% of all identified known miRNAs.
HM-enriched miRNAs of the let-7 family, in particular let-7f-5p which was the miRNA with the highest expression here (Fig 4A-4B; Supplementary 4, Table C in S1 File) and in previous studies (Alsaweed et al., 2015bb), gradually increased in the first 6 months postpartum (Fig 6A), suggesting significant regulatory functions. Further, most of the let-7 miRNA family members have also been found to be in the most highly expressed miRNAs in cow’s skim milk (Chen et al., 2010). Gene target analysis using TargetScan (release 7.0) revealed more than 100 genes that can be targeted by let-7f-5p. GO analysis on these targets showed several cellular metabolic processes regulated by let-7f-5p, including protein, carbohydrate, and triglyceride synthesis. This abundant HM miRNA has also been found to play a critical role in tissue development (Bussing et al., 2008), especially of the nervous system (Kapsimali et al., 2007). Moreover, HM highly expressed miR-22-3p was identified to regulate T lymphocyte differentiation and development (Zhou et al., 2008). Other highly expressed HM miRNAs, such as miR-182-5p/181a-5p, were also found to play different roles in immune response and immune cell differentiation. The latter miRNA (miR-181a-5p) has been found at high levels in thymocytes (Chen et al., 2004), promoting their differentiation into mature T lymphocytes that respond to foreign pathogens (Neilson et al., 2007). Interestingly, it has been recently reported using a murine model that milk stem cells migrate in large numbers to the neonatal thymus, where they integrate and differentiate into thymocytes (Hassiotou et al., 2015), a process that may be further facilitated by milk cellular miRNA such as miR-181a-5p. In addition, the thymus of breastfed infants is known to be larger in size compared to the thymus of formula-fed infants (Moore et al., 2009), further suggesting involvement of HM miRNA in the maturation, development and function of the infant’s thymus.

Other highly expressed HM miRNAs were identified with involvement in numerous biological functions and potential significance for the infant (Supplementary 4, Tables N-Q in S1 File). For example, HM miR-375 acts in pancreatic islets and is required for normal glucose homeostasis in response to insulin increase (Kloosterman et al., 2007). HM miR-148a-3p regulates the DNMT1 enzyme, participating in liver development, and also acts as a tumour suppressor (Gailhouste et al., 2013). Some members of the let-7 family, which are abundant in HM, particularly let-7f-5p, are known to play important roles in various biological functions, such as controlling cell differentiation early in development (Abbott et al., 2005) and influencing growth and development (Johnson et al., 2007, Bussing et al., 2008). In addition to the known miRNAs, the top 20 novel miRNAs in HM cells and lipids were found to be important regulators of cell growth and immune system development, in
particular hematopoietic or lymphoid organ development and somatic diversification of immune receptors (Supplementary 4, Tables P-Q in S1 File).

6.7 CONCLUSION
Our findings highlight HM as one of the richest sources of miRNAs in the human body. Numerous known miRNAs were identified here for the first time in HM cells and lipids, as well as numerous high-confidence novel miRNAs, which may play significant regulatory functions in the lactating mammary gland and/or the breastfed infant. HM cells, exosomes and fat globules are thought to act as protective vehicles transferring these miRNAs to the infant’s bloodstream and different tissues, with particular interest in HM cells. These appear to harbour the greatest number of miRNAs in HM, and have recently been shown to survive the infant’s gastrointestinal tract and be distributed and integrated into various infant tissues (Hassiotou et al., 2015). Certain highly expressed HM miRNAs involved in development, growth and metabolic processes are conserved in the first 6 months of lactation, potentially participating in the maintenance of milk production during established lactation and/or the development of the infant. The variation seen in the composition of approximately a third of HM miRNAs across this period, together with the mother-specific profiles of novel miRNAs, suggests adaptation to infant needs early in life.
Figure 6. Comparison of expression of the top 20 most highly and commonly expressed known (A) and the 20 most highly expressed novel (B) miRNAs in the cell and lipid fractions of HM considered together, during the three stages of lactation examined (months 2, 4 and 6). Due to the variability in the total reads between novel miRNAs (B), novel_mir_189 is presented separately from the rest. From the top 20 known and novel miRNA species, only the most highly expressed known miRNA let-7f-5p was expressed at higher levels from month 2 to month 6 (p<0.01).
CHAPTER 7

GENERAL DISCUSSION
Breastmilk (human milk, HM) facilitates the term infant’s optimal development, protection and growth (Kramer, 2010). Parts of its composition, such as its macronutrient content and immunoprotective molecules including immunoglobulins and lactoferrin, have been extensively investigated (Newburg, 2001). However, further research is still required to characterize and address the functional significance of more recently discovered components of HM. These include cellular and biomolecular elements that may provide important benefits to the infant and may also be used as biomarkers of breast health and performance during lactation (Hassiotou and Geddes, 2013). Amongst these, miRNAs are small non-coding RNA molecules that are present in various tissues and fluids of the body and are crucial regulators of gene expression, and thus cell function. miRNAs with known immunomodulatory and developmental roles are abundant in HM, and may contribute to the growth and development of infant (Kosaka et al., 2010, Munch et al., 2013), with additional potential significance as breast functional biomarkers (Zhang et al., 2014, Alsaweed et al., 2015a). In this thesis, the isolation of these molecules from different fractions of HM was optimized, and HM miRNAs were further characterized using OpenArray and Sequencing technologies to obtain a thorough understanding of the miRNA profile of HM and factors that may influence it.

Extraction of total RNA and miRNA from HM cells, lipids and skim milk was optimized to allow standardization of the procedures measuring their concentrations and quality for subsequent analyses, such as miRNA profiling. Direct comparisons of the miRNA concentration between the three HM fractions using optimized methodology for each fraction had not been done before, which had resulted in some variability in previous literature (Weber et al., 2010, Kosaka et al., 2010, Munch et al., 2013). The previous fractionation of whole milk using high-speed centrifugation or after freezing were two of the main technical issues that may have compromised integrity of each HM fraction resulting in miRNA cross-contamination between fractions (Alsaweed et al., 2015a). Moreover, together with skim milk and milk lipids, milk cells should be used for the miRNA profiling, and not discarded, as they are the richest miRNA fraction in HM. The filter column-based extraction method provides better miRNA yields and quality from all three HM fractions compared to other methods used in the literature, such as phenol/chloroform-based methods. These are important considerations that can improve comparison of different studies via methodology standardization, and should be therefore considered in future miRNA studies of HM, being also applicable, at least to some extent, to miRNA analyses of milk from other mammalian species.
The optimized protocols developed here were first applied to profile miRNAs in HM cells and lipids, and compare the HM miRNA composition of these two less studied fractions with maternal peripheral blood mononuclear cells (PBMCs) and plasma. The majority of miRNAs in HM cells and lipids were not related to PBMCs or plasma, emphasising the uniqueness of miRNA composition of HM and supporting the notion that HM miRNAs primarily originate from the lactating mammary epithelium and are secreted into breastmilk. HM cells and lipids are enriched in different miRNA species compared to previously studied skim milk, with milk cells being richer in miRNAs and showing greater miRNA diversity compared to milk lipids. The synthesis of HM miRNAs in the lactating breast further supports their lactation-specific function and highlight their potential use as biomarkers of breast health and lactation performance. Future work can use breastmilk-derived stem cell cultures to shed light into the role of miRNAs in regulating the different stages of development and expansion of the lactating breast. Moreover, the involvement of miRNAs in the response of the lactating breast to infections, such as mastitis, can be elucidated, revealing a new component of the mammary gland and the milk that could be used as a diagnostic marker.

The studies of this thesis showed that HM, particularly its cellular and lipid components, is one of the richest sources of miRNAs in the human, and it may potentially contain undiscovered (novel) miRNAs expressed at lower levels than the known miRNAs profiled previously. Moreover, factors such as infant feeding (milk removal) have been previously shown to influence the cell and lipid content of HM (Hassiotou et al., 2013ah, Mitoulas et al., 2002), and may thus also affect its miRNA content. To give insight into novel HM miRNAs and the effects of feeding, next generation sequencing was used in the HM cell fraction collected pre- and post-feeding in month 2 of lactation. Indeed, some miRNAs were differentially expressed between pre- and post-feed milk, with post-feed milk containing significantly more cells, and thus miRNAs, than pre-feed milk. The upregulated miRNAs in post-feed milk were found to be associated with synthesis of milk components such as lactose, estrogen, growth hormone, triacylglycerol, and fatty acids, as well as activation of cell division and proliferation, suggesting support of milk synthesis. These findings further emphasised how dynamic the miRNA composition of HM is in the short-term, and that it can be influenced to some extent by milk removal, supporting feeding on demand practices to better meet the changing infant needs.

In contrast to HM, bovine milk- and soy milk-based infant formulae were found to contain significantly fewer human miRNA species. As it has been previously emphasised
Alsaweed et al., 2015a), the manufacturing process of infant formulae discards the milk cells and lipid fractions, which conserve the majority of milk miRNAs.

In a next study, the cell and lipid miRNA profiles of HM were compared during the first six months of lactation in samples collected longitudinally from 10 lactating women. Approximately one third of the HM miRNAs were differentially expressed in the first six months postpartum. Although 71.2% of the commonly identified miRNAs in the first six months were similarly expressed during this period, milk from month 4 of lactation contained more upregulated miRNAs compared to month 2 and 6. These differentially expressed miRNAs may play important roles in the remodelling of the mammary gland in response to changing infant feeding patterns, which usually occur in the transition from exclusive to non-exclusive breastfeeding. Interestingly, let-7f-5p was the most highly expressed miRNA, and it dramatically increased between months 2 and 6, suggesting a significant role in the infant’s development. In addition to let-7f-5p, the top 20 most highly expressed HM miRNAs were found to be involved in the synthesis and regulation of milk nutritional components such as lactose, triacylglycerol, fatty acids, growth hormone and insulin receptor, in immune responses and in development. They are also involved in the control of body fluid balance, thirst, and appetite, implicating a functional significance for the infant. In addition, HM cells and lipids conserved numerous novel miRNAs expressed at lower levels, highlighting the diverse miRNA content of HM, with molecules whose functionality is yet undiscovered.

Together, the findings of this thesis emphasize that HM is a rich source of molecules with bioactivity, amongst which miRNAs may play a central, yet not fully established, role. We have conducted a comprehensive screening of HM miRNAs in healthy breastfeeding women in the first six months of lactation, providing a basis for subsequent studies on the significance of HM miRNAs as biomarkers of breast health and lactation performance as well as in the breastfed infant. HM miRNAs may be used as a non-invasive tool to further investigate and understand the biology of the lactating mammary gland, and its responses to abnormal conditions such as infections of the breast (e.g. mastitis) or low milk supply. Moreover, their high stability at low pH in the gastrointestinal tract (Wolf et al., 2015, Zhou et al., 2012), and their packaging in microvesicles such as exosomes, membrane-bound entities (i.e. LDL and HDL), protein complexes (i.e. Argonaute) (Witwer and Hirschi, 2014) and cells (Alsaweed et al., 2015a) have strongly suggested their survival in the infant (Alsaweed et al., 2015a). The mechanism of exogenous miRNA transfer is more likely transcytosis, i.e. uptake of stable miRNAs by intestinal epithelial cells during digestion.
However, HM miRNAs may also be delivered to the infant via transmembrane channels via endocytosis (May and Plasterk, 2005, Witwer and Hirschi, 2014). Therefore, HM miRNAs may act as critical regulators of infant protection, growth and development.
The supplementary material of each chapter (Chapters 3-6) is available in the CD attached to this thesis.

**Supplementary material 1 (CHAPTER 3)**
Table S1

**Supplementary material 2 (CHAPTER 4)**
Tables S1-S22
Figures S1-S6

**Supplementary material 3 (CHAPTER 5)**
Tables S1-S14
Figures S1-S10

**Supplementary material 4 (CHAPTER 6)**
S1-S3 Figures
S1 File (Tables A-Q)


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APPENDIX

Copies of the published versions of Chapters 2, 3 and 4.
Article

MicroRNAs in Breastmilk and the Lactating Breast: Potential Immunoprotectors and Developmental Regulators for the Infant and the Mother

Mohammed Alsaweed 1,2, Peter E. Hartmann 1, Donna T. Geddes 1 and Foteini Kakulas 1,*

1 School of Chemistry and Biochemistry, The University of Western Australia, Crawley 6009, Western Australia, Australia; E-Mails: mohammed.alsaweed@research.uwa.edu.au (M.A.); peter.hartmann@uwa.edu.au (P.E.H.); donna.geddes@uwa.edu.au (D.T.G.)

2 College of Applied Medical Sciences, Majmaah University, Almajmaah, Riyadh 11952, Saudi Arabia

* Author to whom correspondence should be addressed; E-Mail: foteini.kakulas@uwa.edu.au; Tel.: +61-864-884-467.

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Abstract: Human milk (HM) is the optimal source of nutrition, protection and developmental programming for infants. It is species-specific and consists of various bioactive components, including microRNAs, small non-coding RNAs regulating gene expression at the post-transcriptional level. microRNAs are both intra- and extra-cellular and are present in body fluids of humans and animals. Of these body fluids, HM 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. Potential effects of exogenous food-derived microRNAs on gene expression have been demonstrated, together with the stability of milk-derived microRNAs in the gastrointestinal tract. Taken together, these strongly support the notion that milk microRNAs enter the systemic circulation of the HM fed infant and exert tissue-specific immunoprotective and developmental functions. This has initiated intensive research on the origin, fate and functional significance of milk microRNAs. Importantly, recent studies have provided evidence of endogenous synthesis of HM microRNA within the human lactating mammary epithelium. These findings will now form the basis for investigations of the role of
microRNA in the epigenetic control of normal and aberrant mammary development, and particularly lactation performance.

**Keywords:** human milk; breastmilk; breastfeeding; RNA; microRNA; cells; lipids; skim milk; immune system; development; infant formula

1. Introduction

Since their recent discovery in 1993, microRNAs (also known as miRNAs) have emerged as key regulators of gene expression at the post-transcriptional level in humans, animals and plants [1,2]. They act by binding to an mRNA target to either inhibit the translation of mRNA into protein and/or promote its degradation [3–5]. The microRNA family includes extremely small non-coding RNA (~22 nucleotide in length) that have been isolated from cells, tissues and body fluids of various mammalian species [6,7]. The biogenesis of microRNA comprises three main processes [8]: microRNAs are first transcribed into primary microRNA (pri-microRNA) from specific independent genes on DNA by RNA polymerase II, and are then converted into hairpin precursor microRNA (pre-microRNA) by the Drosha–DGCR8 complex. The enzyme Dicer then produces mature microRNA from pre-microRNA in the cytoplasm (Figure 1) [7,9]. According to miRBase version 21.0 (http://www.mirbase.org) released in June 2014, the number of pre-microRNAs in the human is estimated to be 1881. These correspond to 2588 known mature microRNAs, while the number of human protein-coding genes that are considered to be targets of microRNAs is estimated to be approximately 20,000–25,000 [10]. Therefore, a single mature microRNA can bind and regulate multiple mRNAs (genes) [6]. Importantly, ongoing research is still discovering new microRNAs.

In mammalian cells, various functional studies have demonstrated that microRNAs regulate up to 50% of protein synthesis (gene expression) [4]. Several roles of different microRNAs were investigated experimentally and they are involved in regulating a range of biological processes in plants and mammals (including humans) [3,11,12]. In addition to controlling normal physiological processes, microRNAs have been implicated in pathologies such as cancer, autoimmune diseases, gastrointestinal diseases, and diseases of the reproductive system [3,4]. microRNAs have recently been reported to be important regulators of pluripotency-related genes and they have been used to reprogram somatic cells into induced pluripotent stem cells (iPSCs) [13–15]. This regulation could potentially be an important method for regenerative medicine and biomedical research, as it eliminates the need for viral vectors. Viral vectors are used to reprogram cells into iPSCs, however they have been shown to uncontrollably influence reprogramming via random insertion of exogenous sequences into the genome [16,17].

Further to their role in the epigenetic regulation of stem cell fate and function, microRNAs also regulate the mammalian immune system. Their functions include regulation of T and B cell development [18,19], release of inflammatory mediators [20], proliferation of neutrophils and monocytes [21], and differentiation of dendritic cells and macrophages [22]. microRNAs are also thought to be involved in haematopoiesis [23], cardiac muscle development [24], insulin secretion [25], and neurogenesis [26]. Given their role in numerous physiological processes, deregulation of microRNA function can lead to disease; therefore, increasing evidence supports their use as diagnostic biomarkers.
Either upregulation or downregulation of microRNAs has been found to be associated with initiation and progression of some types of cancers [27]. These include breast cancer [28], where upregulation of oncogene miR-2 has been shown to be involved in both initiation and progression of the disease [29].

**Figure 1.** The predicted biogenesis of microRNA. MicroRNA are first transcribed from specific genes on DNA as primary microRNA (pri-microRNA) by RNA polymerase II (RNAPII). In the nucleus, pri-microRNA are converted into ~70-nucleotide precursor hairpin microRNA (pre-microRNA) by the enzymatic Drosha–DGCR8 complex. Pre-microRNA are then transported from nucleus to the cytoplasm by Exportin 5. There, the Dicer-TRBP complex produces ~20 base pair 3′ microRNA and 5′ microRNA duplex. Dicer with assistance from argonaute 2 (AGO2) generates mature microRNA by cleaving the double strand of pre-microRNA. Only one strand of microRNA (3′ microRNA or 5′ microRNA) can be attached into the RNA-induced silencing complex (RISC). Finally, the microRNA/RISC complex binds to 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.

High levels of microRNAs have been detected in body fluids, such as plasma, urine, saliva, seminal fluid, tears, cerebrospinal fluid (CSF) and more recently, milk [30]. Milk is a non-invasive source of numerous biomolecules that are either synthesized in the lactating breast or are transferred via the
systemic circulation and provide important functions for both the lactating mother and the breastfed infant. To a large extent, milk microRNAs appear to be endogenous to the mammary gland [31] 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. Further, food-derived microRNAs (e.g., exogenous miR-168a) [32] have been suggested to survive the mammalian gastrointestinal (GI) tract and regulate mammalian genes [32,33,34]. As human milk (HM, breastmilk) is highly enriched in microRNAs, it would be of great interest to illuminate the fate and function of this breastmilk 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 HM [35,36], have been shown to survive the GI tract of adult humans and increase in their serum post-consumption [37]. More recently, bovine milk exosomal microRNA transfer was demonstrated in human intestinal colon cells and rat small intestinal cells by endocytosis in vitro [38], further highlighting the important role of vehicle-mediated transfer of milk microRNA [39].

