TITLE PAGE

Title: Association between infant consumption of breast milk microRNAs and atopy protection

Authors: Steven D. Hicks, MD, PhD, Desirae Chandran, Kaitlyn Warren, MS, and Alexandra Confair, BS

Author Affiliation: Department of Pediatrics, Penn State College of Medicine, Hershey PA, USA

NCT#: ID: STUDY00008657

Document Date: 1/26/26

FUNDING

This study was funded by a grant from the Gerber Foundation to SDH (Grant #204135). The funding source had no role in the study design, the collection, analysis or interpretation of data, the writing of the manuscript; or the decision to submit the article for publication.

ABSTRACT

Background: Breastfeeding is thought to reduce infant atopy risk. The biologic mechanism for this protective effect is not fully understood.

Objectives: We tested the hypothesis that infant consumption of three breastmilk microRNAs (miR-146b-5p, miR-21-5p, miR-375-3p) would be associated with reduced atopy risk.

Methods: The Breast Milk IMPACT Study involved a cohort of 221 breastfeeding mother-infant dyads planning to breastfeed beyond four months. Infant consumption of breastmilk microRNAs in the first six months was calculated as the product of breastmilk microRNA concentration and the number of breastmilk feeds, across three lactation stages: early milk (0-4 weeks), transitional milk (4-16 weeks), and mature milk (16-24 weeks). The primary outcome was infant atopy in the first 12 months, defined as atopic dermatitis, food allergies, or wheezing.

Results: Seventy-three infants developed atopy – 41 atopic dermatitis (25%), 33 food allergy (20%), 10 wheezing (6%). Eleven developed more than 1 condition (7%). Infants who did not develop atopy consumed higher levels of breastmilk miR-375-3p (d= -0.33, p=0.007) and miR-146b-5p (d= -0.28, p=0.037). Consumption of miR-375-3p was associated with reduced atopy risk (OR = 0.48, 95% CI: 0.23 – 0.98, p = 0.045). Breastmilk levels of miR-375-3p increased throughout lactation (F = 132.3, p = 8.4×10^{-34}) and were inversely associated with maternal body mass index (t= -2.1, p=0.032).

Conclusions: This study provides evidence that infant consumption of breastmilk miR-375-3p may reduce atopy risk. Such knowledge could be used to develop infant formulas that better reflect breastmilk biology, or to develop interventions to enhance breastmilk miR-375-3p levels.

KEY MESSAGES

- Infant consumption of miR-375-3p is associated with a two-fold reduction in atopy risk.
- Increasing levels of miR-375-3p in maternal breastmilk, or adding miR-375-3p to infant formula may reduce the risk of allergic conditions.

CAPSULE SUMMARY

In this cohort study of 221 mother-infant dyads, consumption of breastmilk miR-375-3p was associated with a reduction in infant atopy. Breast milk miR-375-3p levels increased throughout lactation and were inversely related to maternal body mass.

KEY WORDS: Breastfeeding, microRNA, miR-375, allergies, risk reduction, cohort, body mass

ABBREVIATIONS: AD (Atopic dermatitis), ANOVA (Analysis of variance), BMI (Body mass index), DSQ (Dietary screener questionnaire), IFP (Infant feeding practices), IL (Interleukin), IMPACT (Influence of the micro-transcriptome profile on atopy in children over time), ISAAC-WQ (International study of asthma and allergies in childhood – wheezing questionnaire), miRNA (microRNA), NSLAH (National survey of lead and allergens in housing).

INTRODUCTION

Atopy, the predisposition toward allergic diseases such as atopic dermatitis (AD), food allergies, and asthma, occurs in approximately one-third of children. (1) Atopy results from inappropriate activation of the immune response to benign environmental exposures. (2) The developmental origins of atopy are not fully understood. (3)

Infants who breastfeed beyond three months may have lower risk for certain atopic conditions. (4) (5) Maternal breastmilk contains numerous immunomodulatory components that could convey atopy protection. (6) (7) Bioactive factors in breastmilk, called microRNAs (miRNAs), may play a role. (8) miRNAs are small, non-coding molecules that regulate gene expression across multiple tissues. (9) There are nearly 1,000 different miRNAs in breastmilk. (10) The majority are found in the lipid or cellular fractions of milk, (11) and evidence suggests that they regulate immune pathways (9) (12). Although breastmilk miRNA composition can vary based on maternal weight, diet, or genetics, (13)-(16) breastmilk reliably contains miRNAs, whereas formula does not. (14)

miRNAs are often packaged within protective vesicles, (15) making them stable in breastmilk and transferable to the infant gut, where they may be absorbed and functionally incorporated by epithelial and immunologic cells in the oropharynx. (16)-(21) Animal studies suggest nutritional miRNA influences development of the immune system. (17) (18) Thus, breastmilk miRNA exposure may mitigate infant atopy risk.

