

Immune-related microRNAs in breast milk and their relation to regulatory T cells in breastfed children

Emelie Ahlberg¹  | Magalí Martí¹  | Dhanapal Govindaraj¹  | Elisabet Severin^{1,2} | Karel Duchén^{1,2}  | Maria C. Jenmalm¹  | Lina Tingö^{1,3} 

¹Department of Biomedical and Clinical Sciences, Linköping University, Linköping, Sweden

²Allergy Center, Linköping University Hospital, Linköping, Sweden

³School of Medical Sciences, Nutrition-Gut-Brain Interaction Research Center/Food and Health Program, Örebro University, Örebro, Sweden

Correspondence

Lina Tingö, Department of Biomedical and Clinical Sciences, Lab 1, Linköping University, Linköping, Sweden.
Email: lina.tingo@liu.se

Funding information

Cancer- och allergiförbundet; Region Östergötland, Grant/Award Number: RÖ-930610; Dr P Håkansson's Stiftelse, Eslöv, Sweden; Faculty of Medicine and Health Sciences at Linköping University; Forskningsrådet i Sydöstra Sverige, Grant/Award Number: 2019-00989; Hjärt-lungfonden, Grant/Award Number: 20170365 and 20200301; Joanna Coccozas stiftelse för barnmedicinsk forskning, Grant/Award Number: 2020-01041; Lisa and Johan Grönberg Foundation, Sweden; Vetenskapsrådet, Grant/Award Number: 969326, 940313 and 931756

Editor: Alexandra Santos

Abstract

Background: The immunomodulatory capacity of breast milk may partially be mediated by microRNAs (miRNA), small RNA molecules that regulate gene expression on a post-transcriptional level and are hypothesized to be involved in modulation of immunological pathways. Here, we evaluate the expression of immune-related miRNAs in breast milk after pre- and postnatal supplementation with *Limosilactobacillus reuteri* and omega-3 (ω -3) polyunsaturated fatty acids (PUFAs), and the association to infant regulatory T cell (Treg) frequencies.

Methods: One-hundred and twenty women included in a double-blind, randomized, placebo-controlled allergy intervention trial received *L. reuteri* and/or ω -3 PUFAs daily from gestational week 20. Using Taqman qPCR, 24 miRNAs were analyzed from breast milk obtained at birth (colostrum) and after 3 months (mature milk) of lactation. The proportion of activated and resting Treg cells were analyzed in infant blood using flow cytometry at 6, 12, and 24 months.

Results: Relative expression changed significantly over the lactation period for most of the miRNAs; however, the expression was not significantly influenced by any of the supplements. Colostrum miR-181a-3p correlated with resting Treg cell frequencies at 6 months. Colostrum miR-148a-3p and let-7d-3p correlated with the frequencies of activated Treg cells at 24 months, as did mature milk miR-181a-3p and miR-181c-3p.

Conclusion: Maternal supplementation with *L. reuteri* and ω -3 PUFAs did not significantly affect the relative miRNA expression in breast milk. Interestingly, some of the miRNAs correlate with Treg subpopulations in the breastfed children, supporting the hypothesis that breast milk miRNAs could be important in infant immune regulation.

Trial registration: ClinicalTrials.gov-ID: NCT01542970.

KEYWORDS

breast milk, *Limosilactobacillus reuteri*, microRNA, omega-3 polyunsaturated fatty acids, randomized placebo-controlled trial, regulatory T cell

Maria C. Jenmalm and Lina Tingö should be considered joint senior authors.

This is an open access article under the terms of the [Creative Commons Attribution](https://creativecommons.org/licenses/by/4.0/) License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

© 2023 The Authors. *Pediatric Allergy and Immunology* published by European Academy of Allergy and Clinical Immunology and John Wiley & Sons Ltd.

1 | INTRODUCTION

Maternal supplementation with probiotics and omega-3 (ω -3) polyunsaturated fatty acids (PUFAs) seem to influence offspring immune programming. Hence, these dietary supplements may have allergy preventative effects, potentially mediated in part by the breast milk.¹⁻⁴ Breast milk harbors several factors with well-established immune relevance, for example, microorganisms, oligosaccharides, immunoglobulins, cytokines, and chemokines.⁵ Interestingly, the breast milk is also a rich source of different RNA species, among them microRNAs (miRNAs). miRNAs are small noncoding RNAs (~22 nucleotides), that modulate gene expression on a post-transcriptional level.⁶

Most breast milk-miRNA are encapsulated in extracellular vesicles (EVs). Breast milk EVs are considered to be primarily produced by the mammary glands and other milk cells,⁷ and seem resistant to digestion processes resembling the baby's gastrointestinal system.⁸ Indeed, previous animal studies suggest that milk EVs can be found in a range of different organs, such as the intestine, spleen and heart after suckling.⁹ Hence, it is possible that breast milk EV enclosed miRNAs absorbed in the intestine are subsequently involved in infant gene regulation. Notably, several miRNAs expressed in human breast milk are involved in regulation of immune modulatory pathways.¹⁰ Moreover, milk-derived EVs and their miRNAs may be involved in the development and maturation of regulatory T cells (Treg).¹¹ In support of this, Admyre et al.¹² observed an increase in Tregs after incubating human peripheral blood mononuclear cells with human milk EVs. Through such functions, miRNAs might be an alternate route to facilitate immune programming in infants,¹³ in addition to the more conventional ones.⁵

Interestingly, breast milk miRNAs display a similar abundance of immunomodulatory miRNAs across several mammalian species, suggesting an evolutionary benefit.¹⁴ The effect of maternal diet on milk miRNA expression is, however, scarcely explored.¹⁰ A previous study by Simpson et al.¹⁵ suggests that pre- and postnatal probiotic supplementation may indeed modulate the expression of miRNA in mature breast milk. Concerning ω -3 PUFAs, no previous studies in humans exist; one study carried out in cows suggests that sunflower oil (naturally containing ω -3 PUFA) can modulate milk miRNA expression.¹⁶ Hence, we hypothesized that maternal supplementation with probiotics and ω -3 PUFAs would influence miRNA expression in human breast milk. Little is also known about miRNA expression and milk maturity,¹⁰ an additional matter that needs exploration.