2. MicroRNAs Are Highly Enriched in Milk

2.1. microRNAs in Mammalian Milk

HM is considered the optimal food for term infants in the first six months of life [40], with the World Health Organization recommending exclusive breastfeeding for up to six months, with continuation of breastfeeding for at least the first two years [41]. In addition to providing nutrition, HM has long been known to protect the infant from infections and to play developmental functions integral to the infant, in which microRNAs are likely to be highly involved. microRNAs can be isolated and experimentally studied in the main three fractions of milk, the cells, lipids, and skim milk (Figure 2). Interestingly, HM is one of the richest microRNA source of all body fluids in the human, containing up to ~1400 mature microRNAs (Figure 3) [35,36,42,44,45,46]. Cellular and lipid fractions of HM contain a greater amount of microRNAs compared to the skim milk fraction [44,47], which is important to consider when analyzing milk microRNA. Not surprisingly, a wide variation in microRNA expression amongst lactating women has also been shown [48], with the factors that influence this variation having not been studied to date. Further, animal studies have shown that the type and expression levels of microRNA are distinctly different between the lactating and non-lactating mammary glands in the cow [49]. microRNAs were also found to be in involved in mammary gland development in murine models [50]. These animal studies have suggested a key role of microRNAs in the regulation of the development and performance of the lactating mammary gland, and they therefore have the potential to influence milk synthesis.

2.2. microRNAs in Different Milk Fractions

In 2010, Weber et al. isolated and profiled microRNA from 12 different human body fluids, including human colostrum and milk [30]. In the same year, microRNAs were profiled in skinned HM [48]. Since then, a few studies have profiled microRNA in HM and in the milk of other mammalian species (Table 1) [36,44,48]. This was carried out using different platforms including qPCR, microarray analysis and small RNA sequencing [30,36,44,48,51,52]. Although qPCR is generally the method of choice
providing high sensitivity and specificity, with low RNA input requirements, small RNA sequencing is often employed to screen for all microRNAs present. This allows identification of novel microRNAs and it is sensitive enough for microRNA quantification. Small RNA sequencing is done either using high throughput next generation sequencing (NGS) or small-scale NGS platforms [4].

**Figure 2.** A workflow of microRNA identification in HM. Whole HM can be fractionated by centrifugation for 20 min at 800 g at 20 °C to obtain three fractions including the cells, the lipid layer and skim milk. Total RNA and microRNA can be extracted from each fraction using the optimal kit [47]. Profiling of microRNA after quantification and measurement of its quality can be performed using three different methods [47]: phenol/guanidine, filter column, and a combination of the filter column and phenol/guanidine methods. Small RNA sequencing can determine novel microRNAs and identify all microRNAs in a sample. Microarray analysis and qPCR-based methods can on principle only measure specific microRNAs. Validation of presence and expression patterns of a microRNA of interest is done using qPCR as it is highly sensitive and specific.
Figure 3. Differences in the microRNA content between the different fractions of human milk. (A) Box plots showing the total RNA content (enriched in microRNA) measured using NanoDrop 2000 in HM cells ($n = 30$ milk samples from 20 mothers), lipids ($n = 127$ milk samples from 79 mothers) and skim milk ($n = 116$ milk samples from 79 mothers) obtained from healthy breastfeeding mothers [35,45,47]. (B) Box plot showing the number of mature microRNA species that have been identified in HM cells, lipids and exosomes using deep small RNA sequencing. HM cell microRNAs were profiled in $n = 20$ samples collected from 10 healthy exclusively breastfeeding mothers in month 2 of lactation [35] using Illumina HiSeq2000, with total clean reads of 268,681,616 matched to miRBase version 20.0. This study identified 1467 different mature known microRNAs in HM cells. HM lipid samples ($n = 7$) were sequenced using an Illumina 1G Genome analyzer, with 124,110,646 clean reads mapped to miRBase version 16.0. This study identified 308 mature known microRNAs in HM lipids [44]. HM exosome samples ($n = 4$) were sequenced using an Illumina Genome analyzer II, with 83,520,000 clean reads matched to miRBase version 17.0. This study identified 602 mature known microRNAs in HM exosomes [36].
Amongst the 12 human body fluids analysed, Weber et al. used qPCR to profile 429 known mature microRNAs in mature skimmed HM and 386 known mature microRNAs in skimmed human colostrum (the mammary secretion produced in the first few days postpartum) [30]. This study demonstrated for the first time the high abundance of microRNA in HM, 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) [30]. Microarrays were first used to profile microRNAs in HM by Kosaka et al., who examined 281 different microRNAs in skimmed HM obtained in the first six months of lactation [48]. Although these two pioneering studies focused on microRNA analysis in skimmed HM, it has been argued that the other two main milk fractions (lipids and cells) are likely to harbor large quantities of microRNAs. Indeed, later Munch et al. analyzed the lipid fraction of HM using small RNA sequencing and found high quantities of microRNA, reporting 308 microRNA species that are predicted to target a total of 9074 genes [44]. In addition, a subsequent study performed deep sequencing to profile microRNAs from HM exosomes [36], which are small cell-derived vesicles carrying proteins and molecules present in all body fluids [53]. This study found 639 exosomal mature microRNAs originating from 452 pre-microRNAs [36].

**Table 1.** Number of mature known microRNA species reported for different mammalian species for the three milk fractions (cells, lipids and skim milk).

<table>
<thead>
<tr>
<th>Species</th>
<th>Milk Fraction</th>
<th>microRNAs *</th>
<th>Profiling Method</th>
<th>Reference</th>
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</thead>
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<tr>
<td><strong>Human</strong></td>
<td>Skim milk (mature)</td>
<td>429</td>
<td>qPCR</td>
<td>[30]</td>
</tr>
<tr>
<td></td>
<td>Skim milk (colostrum)</td>
<td>386</td>
<td>qPCR</td>
<td>[30]</td>
</tr>
<tr>
<td></td>
<td>Skim milk (mature)</td>
<td>281</td>
<td>Microarray</td>
<td>[48]</td>
</tr>
<tr>
<td></td>
<td>Milk exosomes</td>
<td>639 **</td>
<td>Solexa deep sequencing</td>
<td>[36]</td>
</tr>
<tr>
<td></td>
<td>Milk lipids</td>
<td>308</td>
<td>Solexa deep sequencing</td>
<td>[44]</td>
</tr>
<tr>
<td></td>
<td>Milk cells</td>
<td>450 ***</td>
<td>TaqMan OpenArray</td>
<td>[45, 46]</td>
</tr>
<tr>
<td></td>
<td>Milk lipids</td>
<td>337 ***</td>
<td>TaqMan OpenArray</td>
<td>[45, 46]</td>
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<tr>
<td></td>
<td>Milk cell pre-feed</td>
<td>1287</td>
<td>Solexa deep sequencing</td>
<td>[35]</td>
</tr>
<tr>
<td></td>
<td>Milk cell post-feed</td>
<td>1308</td>
<td>Solexa deep sequencing</td>
<td>[35]</td>
</tr>
<tr>
<td><strong>Bovine</strong></td>
<td>Skim milk (colostrum)</td>
<td>230</td>
<td>Solexa deep sequencing</td>
<td>[52]</td>
</tr>
<tr>
<td></td>
<td>Skim milk (mature)</td>
<td>213</td>
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<td>[52]</td>
</tr>
<tr>
<td></td>
<td>Skim milk (colostrum)</td>
<td>100</td>
<td>Microarray</td>
<td>[54]</td>
</tr>
<tr>
<td></td>
<td>Skim milk (mature)</td>
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<td>Microarray</td>
<td>[54]</td>
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<tr>
<td><strong>Porcine</strong></td>
<td>Milk exosomes</td>
<td>180 **</td>
<td>Solexa deep sequencing</td>
<td>[51]</td>
</tr>
<tr>
<td></td>
<td>Milk exosomes (colostrum)</td>
<td>491</td>
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<td>[55]</td>
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<tr>
<td><strong>Murine (rat)</strong></td>
<td>Skim milk (colostrum)</td>
<td>128</td>
<td>Microarray</td>
<td>[56]</td>
</tr>
<tr>
<td></td>
<td>Skim milk (mature)</td>
<td>144</td>
<td>Microarray</td>
<td>[56]</td>
</tr>
</tbody>
</table>

* Number of detectable mature microRNAs; ** Precursor microRNAs (pre-microRNAs); *** 8 ≤ Ct ≤ 29.

More recently, we used the Taqman OpenArray Panel system (Applied Biosystems, Foster City, CA, USA) to screen 754 human mature microRNAs in the cellular and lipid fractions of HM. In addition, we performed comparisons with the microRNA content of maternal peripheral blood mononuclear cells (PBMCs) and plasma [46]. This analysis identified 293 and 233 microRNA species in the breastmilk cell and lipid fractions, respectively. Maternal PBMCs contained 345 different microRNAs, whereas only 169 were found in maternal plasma. Breastmilk cells and PBMCs had significantly higher microRNA content compared to breastmilk lipids and plasma, respectively.
Correlation and cluster analyses showed that breastmilk cells and lipids were highly related in terms of microRNA expression patterns and species, however PBMC microRNAs were not correlated with breastmilk microRNAs. In plasma, marked inter-individual variation in expression levels of single microRNA species was observed. This study together with our previously published optimization of microRNA extraction from HM [47] demonstrated the presence of microRNAs in all three fractions of HM (cells, lipids, and skim milk), and revealed that HM conserves more microRNAs with different expression patterns compared to maternal plasma [46], but also other human body fluids [30]. Skim milk (known as the plasma phase of milk) is cell and fat globule free milk, and appeared to be extremely low in microRNAs and total RNAs compared to the cell and lipid milk fractions [47].

In addition to HM, microRNA have been analyzed in other mammals’ milk, particularly the dairy cow, in various studies (Table 1) [51,52,53,54]. Mature bovine skim milk has been shown to contain 213 microRNA species using deep sequencing [52] and 53 using microarrays [54]. Some of these microRNA are enriched in either mature milk or colostrum [52]. Specifically, bovine colostrum has been found to be richer in microRNA (both higher total content and species number) compared to mature bovine milk, where 230 and 100 microRNAs were identified in skimmed bovine colostrum in the above studies, respectively (Table 1) [52,54]. Similarly, bovine milk-derived microvesicle RNA and total milk RNA levels have been shown to be higher in colostrum compared to mature milk [53,54]. Unlikely bovine milk, skimmed rat colostrum was shown to conserve fewer microRNA species (128) than mature rat skim milk (144) (Table 1) [56]. Izumi et al. [54] isolated 53 mature microRNAs from mature bovine skim milk, and 100 from skimmed bovine colostrum (Table 1). Gu et al. examined the exosomes 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 HM (Table 1) [51]. However, 39 pre-microRNA were identified that were not homologues of HM microRNA [51]. This was one of the first efforts to quantify changes in milk microRNA content over time, in this case within the first month of lactation, which can provide insight into both their involvement in the maturation of the mammary gland into an organ that synthesizes copious amounts of milk and their role(s) in the development and protection of the offspring. Another study in porcine milk exosomes identified 491 mature microRNAs by small RNA sequencing in the first 6 days postpartum (Table 1) [55]. These pre-microRNA that are shared between different species potentially have similar physio-pathological mechanisms of action and functions in milk amongst mammals [51]. In all animal milk microRNA studies to date, only a few microRNA were found to be highly expressed [30,36,44,48,51,52]. For example, the top 10 most highly expressed microRNAs in exosomal porcine milk were contributing approximately 87% of the total 234 microRNA [51].

### 2.3. Origin of Milk microRNAs

Our comparisons between breastmilk blood microRNA provided important insight into the origin of milk microRNA, with the mammary gland appearing to be the main source of milk microRNA, with the maternal circulation having a smaller contribution [46]. This was consistent with a recent microRNA analysis in tammar wallaby milk. In the tammar study, most microRNAs were differentially expressed between skim milk and blood serum, although the total number of microRNA species was

\[ p < 0.05 \]
similar in both milk and serum (86 and 82 microRNAs, respectively) [57]. Prior to these findings, it was believed that because ribosomal RNA (18S and 28S) measured by the Bioanalyzer is usually absent or low during the isolation and quantification of microRNA, microRNA are less likely to be secreted from milk cells [51,54]. Nevertheless, taken together, the similarities between milk cell and lipid microRNAs, the differences with PBMCs and plasma both in the human [45] and dairy cow [52], and the known secretion of milk lipids from lactocytes, which are the most abundant cell type in HM under healthy conditions [58], strongly suggest that the milk cell and lipid microRNAs are primarily endogenously synthesized in the mammary gland [45,57]. Moving forward, it will be important to understand the factors controlling mammary microRNA synthesis during pregnancy and lactation, as this is likely to impact the health and development of both the mammary gland and the infant.

2.4. Milk microRNAs as Diagnostic Tools

Although the microRNA content and composition of different milk fractions is being intensively investigated, the understanding of factors influencing them as well as the roles and functions of these molecules in the lactating mammary gland and for the breastfed offspring is still very poor. A number of maternal and/or infant characteristics have previously been reported to influence the composition of HM, including infant feeding, preterm birth, the stage of lactation, parity, maternal body mass index (BMI), infant sex, and the health status of the mother and the infant [59–65]. It is not unlikely that some of these factors may affect the microRNA content of HM, yet very few studies have examined these associations. Recently, the effects of infant feeding and milk removal on HM microRNA content and composition were investigated [45,46]. It is well established that post-feed milk contains more fat and cells compared to pre-feed milk [60,61]. Similarly, additional microRNA species were detected in post-feed milk, however the difference in the total number of microRNA species or the expression of the majority of microRNAs between pre- and post-feed milk was not statistically significant in a group of 10 lactating women examined in month 2 of lactation ($n = 10$, $p > 0.05$). Yet, a subgroup of 27 known and 1 novel microRNAs in this study were expressed more highly post-feeding ($p < 0.05$). From these findings, it can be concluded that milk removal may influence the content and/or expression of certain microRNAs in HM, but the overall microRNA composition appears to remain constant [35]. This is in agreement with Kosaka et al., who showed variable microRNA expression patterns between mothers, but claimed no significant intra-individual variation in microRNA expression [48]. Yet, this study only collected 2–4 samples from each of eight lactating women at different stages of lactation without standardizing the sampling based on infant feeding/milk removal or time of the day. Therefore, further studies are required to shed light into factors that may influence HM microRNA content within a mother. Nevertheless, Kosaka et al. [48] as well as the more recent study by Alsaweed et al. [35] showed great inter-individual variation in HM microRNA content, which could potentially be associated with parity, preterm birth, infant characteristics or environmental factors (e.g., maternal diet) [37,66]. Interestingly, maternal diet has been shown to influence other HM components, such as fatty acids [67] during lactation, but also fetal growth and health during pregnancy [68,69].
Similarly to numerous other immunological components of HM (immune cells, lysozyme, lactoferrin, immunoglobulins [62,70]), immune-related milk microRNA may be influenced by the health status of the mother and/or the infant. Although this has not been investigated in HM, a recent study in the dairy cow examined milk from healthy cows and those infected with *S. uberis* 0140J. It was found that 26 microRNAs isolated from milk cells described as monocytes were differentially expressed between the two cohorts [71]. The majority of the differentially expressed microRNAs are implicated in innate immunity, suggesting that infection of the lactating breast changes the milk microRNA profile to enhance immunoprotection and facilitate recovery. It is of note that in this study, milk monocytes were identified using the marker CD14, which is also expressed by milk epithelial cells [58,70]. Although epithelial cells are not the dominant cell type in bovine milk, in contrast to HM [58,70], the microRNA profiles reported in this study are likely to represent more cell types than just monocytic immune cells of milk.

Although in HM the effects of infection on microRNA profiles are currently under investigation, evidence from animal studies supports the use of milk microRNA as a tool of assessing the health status of the lactating breast as well as the response to treatment, similarly to what has been previously shown for breastmilk immune cells [62,70]. The potential diagnostic value of milk microRNA has also been suggested by other animal studies. miR-148a-3p, which has been found to be the most highly expressed microRNA in exosomes of HM [36], bovine [52] and porcine milk [51], has been proposed as a biomarker for raw milk quality control in the dairy industry, and also for artificial infant formulae [52]. The use of microRNAs as biomarkers for milk quality control was first proposed by Chen et al. due to their high stability in milk, even under very harsh conditions including the sterilizing process during product manufacture and milk processing [52]. However, Weber et al. [30] reported a lower concentration of miR-148a in skimmed HM than what was previously shown in bovine skimmed milk by Chen et al., in HM exosomes by Zhou et al., and in porcine milk exosomes by Gu et al. [36,51,52]. In addition to miR-148-3p, controversies exist over miR-494, which has been identified to be present in high concentrations in both HM [48] and bovine milk by Izumi et al. [54], but in very low concentrations in bovine milk by Chen et al. [52]. It is not clear the degree to which inter- and intra-species variations and factors associated with them have contributed to these differences. Further, differing methodological approaches and lack of standardization of milk collection, storage, processing, milk fractionation (if any), and RNA extraction are also likely sources of potential variation in results. Due to the rapidly evolving techniques in this field, the need is arising for greater emphasis of studies to optimize and standardize the methodology employed in milk microRNA research. These procedures had already been optimized for microRNA extraction and analysis in blood and plasma [72], and only recently this has been done for HM [47].

The optimization of microRNA and total RNA extraction from HM was conducted in three main milk fractions (cell, lipids and skim milk) using different extraction methods and commercially available kits. The most efficient kits and methods were reported for each HM fraction [47]. In this study, microRNAs were found to be enriched in HM, with different milk fractions yielding different microRNA concentrations [45]. Therefore, it became clear that different fractions of HM require different processing for extraction, profiling and functional studies. Importantly, milk samples were fractionated and analysed fresh upon expression and not after storage, enabling extraction and analysis of microRNA specific to each milk fraction. Previous studies on skim milk or milk lipids have typically analysed milk
after freezing [36,48,51], a process that is likely to result in cross-contamination between milk fractions due to membrane lysis (in milk cells and potentially fat globules) that is known to occur during freezing. These are important considerations for future investigations, with this optimization study now providing a standard protocol for HM microRNA analysis [47].

Furthermore, the optimisation and standardization of the methodology for milk microRNA analyses [47] opens new avenues for clinical exploitation of these molecules diagnostically, particularly given their non-invasive access via breastmilk. The suggested origin of many milk microRNA from the mammary gland [45,57] makes them an attractive target as biomarkers of the health status and performance of the lactating breast as well as of breast aberrations such as cancer. Epigenetic modification has been suggested to be involved in the normal development of the mammary gland, although the specific mechanisms are still largely unexplored [73]. For example, miR-29s was found to regulate important lactation-related genes in mammary epithelial cells from the dairy cow, such as casein alpha S1 (CSN1S1), E74-like factor 5 (Elf5), and glucose transporter 1 (GLUT1) [52,74]. Decreasing expression of miR-29s was associated with reduction of lactoprotein, triglycerides (TG) and lactose [52,74]. These findings can form the basis for examination of potential avenues for enhancement and optimisation of milk quality in the dairy cow, as well as improvement of lactation performance in women with insufficient milk supply.