Circulating miRNA levels are dysregulated in atopic conditions. (19)-(28) For example, levels of miR-21 are elevated in the skin of patients with AD and in the bronchial cells of patients with asthma. (19) Up-regulation of miR-375 may prevent inflammatory pathways associated with allergic rhinitis. (20) Increases in miR-146 are associated with reduced inflammation in airway smooth muscle, and protection from asthma and AD. (21) (22) Notably, all three of these miRNAs have a robust presence in breastmilk. (23)

This study, Breast Milk IMPACT (Influence of the Micro-transcriptome Profile on Atopy in Children over Time), followed 221 breastfeeding mother-infant dyads from birth to 12 months to test the hypothesis that infant consumption of three breastmilk miRNAs (miR-146b-5p, miR-21-5p, miR-375-3p) would be associated with reduced atopy risk. To our knowledge, this is the first large-scale, prospective cohort study investigating the relationship between longitudinal breastmilk miRNA consumption and infant atopy. (24)

METHODS

This study was approved by the Independent Review Board at the Penn State College of Medicine (STUDY00008657). Written informed consent was obtained from all participant at enrollment. The study was registered on clinicaltrials.gov (NCT04017520).

Participants

This longitudinal cohort study involved a convenience sample of 221 mother-infant dyads followed from birth to 12 months of age. This sample size was determined by a-priori power analysis estimating that approximately 33% of infants (n = 73) would develop atopy and provide >80% power to detect a 1.5-fold difference in miRNA consumption across atopy and non-atopy groups on Mann Whitney U-testing ($\alpha = 0.05$). Inclusion criteria were mothers who delivered at term (>35 weeks), and intended to breastfeed beyond four months. Exclusion criteria were: 1) maternal morbidities that could impact breastfeeding success or influence breastmilk miRNA composition (e.g. cancer, drug addiction, human immunodeficiency virus infection); 2) plan for infant adoption; 3) presence of neonatal condition that could impact ability to breastfeed (e.g. cleft lip, metabolic disease, NICU admission >7 days); 4) plan for pediatric care outside the medical center, or 5) non-English speaking. Between April, 2018 and October, 2020 research staff screened 2,487 potential participants through the electronic medical record, approached 359 eligible participants (14%), and enrolled 221 participants (61% of those eligible). Recruitment occurred at four pediatric outpatient clinics affiliated with the academic medical center. All participants had access to on-site lactation support for the duration of the study. 163 dyads completed the study (**Figure 1**). The primary medical outcome was presence or absence of atopy in the first 12 months, defined by parent report of AD, food allergies, or wheezing on standardized questionnaires, (25)-29) in accordance with guidelines from the American Academy of Allergy, Asthma, and Immunology. (26) Atopy cases were confirmed through review of the medical record.

Survey collection

Medical and demographic characteristics were collected for all dyads through nurse-administered survey at enrollment. Maternal age, tobacco use, body mass index (BMI), and atopy history were recorded. Infant sex, gestational age, delivery route, birth weight, and race were recorded. Duration of breastfeeding and the proportion of feeds consisting of breastmilk were assessed at 4, 16, and 24 weeks using the Infant Feeding Practices (IFP)-II survey. (27) Infant AD was assessed by trained research nurses at 4, 16, 24 and 48 weeks using the Scoring Atopic Dermatitis (SCORAD) tool. (25) The SCORAD was developed by the European Task Force on Atopic Dermatitis for rapid utility in outpatient clinics and has been validated in infants and children. Infant food allergies were assessed through the IFP-II survey at 4, 16, 24, and 48 weeks. (27) Infant wheezing during the first 12 months was assessed through administration of a standardized wheezing questionnaire from the International Study of Asthma and Allergies in Childhood (ISAAC-WQ) at 48 weeks. (28) The ISAAQ-WQ was developed to measure the prevalence of recurrent wheezing and related risk factors in infants during the first 12 months. Allergens in the infant environment were reported at 4 weeks using a modified form of the National Survey of Lead and Allergens in Housing (NSLAH). (29) This survey was developed by the National Institute of Environmental Health Sciences to assess environmental allergens within the home. In order to assess the relationship between breastmilk miRNAs and maternal diet, the Dietary Screener Questionnaire (DSQ) was administered at each breastmilk

collection (0, 4, and 16 weeks). (30) Missing survey data (42/2608, 1.6%) was imputed using the mean cohort value.

Sample collection

Breastmilk (5 ml) was manually expressed into RNAse-free tubes from a sterilized nipple surface, as we have previously described. (13) (31) Foremilk (pre-feed) samples were collected in weeks 0, 4, and 16. These time points were chosen to reflect important changes in breastmilk miRNA levels that occur during the course of lactation, (23) and to capture the period when atopic benefits attributed to breastfeeding occur. (4) To control for differences between breasts, mothers utilized the same breast for each collection. Time of collection was recorded for all samples. The 163 mothers who completed the study contributed 442 breastmilk samples: 153 “early milk” samples at 0 weeks (4 ± 2 days post-partum), 155 “transitional milk” samples at 4 weeks (39 ± 11 days post-partum), and 134 “mature milk” samples at 16 weeks (128 ± 8 days post-partum). There were 127 mothers that contributed 3 samples, 25 mothers that contributed 2 samples, and 11 mothers that contributed 1 sample. Samples were immediately transferred to -20° C for storage and placed at -80° C within four weeks, undergoing exactly one freeze-thaw cycle prior to RNA extraction.