Here, we wanted to investigate whether maternal intake of probiotics and ω -3 PUFAs modulate miRNA expression in human breast milk, and if certain miRNAs are associated with immunological changes in the child. The specific aims were as follows: (1) to study expression of 24 immune relevant miRNAs in human breast milk after pre- and postnatal *Limosilactobacillus reuteri* (previously called *Lactobacillus reuteri*) and ω -3 PUFA supplementation; (2) to examine their differential expression according to milk maturity (i.e., between

Key Message

Maternal supplementation with *L. reuteri* and ω -3 PUFAs did not significantly affect the relative miRNA expression in breast milk. Interestingly, some of the miRNAs correlate with Treg subpopulations in the breastfed children, supporting the hypothesis that breast milk miRNAs could be important in infant immune regulation.

colostrum and mature milk); (3) to explore associations to maternal characteristics; and (4) to relate the expression of these 24 miRNAs to Treg frequencies in the breastfed infants (Figure 1).

2 | METHODS

Details on experimental procedures and statistical analyses are provided in Supplementary File S1.

2.1 | Study population

Breast milk was collected from women ($n=120$) enrolled in a double-blind, randomized, placebo-controlled, multicenter trial (ClinicalTrials.gov-ID: NCT01542970). Families with history of allergic disease were invited to participate in the study. The definition of allergic disease was atopic eczema, asthma, food allergy, or allergic rhinoconjunctivitis, based on self-reported data, verified by a clinician based on reported symptoms. The women were randomized to either: (1) *L. reuteri* + ω -3 PUFAs ($n=33$), (2) *L. reuteri* + Placebo ($n=33$), (3) ω -3 PUFAs + Placebo ($n=27$), and (4) Placebo + Placebo ($n=27$). *L. reuteri* DSM 17938 (BioGaia AB) oil drops were ingested twice daily (20 droplets) by the pregnant women from gestational week 20 until delivery, corresponding to 10^9 colony forming units (CFUs) per serving. After delivery, the child received 10^8 CFUs per serving (5 droplets \times 1) during its first year of life; the corresponding placebo consisted of refined coconut and peanut oil without *L. reuteri*. The ω -3 PUFA supplement consisted of three 1000mg Pikasol capsules (Orkla Health, one capsule containing 640mg ω -3 PUFAs, of which 35% eicosapentaenoic acid and 25% docosahexaenoic acid) or corresponding placebo (olive oil), ingested twice daily by the pregnant women, from gestational week 20 until 3 months of postpartum.

The study was approved by the Regional Ethics Committee in Linköping (Dnr 2011/45-31), and written informed consent was obtained from all participants. The primary outcome of the trial is to evaluate preventative effects of *L. reuteri* and ω -3 PUFAs on pediatric allergies; the clinical data collection is, however, still ongoing to fulfill the power requirements for this outcome.