3. Functions of Milk microRNAs

3.1. Stability and Uptake of Food-Derived microRNAs

Accumulating evidence confirms that microRNAs are present in all food sources. A number of studies have begun to investigate the fate of food-derived microRNAs, and whether they survive the GI tract and influence gene expression in mammals, including humans [32,33,34,37]. Food-specific microRNAs ingested orally have been found to be present in tissues and sera of different animals. Specifically, exogenous miR-168a, which is a rice-specific microRNA, was present in human sera in a Chinese cohort [32]. miR-168a was found to bind low-density lipoprotein receptor adapter protein 1 (LDLRAP1) in the human and mouse and to inhibit LDLRAP1 expression in the liver [32], demonstrating not only survival and uptake of this food-derived microRNA in humans, but also epigenetic regulation influencing tissue function. However, not all cross-microRNAs from food sources increase after consumption in mammals. For example, miR-167a and miR-824 are highly expressed in broccoli. Extensive consumption of broccoli sprouts by healthy humans did not change the expression pattern of either microRNA in the plasma [37]. Moreover, Dickinson et al. investigated exogenous microRNA uptake in mice by feeding them rice-containing chow. This study failed to report gene-targeting functions of the plant-derived microRNA or change in expression levels in the liver or plasma of the animals [75]. The above discrepancies between studies examining plant-derived microRNA transfer to mammals may reflect the lack and/or minimal contribution of exosomal transfer, since very limited evidence currently supports the production of exosome-like structures by plants that can be uptaken by mammalian cells [76]. Indeed, the packaging of microRNA within “transporting vehicles” may play an important role for their transfer and function in the recipient.
In the case of HM microRNA, it has been suggested that their transfer to the infant’s bloodstream is further facilitated by the known packaging of milk microRNA in “vehicle” structures, such as somatic cells, exosomes and other microvesicles, 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 endosomal transfer via epithelial cells of the intestine [38]. Through these vehicles, milk-derived microRNA are thought to be uptaken by the infant and participate in the epigenetic regulation of various functions including immune protection and development (Figure 4) [34,77]. In particular, recent studies have emphasized the importance of exosomal transfer of milk-derived microRNA. Extracellular vesicles including exosomes were shown to attach to different types of cells by endocytosis and to carry microRNA such as miR-21, which downregulated expression of TGFβRII and TPM1 in the recipient cells [78,79,80]. Extracellular vesicles were further investigated in commercial bovine milk, and were found to carry immunoregulatory microRNAs. These milk-derived extracellular vesicles were resistant to harsh conditions such as low pH [39]. Moreover, uptake and functionality, including therapeutic effects, of milk microRNAs has been recently demonstrated both in vitro and in vivo by Arntz et al. [81].

In this study, immune-related microRNAs (miR-30a, miR-223, miR-92a) were highly expressed in bovine milk-derived extracellular vesicles, which were uptaken in vitro by splenocytes and intestinal cells. When orally administered to BALB/c mice with experimental rheumatoid arthritis, these milk-derived extracellular vesicles were uptaken by RAW264.7 macrophages after 1–3 h as well as by the ileum tissue of the animals after 24 h. After 9 weeks of daily oral administration, arthritis was delayed, with reduction in cartilage depletion and joint inflammation [81].

In addition to exosomal transfer, HM cells may significantly contribute to the transfer of milk-derived microRNA to the infant. It has been recently shown that stem cells and immune cells from milk are transferred to the bloodstream of suckling pups in mice, and from there to different tissues [82,83]. Given that the cells of milk are highly rich in microRNA [35,45], this is likely to be an important source of microRNA for neonates in addition to milk exosomes.

MicroRNAs contained in infant formulae may also, to a small extent, be transferred to the infant’s circulation. Baier et al. investigated bovine milk-derived miR-29b and miR-200c in human adults after consuming cow’s milk and found that both microRNAs were increased 2-fold in human PBMCs and could potentially alter gene expression [37]. Both microRNAs were also highly expressed in the human plasma after few hours of consuming cow’s milk, and returned to the normal baseline expression level after 24 h of the initial milk consumption [37]. 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, a process that was influenced by glycoproteins on the surface of host cells [38]. However, differences exist in the microRNA content between bovine milk and infant formula, with the latter 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 [52,84]) [45]. 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.
Figure 4. A potential scenario depicting the sources of exogenous microRNA for the infant (breastmilk and infant formulae) and uptake of them along with other macro/micronutrients (i.e., fatty acids and amino acids) in the infant’s gastrointestinal (GI) tract. Breastmilk microRNAs can be delivered to the infant either as free molecules in skim milk, or via uptake of breastmilk cells, exosomes and other milk microvesicles in the GI tract. There, absorption is thought to occur through intestinal epithelial cells, from which milk-derived microRNA may reach various organs and tissues via the bloodstream to potentially perform functions, such as immunoprotection and developmental programming. It is of note that infant formulae are extremely poor in microRNA compared to HM, with potential differences also in the biological activity of these molecules in formula that merit further investigation.

One of the major requirements for confirmation of the functionality of food-derived microRNAs in mammals is to demonstrate their survival in the GI tract. Several studies have shown that microRNAs are extremely stable under various harsh conditions in vitro [36,48,85]. For breastmilk microRNA, the main considerations are resistance to RNase digestion and tolerance of low pH, and temperature and freeze/thaw cycles in the case of frozen HM [36,48,53]. Exosomal microRNA has been suggested to be protected [36], but other microvesicles including fat globules are also considered to be involved in microRNA protection, such as apoptotic bodies (small vesicles derived from apoptotic cell death) [48], which have not been investigated yet. 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 to home in different organs [32,57].

With regard to free microRNA in milk (such as those present in skim milk), a few theories have been proposed. Ribonuclease (RNase), which has been found to exist in all body fluids [86], degrades RNA molecules into small fragments, and is thus a key enzyme in the RNA maturation process [87]. Milk is known to have high RNase activities [36,48]. On the other hand, it is known that RNAs are unstable under harsh conditions [88,89]. Furthermore, HM and raw milk-derived microRNAs are found to be extremely stable even after RNase treatment in vitro [48,51,53,54]. The effects of low pH solution on microRNA integrity were examined using qPCR, showing that they are very stable [48,51]. It is important to note that the GI tract of infants is less acidic than that of adults [90], 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 [36,48,51]. As microRNA do not denature if subjected to different temperature cycles (at least those tested), microRNAs in stored HM fed to hospitalized infants are likely to be unaffected [48].

The above observations strongly support the survival of the natural microRNA content of HM 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 HM.

A recent study utilized a mouse model of miR-375 and miR-200c knockout (KO) pups fed by wildtype (WT) foster mothers or KO mothers [91]. The study concluded that no evidence was found for intestinal uptake in KO or WT pups of miR-375 and miR-200c derived from foster mother milk [91]. However, a small increase in the plasma levels of both of these microRNAs was detected in KO pups after nursing, suggesting that some microRNA copies are actually transferred to the bloodstream. It is of note that the examined microRNAs were not highly expressed in the WT mother milk of the murine model used. Further, both miR-375 and miR-200c are known to be involved in the control of endocytosis and/or exocytosis and to modulate epithelial function, which may influence exosomal endocytosis and thus uptake of the examined microRNAs [92,93]. Therefore, this KO mouse model and the chosen microRNAs may be inappropriate for investigating milk microRNA uptake by the nursed offspring. As it has been previously shown [37,38], not all dietary microRNAs are ideal for exogenous microRNA uptake studies [94]. Further studies are required to confirm the findings of Title et al. [91] as well as investigate milk microRNA uptake in more appropriate models.

On the other hand, similar to human serum [89], exogenous spiked-in synthesized (artificial) microRNAs in bovine milk were unstable and degraded compared to natural, endogenous microRNAs in bovine milk, which remained stable [51,54]. Interestingly, it is known that HM contains high quantities of very stable microRNAs, which are resistant to the pasteurization and milk bank storage procedures [36,48]. 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) [95]. In C. elegans, several microRNAs were stably expressed after UV-C treatment, such as miR-57-5p and miR-55-3p [96]. In a study of human primary keratinocytes exposed to UV-A and UV-B for 6 h, most microRNAs survived and no difference in expression was seen, except for few microRNAs such as miR-23b (upregulated) and miR-10a (downregulated) [97]. Yet, other studies have reported upregulation of skin microRNA in response to UV irradiation [98], which may be involved in cancer initiation in the
skin [99]. In summary, although ultraviolet radiation may not affect the majority of microRNA in mammalian cells, current evidence suggests that some microRNA species may be affected, and therefore this requires further investigation. In general, as it is also emphasized by the food industry and the industry of processing raw milk products such as milk powdechen [52,54], microRNAs in food and milk are very stable under and resistant to harsh conditions, and are therefore likely to be taken up by the HM fed infant, especially when protected within vehicle structures such as exosomes and live milk cells.

3.2. microRNAs Act as Immune Regulators

The tolerance of microRNAs of harsh conditions and the evidence that they migrate to the bloodstream and potentially different organs of the breastfed infant, suggest that they may play functional roles in the epigenetic regulation of development. Most of the microRNAs in HM are known for their immunocompetence [54,100,101], and they are particularly abundant (Table 2) [30,36,44,48]. 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 [100,101]. In addition, microRNA can play key roles in autoimmune conditions, such as inflammatory bowel disease (IBD), and regulate the development or prevention of these diseases [102]. Therefore, they could potentially be used as milk biomarkers to diagnose immune disorders such as allergic conditions [103,104].

Kosaka et al. [48] reported high quantities of microRNAs in HM with functions associated with the immune system during the first 6 months of lactation, including, but not limited to miR-181a, miR-17, miR-155, miR-150, and miR-223. In particular, miR-181 and miR-155, which are known to regulate B cell differentiation [48,105,106], are present in high concentrations in HM [44,48], suggesting a function in the development of the infant’s immune system.
Table 2. Immune-related microRNAs expressed in different fractions of milk from different mammalian species that have been highlighted in previous studies.

<table>
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<th>MicroRNA</th>
<th>Regulatory Function(s)</th>
<th>References</th>
<th>Presence in HM</th>
<th>References</th>
<th>Presence in Animal Milk</th>
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<td>MicroRNA</td>
<td>Regulatory Function(s)</td>
<td>References</td>
<td>Presence in HM</td>
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In addition, microRNA clusters miR-17 and miR-92 have been detected at high levels in HM, and given their function in regulating monocyte development as well as B and T cell differentiation and maturation [18,132], 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 [119], is also found at high levels in HM [48]. HM is rich in B cell-related microRNAs, such as miR-181 and miR-155, which potentially induce B cell differentiation [108,110]. On the other hand, miR-150, which is present in lower concentrations in HM, is known to act as a B cell suppressor [121,122]. Interestingly, Zhou and colleagues identified a large number of microRNAs in HM exosomes [36]. 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 [36]. Specifically, miR-30b-5p is known to induce immunosuppression and reduce immune cell activation [123]. In contrast, miR-182-5p induces T cell-mediated immune responses [124]. In the same study, 59 pre-microRNAs out of 87 (67.8%) that were detected in HM exosomes are considered to have immunological functions [36], which is consistent with a previous study in human skim milk microRNA [48]. The miR-17-92 cluster was also highly expressed in HM exosomes, with a speculated function as a developmental regulator of the immune system [19].

Some microRNAs present in milk may have more than one function. Interestingly, miR-17-92, which is known to have immunological functions, has also been implicated in oncogenesis by promoting cell proliferation and inhibiting apoptosis [133], although its role in the breastfed infant is poorly understood. Given that genes known to act as oncogenes have recently also been implicated in normal lactation [58,134,135], it can be hypothesised that miR-17-92, as well as other microRNAs with similar functions may participate in the milk-secretory function of the lactating breast rather than act as oncogenes in the context of lactation and breastfeeding. Some of these microRNAs therefore may be indicators of lactation performance.

In addition to HM, microRNAs with immunological functions have been identified in the milk of other mammalian species [51–54]. Profiling of bovine milk showed a high similarity of microRNA content to HM in respect to immune-related microRNAs [48,52,53], although this does not directly translate to the human infant. miR-181a and miR-155, which play important roles in immune system regulation and inflammation [44,136], were profiled in both bovine colostrum and mature milk, and were detected in high quantities, more so in colostrum [52,53]. More specifically, bovine immune-related microRNAs are present at higher concentrations in colostrum compared to mature milk [52], although this is yet to be investigated in HM. This appears to be one of the factors involved in providing greater immunological support required early in life, and is consistent with the higher numbers of immune cells and concentrations of various humoral immunological factors such as lactoferrin and secretory IgA in colostrum compared to mature HM [58,62,137–139]. Bovine milk 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 [54]. Additionally, the expression levels of a selected bovine microRNA group, including miR-223, miR-106b, miR-15b, miR-155, and miR-34a, have been analyzed using qPCR and compared between colostrum and mature milk, where they were found to be present at significantly different levels [54]. In contrast to bovine milk [52–54], rat skim milk [56] and porcine milk exosomes [51], a study showed that the levels of microRNA concentration and expression in HM.
are lower in colostrum compared to mature milk, where 429 different microRNAs were identified in human mature milk vs. 386 different microRNAs in human colostrum [30]. This warrants validation, together with investigation of differences in immune-related microRNAs between human colostrum and mature HM, since these molecules are likely to contribute in the immunoprotection of the neonate in the first days postpartum when it is most susceptible, as well as in the development of infant’s immune system and long-term protection against infections.

Kosaka et al. [48] and Gu et al. [51] first showed that human skim milk and porcine milk exosomes, respectively, contain microRNAs related to immune responses (Table 2). Gu et al. found that 58 out of 84 immune-related microRNAs listed in the Pathway Central Database (Qiagen, Valencia, CA, USA) were enriched in porcine milk exosomes [51], consistent with another recent study showing that HM exosomes were enriched with immune-related microRNA [36]. This study identified 12 out of 13 high abundance microRNAs in porcine milk exosomes to be expressed at higher levels in the first 3 days postpartum compared to later in month 1 postpartum (days 7, 14, 21 and 28) [51]. These 12 microRNAs (let-7a-5p, miR-182-5p, miR-191-5p, miR-200c-3p, miR-21-5p, miR-25-3p, miR-27b-3p, miR-30a-5p, miR-30c-2-5p & -1-5p, miR-30d-5p, miR-375-3p, and miR-574-3p) [51] are all immune-related and they regulate immune response genes and proteins [140]. More specifically, miR-30c-2-5p and miR-1-5p are immunosuppression regulators [123], whereas let-7a-1-5p regulates inflammation-associated cytokine IL-6 (interleukin-6) that induces STAT3 (signal transducers and activators of transcription 3) signaling [141]. The innate immune receptors can be activated and regulated by porcine milk miR-21-5p, toll-like receptor 4 (TLR4), and a key cytokine receptor via targeting programmed cell death protein 4 (PDCD4) and interleukin 12 (IL-12), respectively [142]. Also, IL-12 that is negatively controlled by miR-21-5p, is also responsible for regulating T cells and natural killer cells [143]. Further, the abundant porcine milk miR-27b was found to induce lipopolysaccharide (LPS), which inhibits and de-stabilizes the peroxisome proliferator-activated receptor c (PPARc), which is important in dampening inflammation via macrophage immune response [144].

Several bovine immune-related microRNAs [100,101] have been isolated from milk-derived microvesicles [53], and were also found to be expressed in the mammary gland using small RNA sequencing (Table 2) [145]. These microRNAs include miR101 and miR150, which are known regulators of T cells [146,147], and also miR-223 that has been reported to modulate innate immune cell (granulocytes and neutrophils) differentiation and activation [100,119,120]. miR-155 and miR-223 have been detected in bovine milk and are both involved in many immune functions [100], and potentially have anti-inflammatory effects especially in bovine colostrum. In addition to this function, miR-155 regulates T and B cell differentiation, and is a known modulator of T helper cells (Th1/Th2 balance) [148]. In contrast, miR-223 negatively regulates neutrophil proliferation and activation [100]. miR-25-3p targets KLF4, which is a potent mediator of inflammation [149], and has a crucial role in the development of the immune system [51]. miR-30a-5p targets GalNAc transferase 7 (GALNT7) to promote cellular invasion and immunosuppression [123]. miR-182-5p promotes T cell-mediated immune responses by inhibiting forkhead box protein O1 (FOXO1) [124], a gene that is also targeted by miR-21 in cancer [150,151]. miR-200c-3p has been identified to regulate T cell differentiation by targeting zinc finger E-box-binding homeobox 1 (ZEB1) [152], and also to regulate CD4 differentiation [153]. Moreover, as mentioned earlier, bovine milk microRNA expression patterns have been recently found to be altered during severe inflammation of mammary gland such as mastitis [71]. Gene target
analysis of the up- and down-regulated milk microRNAs such as miR-223 and miR-15b, respectively, revealed several roles of these microRNAs in response to mastitis [154]. Also, most milk microRNAs were downregulated during mastitis, suggesting that they actively control the mammary immune response to *S. uberis*, which causes mastitis in the dairy cow [154].

Collectively, the current data highlight that breastmilk is a complex system of different microRNA molecules with synergistic and antagonistic relationships, controlling specific immune responses in the infant and the lactating breast [36,42,44,48]. 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 both the infant and the lactating breast, further supporting the potential use of these molecules diagnostically.

### 3.3. microRNAs Are Key Regulators of Milk Lipid Metabolism

MicroRNAs have been isolated from HM lipid vesicles including fat globules in large numbers [44], as well as from milk cells and skim milk [44,46,155]. This has formed the basis for the potential use of both extracellular and intra-vesicle milk microRNAs as biomarkers in molecular diagnostics for a range of diseases [156]. Although lipid metabolism is usually regulated extracellularly, microRNAs have recently been identified to regulate genes associated with lipid metabolism at the post-transcriptional level [155]. These genes control functions related to cholesterol homeostasis, fatty acid oxidation, and lipogenesis, offering new opportunities for the treatment of various diseases such as dyslipidemias [157].