Sample processing

Breastmilk RNA was purified from the lipid fraction using a Norgen Circulating and Exosomal RNA Purification Kit (Norgen Biotech; Ontario, Canada), as previously described. (13) (23) Lipid fractions were selected for their robust concentration of immunologic miRNAs with high potential for maternal-infant transfer (due to the protective effects of lipid encapsulation and micro-vesicles). (10) (11) (13) Samples were processed in batches containing matched ratios of atopic and non-atopic dyads. RNA was sequenced at the SUNY Molecular Analysis Core using the Illumina TruSeq Small RNA Prep protocol and a NextSeq500 instrument (Illumina; San Diego, CA, United States) at a targeted depth of ten million, 50 base, paired-end reads per sample. RNAseq was selected to permit estimation of miRNA concentrations as parts per million, rather than a relative value yielded by polymerase chain reactions. FASTQ files were deposited into the Gene Expression Omnibus repository (GSE192543). Reads were aligned to the hg38 build of the human genome using Partek Flow (Partek; St. Louis, MO, United States) and the Bowtie2 aligner. Samples with raw miRNA counts <10,000 were excluded (n = 10, 2.2%). Individual miRNA features with sparse counts (<10 in >10% of samples) were filtered. Within each sample, the concentration of the three miRNAs of interest (miR-146b-5p, miR-21-5p, miR-375-3p) was determined as reads per million, and mean-center scaled.

Statistical analysis

The primary exposure for the study was infant miRNA consumption in the first six months. Consumption was calculated as the product of milk microRNA concentration and the number of breastmilk feeds, across three lactation stages. For example, consumption of miR-375-3p was calculated as follows: $[\text{miR-375}_{T0}] \times D_{T0} \times P_{T0} + [\text{miR-375}_{T4}] \times D_{T4} \times P_{T4} + [\text{miR-375}_{T16}] \times D_{T16} \times P_{T16}$; where T represents the time period (T0: 0-4 weeks, T4: 4-16 weeks, T16: 16-24 weeks), D represents the number of breastfeeding days during the time period, and P represents the proportion of feeds that included breast milk (**eFigure 1**). There were 24 missing samples (4% of all samples), due to collection failure (i.e., missed appointment, or COVID-related interruptions). For these samples, miRNA concentration was imputed using the mean miRNA value for the specific lactation stage.

The primary medical outcome was presence or absence of atopy in the 12 months after delivery. Medical, demographic, and environmental characteristics were compared between atopic and non-atopic groups with chi-square or student's t tests. Mann-Whitney U-tests were used to assess differences in breastmilk miRNA consumption between groups. Binomial regression with an analysis of variance (ANOVA) omnibus test was used to assess the contribution of breastmilk miRNA consumption to atopy risk, while controlling for relevant medical, demographic, and environmental characteristics. Co-linearity was assessed. Odds ratios with 95% confidence intervals were reported.

The following secondary analyses were used to assess expression patterns for each miRNA candidate: 1) A Kruskal-Wallis test was used to investigate differences in breastmilk miRNA exposure between atopy sub-groups (e.g., AD, food allergy, wheezing); 2) A Friedman repeated measures ANOVA with post-hoc Durbin Conover pairwise comparisons was used to determine whether miRNA candidates displayed significant changes across the three stages of lactation; 3) A mixed effects model was fit by restricted maximum likelihood to examine the impact of modifiable maternal characteristics (i.e., dietary scores, body mass index, and tobacco use) on breastmilk miRNA levels over time. Breastmilk miRNA concentration served as the dependent variable, participant ID was the clustering variable, and maternal characteristics served as covariates. Effects of maternal characteristics were assessed with fixed effects omnibus tests.

RESULTS

Participants

Participating infants were predominantly female (92/163, 56%), Caucasian (132/163, 131/163, 80%), and born via vaginal delivery (132/163, 81%) (**Table 1**). Nearly half had a history of maternal atopy (67/163, 41%), or atopy in a first-degree relative (74/163, 45%). Approximately one-third attended daycare (57/163, 35%). Most families reported pets in the home (99/163, 60%), one-third reported local atmospheric pollution (56/163, 34%), and few reported employing allergen mitigation techniques at home (26/163, 16%). Most families introduced solids prior to six months of age (142/163, 87%) and breastfed for at least six months (132/163, 81%).