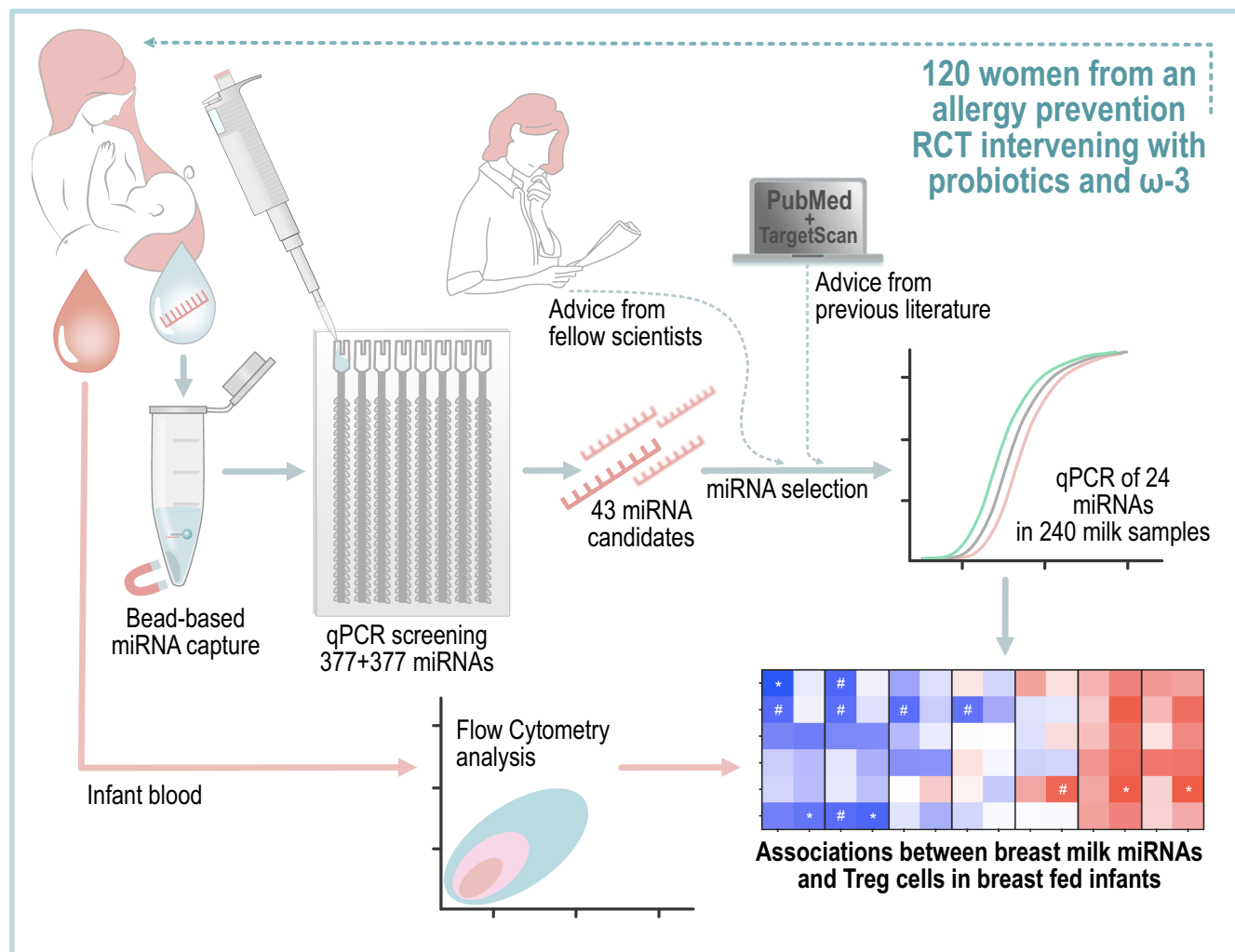


FIGURE 1 Schematic overview of the experimental set up. Breast milk samples at birth (colostrum) and at 3 months were collected from women ($n=120$) enrolled in an ongoing double-blind, randomized, placebo-controlled, multicenter trial, to investigate the protective effects of probiotics and omega-3 polyunsaturated fatty acids. A total of 754 microRNAs (miRNAs) were isolated and screened in four milk samples, from which 43 and later 24 miRNAs were selected for further analysis on basis of their immune relevance, in addition to them being expressed at readable amounts in most of the samples. Regulatory T (Treg)-cell populations were analyzed by flow cytometry from whole blood at 6 ($n=43$), 12 ($n=52$), and 24 ($n=61$) months from the breastfed children. The frequencies of activated (a)Treg ($CD45RA^+Foxp3^{++}$) and resting (r)Treg ($CD45RA^+Foxp3^+$) cells (in proportion to $CD3^+CD4^+$ T cells) were later correlated with the relative expression of the breast milk miRNAs.

2.2 | Breast milk collection and experimental procedures

Skim milk was obtained from milk collected 1–3 days after delivery (colostrum) and at 3 months of postpartum (mature milk) and stored at -70°C . After thawing, miRNA was extracted using TaqMan miRNA ABC Purification Kit–Human Panel A (Applied Biosystems), following the manufacturer protocol with minor modifications.

We initially screened four randomly chosen mature (6 months of postpartum) milk samples (originating from women included in the PROOM-3 RCT) for 754 mature miRNAs (using TaqMan low-density array microfluidic cards) and out of these, 24 miRNAs were

selected for further qPCR analysis on basis of their immune relevance and detectability. Please refer to Figure 1 and see detailed description in the Supplementary File S1 (under the subheading “qPCR TaqMan advanced miRNA human A and B cards”).

Infant Treg subpopulations ($CD45RA^-Foxp3^{++}$ and $CD45RA^+Foxp3^+$) were analyzed by flow cytometry at 6 ($n=43$), 12 ($n=52$), and 24 ($n=61$) months. Complete gating strategy is described in Figure S1.

2.3 | miRNA target prediction

Potential mRNAs targets of the Treg-associated miRNAs were predicted using TargetScan 8.0. All predicted targets were subsequently

uploaded to the Database for Annotation, Visualization and Integrated Discovery (DAVID) 2021 to visualize KEGG pathway enrichment.

2.4 | Data processing and statistics

In brief, amplification curves and cycle threshold (Ct) values were generated using the Thermo Fisher Connect Software (Life Technologies Corp), and endogenous reference miRNAs for normalization were determined by the Normfinder algorithm run in R version 4.2.1. In statistical testing, p -values $< .05$ was considered statistically significant. However, after multiple comparisons testing using the Benjamin-Hochberg method, q -values of $< .05$ were considered as acceptable, while q -value $< .10$ were considered a trend. All analyses regarding the intervention were conducted in R version 4.2.1 by an unblinded researcher (author initials M.M.); all other involved researchers and clinicians remain blinded throughout to uphold the blinding of the ongoing randomized controlled trial (RCT). The analyses of relationship between the relative miRNA expression, maternal characteristics, and proportion of Tregs were performed in GraphPad Prism 9.4.1 (GraphPad software, Inc.).

3 | RESULTS

Comparisons of participant characteristics revealed no substantial differences between the treatment groups, except for higher proportion of maternal atopy in the group receiving double treatment

and a larger proportion of exclusive breastfeeding at 3 months in the ω -3 PUFA + placebo group (Table 1).

3.1 | miRNA expression and milk maturity

The miRNA expression differed significantly between the two lactation time points (PERMANOVA, p -value = .001; Figure 2), but not between supplement received nor the interaction supplementation \times lactation period (Figure 2). Ten of the 24 selected miRNAs showed an increased relative expression, while 10 decreased, in mature milk in comparison with colostrum levels (in at least one of the treatment groups); two miRNAs remained stable, miR-24-3p and miR-145-5p (Figures S2–S5).

3.2 | Maternal characteristics

Maternal *L. reuteri* and/or ω -3 supplementation did not significantly impact the milk miRNA expression. The relative expression of miR-181c-5p was, however, higher in mature milk in both groups receiving *L. reuteri* (i.e., *L. reuteri* + ω -3 [Kruskal-Wallis test, p -value = .05]; *L. reuteri* + placebo [Kruskal-Wallis test, p -value = .045]) compared with placebo, but the p -values did not withstand multiple comparison adjustment (Figure S6).

Interestingly, let-7e-3p tended to be less expressed in colostrum from mothers with atopy in comparison with healthy mothers (p -value = .003, q -value = .068); no similar associations were observed in mature milk (Figure S7). Parity, gender of baby, or maternal age did

TABLE 1 Participants characteristics.