The known lipid regulatory microRNAs are few and include amongst others miR-335, miR-33, miR-122, miR-370, miR-378-3p, and miR-125a-5p [157]. Interestingly, these microRNAs have been identified in abundance in the HM lipid fraction [44], human skim milk [30], HM cells [35], human colostrum [30], HM exosomes [36], as well as in bovine skim milk and colostrum [52], suggesting that they play critical roles in the lipid metabolism and/or synthesis in the lactating breast. For example, miR-33 has been shown to regulate cholesterol homeostasis at the cellular level [158,159]. One of the most significant predicted gene targets of miR-33 is ABCA1, which produces cholesterol efflux regulatory protein (CERP). CERP is responsible for regulating cellular cholesterol and phosphate homeostasis, and also transporting cholesterol outside of the cell [160]. miR-33 also targets ABCG1, which reduces the efflux of cholesterol to high-density lipoprotein (HDL) and serum in macrophages [156,161].

miR-125a-5p is another microRNA found abundantly in human and other species milk, regulating oxysterol binding protein-related Protein 9 (ORP9) [162], which is involved in various processes of lipid metabolism [163,164] including induction of lipid uptake by macrophages [162]. Furthermore, HM miR-103 [30,44] is known to regulate milk fat synthesis, promoting fat globule synthesis and accumulation of triglyceride and unsaturated fatty acids [165]. Overexpression of miR-103 has been identified as a crucial regulator of milk fat synthesis and composition as well as milk nutrient levels [165]. Interestingly, downregulation of miR-103 was not shown to affect fat accumulation in caprine milk lactocytes, suggesting that there may be alternative and/or compensatory mechanisms controlling mammary fat metabolism [165]. Further, miR-193b and miR-365, also present in milk, were shown to control lipid synthesis, upregulating brown fat differentiation via enhancing expression of Runt-related transcription factor 1 translocated to 1 (Runx1t1) [166].
Interestingly, in addition to fat globule-related microRNA, many microRNA have been found to be packaged into other lipid-based carriers, such as exosomes, microvesicles and apoptotic bodies, which are secreted by various cell types, such as immune cells [167], and many of which are found in HM [36]. These are known as lipid particle carriers [168,169], packaging not only microRNA, but also lipoproteins [155], and have the important function of delivering extracellular microRNA to recipient cells. For example, miR-150 is transported via microvesicles from macrophage-like cells to human microvascular endothelial cells, where it is thought to target c-Myb and regulate cell migration [122,170,171]. Moreover, adipocyte-derived microRNA such as miR-27a, miR-146b and miR-16, are transported to other cell recipients via microvesicles [171]. Therefore, it can be postulated that microRNAs contained in milk microvesicles/exosomes as well as fat globules are transferred to recipient cells in the GI tract of infants, and a proportion may transfer to the blood circulation from where they are transported to the infant’s tissues, playing regulatory functions.

3.4. Various Potential Benefits of Human Milk microRNAs

The function of extracellular microRNA is still poorly understood [48]. Current evidence supports the notion that extracellular microRNAs play crucial roles in cell-cell communication [42,172,173]. microRNAs have been shown to be exported by cells in culture [42]. Moreover, proteins and mRNA can be taken up by neurons through exosomes from adjacent cells, suggesting that the same is possible for microRNA [42]. The existence of microRNA in exosomes and their potential function as extracellular regulators have opened up a new field of possibilities for use of microRNAs as biomarkers in health and disease [86,88] as well as in therapeutic modeling [174,175].

HM microRNA are potentially involved in many physiopathological functions, including regulating cell growth and differentiation [48] as well as influencing development in the infant [52]. For example, one of the most highly expressed microRNA in HM, miR-148a-3p [35,44], which is also found in other species’ milk [51,52], targets DNA methyltransferase 3b (DNMT3B) and suppresses its expression, potentially to facilitate DNA methylation during development [176]. At the same time, given that the majority of cells in mature HM under healthy conditions are lactocytes [58], HM microRNA are reflective of the microRNA composition and function of the lactating mammary epithelium, and this can form the basis for further explorations of their use as non-invasive, easily accessible biomarker of the functionality of the lactating breast.

Moreover, some tissue-related microRNAs have been found in HM [48], but less abundantly than in tissue and organs [36]. For example, miR-142-5p and miR-142-3p (hematopoietic system), miR-122 (liver), and miR-216 and miR-217 (pancreas) were highly expressed in these organs and less abundantly in HM [48,177], suggesting that these HM microRNAs may originate from the maternal bloodstream to specifically target the development, growth and function of the corresponding organs in the HM fed infant. At the same time, they may have specialized functions in the breast during lactation. Similarly in bovine milk, microRNA have been identified as tissue-specific microRNA present in low quantities in milk and with lower expression in both bovine colostrum and mature milk [52]. These include for example muscle miR-1 and miR-133 [173], brain miR-9 and miR-124a [178], pancreatic miR-216 and miR-217 [179], liver miR-122 [21,173], blood cell miR-451 [180], and endothelial cell miR-126 [181].
MicroRNAs isolated from HM fat globules have been shown to be regulated by a maternal high-fat diet, and this may modify metabolic pathways in HM fed infants [44]. This is in agreement with the putative roles of circulating microRNAs, which when altered in either composition or concentration can be associated with cardiovascular morbidity and mortality [182,183]. Munch et al. found that gene targets of 308 microRNAs in HM lipids have a wide range of functions, particularly in the regulation of gene expression and metabolism, and immune responses [44], suggesting the potential importance of these microRNAs for HM fed infants [44,184]. Further to these functions, some HM microRNA are thought to participate in the regulation of the central nervous system (CNS). For example, Munch et al. [44] showed that HM miR-118.2 targets Teneurin Transmembrane Protein 2 (TENM2), the encoding protein of which is found at high levels in the CNS [185], suggesting a regulatory function in the infant’s neural development and promotion of connection formation within the nervous system [186,187]. Adipogenesis may also be targeted in the infant via milk-derived microRNAs. Overexpression of miR-155 [54] has been speculated to decrease brown adipose tissue mass by targeting the adipogenic transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) [188]. Milk-derived miR-29a inactivates the INSIG-1 gene [30,44,52], which is likely to regulate adipogenesis [189], and this was positively associated with body mass index (BMI) [190]. In addition to direct effects on fat deposition, milk microRNA may be involved in the short- and/or long-term appetite control conferred to the infant via breastfeeding, together with the numerous appetite regulatory hormones of breastmilk, such as leptin, adiponectin, ghrelin, insulin and others [191].

In addition to their involvement in normal metabolism and tissue function, many microRNAs have been shown to target genes related to cancer [192,193], 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 [43] and some of them are present in milk, and more specifically in HM. Although epidemiological evidence has previously associated bovine milk consumption with increased risk of certain cancers in adults [194–199], and this could be related to the content of bovine milk in oncogenic microRNA [200], the microRNAs in HM appear to have normal lactation-specific functions for the lactating mammary gland and the infant [45]. Interestingly, HM microRNA have been proposed to protect the infant against cancer through to adulthood [44]. For example, miR-21, which is present in both HM and bovine milk [36,52], is also known to be overexpressed in human hepatocellular cancer (HCC). Therefore, any deregulation of miR-21 can be associated with HCC growth by modulating mTORC1 signaling, i.e., PTEN expression [201]. miR-21 is an abundant microRNA in bovine milk [52] and has been isolated from both colostrum and mature HM [30]. It is also abundant in human plasma [202], and in infants it is thought to be involved in promoting postnatal growth [203]. In addition, miR-21 has other normal tissue functions, including regulation of adipogenic differentiation in mesenchymal stem cells (MSCs) of human adipose tissue [204]. Further, HM microRNAs may directly regulate tumor suppressor genes [205], such as the let-7 family, which is involved in decreasing lung tumor growth by directly targeting the RAS oncogene [206]. The specific normal functions of HM microRNA for the infant and in the lactating breast warrant further investigation.
4. Infant Formula is Poor in microRNAs Compared to Human Milk

HM is much more than nutrition for the infant, containing fat, carbohydrates, proteins, vitamins and minerals, but also immunoprotective and regulatory biomolecules as well as viable cells that provide essential signals for the infant’s optimal growth, development and protection [70,191,207–209]. However, a rapid worldwide population growth over the last 100 years, and the high demand to provide artificial milk for infants has led scientists and industrial companies to successfully produce infant formula from bovine milk as an alternative or complementary food for infants initially without access to HM [210]. And although many have expressed the view that infant formula should only be made available to the infant if mother’s own milk is not sufficient, infant formula has recently become controversially more popular within some communities for non-medical reasons [211].

HM is a complex biofluid containing maternal somatic cells, beneficial microbiota, and molecules including microRNAs with functional roles [30,134,212–214]. Most infant formulae are bovine milk-based [44], and similar to HM, bovine milk microRNAs are most likely to be highly conserved in both fat globules (lipid fraction) and cells. Due to their stability, microRNAs may largely survive the industrial milk preparation procedures, however the milk cell and lipid fractions are usually discarded from formula [215,216], so the microRNA presence in formulae is significantly reduced. This has been confirmed by studies showing that the expression level of microRNAs in formulae is much lower than that of raw bovine milk (Table 3) [52,54,217]. Chen et al. selected and surveyed seven microRNAs as quality control markers for raw milk and different brands of infant formula [52]. The expression level of these seven microRNAs was significantly lower in formulae compared to raw milk [52]. Izumi et al. found that the total RNA concentration of three types of infant formulae (standard formula, follow-on formula, and extensively hydrolyzed formula) was significantly lower than in raw bovine milk [54]. Also, two highly expressed microRNAs (miR-148a and miR-200c) in bovine milk were differentially expressed among the three different types of infant formula [54]. In a recent study, we have compared the human microRNA content and expression levels of two infant formulae from the Australian market, a bovine-milk based and a soy-based formula [46]. Out of 754 human mature microRNAs tested using Taqman Openarray (Applied Biosystems), only 45 microRNAs were identified in the bovine milk formula, and only 22 microRNAs in the soy formula [46], (Table 3). Moreover, the biological activity of the remaining few formula microRNAs may be altered by the formula processing procedures, something that requires further investigation. Although the functional effects of non-human milk microRNAs on infants have not been investigated, it is possible that some microRNAs that are shared between bovine and human milk may play similar beneficial functions for the offspring across mammals, emphasizing the potential detrimental effects for infants of the low microRNA content of artificial formulae. This, together with the near absence in formulae of other immunoprotective factors of HM are likely to at least partially explain the reduction in protection from disease in infants fed artificial formulae [217]. Donor milk is the preferred alternative to formula, and even if pasteurized, donor milk is likely to retain many HM microRNA, potentially conferring more benefits to the infant than formula.
Table 3. Comparison of selected microRNAs and their abundance in infant formulae, bovine milk, and human milk (HM).

<table>
<thead>
<tr>
<th>MicroRNA</th>
<th>Mature Sequence (miRBase 20.0)</th>
<th>Existence</th>
<th>Expression Level</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>bta-miR-26a</td>
<td>UUCAAGUAUCCAGGAUAGGCUCU</td>
<td>Bovine milk. Infant formula. HM.</td>
<td>Low in formulae compared to raw bovine milk. Highly expressed in HM lipid fraction.</td>
<td>[44,52]</td>
</tr>
<tr>
<td>hsa-miR-26a-5p</td>
<td>UUCAAGUAUCCAGGAUAGGCUCU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bta-miR-26b</td>
<td>UUCAAGUAUUCAGGAUAGGUU</td>
<td>Bovine milk. Infant formula. HM.</td>
<td>Low in formulae compared to raw bovine milk. Highly expressed in HM lipids and exosomes.</td>
<td>[36,44,52]</td>
</tr>
<tr>
<td>hsa-miR-26b-5p</td>
<td>UUCAAGUAUUCAGGAUAGGUU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bta-miR-200c</td>
<td>UAAUACUGCCGGUAAUGAUGGA</td>
<td>Bovine milk. Infant formula. HM.</td>
<td>Low in formulae compared to raw bovine milk. Highly expressed in HM lipid fraction.</td>
<td>[44,52,54]</td>
</tr>
<tr>
<td>hsa-miR-200c-3p</td>
<td>UAAUACUGCCGGUAAUGAUGGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bta-miR-21-5p</td>
<td>UAGCUUAAUCAGACUGUUGACU</td>
<td>Bovine milk. Infant formula. HM.</td>
<td>Low in formulae compared to raw bovine milk. Highly expressed in HM lipids, skim milk and exosomes.</td>
<td>[30,36,44,48,52]</td>
</tr>
<tr>
<td>hsa-miR-21-5p</td>
<td>UAGCUUAAUCAGACUGUUGACU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bta-miR-30d</td>
<td>UGUAACAUCCCGACUGGAAGCU</td>
<td>Bovine milk. Infant formula. HM.</td>
<td>Low in formulae compared to raw bovine milk. Highly expressed in HM lipids and skim milk.</td>
<td>[30,44,52]</td>
</tr>
<tr>
<td>hsa-miR-30d-5p</td>
<td>UGUAACAUCCCGACUGGAAGCU</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bta-miR-99a-5p</td>
<td>AACCCGUAGAUCCCGAUCUUGU</td>
<td>Bovine milk. Infant formula. HM.</td>
<td>Low in formulae compared to raw bovine milk. Highly expressed in HM lipids and skim milk.</td>
<td>[30,44,52]</td>
</tr>
<tr>
<td>hsa-miR-99a-5p</td>
<td>AACCCGUAGAUCCCGAUCUUGU</td>
<td></td>
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</tr>
<tr>
<td>bta-miR-148</td>
<td>UCAGUGCAUCACAGACUUUGU</td>
<td>Bovine milk. Infant formula. HM.</td>
<td>Low in formula compared to raw bovine milk. Highly expressed in HM lipid, skim milk and exosomes.</td>
<td>[30,36,44,52,54]</td>
</tr>
<tr>
<td>hsa-miR-148a-3p</td>
<td>UCAGUGCAUCACAGACUUUGU</td>
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</tr>
</tbody>
</table>

Notes: hsa refers to human, bta refers to *Bos taurus* (bovine) species.
5. Conclusions and Outlook

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 [218]. Deregulation of microRNAs has also been shown to be associated with disease, for which they are useful diagnostic biomarkers [192,219]. The recent discovery and identification of microRNA in HM requires further study to elucidate the biology of microRNA and their normal functions in the development and protection of the human breast and the infant. Although the use of circulating microRNA as biomarkers is still in its infancy [156], microRNA have been proposed as biomarkers for various abnormalities [86], including breast cancer [220,221] and more recently, milk microRNA as biomarkers for lactation performance [52] and mastitis [71]. This is of particular interest since milk can be accessed easily and non-invasively and is plentiful. Importantly, microRNAs are extremely stable, and are transferred to humans via food, and also to infants via HM. 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. The investigation of the roles of HM microRNA for the infant and the mother will not only reveal novel attributes of breastfeeding, but it may also open new diagnostic and therapeutic avenues.

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Author Contributions

Mohammed Alsaweed and Foteini Kakulas wrote the manuscript. Peter E. Hartmann and Donna T. Geddes critically reviewed the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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Human Milk MicroRNA and Total RNA Differ Depending on Milk Fractionation

Mohammed Alsaweed,1,2 Anna R. Hepworth,1 Christophe Lefèvre,3 Peter E. Hartmann,1 Donna T. Geddes,1 and Foteini Hassiotou1*  

1School of Chemistry and Biochemistry, The University of Western Australia, Crawley, Western Australia, Australia  
2College of Applied Medical Sciences, Majmaah University, Almajmaah, Riyadh, Saudi Arabia  
3Centre for Biotechnology and Interdisciplinary Sciences, Deakin University, Victoria, Australia

ABSTRACT

MicroRNA have been recently discovered in human milk signifying potentially important functions for both the lactating breast and the infant. Whilst human milk microRNA have started to be explored, little data exist on the evaluation of sample processing, and analysis to ensure that a full spectrum of microRNA can be obtained. Human milk comprises three main fractions: cells, skim milk, and lipids. Typically, the skim milk fraction has been measured in isolation despite evidence that the lipid fraction may contain more microRNA. This study aimed to standardize isolation of microRNA and total RNA from all three fractions of human milk to determine the most appropriate sampling and analysis procedure for future studies. Three different methods from eight commercially available kits were tested for their efficacy in extracting total RNA and microRNA from the lipid, skim, and cell fractions of human milk. Each fraction yielded different concentrations of RNA and microRNA, with the highest quantities found in the cell and lipid fractions, and the lowest in skim milk. The column-based phenol-free method was the most efficient extraction method for all three milk fractions. Two microRNAs were expressed and validated in the three milk fractions by qPCR using the three recommended extraction kits for each fraction. High expression levels were identified in the skim and lipid milk fractions for these microRNAs. These results suggest that careful consideration of both the human milk sample preparation and extraction protocols should be made prior to embarking upon research in this area. J. Cell. Biochem. 116: 2397–2407, 2015. © 2015 The Authors. Journal of Cellular Biochemistry Published by Wiley Periodicals, Inc. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

KEY WORDS: BREASTMILK; HUMAN MILK; RNA; microRNA; CELLS; LIPIDS; FAT; FAT GLOBULE; SKIM MILK; MILK FRACTIONS

Human milk (breastmilk) is a complex secretion of the mammary gland that is the main source of nutrition, immune protection, and developmental programming for the infant [Hassiotou and Geddes, 2013]. In addition to being a balanced food for infants containing water, minerals, vitamins, proteins, carbohydrates, and lipids, human milk is a potent source of immunomodulatory factors [Hanson et al., 1997; Kramer, 2010]. These include bioactive molecules, such as immunoglobulins and lactoferrin [Lonnerdal, 2003; Hassiotou et al., 2013], and immune cells amongst stem cells, progenitor cells, and epithelial cells that constitute a cellular hierarchy in human milk [Hassiotou et al., 2012]. Further to these components, milk is a rich source of RNAs, and microRNAs [Lemay et al., 2013]. The latter have been recently discovered in human milk [Kosaka et al., 2010; Weber et al., 2010; Zhou et al., 2012; Munch et al., 2013] and in the milk of other mammalian species [Chen et al., 2010; Gu et al., 2012; Bai et al., 2013; Izumi et al., 2014], suggesting that they may play crucial roles both in the lactating mammary gland and for the breastfed infant [Zhou et al., 2012; Munch et al., 2013].