Atopy characteristics

Seventy-three infants developed atopy. AD was the most common atopic condition (41/163, 25%), followed by food allergy (33/163, 20%), and wheezing (10/163, 6%). Eleven infants (6%) developed more than one atopic condition. The majority of atopic infants (42/73, 57%) experienced symptom onset after six months of age. There were no differences ($p < 0.05$) in maternal traits, infant traits, or environmental exposures between atopic (n = 73) and non-atopic (n = 90) infants.

Breastmilk miRNA consumption

Breastmilk samples collected from atopic and non-atopic dyads did not differ ($p < 0.05$) in collection time (12:39 ± 3:33 vs. 12:38 ± 3:07), total RNA counts ($6.4 \times 10^6 \pm 5.0 \times 10^5$ vs. $7.1 \times 10^6 \pm 6.0 \times 10^5$), or RNA quality (34.6 ± 0.1 vs. 34.6 ± 0.2). Infants who did not develop atopy consumed higher levels of breastmilk miR-375-3p ($d = -0.33$, $p = 0.007$) and miR-146b-5p ($d = -0.28$, $p = 0.037$), but not miR-21-5p ($d = 0.01$, $p = 0.21$) (**Figure 2A**). Atopy subgroups displayed no difference in consumption of miR-375-3p ($X^2 = 8.07$, $p = 0.089$), miR-146b-5p ($X^2 = 9.51$, $p = 0.050$), or miR-21-5p ($X^2 = 4.25$, $p = 0.37$) (**Figure 2B**). Consumption of breastmilk miR-375-3p was associated with reduced atopy risk ($Z = -2.0$, $p = 0.045$, OR = 0.48, 95% CI: 0.23 – 0.98) (**Figure 2C**). Timing of solid food introduction ($Z = 1.9$, $p = 0.048$, OR = 3.16, 95% CI: 1.01 – 9.94) was the only other factor associated with atopy risk (**eTable 1**).

Factors impacting breastmilk miRNA levels

All three candidate miRNAs displayed significant changes ($p < 0.05$) in breastmilk levels over the course of lactation (**Figure 3A-C**). Levels of miR-375-3p increased from early milk (0 weeks) to transitional milk (4 weeks), and remained elevated in mature milk (16 weeks; $F = 132.3$, $p = 8.4 \times 10^{-34}$). In contrast, levels of miR-21-5p were highest in early milk ($F = 23.7$, $p = 1.4 \times 10^{-9}$), whereas miR-146b-5p levels peaked in transitional milk ($F = 55.4$, $p = 4.5 \times 10^{-14}$). Only miR-375-3p displayed an interaction between lactation stage and atopy status ($F = 6.5$, $p = 0.002$). (**Figure 3D**).

The relationship between breastmilk miRNA levels and modifiable maternal characteristics (i.e., tobacco use, diet, BMI) were assessed with linear mixed effects models (**eTable 2**). Levels of miR-375-3p were inversely associated with maternal BMI ($F = 4.7$, $t = -2.1$, $p = 0.032$) (**Figure 4A**). Levels of miR-146b-5p were directly associated with maternal calcium intake ($F = 5.3$, $t = 2.3$, $p = 0.021$) and inversely associated with maternal tobacco use ($F = 6.8$, $t = -2.6$, $p = 0.009$) (**Figure 4B-C**). There were no associations between maternal traits and miR-21-5p levels.

DISCUSSION

This study is the first to demonstrate that infant consumption of breastmilk miRNA may provide protection against atopy development. Specifically, consumption of miR-375-3p over the first six months was associated with a two-fold risk reduction for AD, food allergies, and wheezing in the first year of life. Consumption of miR-375-3p was more strongly associated with atopy outcomes than oft-cited risk factors, such as family history and maternal tobacco use. (3) This knowledge has important applications for infant health, including the optimization of formula to better reflect the biologic characteristics of breastmilk.

Currently, infant formula contains no human miRNA, (32) and what little bovine miRNA survives the pasteurization process is unlikely to have a bioactive impact on the developing infant. (33) Addition of synthetic miR-375-3p to formula might ameliorate the disparities in atopy outcomes reported between formula- and breast-fed infants, (4)-(7) In contrast to formula, miR-375-3p is present in over 99% of all breastmilk samples and constitutes just under 1% of all miRNA in breastmilk. (23)

This study shows that breastmilk levels of miR-375-3p increase over the course of lactation, which may explain why sustained breastfeeding is important for atopy protection. (5) Intriguingly, miR-375-3p levels were lower in mothers with elevated BMI. This could explain previous findings linking maternal weight with infant AD and wheezing outcomes. (34) It could also provide an opportunity to enhance the atopic benefits associated with breastfeeding through targeted interventions aimed at increasing miR-375-3p levels via maternal weight control. (35)

A growing body of literature suggests that orally administered exosomal miRNAs survive digestion, impact immunologic responses in local mucosa, and are readily absorbed into circulation. (8) (16) (21) (32) (36) These foundational investigations have predominantly relied on cell culture, animal models, or bovine milk consumption. The current study adds to this body of evidence by demonstrating that consumption of miR-375-3p in human milk is associated with reduced atopy risk.