Treatment groups	<i>L. reuteri</i> + ω -3 ($n = 33$)	<i>L. reuteri</i> + placebo ($n = 33$)	ω -3 + placebo ($n = 27$) ^a	Placebo + placebo ($n = 27$)
Mothers age, years	30 (4.0)	32 (5.0)	30 (5.8)	32 (5.0)
Supplement intake, weeks	20 (2)	20 (2)	20 (2)	20 (2)
Partus, week	40 (2)	40 (1)	40 (2)	40 (2)
Caesarean section	4/32 (12.5%)	2/33 (6.1%)	4/26 (15.4%)	2/27 (7.4%)
Birth weight, kg	3.6 (0.7)	3.4 (0.5)	3.7 (0.6)	3.6 (0.6)
Birth length, cm	50 (2.0)	50 (3.0)	51 (3.0)	51 (1.5)
Gender of baby, male	10/33 (30.3%)	19/33 (57.6%)	12/26 (46.2%)	15/27 (55.6%)
First born	15/33 (45.5%)	15/33 (45.5%)	16/26 (61.5%)	15/27 (55.6%)
Maternal atopy ^b	27/33 (81.8%) [†]	19/33 (57.6%) [†]	11/26 (42.3%) ^{†‡}	20/27 (74.1%) [‡]
Breastfeeding exclusive 3 months	29/32 (90.6%)	25/32 (78.1%) [§]	26/26 (100%) [§]	21/26 (80.8%)
Animal in household	8/33 (24.2%)	12/33 (36.4%)	8/26 (30.8%)	5/27 (18.5%)

Note: Continuous variables are expressed as median and interquartile range and were analyzed using the Shapiro-Wilk's test followed by Kruskal-Wallis test. Categorical variables are expressed as n/N (%) and were analyzed using Chi-square test or Fisher exact test. [†]The *Limosilactobacillus reuteri* + ω -3 group had more mothers with atopy compared to *L. reuteri* + placebo and ω -3 + placebo, $p < .05$. [‡]The placebo + placebo group had more mothers with atopy compared to ω -3 + placebo, $p < .05$. [§]The ω -3 + placebo group had more mothers that were exclusively breastfeeding at 3 months compared to *L. reuteri* + placebo, $p < .05$. The displayed data was retrieved from parentally reported questionnaire data.

^aMissing data from one participant.

^bMaternal atopy was defined by self-reported allergic disease, verified by a clinician based on reported symptoms, and categorized as eczema, asthma, food allergy, or allergic rhinoconjunctivitis.

FIGURE 2 Principal component analysis of the miRNA Δ Ct value in colostrum and mature milk (3 months), from mothers receiving *Limosilactobacillus reuteri* and/or ω -3 fatty acids from gestational week 20 ($n = 119$). Circles describe samples from colostrum (col) and triangle describe samples from mature milk (mo3). OL (red): *L. reuteri* + ω -3, OP (blue): ω -3 + Placebo, PL (green): *L. reuteri* + Placebo and PP (black): Placebo + Placebo.

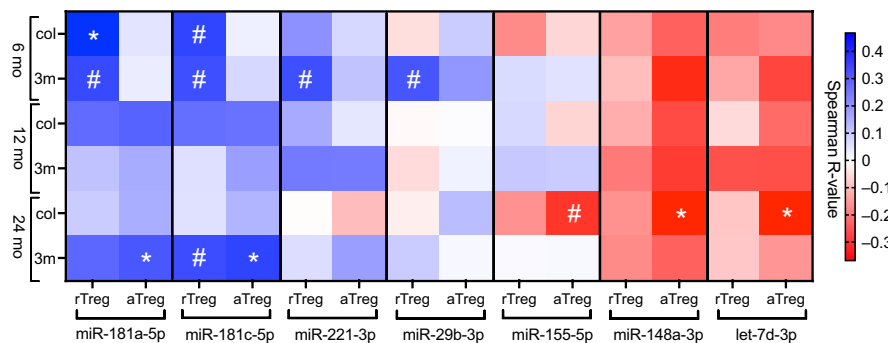
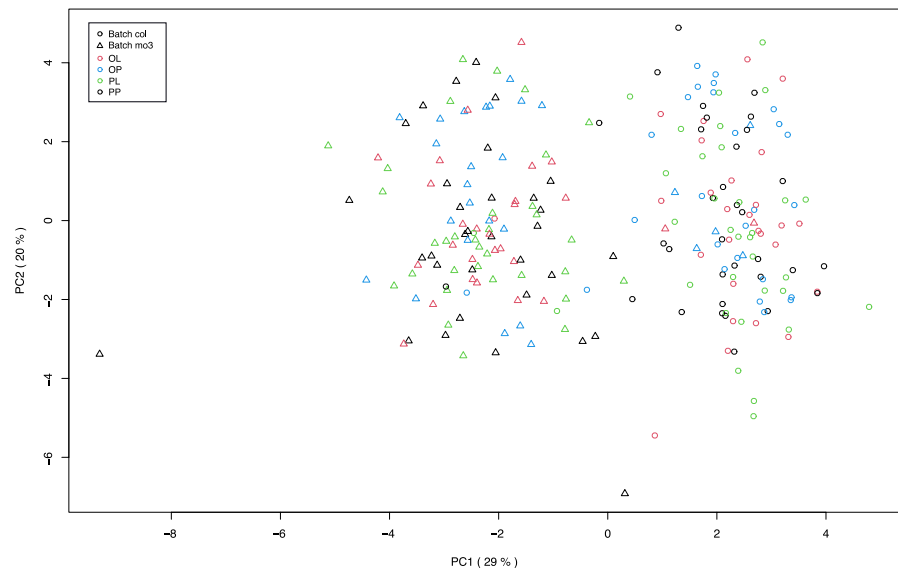


FIGURE 3 Heatmap displays the Spearman's rho correlation coefficients between the relative miRNA expression in colostrum (col) and mature milk (3 months) with the proportions of activated regulatory T cells (aTreg, CD45RA⁺Foxp3⁺) and resting regulatory T cells (rTreg, CD45RA⁺Foxp3⁺) at 6, 12 and 24 months. Blue indicates a low relative miRNA expression correlating with a high proportion of cells and red indicate a high relative miRNA expression and high proportion of cells. #q-value < .1, *q-value < .05.

not correlate to miRNA expression at any of the time points (data not shown).