MicroRNAs are small non-coding RNA molecules found in plants and animals [Ambros, 2004]. First discovered in 1993 in Caenorhabditis elegans [Lee et al., 1993; Wightman et al., 1993], they are considered to be crucial regulators of gene expression at the post-transcriptional level by attaching to messenger RNA (mRNA) to either...
inhibit protein translation and/or induce mRNA degradation [He and Hannon, 2004; Pritchard et al., 2012]. They are thus involved in a number of developmental and physiological processes, including cellular differentiation, apoptosis, proliferation, immune response, and maintenance of cell and tissue identity [Bartel, 2004]. Deregulation of microRNAs is associated with aberrant cell functions leading to cancers [Calin and Croce, 2006; Esquela-Kerscher and Slack, 2006] and other diseases [Lu et al., 2008]. MicroRNA exhibit diversified expression patterns, with some of them being specific to certain organs, such as miR-122, which is primarily found in the liver [Lagos-Quintana et al., 2002], or miR-1, which predominantly exists in the mammalian heart [Lee and Ambros, 2001]. The number of new microRNA molecules discovered is increasing, with over 2,000 having been identified in humans thus far (http://www.mirbase.org). In addition to cells and tissues, microRNA are present in body fluids, such as plasma [Sourvinou et al., 2013] urine, saliva, and tears [Cortez et al., 2011] as well as abundantly in milk [Weber et al., 2010; Munch et al., 2013]. The few studies that have examined microRNA in human milk have largely focused on its skimmed fraction. In 2010, Kosaka et al. reported 281 novel microRNA in skim milk [Kosaka et al., 2010], while Weber et al. found 429 microRNA in mature skim milk and 368 in skimmed colostrum [Weber et al., 2010]. In addition to skim milk microRNAs, human milk contains microRNA packaged in vesicles. These have been examined more recently, with 452 pre-microRNA detected in human milk exosomes [Zhou et al., 2012]. Exosomes are small membrane vesicles secreted from mammalian cells that protect molecules and proteins, which are then transported into the extracellular environment participating in cell–cell communication [Admyre et al., 2007]. Human milk exosomes are rich in microRNA and immune-associated proteins, particularly these that have been isolated from skim milk [Admyre et al., 2007]. So far, 59 immune-related microRNA have been described within human milk exosomes [Zhou et al., 2012]. Further to those, other particles in human milk also contain microRNA. Recently, Munch et al. showed that the milk fat globule encompasses novel microRNAs [Munch et al., 2013]. Similar to adults [Baier et al., 2014], most likely human milk carries these microRNAs to the infant. Although some hypotheses were raised that oral microRNAs do not survive in the human gastrointestinal tract [Dickinson et al., 2013; Witwer and Hirschi, 2014], milk microRNAs are protected within fat globules, exosomes, or cells, and therefore are likely to be transferred intact across to the infant’s blood. Moreover, human milk is known to contain maternal cells [Hassiotou et al., 2013], which are transferred to the infant [Hassiotou et al., 2014]. These cells are rich in microRNA [Alsaweed et al., 2015]. Exogenous microRNAs have been previously described to play functional roles in adults [Zhang et al., 2012; Baier et al., 2014], and therefore it is likely that cellular microRNAs from human milk contribute regulatory functions in the infant.

The properties and regulation of microRNA in the different cell types present in human milk as well as in the milk fat globule, skim milk, and exosomes remain unknown. Early evidence suggests that certain human milk microRNA support the immune system of the infant, especially in the first six months of life, such as the highly abundant in milk miR-155, which regulates T and B cells and has a role in the innate immune response [Kosaka et al., 2010]. Prior to investigating the properties and roles of human milk microRNA in either the mammary gland or the infant, it is critical to establish appropriate methodology that allows consistent isolation and quantification of these molecules, similar to that of plasma [Sourvinou et al., 2013], serum [Farina et al., 2014], and blood cells [Hammerle-Fickinger et al., 2010; Monneau et al., 2014]. However, in this study, 8 extraction kits from different were used.

Given that only the skim milk fraction of human milk has been widely investigated, it is not known what contribution the lipid or cell fractions make to the total microRNA population of human milk.

Considering that handling and isolation protocols play critical roles in the reliable quantification of microRNA in plasma [Sourvinou et al., 2013], it is logical to expect that special protocols, and handling procedures may apply to human milk. In addition, different methods have been utilized, contributing to the wide variation of total RNA, and microRNA published in human milk studies. In this study, we determined whether the microRNA content differed between the skin, lipid, and cell fractions of human milk and investigated the efficacy (yield and quality) of microRNA extraction for eight commercially available kits in each milk fraction. This provided valuable insight into the abundance and content of microRNA in different human milk fractions, setting the basis for profiling, and functional microRNA studies in human milk.

**MATERIALS AND METHODS**

**HUMAN MILK COLLECTION**

The study was approved by the Human Research Ethics Committee of The University of Western Australia. All participants provided informed written consent. Fresh human milk samples (n = 49) were collected from n = 29 breastfeeding mothers on 1–4 occasions under sterile conditions. Symphony pumps (Medela AG, Switzerland) were used for Human milk expression and sample volumes ranged 14–115 mL. All participants and their infants were healthy at the time of milk collection, with current smoking, and medication use being exclusion criteria for participation. Lactation stages at sample collection ranged from 3 to 158 weeks.

**HUMAN MILK FRACTIONATION**

All human milk samples were fractionated immediately after expression by centrifugation at 720g for 20 min. Three fractions were obtained from each sample (cells, lipids, and skim milk), and were transferred to 15-mL RNase free tubes (Fig. 1). Purification of the milk fractions involved an additional centrifugation step in the lipid and skim milk fractions, whilst cells were washed twice in sterile phosphate-buffered saline (PBS, Gibco).

**TOTAL RNA/microRNA EXTRACTION**

All RNA/microRNA extractions were done on ice immediately after separation and purification of the three milk fractions (Fig. 1) using eight commercially available kits (Table I). Each fraction of each milk sample was separated into 2–5 identical aliquots depending on the original sample volume and the size of the cell pellet. Each aliquot was used for extraction by a different kit such that the number of aliquots obtained per sample reflected the number of kits used for this sample. Table II shows the number of milk samples tested with...
each extraction kit and for each milk fraction (cells, lipids, and skim milk). Specifically for purified lipids and skim milk, 100–350 μL were used for microRNA extraction, based on the manufacturer's recommendation for each kit. Briefly, samples were lysed using the lysis reagent provided by the kit, and were homogenized by gentle movement 10 times into and out of a sterile syringe and needle system. Separation of large (DNA, large RNA, and debris) and small (small RNA) phases and precipitation of the former was done with either chloroform or alcohol. After that, isopropanol or column separation was used for RNA precipitation. Then, different washing steps depending on the kit were required to obtain pure small RNA, which was eluted either in elution buffer or suspended in RNase-free water.

MicroRNA Analysis and Quantification
Concentration and purity (260:280 ratio) of the extracted total RNA was measured using a spectrophotometer (NanoDropTM 1000, Wilmington, DE). The microRNA concentration and microRNA/small RNA ratios were quantified by capillary electrophoresis using the small RNA Chip kit (Agilent, CA) in an Agilent Bioanalyzer 2100 instrument. The amount of small RNA was normalised to 100, and the amount of microRNA was presented as a percentage of this value. Using this kit, we were able to quantify the small RNA in a sample including microRNA, which ranged in size between 6 and 150 nucleotides for small RNAs, and 10–40 nucleotides for microRNAs.

Reverse Transcription and Quantitative RT-PCR
Two whole human milk samples from two different mothers were fractionated as described above. RNA including microRNA was extracted using recommended kits for each fraction (Table III). RT-PCR was used to validate the presence of two mature microRNAs using the TaqMan miRNA assay (Life Technologies, Foster City, CA). The expression level of hsa-miR-148a-3p and hsa-miR-30a-5p was done in two steps according to the manufacturer's protocol. The reverse transcription was performed using 600 ng of input RNA using the TaqMan miRNA reverse transcription kit and pooled both microRNA primers (5′/C2′ primers) and endogenous control (RNU48). The RT reaction was processed using BioRad C-1000 thermo cycler (Hercules, CA) as follows: 16°C for 30 min; 42°C for 30 min; 85°C for 5 min, then the sample was held at 4°C. The PCR reaction was performed using Fast advanced master mix in triplicates and TaqMan microRNA probes for both examined microRNAs and the endogenous control (20′/C2′) using 7500 Fast Real Time-PCR system as follows: 50°C for 2 min; 95°C for 20 s followed by 40 cycles at 95°C for 3 s; and finally 60°C for 30 s. Comparative Ct (RQ) analysis was performed using 7500 software V2.0.6 by normalizing all samples to milk cell A sample.

Statistical Analysis
Statistical analysis was done using R 2.9.0 for MacOSX (Team Development Core R, 2009) using the base packages, and the libraries nlme, multcomp, and lattice for linear mixed effects models, general linear hypothesis tests, and graphical presentation of data, respectively. Extraction kits were compared in terms of: (a) their efficiency in extracting total RNA, (b) the purity of the extracted RNA (260/280 ratio, whereby values of 1.80–2.19 were considered good; values of 1.50–1.79 were considered moderate; and values >2.20 or <1.50 were considered poor), (c) their efficiency in extracting microRNA, and (d) the ratio of microRNA to small RNA. This was done separately for each human milk fraction (cells, lipids, and skim milk).

Comparison of the kits was done in two sets reflecting the kits tested in aliquots of the same milk samples (Table I). In the first set
Overall, where found to be more appropriate, and regression otherwise. were compared using analysis of variance. LME models are reported with random effects of different baseline levels per sample. Models squares (OLS) regression, and a linear mixed effects (LME) model combination of measure, kit set, and milk fraction. Ordinary least levels) as the predictor. Two models were created for each measure of interest as the response and the kit (factor with either 3 or

Patterns of RNA purity (Table IV; Suppl. Table 1) were found to be significantly different among the RNA extraction kits examined \( (P \leq 0.001) \), with the column-based/phenol-free kits being the best performers and the phenol/guanidine–based kits being the worst performers. Moreover, each kit performed differently for different human milk fractions. Notably, the mirPremier microRNA Isolation Kit yielded good 260/280 ratios for the lipid and cellular fractions, but low ratios for the skim milk fraction. In general, all tested kits performed well in the cellular and lipid fractions, with more variation and lower RNA purity seen for the skim milk fraction.

In the milk lipid fraction and comparison Set 1, 260/280 ratios of mirVana and mirNeasy micro kits were significantly higher than those of RNAzol-RT \( (P < 0.001 \) for both), which had a mean 260/280 ratio that was outside the acceptable range \( (1.65) \). Similarly, differences were seen among the TRIzol-LS, miRNeasy mini and miRCURY-Cell&Plant kits, where 260/280 ratios for TRIzol-LS were lower than in the other two kits \( (P = 0.016 \) and \( P = 0.019 \), respectively). However, the mean 260/280 ratio for TRIzol-LS was within the acceptable range \( (1.84) \), but lower than in the other two kits. Given the 260/280 cut-offs used, RNAzol-RT, and Trizol-LS were outside the acceptable range for lipids, indicating that these are not optimal choices for extracting total RNA or microRNA from milk lipids.

In skim milk and comparison Set 1, 260/280 ratio for mirVana was significantly higher than those of miRNeasy micro \( (P < 0.001) \) and RNAzol-RT \( (P = 0.041) \), both of which were outside the acceptable range. In comparison Set 2, although a wide range of average values was obtained among miRNeasy mini, TRIzol-LS, miRCURY-Cell&Plant, miRCURY-Biofluids, and mirPremier, no significant differences were found between the five kits tested \( (P = 0.631) \). Notably, the mean 260/280 values of TRIzol-LS and mirPremier were outside the acceptable range \( (1.60 \) and \( 1.41, \) respectively).

In the cellular fraction and comparison Set 1, 260/280 ratios for miRNeasy micro and mirVana were significantly higher than those for RNAzol-RT \( (P < 0.001 \) and \( P < 0.003, \) respectively), where RNAzol-RT ratio averaged at 1.61, which is considered to be a low ratio. In comparison Set 2, only the 260/280 ratios for TRIzol-LS were significantly lower than all other kits \( (P < 0.001) \), and they were also outside the acceptable 260/280 range \( (1.70) \).

### RESULTS

#### PURITY OF EXTRACTED RNA

Patterns of RNA purity (Table IV; Suppl. Table 1) were found to be significantly different among the RNA extraction kits examined \( (P \leq 0.001) \), with the column-based/phenol-free kits being the best performers and the phenol/guanidine–based kits being the worst performers. Moreover, each kit performed differently for different human milk fractions. Notably, the mirPremier microRNA Isolation Kit yielded good 260/280 ratios for the lipid and cellular fractions, but low ratios for the skim milk fraction. In general, all tested kits performed well in the cellular and lipid fractions, with more variation and lower RNA purity seen for the skim milk fraction.

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### TABLE II. Number of the Three-breastmilk Fraction Samples Used for RNA/microRNA Extraction in Evaluating Their Performance Using the Eight Kits.

<table>
<thead>
<tr>
<th>Kit/Fraction</th>
<th>Cells</th>
<th>Lipids</th>
<th>Skim milk</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>miRNeasy micro Kit</td>
<td>14</td>
<td>27</td>
<td>29</td>
<td>70</td>
</tr>
<tr>
<td>mirVana microRNA Kit</td>
<td>11</td>
<td>23</td>
<td>26</td>
<td>60</td>
</tr>
<tr>
<td>RNAzol-RT Reagent</td>
<td>11</td>
<td>22</td>
<td>25</td>
<td>58</td>
</tr>
<tr>
<td>miRNeasy mini Kit</td>
<td>14</td>
<td>22</td>
<td>22</td>
<td>58</td>
</tr>
<tr>
<td>TRIzol-LS Reagent</td>
<td>12</td>
<td>21</td>
<td>17</td>
<td>50</td>
</tr>
<tr>
<td>miRCURY RNA IsoBiofluids Kit</td>
<td>15</td>
<td>19</td>
<td>23</td>
<td>57</td>
</tr>
<tr>
<td>miRCURY RNA Iso-Biofluids Kit</td>
<td>15</td>
<td>24</td>
<td>23</td>
<td>62</td>
</tr>
<tr>
<td>mirPremier microRNA Kit</td>
<td>14</td>
<td>24</td>
<td>19</td>
<td>57</td>
</tr>
<tr>
<td>Total</td>
<td>106</td>
<td>182</td>
<td>184</td>
<td>472</td>
</tr>
</tbody>
</table>
### TABLE III. The Three Most Effective Kits for Each Breastmilk Fraction in Each of the Four Evaluation Criteria (Total RNA Extracted, Quality of Extracted RNA, Total microRNA Extracted, and microRNA/small RNA Ratio). Total RNA and microRNA Are Presented in ng for the Cellular Fraction and in ng/μL for the Lipid and Skim Milk Fractions. All Values Represented Are Means ± standard Deviations.

<table>
<thead>
<tr>
<th>Comparison Set 1</th>
<th>Comparison Set 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mIRNeasy RNA isolation kit-Cell &amp; Plant (90.00-95.00% good, 17.00 moderate, 13.00 poor); (1.98 ± 0.11) TRIzol LS (25.00% good, 14.00 moderate, 61.00 poor)</td>
</tr>
<tr>
<td>mIRNeasy mini (75.00 ± 0.05 ng)</td>
<td>mIRNeasy mini (100%; 1.00); (2.04 ± 0.03) TRIzol LS (15.00-25.00 ng/mL)</td>
</tr>
<tr>
<td>miRCURY RNA isolation kit-Cell &amp; Plant (100%; 1.00); (2.04 ± 0.04) TRIzol LS (15.00-25.00 ng/mL)</td>
<td></td>
</tr>
<tr>
<td>miRNeasy mini (75.00 ± 0.05 ng)</td>
<td>miRNeasy mini (100%; 1.00); (2.04 ± 0.03) TRIzol LS (15.00-25.00 ng/mL)</td>
</tr>
<tr>
<td>miRCURY RNA isolation kit-Cell &amp; Plant (100%; 1.00); (2.04 ± 0.04) TRIzol LS (15.00-25.00 ng/mL)</td>
<td></td>
</tr>
<tr>
<td>miRNA microRNA isolation kit (100%; 1.00); (2.04 ± 0.07) TRIzol LS (15.00-25.00 ng/mL)</td>
<td></td>
</tr>
<tr>
<td>miRNeasy mini (75.00 ± 0.05 ng)</td>
<td>miRNeasy mini (100%; 1.00); (2.04 ± 0.03) TRIzol LS (15.00-25.00 ng/mL)</td>
</tr>
<tr>
<td>miRCURY RNA isolation kit-Cell &amp; Plant (100%; 1.00); (2.04 ± 0.04) TRIzol LS (15.00-25.00 ng/mL)</td>
<td></td>
</tr>
<tr>
<td>miRNA microRNA isolation kit (100%; 1.00); (2.04 ± 0.07) TRIzol LS (15.00-25.00 ng/mL)</td>
<td></td>
</tr>
<tr>
<td>miRNeasy mini (75.00 ± 0.05 ng)</td>
<td>miRNeasy mini (100%; 1.00); (2.04 ± 0.03) TRIzol LS (15.00-25.00 ng/mL)</td>
</tr>
<tr>
<td>miRCURY RNA isolation kit-Cell &amp; Plant (100%; 1.00); (2.04 ± 0.04) TRIzol LS (15.00-25.00 ng/mL)</td>
<td></td>
</tr>
<tr>
<td>miRNA microRNA isolation kit (100%; 1.00); (2.04 ± 0.07) TRIzol LS (15.00-25.00 ng/mL)</td>
<td></td>
</tr>
</tbody>
</table>

*Results from linear mixed effects models, indicating that there are significant correlations between measures within a sample.

### TABLE IV. Mean and Standard Deviation of Quantity and Quality of RNA and microRNA Extracted From Different Fractions of Breastmilk (cells, Lipids, and Skim milk) by the Eight Kits in Two Separate Comparison Sets. Total RNA and 260/280 Ratio Were Obtained by Nanodrop, While Total Microrna, and microRNA/small RNA Ratio Were Measured by Bioanalyzer Using Small RNA Kit.

<table>
<thead>
<tr>
<th>Milk fraction</th>
<th>microRNA</th>
<th>260/280 ratio</th>
<th>RNA</th>
<th>microRNA Small RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>miRNeasy mini (100%; 1.00); (2.04 ± 0.03) TRIzol LS (15.00-25.00 ng/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipids</td>
<td>miRNeasy mini (100%; 1.00); (2.04 ± 0.03) TRIzol LS (15.00-25.00 ng/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skim milk</td>
<td>miRNeasy mini (100%; 1.00); (2.04 ± 0.03) TRIzol LS (15.00-25.00 ng/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TOTAL RNA CONCENTRATION

Total RNA concentration differed both among kits and human milk fractions for the same kit (Table IV, Fig. 2). The lipid fraction contained significantly higher concentration of RNA compared to skim milk ($P < 0.05$) (Fig. 3). In the lipid fraction, the mean ± S.D. of total RNA concentration ranged from 7.02 ± 3.99 ng/μL in the mirPremier kit to 49.90 ± 61.06 ng/μL in the miRCURY–Cell&Plant kit. In comparison Set 1, no significant difference was found between miRNeasy micro, mirVana, and RNAzol-RT ($P = 0.600$). In comparison Set 2, mirPremier yielded significantly lower total RNA values than TRizol-LS ($P = 0.05$), miRNeasy micro ($P = 0.007$) and miRCURY–Cell&Plant kits ($P = 0.005$). No significant differences were found among the remaining four kits ($P > 0.05$). TRizol-LS, miRNeasy mini kit and miRCURY–Cell&Plant kit yielded high amount of total RNA from the human milk lipid fraction.