Several prior studies have established the importance of miR-375 in atopy pathophysiology. (21) (37)-(43) A study of cultured esophageal tissue from children with eosinophilic esophagitis found that miR-375 levels were repressed by interleukin (IL)-13, and that miR-375 was inversely related to the level of eosinophils and the expression of mast-cell-specific genes. (37) Another study employing a mouse model of allergic rhinitis showed that miR-375 expression was decreased in nasal mucosa, but administration of miR-375 could prevent epithelial inflammation by inhibiting IL-6. (21) Although the precise mechanisms connecting miR-375 with atopy are still being defined, it appears that frequent miR-375 exposure (through breastmilk ingestion) is a plausible explanation for reduced inflammation in the oropharynx, and reduced atopy risk.

To our knowledge, only one prior study has investigated the relationship between breastmilk miRNAs and child atopy. (38) A retrospective study by Simpson and colleagues (2015) used RNA sequencing to measure breastmilk miRNA levels in mature milk from 54 women, and found no association with infant AD at 2 years of age. However, this important study established that atopy related miRNAs (i.e., miR-21-5p, miR-146b-5p, miR-375-3p) were highly present in breastmilk and formed the basis for our hypothesis-driven investigation. Our ability to detect a relationship between breastmilk miRNAs and infant atopy outcomes may be attributed to a larger sample size, longitudinal collection of milk samples at various lactation stages, inclusion

of multiple atopic conditions, or a novel approach to control for total milk consumption in the first six months.

There are, however, several limitations of this study. We did not include allergic rhinitis in the definition of atopy due to the limited prevalence of this condition among infants. Impacts of miR-375-3p on allergic rhinitis or atopic outcomes beyond 12 months cannot be inferred. The study's drop-out rate (24%) was exacerbated by the COVID-19 pandemic, and may have contributed attrition bias. Missing data (4%) was imputed using mean values from the entire cohort. Such an approach may enhance the likelihood of false-negative results. Despite the importance of exosomes in miRNA transport, (39) (32) we did not specifically isolate exosomal RNA. However, we note that the majority of miRNAs in breastmilk are contained within exosomes, (33) and that lipid fractions are likely to contain additional sources of encapsulated miRNAs that may survive the digestive tract. Finally, we recognize that our approach for estimating miRNA consumption assumes that total RNA content is relatively stable across milk samples. In the absence of technologies capable of measuring absolute miRNA levels, this assumption is unavoidable, but likely provides a more accurate estimate than approaches that ignore duration and frequency of breastfeeding completely.

In conclusion, this study demonstrates that infant consumption of breastmilk miR-375-3p is associated with reduced atopy risk in the first year of life. Increases in miR-375-3p over the course of lactation support the idea that sustained breast feeding enhances atopy protection. Additional studies are necessary to confirm this important relationship and determine if oral administration of synthetic miR-375 conveys atopy protection in translational models.

ACKNOWLEDGEMENTS

SDH had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Data from this study has been made publicly available through the Gene Expression Omnibus repository (GSE192543).

All authors made substantial contributions to design of the work, or the acquisition, analysis, or interpretation of data. All authors played a role drafting the work or revising it critically for intellectual content. All authors approve of the manuscript and agree to be accountable for all aspects of the work.

The authors thank Faoud Ishmael, MD, PhD (Penn State College of Medicine, Hershey, PA) and Nicole Hackman, MD (Penn State College of Medicine, Hershey, PA) for assistance with study design. We wish to acknowledge Jessica Beiler, MS (Penn State College of Medicine, Hershey, PA) for assistance with data and sample acquisition. We thank Frank Middleton, PhD (SUNY Upstate Medical University, Syracuse, NY), Karen Gentile, MS (SUNY Upstate Medical University, Syracuse, NY), and Susan DiAngelo, BS (Penn State College of Medicine, Hershey, PA) for assistance with sample processing.

REFERENCES

1. Spergel JM, Paller AM. Atopic dermatitis and the atopic march. Journal of Allergy and Clinical Immunology. 2003; 112(6): S118-S127. https://doi.org/10.1016/j.jaci.2003.09.033.