3.3 | Infant regulatory T cells and breast milk miRNA

Four breast milk miRNAs (miR-181a/c-5p, miR-148a-3p, and let-7d-3p) correlated significantly (q -value < .05, Spearman R -value ranging between $-.36$ and $.47$) with the frequencies of infant aTreg and/or rTreg (defined as CD45RA⁺Foxp3⁺ and CD45RA⁺Foxp3⁺, respectively). A detailed overview of the associations with a Spearman q -value < 0.1 is shown in Figure 3; for all associations in the entire data set refer to Tables S2 and S3.

Interestingly, the significant associations seem to maintain their direction of correlation throughout the first 24 months. For miR-181a/c-5p, negative correlations were consistently found, that is, lower miRNA expression in colostrum and mature milk correlated with higher frequencies of infant Tregs (Figure 3). For miR-148a-3p

and let-7d-3p, the direction of association was instead positive, that is, higher milk miRNA expression correlated with a larger proportion of circulating aTregs.

To further explore potential functions of the miRNAs associated with infant Tregs, we conducted a target prediction and pathway analysis for the six miRNAs correlating with infant Tregs (i.e., Spearman q -values < 0.1). Taken together, these six miRNAs (i.e., miR-181-5p, miR-148a-3p, let-7d-3p, miR-221-3p, miR-29-3p, and miR-155-5p) target 2598 mRNAs (Table S4). Notably, three of the top five predicted KEGG pathways were related to T-cell function: PI3K-Akt signaling-, MAPK signaling-, and FoxO signaling pathway. Two pathways were connected to cancer: pathways in cancer and small cell lung cancer (including immunological pathways, such as cytokine- and Jak-STAT signaling). Several other KEGG pathways related to T-cell function were also implicated, such as mTOR-, Jak-STAT-, TGF- β -, and T cell receptor signaling pathways. All the indicated KEGG pathways are listed in Table S5. After reviewing all individual targets, it was also revealed that several of them were directly involved in control of T cell and Treg function, for example CD4, SOCS1, IL-10, and IL-13, see Table S4.

4 | DISCUSSION

We investigated breast milk miRNA expression after maternal intake of probiotics and ω -3 PUFA, and their association with Tregs in the breastfed child. Although no significant modifications by maternal supplementation were found, we observed that several milk miRNAs changed over time (from colostrum to mature milk) and that some correlated with the proportion of activated and resting Tregs in the infant.

On basis of the trends presented here, taken together with previous research on mammal supplementation with ω -3 PUFA containing oils¹⁶ and probiotic supplementation in pregnant women,¹⁵ we believe there is reason to further explore this area. The scarcity of research and the variation in lactating species studied, sources and dosage of ω -3 PUFAs, and probiotic strains, however, makes it difficult to combine results from different studies to infer definite conclusions. Much also remains to be clarified in terms of underlying biological mechanisms. Studies have shown that orally consumed bacteria can be isolated from the milk of lactating animals,¹⁷ indicating that an enteromammary pathway exists.¹⁸

It may be worth to consider the biological relevance of probiotic organisms when designing future studies. *L. reuteri*, as used here, is naturally present in breast milk,¹⁹ perhaps indicating particular biological relevance to the perinatal phase. On this note, our group have previously shown¹ that a combination of pre- and postnatal supplementation with *L. reuteri* decrease the risk of developing IgE-associated eczema in children¹; this might be ascribed to the ability of *L. reuteri* to induce Tregs.²⁰ The present study indicates miR-181c-5p modulation in the probiotic supplemented groups (although not withstanding FDR correction), and miRNAs from this particular family were also significantly associated with infant Treg populations. Speculatively, this indicates an association between the *L. reuteri* supplementation and miR-181a/c-5p expression in breast milk, which is seemingly associated with Treg populations in the breastfed children. Interestingly, miR-181a/b-1-deficient Treg cells displayed elevated suppressive capacity in vivo,²¹ and miR-181a-3p potentially inhibits Treg cell activity in vitro.²²

Whether or not breast milk is allergy protective remains under debate.^{23,24} Previous research from our group has shown that concentrations of Th2-associated cytokines (IL-4, IL-5, and IL-13) are higher in breast milk from allergic mothers.²⁵ Also, maternal atopy¹⁵ and asthma²⁶ appear to affect breast milk miRNA profile. In the current study, let-7e-3p tended to be lower in milk from allergic mothers (Figure S6). Interestingly, downregulation of milk let-7f-5p has been associated with infant atopy.¹⁵ This may suggest that the let-7 family is dysregulated in allergic disease. In support of this, a previous study observed that let-7e is lower in nasal mucosa tissue from allergic rhinitis patients.²⁷ Hence, the inconclusive findings regarding breast milk and allergy prevention may in part be due to differing breast milk composition, including varying levels miRNAs.