In the skim milk fraction, the mean ± S.D. of RNA concentration ranged 0.60 ± 0.46 ng/μL (mirPremier) to 9.26 ± 18.23 ng/μL (TRizol-LS). In comparison Set 1, RNAzol-RT was clearly a better performer than miRNeasy micro and mirVana in terms of amount of extracted RNA ($P < 0.001$). In comparison Set 2, TRizol-LS was the best performer (mean = 9.26 ng/μL), although this was only

Fig. 2. Total RNA (A), microRNA (B) and microRNA to small RNA ratio (C) obtained by the eight kits tested. Different shades of grey represent different individuals (human milk samples). RNA and microRNA (A and B, respectively) are presented in ng for the cellular fraction and in ng/μL for the lipid and skim milk fractions. All values represented are means ± standard deviations.
significantly different from mirPremier \( (P = 0.064) \), which had the lowest performance amongst those tested (mean \( \mu = 0.60 \) ng/\( \mu \)L). In human milk cells, the mean \( \pm S.D. \) of total RNA amount extracted from equal cell aliquots ranged from 1,907 \( \pm 3,321 \) ng in the mirPremier kit to 11,083 \( \pm 15,106 \) ng in the miRNeasy mini kit. In comparison Set 1, total RNA extracted with miRNeasy micro kit was significantly higher than that obtained with RNAzol-RT \( (P = 0.110 \) to \( P = 0.720) \) (Table IV, Fig. 2). In the lipid fraction, the highest mean microRNA concentration was obtained by miRCURY-Biofluids \( (12.74 \pm 24.44 \) ng/\( \mu \)L), followed by mirPremier \( (9.43 \pm 8.06 \) ng/\( \mu \)L), while the lowest by mirVana \( (0.80 \pm 1.70 \) ng/\( \mu \)L). In comparison Set 1, no overall difference was seen among the three kits \( (P = 0.169) \). Tukey’s HSD comparison showed a borderline difference between miRNeasy micro kit and mirVana, with a tendency for higher levels in the former \( (P = 0.092) \). In comparison Set 2, there was no overall difference among the kits \( (P = 0.216) \). Further, multiple comparisons of means/Tukey’s HSD showed no significant differences, with the smallest \( P \)-value being 0.210. Thus, all the tested kits performed relatively well in extracting high levels of microRNA from the lipid fraction of human milk.

In skim milk, all kits yielded relatively low quantities of microRNA. Specifically, miRCURY–Cell&Plant kit yielded the highest mean concentration of extracted microRNAs \( (2.17 \pm 5.66 \) ng/\( \mu \)L), whereas the lowest mean concentration was obtained with mirVana.
[0.10 ± 0.15 ng/μL] and miRNasy micro kits [0.10 ± 0.9 ng/μL]. In comparison Set 1, there was no evidence of significant difference among either the three kits overall (P = 0.342) or using multiple comparisons of means (P ≥ 0.386). In comparison Set 2, no evidence of difference was found among the five kits either overall (P = 0.363) or using multiple comparisons of means (all P > 0.449). Therefore, similar to the lipid fraction, in skim milk all eight tested kits performed similarly in extracting microRNA, although in almost all cases the extracted quantities of microRNA were very low in this fraction compared to the other two human milk fractions.

In human milk cells, TRizol-LS showed the largest mean amount of microRNA (1.443 ± 3.448 ng), while mirVana yielded the smallest mean amount of microRNA (65.67 ± 84.38 ng). In comparison Set 1, there was no evidence of difference among the miRNasy micro kit, mirVana, and miRNAeasy kit either overall (P = 0.308) or for multiple comparisons of means (all P > 0.241). Similarly, in comparison Set 2, no significant difference was found among the five kits in terms of levels of extracted microRNA either overall (P = 0.722) or for multiple comparisons of means (P > 0.614).

**MicroRNA/SMALL RNA RATIO (%)**

To further assess the efficiency of the examined kits to extract microRNA from human milk fractions, we compared the percentage ratio of microRNA to small RNA obtained with each kit (Table 4, Fig. 2). In the lipid fraction, the highest mean microRNA/small RNA ratio was seen with the miRNasy micro kit (54.4%) and RNAzol-RT (50%). In comparison Set 1, there were significant differences among kits (P = 0.041), while in comparison Set 2 there was no overall evidence of significant differences among the five kits compared (P = 0.217). Thus, although all the examined kits performed similarly in terms of microRNA/small RNA ratios in the human milk lipid fraction, the highest mean was obtained by the miRNasy micro kit.

In the skim milk fraction, the highest microRNA/small RNA ratio was obtained by mirPremier (57.8%), followed by RNAzol-RT (48.5%). In comparison Set 1, significant differences were seen among kits (P < 0.001). The highest ratios were observed with RNAzol-RT, which were significantly higher than both miRNasy micro and mirVana (P < 0.001), which did not significantly differ from one another (P = 0.732). Similarly, comparison Set 2 yielded significant differences among kits (P < 0.001). mirPremier kit was higher compared to all other kits (57.8%), and yielded significantly higher microRNA/small RNA ratios than either miRNasy-Cel&Plant or miRNAeasy mini kit (P < 0.001). Moreover, ratios obtained by miRCURY-Biofluids and TRizol-LS were significantly higher than those obtained by miRCURY-Cel&Plant (P = 0.049 and P = 0.063, respectively), although the significance of the difference between TRizol-LS and miRCURY-Cel&Plant was much weaker, suggesting that TRizol-LS yields more variable microRNA/small RNA ratios than miRCURY-Biofluids. Finally, no significant differences were seen between miRCURY-Biofluids and mirPremier (P = 0.196). Overall, all examined kits performed similarly in terms of microRNA/small RNA ratios in the skim milk fraction of human milk, with the preference to three kits being mirPremier, RNAzol-RT, and TRizol-LS.

In human milk cells, TRizol-LS and miRNAeasy mini kit showed the highest mean microRNA/small RNA ratio with 51.1% and 36.1%, respectively. In comparison Set 1, no significant difference was seen among the kits overall (P = 0.152). In comparison Set 2, the kits differed significantly (P < 0.001). TRizol-LS yielded higher ratios than miRCURY-Biofluids, miRCURY-Cel&Plant, and mirPremier (P < 0.001), while ratios for miRNAeasy mini were significantly higher than those of miRCURY-Cel&Plant (P = 0.034).

**VALIDATION OF microRNA EXPRESSION IN DIFFERENT HUMAN MILK FRACTIONS**

RT-PCR was used to validate the efficiency of the recommended kits (Table 3) in extracting microRNA using two different samples of each fraction (milk cells, lipids, and skim milk). Using comparative Ct (RQ) analysis, it was found that hsa-miR-148a-3p and hsa-miR-30a-5p were expressed in all three fractions for both milk samples tested (Fig. 5). These two microRNAs showed a similar relative quantification (RQ) value in two different milk lipid samples (A and B samples) [Fig. 5]. Despite the low total RNA and microRNA concentration of skim milk compared to the other two human milk fractions, hsa-miR-30a-5p was highly expressed in one of the skim milk samples tested (B); however, the other skim milk sample showed much lower expression for both microRNAs. In the milk cell fraction, both microRNAs were expressed at relatively low levels compared to lipids and skim milk [Fig. 5]. In the milk lipid fraction, both microRNAs were expressed more consistently in the two mothers compared to the cellular and skim milk fractions.

**DISCUSSION**

Recent advances in human milk compositional studies have revealed the presence of RNA [Lemay et al., 2013] and microRNA molecules [Weber et al., 2010; Munch et al., 2013], similar to those previously found in other biological fluids [Bassiotou et al., 2012]. With breakthrough studies demonstrating an active role of food-derived microRNAs in regulating gene expression in adults [Zhang et al., 2012; Baier et al., 2014], the discovery of these molecules in human milk is of great interest, as they may play a role in the development of the infant immune system. The findings of this study suggest that the choice of extraction kit can significantly impact the quantification of microRNAs in human milk, with TRizol-LS and mirPremier showing the highest efficiency. Further research is needed to determine the biological significance of these microRNAs in human milk and their potential role in infant health.
milk highlights their potential significance for the breastfed infant. In addition, these milk molecules may provide novel diagnostic opportunities in relation to disease [Chen et al., 2008; Turchinovich et al., 2011]. Extensive profiling and quantification of microRNA is therefore essential to the understanding and exploration of these molecules and their functions in human milk. The complexity of human milk composition, including lipid, cellular, and skim milk fractions, also suggests that each fraction potentially requires a different handling procedure and extraction kits for optimal RNA and microRNA isolation. In previous studies of human milk microRNA, the expression and type of microRNAs differed between milk lipids and skim milk, with the latter showing lower levels of microRNA expression than the lipid fraction [Kosaka et al., 2010; Weber et al., 2010; Munch et al., 2013]. This suggests that microRNA content may also be different in human milk cells, as we showed in this study. MicroRNAs in the three human milk fractions could be used for different diagnostic and prognostic purposes, especially in monitoring the performance and related pathologies of the lactating mammary gland.

Studies investigating human milk microRNA content have focused mainly on the skim milk fraction and no comparisons have been made with the milk lipid fraction within the same sample. In this study, the human milk lipid and cell fractions clearly contained higher quantities of both total RNA and microRNA ($P < 0.001$) (Fig. 2) compared to skim milk. Specifically, the lipid fraction was on average 10- and 8-fold richer in microRNA and total RNA, respectively, than skim milk (Fig. 3).

With respect to total RNA in milk lipids, all methods performed similarly in our hands (Table 4), except one of the filter column kits (miRPremier), which yielded significantly lower total RNA than the TRIzol-LS ($P = 0.010$), miRNAeasy mini ($P = 0.007$), and miRCURY-Cell&Plant ($P = 0.005$) kits. Moreover, RNAzol-RT gave significantly higher total RNA yields than mirVana ($P = 0.002$) and the miRNAeasy micro kits ($P = 0.0002$). The above suggest that the phenol/guanidine-based method (RNAzol-RT and TRIzol-LS) yields higher total RNA concentrations in the milk lipid fraction.

Sample RNA purity in milk lipids varied according to the method used. The optimal range considered was between 1.8 and 2.2 (Supp. Table 1). We found that the 260/280 ratios for TRIzol-LS (Table IV) were significantly lower to those of mirNAeasy mini ($P = 0.019$) and miRCURY-Cell&Plant ($P = 0.016$). Generally, the phenol/guanidine method (TRIzol-LS) yielded less pure RNA than the other methods examined. However, when phenol/guanidine was combined with filter column (e.g., mirVana), higher microRNA to small RNA ratios were obtained ($P = 0.018$), suggesting it as an appropriate method for extracting high quantities of microRNA from milk lipids.

With respect to microRNA content in the milk lipid fraction, significantly higher microRNA/small RNA ratios were obtained with the miRNAeasy micro kit compared to mirVana ($P < 0.035$). No differences were seen between RNAzol-RT and miRNAeasy micro kit ($P < 0.190$). TRIzol-LS phenol/guanidine was not different to other filter column kits ($P > 0.050$). These findings suggest that both the phenol/guanidine and the filter column methods extract more microRNA than the filter column combined with phenol/guanidine method.

For the cellular portion of the human milk, we present our results as amounts as it was not possible to determine the concentration in this fraction without cell counts of the total sample (whole milk). This limits the comparison to the lipid and skim milk fractions. Nevertheless, RNA/microRNA amounts of the human milk cell fraction were relatively high and comparable to other cells such as mast cells [Edih et al., 2012]. In the first set of samples, we found that RNAzol-RT yielded less total RNA compared to miRNAeasy micro ($P = 0.017$). In the second sample set, miRPremier yielded less total RNA than miRNAeasy mini ($P = 0.003$). It is prudent to note that the miRPremier kit yielded the lowest amounts of RNA of all kits. However, no significant difference was seen between kits in the microRNA content. In terms of purity of RNA, the phenol/guanidine method yielded significantly lower values compared to the other methods tested (RNAzol-RT: $P < 0.001$; TRIzol-LS: $P < 0.001$). In terms of microRNA/small RNA ratio, TRIzol-LS yielded significantly more small RNA ($P < 0.001$). In summary, it appears that the filter column-based kits yield similar amounts of microRNA and total RNA with good purity in the milk cell fraction.

In the skim milk fraction, significantly purer RNA was obtained using mirVana than either miRNAeasy micro or RNAzol-RT kits ($P < 0.001$). Nevertheless, RNAzol-RT yielded significantly higher total RNA than mirVana ($P = 0.001$). No differences were seen in extracted microRNA levels amongst all kits tested. However, significant differences were observed between kits in the microRNA/small RNA ratios ($P < 0.001$). RNAzol-RT had significantly higher microRNA/small RNA ratio than either miRNAeasy micro or mirVana ($P < 0.001$). Further, microRNA/small RNA ratio of mirPremier was higher than of miRCURY-Cell&Plant or miRNAeasy mini kits ($P < 0.001$). Interestingly, although the phenol/guanidine method (RNAzol-RT and TRIzol-LS) was very efficient in extracting high amounts of total RNA and microRNA from skim milk, the purity of the extracted RNA was rather poor.

By using RT-PCR, the validation of microRNA presence in biological samples has been conducted [Chen et al., 2005; Shi and Chiang, 2005; Doleshal et al., 2008]. MicroRNA expression patterns do not correlate with total RNA concentration that is usually measured by Bioanalyzer or NanoDrop [Doleshal et al., 2008; Moret et al., 2013]. We confirmed this in this study by examining hsa-miR-148a-3p and hsa-miR-30a-5p expression in different human milk fractions. These microRNAs were detected in all human milk fractions using the most effective extraction kits (Table 3). As expected, both microRNAs were expressed at high levels consistently in two different milk lipid samples; in particular, hsa-miR-30a-5p was expressed at higher levels than hsa-miR-148a-3p in the lipid fraction. In contrast, one skim milk sample had low expression of these microRNAs, whereas the other skim milk sample showed high expression. Moreover, these microRNAs were not expressed at high levels in both milk cell fraction samples compared to the other milk fractions. These findings suggest an enrichment for these microRNAs in the fat globules secreted by lactocytes as well as secretion in the skim milk. Expression levels may change rapidly in cells, whereas the same is not expected for either the fat globule or the skim milk. The above merit further investigation.

Our findings indicate that microRNA in human milk are conserved and protected either within cells or fat globules/other vesicles such as exosomes, and very few can be isolated from the skim milk fraction, which has also been called the plasma phase of milk. Importantly,
most previous studies examined skim milk and not the cellular or the lipid fraction [Kosaka et al., 2010; Weber et al., 2010], and they have therefore excluded the fractions of human milk that appear to be richer in microRNA. Most recently, Munch and colleagues (2013) stated that human milk lipids are richer in microRNAs than skim milk [Munch et al., 2013]. Our findings are in agreement with this and strongly suggest that it is necessary to examine microRNA in all three fractions of human milk and not just in one, to allow complete analysis of this component of human milk, its origin, properties and functions. Also, the microRNA content of the human milk cell fraction has not been profiled as done in human milk lipids, although it potentially conserves more novel microRNAs [Munch et al., 2013]. Finally, our results support the rigorous investigation and standardization of sampling, processing, extraction and storage criteria for the investigation of microRNA in different milk fractions. The recommended kits for each human milk fraction based on the quantity and quality of RNA/microRNA were listed in Table 3, and could be applied for highly efficient extraction of RNA/microRNA from exosomes, fat globules, and human fluid cells in addition to human milk fractions.

A potential explanation for the differences between the kits in extraction performance is that the differences in the lysis solution between kits, which is an important step to release intact RNA/microRNA from cells and fat globules. Therefore, the composition and efficiency of the lysis solution must be carefully selected based on the requirements of RNA/microRNA for subsequent studies, such as profiling using qPCR or Microarrays. Importantly, although most of the kits were designed for cellular fractions, we show that they can be used for body fluid samples such as skim milk, with good performance. However, using higher amounts (than those recommended by the manufacturer for fluids) of skim milk for extraction may help to increase the concentration of RNA/microRNA. It is also suggested to use smaller amounts of lysis solution in extracting RNA from skim milk because they are already free in skim milk, and the lysis solution may influence the integrity of RNA transcripts. Another consideration is that microRNAs may be fragmented into smaller pieces during the washing steps, and may not be subsequently conserved in the filter columns. On the other hand, in the phenol/guanidine-based kits, the main issue in our findings was the poor RNA quality, suggesting that RNAs may be influenced during precipitation due to the long term exposure to ethanol and phenol.

This will now generate new avenues for examination of the types, properties and functions of these human milk molecules. Further, the variability amongst and within lactating women and factors that may influence them, such as the stage of lactation or milk removal, can be now robustly and consistently investigated. Opportunities arise for the use of these molecules as diagnostic markers of disease during lactation. Given the recently postulated function of human milk microRNA in providing immunological support to the infant [Kosaka et al., 2010; Zhou et al., 2012]. Although the benefits of oral microRNAs have been recently challenged [Dickinson et al., 2013; Wittwer and Hirschli, 2014], our study sets the basis for further examination using sound methodology of the potential significance of microRNAs in the lactating mammary gland and/or in the infant [Hennehan et al., 2009; Gotte, 2010]. Future studies should consider the methodology developed herein to address important questions of immunological as well as developmental benefits conferred to the infant by human milk microRNA, and the potential to use them as diagnostic markers for the human mammary gland.

CONCLUSIONS

We demonstrated the presence of RNA and specifically microRNA in all three fractions of human milk, including the cells, lipids and skim milk, with the highest levels of both RNA and microRNA obtained in the lipid and cellular fractions. We presented a comparison analysis in a comprehensive dataset of 472 human milk samples, assessing three different extraction methods in eight commercially available kits. These results allow researchers to choose the most appropriate method for measurement of microRNA for their sample composition and fraction of human milk.

ACKNOWLEDGMENTS

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REFERENCES


Human milk miRNAs primarily originate from the mammary gland resulting in unique miRNA profiles of fractionated milk

Mohammed Alsaweed1,2, Ching Tat Lai1, Peter E. Hartmann1, Donna T. Geddes1 & Foteini Kakulas1

Human milk (HM) contains regulatory biomolecules including miRNAs, the origin and functional significance of which are still undetermined. We used TaqMan OpenArrays to profile 681 mature miRNAs in HM cells and fat, and compared them with maternal peripheral blood mononuclear cells (PBMCs) and plasma, and bovine and soy infant formulae. HM cells and PBMCs (292 and 345 miRNAs, respectively) had higher miRNA content than HM fat and plasma (242 and 219 miRNAs, respectively) ($p < 0.05$).