2. Hopkin JM. Genetics of atopy. Pediatric Allergy and Immunology. 1995; 6(3): 139-144. **https://doi.org/10.1111/j.1399-3038.1995.tb00273.x**

3. Tariq SM, Matthews SM, Hakim EA, Stevens M, Arshad SH, Hide DW. The prevalence of and risk factors for atopy in early childhood: a whole population birth cohort study. Journal of Allergy and Clinical Immunology. 1998; 101(5): 587-593. https://doi.org/10.1016/S0091-6749(98)70164-2

4. Kull I, Almqvist C, Lilja G, Pershagen G, Wickman M. Breast-feeding reduces the risk of asthma during the first 4 years of life. Allergy Clinical Immunology. 2004; 114(4): 755-760. https://doi.org/10.1016/j.jaci.2004.07.036

5. Mimouni BA, Mimouni M, Gdalevich M, Mimouni D. Does breastfeeding protect against allergic rhinitis during childhood? A meta-analysis of prospective studies. Acta Paediatrica. 2002; 91(3): 275-279. https://doi.org/10.1111/j.1651-2227.2002.tb01714.x

6. Miliku K, Azad MB. Breastfeeding and the developmental origins of asthma: current evidence, possible mechanisms, and future research priorities. Nutrients. 2018; 10(8): 995. https://doi.org/10.3390/nu10080995

7. Nuzzi G, Di Cicco ME, Peroni DG. Breastfeeding and Allergic Diseases: What's New? Children (Basel). 2021; 8(5): 330. https://doi.org/10.3390/children8050330

8. Melnik BC, John SM, Schmitz G. Milk: an exosomal microRNA transmitter promoting thymic regulatory T cell maturation preventing the development of atopy? Journal of Translational Medicine. 2014; 12(1): 1-1. https://doi.org/10.1186/1479-5876-12-43

9. Alsaweed M, Hartmann PE, Geddes DT, Kakulas F. MicroRNAs in breastmilk and the lactating breast: potential immunoprotectors and developmental regulators for the infant and the mother. International Journal of Environmental Research and Public Health. 2015; 12: 13981-14020. https://doi.org/10.3390/ijerph121113981

10. Munch EM, Harris RA, Mohammad M, Benham AL, Pejerrey SM, Showalter L, et al. Transcriptome profiling of microRNA by Next-Gen deep sequencing reveals known and novel miRNA species in the lipid fraction of human breast milk. PLoS One. 2013; 8(2): e50564. https://doi.org/10.1371/journal.pone.0050564

11. Alsaweed M, Lai CT, Hartmann PE, Kakulas F. Human milk miRNAs primarily originate from the mammary gland resulting in unique miRNA profiles of fractionated milk. Scientific Reports. 2016; 6: 1-13. https://doi.org/10.1038/srep20680

12. Zhou Q, Li M, Wang X, Li Q, Wang T, Zhu Q, et al. Immune-related microRNAs are abundant in breast milk exosomes. International Journal of Biological Sciences. 2012; 8: 118-123. doi:10.7150/ijbs.8.118

13. Carney M,TA,DSea., Carney MC, Tarasiuk A, DiAngelo SL, Silveyra P, Podany A, et al. Metabolism-related microRNAs in maternal breast milk are influenced by premature delivery. Pediatric Research. 2017; 82: 226–236. https://doi.org/10.1038/pr.2017.54

14. Chen X, Gao C, Li H, Huang L, Sun Q, Dong Y, et al. Identification and characterization of microRNAs in raw milk during different periods of lactation, commercial fluid, and powdered milk products. Cell Research. 2010; 20(10): 1128-1137. https://doi.org/10.1038/cr.2010.80

15. Xu L, Yang BF, Ai J. MicroRNA transport: a new way in cell communication. Journal of Cellular Physiology. 2013; 228(8): 1713-1719. https://doi.org/10.1002/jcp.24344

16. Hirschi KD, Pruss GJ, Vance V. Dietary delivery: a new avenue for microRNA therapeutics? Trends in Biotechnology. 2015; 33(8): 431-432. https://doi.org/10.1016/j.tibtech.2015.06.003

17. Arntz OJ, Pieters BC, Oliveira MC, Broeren MG, Bennink MB, de Vries M, et al. Oral administration of bovine milk derived extracellular vesicles attenuates arthritis in two mouse models. Molecular Nutrition & Food Research. 2015; 59(9): 1701-1712. https://doi.org/10.1002/mnfr.201500222

18. Lu TX, Rothenberg ME. Diagnostic, functional, and therapeutic roles of microRNA in allergic diseases. Journal of Allergy and Clinical Immunology. 2013; 132(1): 3-13. https://doi.org/10.1016/j.jaci.2013.04.039

19. Sawant DV, Yao W, Wright Z, Sawyers C, Tepper RS, Gupta SK, et al. Serum microRNA-21 as a biomarker for allergic inflammatory disease in children. Microrna. 2015; 4(1): 36-40. https://dx.doi.org/10.2174/2211536604666150220232507

20. Rebane A, Akdis CA. MicroRNAs in allergy and asthma. Current Allergy and Asthma Reports. 2014; 14(4): 1-9. https://doi.org/10.1007/s11882-014-0424-x

21. Wang T, Chen D, Wang P, Xu Z, Li Y. miR-375 prevents nasal mucosa cells from apoptosis and ameliorates allergic rhinitis via inhibiting JAK2/STAT3 pathway. Biomedicine & Pharmacotherapy. 2018; 103: 621-627. https://doi.org/10.1016/j.biopha.2018.04.050