Ten of the 24 studied miRNAs changed over time, from colostrum to mature milk. Time-dependent fluctuations like these have been reported previously¹⁰ and likely reflect the changing requirements of the infant as it develops from a newborn to a three-month-old baby. As the 24 miRNAs we study here were specifically selected on basis of their immune relevance (i.e., their involvement in immunomodulatory pathways and specifically in T helper cell subset regulation, mitochondrial function, and epigenetic modifications, see supporting information "qPCR TaqMan advanced miRNA human A and B cards"), we did not make any further attempt to summarize their predicted functions. If further interested, please refer to a recent review.²⁸

Human milk exosomes have been shown to increase the number of Tregs and downregulate IL-2 production in vitro.¹² In the current study, we show that seven breast milk miRNAs are associated with aTreg and rTreg frequencies in the child, at 6 and 24 months. In this context, it is noteworthy that colostrum levels of miR-181a/c-5p showed significant associations before FDR correction to more than one Treg populations also at the 12-months' time point; as did miR-148a-3p, let-7d-3p miR-221-3p in mature milk (presented in detail in Table S1). The fact that the same miRNAs stand out at multiple time points, consistently with associations in the same directions, points to their relevance in infant Treg regulation.

miR-148a-3p is the most abundant miRNA in human breast milk,²⁸ regardless of milk fraction.¹⁰ Milk miR-148a-3p has been suggested to function as an epigenetic regulator targeting DNA methyltransferase 1 (DNMT1).^{29,30} DNA de/methylation regulates Foxp3 expression, the key transcription factor in the differentiation of CD4+ T cells into Tregs.^{31,32} Stable Foxp3 expression is associated with selective demethylation of an evolutionarily conserved element within the FOXP3 locus named TSDR (Treg-specific demethylated region)³³; DNMT1 and DNMT3b seem to be in control of this transcriptional "switch."^{34,35} Interestingly, DNMT1 deficiency results in Foxp3 induction following T-cell receptor stimulation³⁵ and Foxp3 expression correlates with the TSDR demethylation rate in a linear fashion.³⁶ Furthermore, FOXP3 TSDR demethylation was significantly lower in children with active IgE-mediated cow's milk allergy than in either children who outgrew the allergy or in healthy children.³⁶ In contrast, strong T-cell receptor signaling suppresses miR-148a to derepress DNMT1 mRNA translation.³⁷ Thus, accumulating evidence supports the view that DNA methylation plays a key role in Treg cell differentiation and function³⁸ and, as previously postulated by Melnik et al., breast milk-derived activation of such pathways may work to increase the expression of Foxp3.³⁹ Indeed, a study by Golan-Gerstl et al.⁴⁰ provides experimental evidence that human breast milk-derived exosomes can increase miR-148a in cell cultures, with subsequent DNMT1 suppression. Furthermore, Admyre et al.¹² demonstrated that incubating naïve T cells with breastmilk exosomes increase the expression of Foxp3+ T cells in a dose-dependent manner. Thus, breastmilk miR-148a-3p, via targeting DNMT1, may be a

key mechanism for inducing Foxp3+ Tregs shaping oral tolerance against introduced external food antigens.⁴¹

To gain further insight into the potential effects the miRNAs in question might have on the Tregs, we explored their predicted mRNA targets (Table S3). Functional analysis revealed multiple KEGG pathways related to T-cell function and after reviewing individual targets, we also found several direct interactions with Treg function. For example, miR-155-5p can modulate the IL2/STAT5 pathway by targeting SOCS1.⁴² In addition, CD4 is a target of miR-181-3p, as is mRNAs involved in the MAPK pathway downstream from the T-cell receptor. Furthermore, loss of miR-181-3p seems to alter thymic T-cell selection as well as upregulating of CTLA-4 by Treg cells.²¹ Also, decreased levels of miR-155-5p and miR-181-3p in Treg cells have been previously correlated with a lower frequency of the same cell population in children with atopic dermatitis.⁴³ Bearing in mind that miRNA target prediction is rather speculative, the importance of miRNAs for Treg function has been supported by several animal studies. For example, knockout models of Dicer, an enzyme crucial for miRNA biogenesis, demonstrate dysregulation of the Treg population resulting in autoimmunity and lack of tolerance maintenance.⁴⁴

We would like to acknowledge some potential limitations to this study. Perhaps, increasing the current sample size of 120 would have powered the study to show more pronounced effects on breast milk miRNA expression. In addition, specifically isolating miRNAs from the EV-fraction, as opposed to skim milk, might have produced a different miRNA profile in the initial screening. With that noted, however, the top expressed breast milk miRNAs are rather consistent throughout different breast milk fractions.¹⁰ Moreover, the initial screening involved a small number of samples ($n=4$); hence, we could potentially have missed some important miRNAs that were differentially expressed between samples. For example, we ended up not including miR-30b-5p, a rather commonly expressed breast milk miRNA¹⁰ with potential immunological effects.⁴⁵ This miRNA would potentially have had relevance to the ω -3 intervention, as well as to Treg modulation: First, its expression has been shown to be up-regulated by the ω -3 fatty acid eicosapentaenoic acid via interaction with free fatty acid receptor 4,⁴⁶ and second, miR-30b-5p targets m6A RNA-demethylase FTO,⁴⁷ and m6A mRNA methylation has been shown to sustain Treg suppressive functions.⁴⁸ The potential importance of this particular breast milk miRNA in infant development has been previously highlighted by Melnik et al.,⁴¹ and future studies may benefit from including it.

In conclusion, maternal supplementation of *L. reuteri* and/or ω -3 PUFAs during pregnancy does not significantly affect the expression of 24 immune-related miRNAs in colostrum or in mature milk. However, some miRNAs moderately correlated with the proportions of active and resting Tregs in the infant at 6 and 24 months, suggesting a direct or indirect modulation of the milk on tolerance development through miRNAs. Future studies are needed to confirm these findings, preferably in in vivo settings similar to this study, but also through functional experiments uncovering mechanisms and directions of action in vitro.

AUTHOR CONTRIBUTIONS

Emelie Ahlberg: Conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing – original draft; writing – review and editing. **Magalí Martí:** Data curation; formal analysis; methodology; software; visualization; writing – review and editing. **Dhanapal Govindaraj:** Investigation; writing – review and editing. **Elisabet Severin:** Data curation; resources; writing – review and editing. **Karel Duchén:** Methodology; resources; project administration; writing – review and editing. **Maria C. Jenmalm:** Writing – review and editing; conceptualization; funding acquisition; project administration; methodology; resources; supervision. **Lina Tingö:** Conceptualization; funding acquisition; investigation; methodology; writing – review and editing; supervision; visualization; writing – original draft; resources.