A strong association in miRNA profiles was found between HM cells and fat, whilst PBMCs and plasma were distinctly different to HM, displaying marked inter-individual variation. Considering the dominance of epithelial cells in mature milk of healthy women, these results suggest that HM miRNAs primarily originate from the mammary epithelium, whilst the maternal circulation may have a smaller contribution. Our findings demonstrate that unlike infant formulae, which contained very few human miRNA, HM is a rich source of lactation-specific miRNA, which could be used as biomarkers of the performance and health status of the lactating mammary gland. Given the recently identified stability, uptake and functionality of food- and milk-derived miRNA in vivo, HM miRNA are likely to contribute to infant protection and development.

Human milk (HM) is the optimal nutrition for term infants1. In addition to being a food source, HM confers developmental programming to the infant and protection against infections, resulting in decreased risk of sudden infant death syndrome and reduced mortality and morbidity both in the short- and long-term2–4. These effects are mediated by HM-specific regulatory factors including both cellular and biochemical components5–10. In contrast, artificial infant formula cannot confer such protective and developmental functions as it lacks important HM components with bioactivity11,12. An additional unique bioactive component of HM that has been recently discovered is miRNAs13,14.

miRNAs are small non-coding RNAs, which regulate gene expression, thus control protein synthesis at the post-transcriptional level in eukaryotic cells15. They have been identified as key regulators of diverse biological and developmental processes in eukaryotes (cell proliferation and differentiation, apoptosis, immune system development and immune response16–18) by targeting messenger RNA (mRNA) during its translation into protein, either degrading the mRNA or inhibiting the translation process18. Aberrant miRNA expression has been found to be associated with pathologies, including different types of cancer, inflammation and diabetes19. Importantly, food-derived miRNA have been recently shown to be very stable in the gastrointestinal tract and be transferred to the blood circulation of adults, influencing gene expression in different tissues20. In addition to tissues and cells, miRNAs have been isolated from body fluids, such as plasma, urine, saliva and tears21. Further, exosomes, small cell-derived vesicles present in body fluids and carrying proteins and molecules, have been shown to take up miRNAs mediating their protection against digestion and facilitating their regulatory functions in different tissues and organs22.

Most recently, miRNAs have been isolated in high quantities from both animal and HM, and were shown to be present both as free molecules in skim milk13,14,22–24 and packaged in vesicles such as milk exosomes and the
fat globules. Bovine milk exosomes can be transported by intestinal cells via endocytosis in the human and rat colon. Moreover, studies of bovine milk consumed by adult humans showed that at least some bovine milk miRNA can be transferred to the bloodstream. This was demonstrated in vitro uptake by splenocytes and intestinal cells of miRNA derived from bovine milk, and their therapeutic role in delaying the onset of experimental arthritis when delivered orally to mice. Collectively, these findings strongly suggest that HM miRNA survive the gastrointestinal tract of the infant to exert regulatory functions during breastfeeding, similar to what has been recently shown for maternal milk-derived stem cells. It is therefore important to elucidate the origin, properties, distribution and functional significance of HM miRNA as a novel regulatory component of milk.

Most previous research in milk miRNA has focused on animal milk, including the bovine, porcine and murine. In these animal studies, next generation sequencing (NGS) has been mainly employed as a miRNA profiling method. Studies using NGS and other global miRNA profiling methods in HM are scarce, with many previous investigations mainly using qPCR-based technology for a limited number of miRNAs. Further, skin milk and to a lesser extent, milk fat have been the milk fractions of choice in previous milk miRNA studies, whilst the milk cellular fraction has been largely neglected despite being a rich source of RNA. Although Munch et al. (2015) stated that RNA in milk fat was the richest in miRNA, this was not compared with HM cells, which potentially conserve high quantities of miRNAs. Recently, we have shown that skin milk has the lowest miRNA content amongst the three HM fractions (cells, fat and skim milk). Because of this and considering that breastfed infants consume whole HM, it is imperative that further to skin milk, the miRNA content of HM cells and fat is rigorously examined to illuminate the true contributions of HM miRNA to infant health. At the same time, comparisons with maternal blood may shed light into the origin of HM miRNA, which is still unexplored.

In this study, we profiled 681 HM mature miRNAs using the TaqMan miRNA OpenArray system (Applied Biosystems), with the aim to determine the miRNA composition of the cell and fat fractions of HM and compare it with maternal peripheral blood mononuclear cells (PBMCs) and plasma collected from exclusively breast-feeding women as well as two commercial infant formulae. In addition, gene ontology for miRNA targets and pathway analyses were conducted for the miRNAs that were differentially expressed between HM and maternal blood. Our study elucidates the origin of HM miRNAs and reveals the contributions of the cells and fat fractions to the total content of miRNAs in HM.

### Results

#### Concentration and quality of RNA enriched in miRNA in maternal milk and blood.

Total RNA was extracted using the mirNeasy mini kit for milk cells and PBMCs, miRCURY RNA Isolation Bioanalyzer Kit for milk fat, and mirVana PARIS Kit for plasma, which have been shown to enrich for miRNA. Therefore, the total RNA concentration in the text below refers to total RNA enriched in miRNA. A high correlation between RNA Integrity Number (RIN) using the Bioanalyzer and 260/280 ratio using the NanoDrop was obtained (ρ < 0.001). The total RNA concentration of PBMCs (ng/10^6 cells) was not different to that of milk cells using Bioanalyzer (ρ = 0.960) or the NanoDrop (ρ = 0.800) (Table 1A, Fig. 1A). However, both the total RNA concentration and RNA quality of plasma were significantly lower than that of milk fat using both instruments (ρ < 0.001) (Fig. 1B–D). The quality of RNA obtained from milk fat was also lower than that of milk cells and PBMCs when using Bioanalyzer (ρ < 0.001), although still within the acceptable range when using NanoDrop (Fig. 1C,D).

High HM cell content was associated with high total RNA concentration of milk (ρ = 0.004 and ρ = 0.005, using Bioanalyzer and NanoDrop, respectively), although the same was not observed for either maternal PBMCs and their total RNA content (ρ = 0.502 and ρ = 0.762, using Bioanalyzer and NanoDrop, respectively) or HM fat content and total RNA (ρ = 0.664 and ρ = 0.823 using the Bioanalyzer and NanoDrop, respectively) (Fig. 1E–G). A trend for a positive association was seen between the volume of milk expressed and the total RNA extracted from milk cells as well as the maternal blood volume collected and the total RNA extracted from PBMCs, although these were not statistically significant (n = 10) (ρ = 0.172, 0.712 and ρ = 0.482, 0.117 using Bioanalyzer and NanoDrop for HM and blood, respectively) (Fig. 1H,I). Further, no relationship existed between the total number of milk cells and PBMCs (ρ = 0.459) (Fig. 1J).

#### Human milk has a diverse miRNA composition that differs from maternal blood.

To investigate the miRNA composition of HM and maternal blood, TaqMan OpenArray was used to measure Ct values for 681 human known mature miRNAs. Using a first criterion of 8 ≤ Ct ≤ 29, HM cells and fat, and PBMCs and plasma were found to conserve 450, 337, 488, and 319 miRNAs, respectively. To determine the most reliably measured miRNAs, we used a novelty criterion stating 8 ≤ Ct ≤ 29 and miRNA presence in ≥ 4 out of the 10 samples tested for each of milk cells, milk fat, PBMCs and plasma. Unreliable miRNAs were determined based on Ct < 5 or excessive variation between replicates. Undermined miRNAs (miRNAs that were tested in the OpenArray panel but were not detected in any of the samples examined) did not match the above criteria (Fig. 2A). Using this novelty criterion, 292 miRNA species were determined in HM cells, 242 miRNAs in HM fat, 345 miRNAs in PBMCs, and 219 miRNAs in blood plasma (Table 1, Supplementary Table S1A). Plasma was diverse in miRNA composition, with great variation between participants, and contained fewer miRNA species than PBMCs and HM cells (ρ < 0.001) or HM fat (ρ = 0.015). HM cells contained more miRNA species than HM fat (ρ = 0.038). PBMCs conserved a high number of miRNAs in most samples, which was significantly higher than in HM fat (ρ = 0.001), but not statistically different to HM cells (ρ = 0.084) (Fig. 2B). Of the miRNA identified, the top 10 and 20 most highly expressed in all four sample groups (HM cells and fat, and maternal PBMCs and plasma) are listed in Table 1 and Supplementary Table S2, respectively. All the miRNA species detected in each sample are listed in Supplementary Table S1B.

Heat map analysis and plotting of miRNA profiles showed that most miRNAs in HM cell and fat samples are clustered together, whilst PBMC and plasma miRNAs do not significantly relate to any of HM cells or fat samples.
Table 1. Sample characteristics for HM cells and fat, and maternal PBMCs and plasma (n = 10 for each sample group), including quantity and quality of total RNA enriched in miRNA extracted from using NanoDrop 2000 and Bioanalyzer 2100, total number of miRNA species detected, and top 10 most highly expressed miRNAs. (SD: standard deviation). 1Total RNA (ng) per 1 NanoDrop 2000 and Bioanalyzer 2100, total number of miRNA species detected, and top 10 most highly expressed miRNAs.

<table>
<thead>
<tr>
<th>Extraction kit used</th>
<th>Milk cells</th>
<th>Milk fat</th>
<th>PBMCs</th>
<th>Plasma</th>
</tr>
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<tr>
<td>miRCURY mini</td>
<td>miRCURY mini</td>
<td>miRCURY mini</td>
<td>miRCURY mini</td>
<td>miRCURY mini</td>
</tr>
<tr>
<td>Cell content/ml milk or blood (mean ± SD)</td>
<td>435.78 ± 311.96</td>
<td>7 (92.5)</td>
<td>724.07 ± 309.44</td>
<td>9 (85.1)</td>
</tr>
<tr>
<td>Fat content of milk (%) to (mean ± SD)</td>
<td>–</td>
<td>8.4 ± 3.0</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Total RNA (mean ± SD) using NanoDrop 2000</td>
<td>2.525 ng/10⁶</td>
<td>29 ng/µl of fat</td>
<td>2.648 ng/10⁶</td>
<td>2.624 ng/10⁶</td>
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<tr>
<td>RNA purity (OD 260/280)</td>
<td>1.91</td>
<td>0.3</td>
<td>1.85</td>
<td>1.82</td>
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<tr>
<td>RNA Integrity Number (RIN) (mean ± SD) using Bioanalyzer 2100</td>
<td>2.04 ± 0.02</td>
<td>0.97 ± 0.08</td>
<td>2.00 ± 0.04</td>
<td>2.07 ± 0.01</td>
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<tr>
<td>Total RNA (mean ± SD) using Bioanalyzer 2100</td>
<td>2.674 ng/10⁶</td>
<td>25 ng/µl of fat</td>
<td>2.709 ng/10⁶</td>
<td>2.687 ng/10⁶</td>
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<tr>
<td>RNA Integrity Number (RIN) (mean ± SD) using Bioanalyzer 2100</td>
<td>7.2 ± 0.97</td>
<td>3 ± 1.2</td>
<td>7.5 ± 1.04</td>
<td>6.8 ± 1.6</td>
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<tr>
<td>Number of detectable miRNA species</td>
<td>292</td>
<td>242</td>
<td>345</td>
<td>219</td>
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Highly expressed miRNAs

<table>
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<tr>
<td>hsa-miR-146b</td>
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<td>has-miR-106a</td>
<td>has-miR-17</td>
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<td>hsa-miR-20a</td>
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<td>hsa-miR-191</td>
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<td>has-miR-106a</td>
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<tr>
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<td>has-miR-155</td>
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<tr>
<td>hsa-miR-30a</td>
<td>hsa-miR-10b</td>
<td>has-miR-10c</td>
<td>has-miR-199a-3p</td>
</tr>
</tbody>
</table>

The full set of miRNAs and their differential expression p values are shown in Supplementary Tables S3–S13. HM cell and fat miRNA expression was highly correlated, with the exception of hsa-miR-564, which was higher in fat than in milk cells. Most of the detected miRNAs in the other sample groups were differentially expressed amongst them. These findings were confirmed using correlation analysis in DataAssist, which showed a high correlation in miRNA expression between HM cells and fat (r = 0.90), whilst the correlation between PBMC miRNAs and miRNAs in HM cells or fat was poorer (r = 0.62; r = 0.57, respectively). Plasma miRNAs were expressed at distinctly different levels than all other three sample groups (r = −0.08, −0.003, −0.27 for HM cell, fat, and PBMCs comparisons respectively).

Further, a comparative analysis of the miRNA species present in each group was performed using the most reliable miRNA identified. This also confirmed the high correlation in miRNA content between HM cells and fat (Fig. 3B). PBMC miRNAs were weakly related to HM miRNAs and also to plasma miRNAs (Fig. 3B). Eulier diagrammatic analysis revealed the overlapping (most reliable) miRNAs between the different sample groups examined, showing 43 miRNAs that were specific to HM and were not found in maternal blood (Fig. 4A, Supplementary Table S14A). Of these, 14 miRNA were specific to HM cells, 6 were specific to HM fat, and 23 were commonly specific between HM cells and fat. Similarly, 84 miRNA species were specific to maternal PBMCs, and 11 to plasma (Fig. 4A, Supplementary Table S14A). In total, 221 miRNA species were shared between HM cells and fat (Fig. 4A, Supplementary Table S14A). Of the 681 miRNAs assayed, 114 were detected in all four sample groups, 34 were specific to HM cells and PBMCs, 2 were specific to HM fat and PBMCs, and 12 were specific to PBMCs and plasma (Fig. 4A, Supplementary Table S14A). Interestingly, no miRNAs were identified that were specific to plasma and HM only. These data provide evidence supporting the endogenous synthesis of the majority of HM miRNA in the lactating mammary epithelium, with a small contribution of the maternal circulation. A proportion of HM miRNA appear to be unique to lactation, as they were not detected in maternal blood.
Figure 1. RNA enriched in miRNA in HM cells and fat, and maternal PBMCs and plasma, and associations with HM components. (A,B) RNA concentration of HM cells, PBMCs, HM fat, and plasma, obtained with NanoDrop 2000 (N) and the Bioanalyzer 2100 (B). (C,D) RNA integrity measured by the Bioanalyzer 2100, and RNA purity (260/280 ratio) using NanoDrop 2000 in all four sample groups. (E,F) Associations between total RNA enriched in miRNA and HM cell content or maternal blood PBMC content using Bioanalyzer 2100 and Nanodrop 2000. (G) HM fat content (%) and RNA concentration of HM fat (ng). (H,I) Associations between HM volume or maternal blood volume with the total RNA enriched in miRNA. (J) Association between PBMC content of blood and HM cell content.
Infant formulae are low in human miRNA compared to human milk. Both infant formulae examined (bovine milk-based and soy-based) contained very few human mature miRNA species, and those were present at very low expression levels compared to HM fractions (Fig. 2A). Although the RNA input of the soy-based formula was higher than that of the bovine milk-based formula (Table 2), the latter contained 45 mature human miRNAs compared to 22 in the soy-based formula with \(8 \leq \text{Ct} \leq 35\), whilst only 26 and 19 respectively with the more reliable \(8 \leq \text{Ct} \leq 29\) (Supplementary Table S15). These differences between the two formulae potentially reflect differences between animal and plant miRNA. The miRNA content and expression patterns of the bovine milk-based infant formula were more similar to that of HM than the maternal PBMCs or plasma, and less so for the plant-based formula (Fig. 3B). Of the miRNA detected in the formulae, 33 were common between the bovine milk-based formula and HM cells and fat, whilst only 8 miRNAs were common between the soy-based formula and HM fractions (Fig. 4B, Supplementary Table S14B). Interestingly, miR159a, which is known to be plant-specific and was not expressed in any of the HM samples, was detected in not only the soy-based formula, but also in the bovine milk-based formula, at 16 replicates and high expression levels compared to the other identified miRNAs in this formula (Fig. 4C, Supplementary Table S16).

Pathway enrichment and ingenuity pathway analyses reveal a plethora of biological functions associated with human milk miRNAs. Pathway enrichment analysis of some of the significantly
Differentially expressed miRNAs in HM cells and fat (miR-200a; 205; 200c; 141; 429; 200b; 106b; 20a; 17; 34a; 34c; 340–5p; 137–3p; 195) was done using the MetaCore pathway analysis tool pipelines by GeneGO (GO) 36. Several of these miRNAs were found to interact in the molecular pathways regulating the inflammation action of Endothelin-1, cardiovascular disease, and sickle cell disease. They are also known to be involved in microphthalmia-associated transcription factor (MITF) in melanoma, epithelial-mesenchymal transition (EMT), lung epithelial progenitor cell differentiation, and tumor protein p53 signaling in prostate cancer (Fig. 5, Supplementary Figures S2–S5).

Due to the limited number of miRNA that are recognized and can be analyzed using MetaCore, we also employed ingenuity pathway analysis to further explore functional and disease pathways associated with the reliably expressed miRNAs (8 ≤ Ct ≤ 29) across all samples through 6 mir seed regions, which is the miRNA-miRNA binding site. All the identified miRNAs were mapped to biological processes to determine their contribution in a variety of normal and abnormal conditions (Supplementary Tables S17–S21). Due to the overrepresentation of knowledge associated with some diseases including cancer and inflammation, compared to normal biological functions, many miRNAs in this study have been identified to be associated with abnormal conditions.
Numerous miRNAs also have important roles in normal conditions. As shown by the differential expression analysis (Supplementary Table S6), miR-564 was differentially expressed between HM cells and fat, where it was upregulated in HM fat. This miRNA is known to regulate the differentiation of adipose tissue-derived stem cells (ADSCs)\(^3\) and, in the case of HM, it may participate in milk or fat synthesis in the lactocyte. A plethora of biological functions across all sample groups examined were regulated by the reliably identified miRNAs.

**Figure 4.** Shared reliable miRNAs (\(8 \leq \text{Ct} \leq 29\) and present in at least 4 samples per group) between the four sample groups examined. The bovine milk- and soy-based formulae are the results of a single assay and the observations are only illustrative (\(8 \leq \text{Ct} \leq 35\)). (A) Euler diagram showing overlapping reliable miRNA species between sample groups. (B) Euler diagram showing the number of reliable miRNA species in the HM cell and fat samples and their overlap with infant formulae. (C) Box plot showing high expression of plant-based miR-159a (16 replicates in each infant formula) in the two formulae tested.