22. Comer BS, Camoretti-Mercado B, Kogut PC, Halayko AJ, Solway J, Gerthoffer WT. MicroRNA-146a and microRNA-146b expression and anti-inflammatory function in human airway smooth muscle. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2014; 307(9): 727-734. https://doi.org/10.1152/ajplung.00174.2014

23. Hicks SD, Confair A, Warren K, Chandran D. Levels of Breast Milk MicroRNAs and Other Non-Coding RNAs Are Impacted by Milk Maturity and Maternal Diet. Frontiers in Immunology. 2022; 12: 1-10. https://doi.org/10.3389/fimmu.2021.785217

24. Tingö L, Ahlberg E, Johansson L, Pedersen SA, Chawla K, Sætrom , et al. Non-Coding RNAs in Human Breast Milk: A Systematic Review. Frontiers in Immunology. 2021; 12: 1-31. https://doi.org/10.3389/fimmu.2021.725323

25. Pucci N, Novembre E, Cammarata MG, Bernardini R, Monaco MG, Calogero C, et al. Scoring atopic dermatitis in infants and young children: distinctive features of the SCORAD index. Allergy. 2005;: 113-116. https://doi.org/10.1111/j.1398-9995.2004.00622.x

26. Muraro A, Lemanske Jr RF, Hellings PW, Akdis CA, Bieber T, Casale TB, et al. Precision medicine in patients with allergic diseases: airway diseases and atopic dermatitis—PRACTALL document of the European Academy of Allergy, Asthma & Immunology. Journal of Allergy and Clinical Immunology. 2016; 137(5): 1347-1358. https://doi.org/10.1016/j.jaci.2016.03.010

27. Fein SB, Labiner-Wolfe J, Shealy KR, Li R, Chen J, Grummer-Strawn LM. Infant feeding practices study II: study methods. Pediatrics. 2008; 122: Suppl 2:S28-S35. https://doi.org/10.1542/peds.2008-1315c

28. Bianca AC, Wandalsen GF, Miyagi K, Camargo L, Cezarin D, Mallol J, et al. International Study of Wheezing in Infants (EISL): validation of written questionnaire for children below 3 years. Journal of Investigational Allergology and Clinical Immunology. 2009; 19(1): 35-42.

29. Vojta PJ, Friedman W, Marker DA, Clickner R, Rogers JW, Viet SW, et al. First National Survey of Lead and Allergens in Housing: survey design and methods for the allergen and endotoxin components. Environmental Health Perspectives. 2002; 110(5): 527-532. https://doi.org/10.1289/ehp.02110527

30. Thompson FE, Midthune D, Kahle L, Dodd KW. Development and evaluation of the National Cancer Institute's Dietary Screener Questionnaire scoring algorithms. The Journal of Nutrition. 2017; 147(6): 1226-1233. https://doi.org/10.3945/jn.116.246058

31. Kelleher SL, Gagnon A, Rivera OC, Hicks SD, Carney MC, Alam S. Milk-derived miRNA profiles elucidate molecular pathways that underlie breast dysfunction in women with common genetic variants in SLC30A2. Scientific Reports. 2019; 9(1): 1-3. https://doi.org/10.1038/s41598-019-48987-4

32. Leiferman A, Shu J, Upadhyaya B, Cui J, Zempleni J. Storage of extracellular vesicles in human milk, and microRNA profiles in human milk exosomes and infant formulas. Journal of Pediatric Gastroenterology and Nutrition. 2019; 69(2): 235-238. https://doi.org/10.1097/MPG.0000000000002363

33. Mutai E, Ramer-Tait AE, Zempleni J. MicroRNAs in bovine milk exosomes are bioavailable in humans but do not elicit a robust pro-inflammatory cytokine response. ExRNA. 2020; 2(1): 1-9. https://doi.org/10.1186/s41544-019-0041-x

34. Wei X, Huang P, Gao C, Shen S, Tu S, Guo Y, et al. Associations of maternal weight status with the risk of offspring atopic dermatitis and wheezing by 1 year of age. Pediatric Allergy and Immunology. 2022; 33(1): e13703. https://doi.org/10.1111/pai.13703

35. Munblit D, Verhasselt V. Allergy prevention by breastfeeding: possible mechanisms and evidence from human cohorts. Current Opinion in Allergy and Clinical Immunology. 2016; 16(5): 427-433. https://doi.org/10.1097/ACI.0000000000000303

36. Liao Y, Du X, Li J, Lönnerdal B. Human milk exosomes and their microRNAs survive digestion in vitro and are taken up by human intestinal cells. Molecular Nutrition & Food Research. 2017; 61(11): Epub. https://doi.org/10.1002/mnfr.201700082