FUNDING INFORMATION

This study was financial support by grants from the Swedish Research Council (2019-00989), the Hjärt-lungfonden (20170365 and 20200301), the Joanna Coccozas stiftelse för barnmedicinsk forskning (2020-01041), the Cancer- och allergiförbundet, the Faculty of Medicine and Health Sciences at Linköping University, Region Östergötland (RÖ-930610), the Research Council of Southeast Sweden (969326, 940313, 931756) and the Lisa and Johan Grönberg Foundation, Sweden and Dr P Håkansson's Stiftelse, Eslöv, Sweden.

CONFLICT OF INTEREST STATEMENT

MC Jenmalm has received honoraria and consultant fees from BioGaia AB, ABIGO Medical and Nutricia/Danone. The other authors declare no conflict of interest.

PEER REVIEW

The peer review history for this article is available at <https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/pai.13952>.

ORCID

Emelie Ahlberg  <https://orcid.org/0000-0002-7119-6114>

Magalí Martí  <https://orcid.org/0000-0002-1927-4656>

Dhanapal Govindaraj  <https://orcid.org/0000-0003-1303-8254>

Karel Duchén  <https://orcid.org/0000-0002-0570-8898>

Maria C. Jenmalm  <https://orcid.org/0000-0002-2117-5366>

Lina Tingö  <https://orcid.org/0000-0002-2482-9281>

REFERENCES

1. Abrahamsson TR, Jakobsson T, Böttcher MF, et al. Probiotics in prevention of IgE-associated eczema: a double-blind, randomized, placebo-controlled trial. *J Allergy Clin Immunol*. 2007;119:1174-1180.
2. Furuholm C, Warstedt K, Larsson J, et al. Fish oil supplementation in pregnancy and lactation may decrease the risk of infant allergy. *Acta Paediatr*. 2009;98:1461-1467.
3. Warstedt K, Furuholm C, Fälth-Magnusson K, Fagerås M, Duchén K. High levels of omega-3 fatty acids in milk from omega-3 fatty acid-supplemented mothers are related to less immunoglobulin E-associated disease in infancy. *Acta Paediatr*. 2016;105:1337-1347.