<table>
<thead>
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<th>miRNA</th>
<th>Bovine milk-based formula</th>
<th>Soy-based formula</th>
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<tbody>
<tr>
<td>hsa-miR-320</td>
<td>1.37 ng/µl</td>
<td>116.4 ng/µl</td>
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<td>hsa-miR-200c</td>
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<td>hsa-miR-106a</td>
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<td>ath-miR159a</td>
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<td>hsa-miR-520c-3p</td>
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**Table 2.** Characteristics of extracted miRNA from bovine milk- and soy-based infant formulae. Although the criterion of miRNA presence in \(\geq 4\) samples with \(8 \leq \text{Ct} \leq 29\) cannot be applied in the formula samples tested due to examination of only one sample for each formula, here we report the top 10 most highly expressed miRNAs in each formula, which had \(8 \leq \text{Ct} \leq 35\). The miRCURY Biofluids kit was used to extract miRNA from both formulae. 12 grams of formula powder dissolved in 4 mL of Trizol LS reagent.
These functions included cellular development and movement, cell cycle, growth and proliferation, and immune responses (Supplementary Tables S17–S21).

Discussion
The discovery of novel bioactive components in HM, such as stem cells and miRNAs, re-emphasizes its importance as a nutritional, developmental and protective agent for infants. Increasing evidence suggests the involvement of miRNAs in both normal mammary development and function and infant health via HM. The abundance of miRNA in milk and their stability in the GI tract in adults further support their survival and functional significance in the breastfed infant. Over the past few years, HM miRNAs have been investigated in either skim milk or milk fat and exosomes, whilst the cellular component of milk has been largely ignored despite its high RNA content. We used the OpenArray Taqman technology to profile 681 human mature miRNAs in the cell and fat fractions of HM and compare it with maternal blood (PBMCs and plasma) as well as with two commercially available bovine milk- and plant-based formulae. HM cells were found to conserve higher quantities of miRNA, both in total content and miRNA species composition, compared to previously studied HM fat and skim milk. The miRNA composition of the cell and fat fractions of HM was similar, whilst maternal PBMCs and plasma displayed distinctly different miRNA profiles to HM. This finding, together with the previously shown dominance of lactocytes in mature HM of healthy mothers and infants, suggest endogenous synthesis of the majority of HM miRNA in the mammary epithelium, with a small contribution of the maternal circulation.

The total RNA content enriched in miRNA was not significantly different between PBMCs and HM cells (Fig. 1E,F), indicating similar transcription activity in both milk-derived mammary epithelial cells and maternal blood-derived PBMCs. In contrast, HM fat conserved higher RNA concentration compared to maternal plasma (Fig. 1B), with similar miRNA content to that of HM cells. This is in accordance with the origin of milk fat globule from the lactocyte and with previous milk fat miRNA studies. Thus, HM fat, in addition to being an essential nutritive component for the infant, is also an important carrier of bioactive miRNA molecules to infants. No relationship was seen between HM fat content and the total RNA of HM fat, which is in agreement with the fact that total HM fat content does not necessarily reflect the number of fat globules. This suggests that the fat miRNAs are primarily packaged within the fat globule or other microvesicles such as exosomes contained within...
the milk fat. A positive relationship between HM cells or PBMCs and total RNA content was seen as expected. However, this was stronger for PBMCs, which represent a specific cell type in contrast to HM cells, which are a heterogeneous population of cells including primarily lactocytes (in mammalian milk from healthy mothers and infants), but also smaller populations of stem cells, progenitor cells, and immune cells\(^{24,39}\). The weaker association between HM cells and total RNA content may reflect differing RNA transcription between these different cell populations of HM.

Although both HM cells and PBMCs were rich in different miRNA species (292 and 345 miRNAs, respectively) and not significantly different to each other in our study cohort (\(p = 0.084\), HM fat had a lower miRNA species number (242 miRNAs) (\(p = 0.038\) for the comparison with HM cells; \(p < 0.001\) for the comparison with PBMCs), followed by maternal plasma (219 miRNAs) (\(p = 0.001\) for the comparison with HM cells and PBMCs; \(p = 0.015\) for the comparison with HM fat). The poor miRNA species content of maternal plasma has also been seen in other body fluids, such as human urine\(^{44}\), and also in skim milk\(^{45}\). Therefore, the HM cellular component conserves more miRNA species than the HM fat and skim milk fractions, emphasizing the need to include it in milk miRNA investigations, particularly since it better represents what the infant receives. However, caution needs to be exercised when analyzing whole milk samples to ensure complete lysis of HM cells and membranous microvesicles for miRNA extraction. This is better performed when fractionating the HM into cells, skim milk, and fat immediately upon expression and prior to freezing, and then extracting miRNA after rigorous cell lysing, as has been previously described\(^{44,45}\).

Differential miRNA expression analysis together with heat mapping and comparative analysis of miRNA species showed strong similarities in miRNA composition and profiles, including expression levels, between HM cell and fat fractions. At the same time, maternal blood, both its PBMCs and plasma, had distinctly different miRNA profiles to HM cells and fat (Fig. 3A–C). It has been previously shown that mature HM of healthy mother-infant dyads, which is what was analysed in our study, the dominant cell type is the lactocyte (secretory mammary epithelial cell), which also secretes the HM fat\(^{46,47}\). Therefore, our findings strongly support the origin of HM cell and fat miRNAs primarily from the mammary epithelium via endogenous synthesis in the lactocytes. The maternal circulation may still likely have a small contribution to milk miRNA. This is in agreement with a recent study in the tammar wallaby reporting a weak correlation between maternal serum and skim milk miRNAs\(^{46,47}\), suggesting that milk miRNAs are primarily synthesized in the mammary gland\(^{46,47}\).

The origin of HM miRNA primarily from the mammary gland emphasizes their potential use as biomarkers of both lactation performance and the health of the gland. Indeed, HM immune cells have been shown to rapidly respond to maternal infections, with the most pronounced responses seen in abnormal conditions of the lactating breast such as mastitis\(^{39}\), which, if left untreated or are managed late, can result in early cessation of breastfeeding, with detrimental effects to both the infant and the mother\(^{41}\). Recent studies have shown distinct miRNA responses in the milk of the dairy cow during mastitis, consistent with the immune cell response\(^{44}\). Moreover, family members of miR-29 (miR-29a/b/c), an abundant miRNA in mammalian milk, which was also found to be highly expressed in HM cell and fat fractions in our study, was shown to epigenetically regulate lactation performance in the dairy cow\(^{45}\). The distinct changes of milk miRNA in response to the status of the mammary gland together with our data supporting the mammary origin of milk miRNA highlight their potential diagnostic value as non-invasive and easily accessible biomarkers of mammary gland function and health to facilitate timely management of lactation difficulties and maintenance of breastfeeding for longer periods. In a similar context, circulating miRNA in plasma have been successfully used as early biomarkers\(^{34,42}\) of aberrant growth in breast cancer\(^{37,47}\) and of other diseases such as type 2 diabetes\(^{44,46}\).

To give insight into the content of artificial infant formulae in miRNA, we compared the miRNA profiles of a bovine milk-based and a soy-based formulae that are in high demand in the Australian market. We found very few human mature miRNAs in both of these formulae and a poor similarity in expression compared to HM (Fig. 2A). The bovine milk-based formula clustered more closely with HM cell and fat miRNAs than the soy-based formula (Fig. 2A,B), likely due to the mammalian milk basis of the former versus the plant basis of the latter, yet still very poorly correlated with HM. Skim bovine milk has been shown to harbor 245 miRNAs\(^{22}\), soy-based formula (Fig. 4C). This is a plant-specific miRNA not detected in any of the HM samples analysed. It is possible that it originated from the nutrition of the animals from which milk was sourced for the bovine formula. Moreover, it may come from vegetable fats, such as soybean oil, that are added to the bovine formula during manufacturing, with detrimental effects to both the infant and the mother\(^{43}\). Recent studies have shown distinct miRNA expression gene expression in adult humans\(^{20}\), which is in agreement with a more maternal circulation may still likely have a small contribution to milk miRNA. This is in agreement with a recent study in the tammar wallaby reporting a weak correlation between maternal serum and skim milk miRNA, suggesting that milk miRNAs are primarily synthesized in the mammary gland\(^{46,47}\).

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Interestingly, miR-159a was the most highly expressed miRNA in both the bovine milk-based and the soy-based formulae (Fig. 4C). This is a plant-specific miRNA not detected in any of the HM samples analysed. It is possible that it originated from the nutrition of the animals from which milk was sourced for the bovine formula. Moreover, it may come from vegetable fats, such as soybean oil, that are added to the bovine formula during manufacturing as per its ingredients, though intentional addition of this miRNA or contamination during preparation and processing cannot be excluded either. This requires validation with further samples of this formula. It is not known whether this miRNA survives in and can be absorbed by the GI tract of the infant to exert gene regulatory functions, and this warrants further investigation. This is likely given that other plant food-derived miRNAs, such as miR-168a, have been shown to influence gene expression in adult humans.

Recently, exogenous miRNAs have been experimentally proven to regulate gene expression in mammalian cells\(^{20,24}\). These miRNAs are transferred to humans via consumption of food, therefore it is highly likely that the same transfer of HM miRNA to the breastfed infant occurs, especially since the neonatal stomach is less acidic and has a highly leaky early in life. A recent study in adult human cells demonstrated that consumption of bovine milk, miR-29b and miR-200c increases in the plasma, returning to baseline levels after 24 hours of the initial consumption. Further, after bovine milk consumption, the expression of runt-related transcription factor 2 (RUNX2), targeted by miR-29b, was elevated in PBMCs. A recent study by Arruza et al. further
demonstrated uptake of milk-derived miRNA by mammalian cells and a therapeutic function in ameliorating experimental arthritis in mice\textsuperscript{31}. Therefore, the high presence of miRNAs in HM further supports its function as a biofluid initiating epigenetic signals in infants that could potentially influence infant development and health. In our study, GeneGo analysis and Ingenuity Pathway Analysis (IPA) revealed a number of biological functions and pathways that are controlled by miRNA enriched in HM, including immunity, growth and development, cell proliferation and apoptosis, lung epithelial progenitor cell differentiation, and epithelial-to-mesenchymal transition (EMT) (Fig. 5). EMT has been found to be a key player in cell differentiation, motility and migration in multiple tissues and organs, particularly during embryogenesis and in cancer\textsuperscript{55}. It has been shown to be normally present in both the human\textsuperscript{55} and the murine mammary gland\textsuperscript{32}, with potential important functions in normal breast remodeling required for milk synthesis. Our results suggest that the EMT process in the normal lactating breast may be mediated by pregnancy- and lactation-specific miRNAs, which requires further investigation.

Consistent with previous studies in HM, some of the most abundant milk miRNAs detected here are known to contribute to metabolic processes, decrease the cancer risk of infants\textsuperscript{33}, participate in the development of the infant's immune system, and protect infants from infections\textsuperscript{12,15,55}. Moreover, miRpath\textsuperscript{56} and KEGG\textsuperscript{57} analyses showed that the majority of the top 10 highly expressed miRNAs in both HM fractions examined here (cells and fat) are involved in the regulation of the cell cycle and the RNA transportation process during development (Supplementary Figure SIA). Normal cell cycle and RNA transport in the lactating breast is required for normal cell differentiation and proliferation of the lactocyte\textsuperscript{1}, and also to prevent breast cancer initiation by controlling cell proliferation\textsuperscript{32}. In contrast, the most highly expressed miRNAs in PBMCs were found to be involved in different molecular pathways to those of HM, such as the maintenance of normal gene activity of K-Ras, HER2 and CDK4, which are known oncogenes\textsuperscript{49} (Supplementary Figure S1B). Further, miRNAs found to be highly expressed in the maternal plasma are known to be involved in the control of oncogenes in melanoma, pancreatic and colorectal cancers\textsuperscript{59}.

Collectively, our data together with previous studies demonstrate that miRNAs are abundant in HM and likely play significant roles in the development and normal function of the lactating mammary gland, and in the HM fed infant. We have provided evidence that HM miRNA are primarily synthesized in the mammary epithelium, and may therefore be used as novel diagnostic biomarkers of lactation performance and breast infection. Further research is required to identify the functions of these miRNAs and examine potential novel miRNAs that may be present in the milk and the breast. Moreover, factors that may influence the expression levels of miRNA in HM should be investigated in an effort to standardize milk miRNA studies and elucidate the maternal-infant interaction in the regulation of these molecules during breastfeeding.

Materials and Methods

Ethics, sample collection and processing. This study was approved by the Human Research Ethics Committee of The University of Western Australia, and the methods were carried out in accordance with the approved guidelines. Informed written consent was provided by all participants, which included 10 exclusively breastfeeding dyads in month 2 postpartum (week 4–8) to ensure established lactation. All participating dyads and their infants were healthy at the time of collection. The workflow from sample collection to analysis is shown in Supplementary Figure S6. Fresh HM samples (24–78 mL) were collected early in the morning. Aseptic collection of the samples was carried out using a breast pump, sterile bottles and other accessories (Medela AG, Switzerland). Maternal blood samples were collected at the time of milk collection by an accredited phlebotomist. All the blood samples were collected into EDTA tubes (Becton Dickinson, Mountain View, CA, USA). Samples were transferred immediately to the laboratory in the dark for processing. HM samples were processed for miRNA analyses as previously described\textsuperscript{12,15,55}. Briefly, fresh milk samples were diluted 1:1 with phosphate buffered saline (PBS; Gibco, Life Technologies, Foster, CA), and were then centrifuged at 800 g for 20 min at 20 °C for centrifugation. HM cells and fat were transferred separately into new RNase free tubes. Cells were then washed three times with PBS, stained with Trypan blue (ProSciTech, Queensland, Australia) and counted using a hemocytometer as previously described\textsuperscript{32}. Fat samples were centrifuged twice at 450 g for 20 min at 20 °C to obtain a pure fat fraction. For blood fractionation, the whole blood samples were collected in EDTA-coated centrifuge tubes and were centrifuged at 800 g at 20 °C for 10 min to separate the plasma from cells. The plasma was then transferred to a new tube and centrifuged further at 3,500 g for 20 min at 4 °C to remove all residual cells and other debris. Blood peripheral mononuclear cells (PBMCs) were isolated from blood cell samples based on SeroCore separation (SeraLab, Australia). Briefly, after transferring the plasma into a new tube, the buffy coat in the top layer of the whole blood was transferred to a new tube with PBS. PBMCs in PBS were gently overlaid onto 4 mL of SeroCore in a new tube and centrifuged at 800 g for 15 min at 20 °C. The middle layer containing PBMCs was collected and was washed three times in PBS, then counted as described for HM cells above. Plasma samples were centrifuged for 15 min at 4 °C at 14,000 g to further purify plasma. miRNAs were extracted from all samples immediately without cryopreservation.

Infant milk formulae. Equal amounts (2 g) of two different types of infant formula powder were dissolved in 4 mL of Trizol LS Reagent (Invitrogen, CA, USA). Standard, whey dominant, infant formula known as bovine milk-based formula (S-26 Gold) and soy-based infant formula (S-26 Gold Soy) manufactured by Aspen Nutritional Australia, were used. As per the manufacturer's bottle instructions, both are considered to be suitable for infants from birth to one year old. The semi-dissolved powder in Trizol was incubated for 30 min at 37 °C for complete dissolution.

Milk fat content. Fat content of whole fresh HM samples was measured using Crematocrit Plus (Medela, Inc, McHenry, Illinois) as previously described\textsuperscript{33}. Briefly, whole HM was taken up by a capillary tube, then centrifuged for 10 min at 11,731 g in a microcentrifuge (BHF Hermle, Germany) to separate the milk fat from skim milk and cells.
Extraction and quantification of miRNA. miRNA were isolated from different fractions of HM according to our previous study\(^6\), where the miRNeasy mini kit (Qiagen, Hilden, Germany) was used to extract miRNAs from HM cells and maternal PBMCs, the mirCURY RNA Isolation-Biofluids Kit (Exiqon, Vedbaek, Denmark) for HM fat and both infant formulae, and the mirVana PARIS Kit (Ambion, Austin, TX, USA) for maternal plasma. These three miRNA extraction kits all use filter column-based methods. All the extractions were done according to the manufacturer’s protocol and as described previously concerning lysis of cells and membranous components via\(^8\). The volume of milk fat and lysis reagent for the miRCURY RNA Isolation-Biofluids Kit were increased to 400 μL and 120 μL, respectively. The concentration and purity of the extracted miRNAs were measured using a NanoDrop 2000 Spectrophotometer (Wilmington, DE, USA) and Agilent Bioanalyzer 2100 (Agilent, CA, USA) with the RNA 6000 NanoChip kit. All miRNA samples were then stored at −80°C until further analyses incubated for 30 min at 37°C for complete dissolution.

Reverse transcription (RT), preamplification and TaqMan OpenArray analysis. The expression levels of 681 human mature miRNAs that have been functionally validated with miRNA artificial templates were proﬁled in the milk, formulae and maternal PBMCs and plasma samples using the TaqMan miRNA OpenArray panel system (Life Technologies, CA, USA). The panel originally proﬁled 758 human mature miRNAs, however since its release, recent updates of miRNA integrity and function conﬁrmed 681 out of the 758 targets as true human miRNAs (Supplementary Table S22). Human ath-miR159a was used as a negative control for the human miRNAs since its release, recent updates of miRNA integrity and function concerning its release, recent updates of miRNA integrity and function concerning the human ath-miR159a was used as a negative control for the human miRNAs (Supplementary Table S22). Human ath-miR159a was used as a negative control for the human miRNAs (Supplementary Table S22). Human ath-miR159a was used as a negative control for the human miRNAs (Supplementary Table S22). Human ath-miR159a was used as a negative control for the human miRNAs (Supplementary Table S22). Human ath-miR159a was used as a negative control for the human miRNAs (Supplementary Table S22). Human ath-miR159a was used as a negative control for the human miRNAs (Supplementary Table S22).

miRNA species between different sample groups (HM cells and fat, and maternal PBMCs and plasma). P < 0.05 was considered statistically significant. Reliable miRNA were 8 ≤ Ct ≤ 29 and detected in at least 4 samples per group, whilst unreliable miRNA correspond to markedly low Ct value (< 5) showing excessive variation between replicates. Underdetermined miRNA correspond to a failed assay or a rare miRNA species (Ct > 29). The DataAssist software (Life Technologies, Foster, CA) was used to calculate the delta Ct value and fold change of each identified miRNA and to perform the differential expression analyses to conﬁrm the results obtained by the HqPCR.

References

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Author Contributions
M.A. collected samples, conducted experiments and data analyses, interpreted results, and wrote the manuscript; C.T.L. conducted statistical analyses and critically reviewed the manuscript; P.E.H. contributed to study design and critically reviewed the manuscript; D.T.G. designed the study, interpreted results and critically reviewed the manuscript; F.K. designed the study, collected samples, interpreted results, wrote and critically reviewed the manuscript. All authors approved the final manuscript.

Additional Information
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