4. Dzidic M, Mira A, Artacho A, Abrahamsson TR, Jenmalm MC, Collado MC. Allergy development is associated with consumption of breastmilk with a reduced microbial richness in the first month of life. *Pediatr Allergy Immunol*. 2020;31:250-257.
5. Andres SF, Scottoline B, Good M. Shaping infant development from the inside out: Bioactive factors in human milk. *Semin Perinatol*. 2022;47:151690.
6. O'Brien J, Hayder H, Zayed Y, Peng C. Overview of microRNA biogenesis, mechanisms of actions, and circulation. *Front Endocrinol (Lausanne)*. 2018;9:402.
7. Alsaweed M, Lai CT, Hartmann PE, Geddes DT, Kakulas F. Human milk miRNAs primarily originate from the mammary gland resulting in unique miRNA profiles of fractionated milk. *Sci Rep*. 2016;6:20680.
8. Kahn S, Liao Y, Du X, Xu W, Li J, Lönnérdal B. Exosomal microRNAs in milk from mothers delivering preterm infants survive in vitro digestion and are taken up by human intestinal cells. *Mol Nutr Food Res*. 2018;62:e1701050.
9. Manca S, Upadhyaya B, Mutai E, et al. Milk exosomes are bioavailable and distinct microRNA cargos have unique tissue distribution patterns. *Sci Rep*. 2018;8:11321.
10. Tingö L, Ahlberg E, Johansson L, et al. Non-coding RNAs in human breast milk: a systematic review. *Front Immunol*. 2021;12:725323.
11. Melnik BC, John SM, Schmitz G. Milk: an exosomal microRNA transmitter promoting thymic regulatory T cell maturation preventing the development of atopy? *J Transl Med*. 2014;12:43.
12. Admyre C, Johansson SM, Qazi KR, Filén JJ, et al. Exosomes with immune modulatory features are present in human breast milk. *J Immunol*. 2007;179:1969-1978.
13. Kosaka N, Izumi H, Sekine K, Ochiya T. microRNA as a new immune-regulatory agent in breast milk. *Silence*. 2010;1:7.
14. van Herwijnen MJC, Driedonks TAP, Snoek BL, et al. Abundantly present miRNAs in milk-derived extracellular vesicles are conserved between mammals. *Front Nutr*. 2018;5:81.
15. Simpson MR, Brede G, Johansen J, et al. Human breast milk miRNA, maternal probiotic supplementation and atopic dermatitis in offspring. *PLoS One*. 2015;10:e0143496.
16. Mbouchon L, Le Guillou S, Marthey S, et al. Sunflower oil supplementation affects the expression of miR-20a-5p and miR-142-5p in the lactating bovine mammary gland. *PLoS One*. 2017;12:e0185511.
17. de Andrés J, Jiménez E, Chico-Calero I, Fresno M, Fernández L, Rodríguez JM. Physiological translocation of lactic acid bacteria during pregnancy contributes to the composition of the milk microbiota in mice. *Nutrients*. 2017;10:14.
18. Rodríguez JM. The origin of human milk bacteria: is there a bacterial entero-mammary pathway during late pregnancy and lactation? *Adv Nutr*. 2014;5:779-784.
19. Sinkiewicz G, Ljunggren L. Occurrence of *Lactobacillus reuteri* in human breast milk. *Microb Ecol Health Dis*. 2008;20:122-126.
20. Mu Q, Tavella VJ, Luo XM. Role of *Lactobacillus reuteri* in human health and diseases. *Front Microbiol*. 2018;9:757.
21. Łyszkiewicz M, Winter SJ, Witzlau K, et al. miR-181a/b-1 controls thymic selection of Treg cells and tunes their suppressive capacity. *PLoS Biol*. 2019;17:e2006716.
22. Jung D, Shin S, Kang SM, et al. Reprogramming of T cell-derived small extracellular vesicles using IL2 surface engineering induces potent anti-cancer effects through miRNA delivery. *J Extracell Vesicles*. 2022;11:e12287.
23. Mennini M, Arasi S, Fiocchi AG. Allergy prevention through breast-feeding. *Curr Opin Allergy Clin Immunol*. 2021;21:216-221.
24. Lin B, Dai R, Lu L, Fan X, Yu Y. Breastfeeding and atopic dermatitis risk: a systematic review and meta-analysis of prospective cohort studies. *Dermatology*. 2020;236:345-360.
25. Böttcher MF, Jenmalm MC, Garofalo RP, Björkstén B. Cytokines in breast milk from allergic and nonallergic mothers. *Pediatr Res*. 2000;47:157-162.
26. Bozack AK, Colicino E, Rodosthenous RS, et al. Breast milk-derived extracellular vesicle miRNAs are associated with maternal asthma and atopy. *Epigenomics*. 2022;14:727-739.
27. Suojalehto H, Toskala E, Kilpeläinen M, et al. MicroRNA profiles in nasal mucosa of patients with allergic and nonallergic rhinitis and asthma. *Int Forum Allergy Rhinol*. 2013;3:612-620.
28. Ahlberg E, Al-Kaabawi A, Thune R, et al. Breast milk microRNAs: potential players in oral tolerance development. *Front Immunol*. 2023;14:1154211.
29. Melnik BC. Milk disrupts p53 and DNMT1, the guardians of the genome: implications for acne vulgaris and prostate cancer. *Nutr Metab (Lond)*. 2017;14:55.
30. Melnik BC, Schmitz G. Milk's role as an epigenetic regulator in health and disease. *Diseases*. 2017;5:12.
31. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol*. 2007;8:277-284.
32. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol*. 2010;10:490-500.
33. Polansky JK, Kretschmer K, Freyer J, et al. DNA methylation controls Foxp3 gene expression. *Eur J Immunol*. 2008;38:1654-1663.
34. Lal G, Bromberg JS. Epigenetic mechanisms of regulation of Foxp3 expression. *Blood*. 2009;114:3727-3735.
35. Josefowicz SZ, Wilson CB, Rudensky AY. Cutting edge: TCR stimulation is sufficient for induction of Foxp3 expression in the absence of DNA methyltransferase 1. *J Immunol*. 2009;182:6648-6652.
36. Paparo L, Nocerino R, Cosenza L, et al. Epigenetic features of FoxP3 in children with cow's milk allergy. *Clin Epigenetics*. 2016;8:86.
37. Li C, Ebert PJ, Li QJ. T cell receptor (TCR) and transforming growth factor β (TGF- β) signaling converge on DNA (cytosine-5)-methyltransferase to control forkhead box protein 3 (foxp3) locus methylation and inducible regulatory T cell differentiation. *J Biol Chem*. 2013;288:19127-19139.
38. Bai L, Hao X, Keith J, Feng Y. DNA methylation in regulatory T cell differentiation and function: challenges and opportunities. *Biomolecules*. 2022;12:1282.
39. Melnik BC, John SM, Carrera-Bastos P, Schmitz G. Milk: a postnatal imprinting system stabilizing FoxP3 expression and regulatory T cell differentiation. *Clin Transl Allergy*. 2016;6:18.
40. Golan-Gerstl R, Elbaum Shiff Y, Moshayoff V, Schecter D, Leshkowitz D, Reif S. Characterization and biological function of milk-derived miRNAs. *Mol Nutr Food Res*. 2017;61:201700009.
41. Melnik BC, Stremmel W, Weiskirchen R, John SM, Schmitz G. Exosome-derived microRNAs of human milk and their effects on infant health and development. *Biomolecules*. 2021;11:851.
42. Lu LF, Thai TH, Calado DP, et al. Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. *Immunity*. 2009;30:80-91.
43. Liu W, Ouyang H, Zeng Q, Luo R, Lu G. Decreased Treg-derived miR-181a and miR-155 correlated with reduced number and function of Treg cells in allergic rhinitis children. *Eur Arch Otorhinolaryngol*. 2019;276:1089-1094.
44. Liston A, Lu LF, O'Carroll D, Tarakhovsky A, Rudensky AY. Dicer-dependent microRNA pathway safeguards regulatory T cell function. *J Exp Med*. 2008;205:1993-2004.
45. Zhou Q, Li M, Wang X, et al. Immune-related microRNAs are abundant in breast milk exosomes. *Int J Biol Sci*. 2012;8:118-123.
46. Kim J, Okla M, Erickson A, Carr T, Natarajan SK, Chung S. Eicosapentaenoic acid potentiates brown thermogenesis through FFAR4-dependent up-regulation of miR-30b and miR-378. *J Biol Chem*. 2016;291:20551-20562.

47. Sun L, Gao M, Qian Q, et al. Triclosan-induced abnormal expression of miR-30b regulates fto-mediated m⁶A methylation level to cause lipid metabolism disorder in zebrafish. *Sci Total Environ.* 2021;770:145285.
48. Tong J, Cao G, Zhang T, et al. m⁶A mRNA methylation sustains Treg suppressive functions. *Cell Res.* 2018;28:253-256.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Ahlberg E, Marti M, Govindaraj D, et al. Immune-related microRNAs in breast milk and their relation to regulatory T cells in breastfed children. *Pediatr Allergy Immunol.* 2023;34:e13952. doi:[10.1111/pai.13952](https://doi.org/10.1111/pai.13952)