37. Lu TX, Lim EJ, Wen T, Plassard AJ, Hogan SP, Martin LJ, et al. MiR-375 is downregulated in epithelial cells after IL-13 stimulation and regulates an IL-13-induced epithelial transcriptome. Mucosal Immunology. 2012; 5(4): 388-396. https://doi.org/10.1038/mi.2012.16

38. Simpson MR, Brede G, Johansen J, Johnsen R, Storrø O, Sætrom P, et al. Human Breast Milk miRNA, Maternal Probiotic Supplementation and Atopic Dermatitis in Offspring. PLoS One. 2015; 10(12): e0143496. https://doi.org/10.1371/journal.pone.0143496

39. Baier SR, Nguyen C, Xie F, Wood JR, Zempleni J. MicroRNAs are absorbed in biologically meaningful amounts from nutritionally relevant doses of cow milk and affect gene expression in peripheral blood mononuclear cells, HEK-293 kidney cell cultures, and mouse livers. The Journal of Nutrition. 2014; 144(10): 1495-1500. https://doi.org/10.3945/jn.114.196436

40. Xi Y, Jiang X, Li R, Chen M, Song W, Li X. The levels of human milk microRNAs and their association with maternal weight characteristics. European Journal of Clinical Nutrition. 2015; 70(4): 445-449. https://doi.org/10.1038/ejcn.2015.168

41. Izumi H, Tsuda M, Sato Y, Kosaka N, Ochiya T, Iwamoto H, et al. Bovine milk exosomes contain microRNA and mRNA and are taken up by human macrophages. Journal of Dairy Science. 2015; 98(5): 2920-2933. https://doi.org/10.3168/jds.2014-9076

42. Rebane A, Runnel T, Aab A, Maslovskaja J, Rückert B, Zimmermann M, et al. MicroRNA-146a alleviates chronic skin inflammation in atopic dermatitis through suppression of innate immune responses in keratinocytes. Journal of Allergy and Clinical Immunology. 2014; 134(4): 836-847. https://doi.org/10.1016/j.jaci.2014.05.022

43. Wang T, Wang P, Chen D, Xu Z, Yang L. circARRDC3 contributes to interleukin 13 induced inflammatory cytokine and mucus production in nasal epithelial cells via the miR 375/KLF4 axis. Molecular Medicine Reports. 2021; 23(2): ePub 1-8. https://doi.org/10.3892/mmr.2020.11780

44. Xu G, Xie Q, Zhou H. [Changes of serum miR-375 and blood target genes in patients with allergic rhinitis before and after treatment and its significance] Zhong Nan Da Xue Xue Bao Yi Xue Ban. Journal of Central South University. 2019; 44(7): 767-774.

Table 1. Participant Characteristics

	All (n = 163)	Atopy (n = 73)	No Atopy (n = 90)	P-val (d or X^2)
Maternal Traits				
Maternal age in years, mean (SD)	30 (4)	30 (4)	30 (3)	0.61 (0.5)
Tobacco use, n (%)	19 (11)	7 (9)	12 (13)	0.45 (0.5)
BMI in kg/m^2, mean (SD)	27.6 (6)	28.1 (6)	27.2 (6)	0.50 (0.6)
Maternal atopy, n (%)	67 (41)	35 (48)	32 (36)	0.11 (2.5)
Infant Traits				
Female sex, n (%)	92 (56)	42 (57)	50 (56)	0.80 (0.06)
Gest. age in weeks, mean (SD)	39.0 (1)	39.0 (1)	38.9 (1)	0.50 (0.1)
Vaginal delivery, n (%)	132 (81)	56 (77)	76 (84)	0.21 (1.5)
Birth weight in grams, mean (SD)	3358 (442)	3362 (440)	3355 (447)	0.35 (4.4)
Caucasian, n (%)	131 (80.4)	55 (75)	76 (84)	0.88 (0.1)
African American, n (%)	7 (4.3)	3 (5)	4 (5)	0.96 (0.04)
Asian, n (%)	8 (4.9)	6 (8)	2 (2)	0.089 (1.7)
Bi-racial, n (%)	8 (4.9)	5 (7)	3 (3)	0.26 (1.1)
Other, n (%)	9 (5.5)	4 (5)	5 (6)	0.88 (0.1)
Atopy in 1st degree relative, n (%)	74 (45)	37 (50)	37 (41)	0.22 (1.4)
Environment				
Daycare attendance, n (%)	57 (35)	24 (33)	33 (37)	0.61 (0.2)
Allergen mitigation, n (%)	26 (16)	13 (18)	13 (14)	0.56 (0.3)
Pet(s) in home, n (%)	99 (60)	44 (60)	55 (61)	0.91 (0.01)
Atmospheric pollution, n (%)	56 (34)	24 (33)	32 (36)	0.72 (0.1)
Solid food intro. <6 months, n (%)	142 (87)	60 (82)	82 (91)	0.091 (2.8)
Breastfeeding ≥6 months, n (%)	132 (81)	57 (78)	75 (83)	0.39 (0.7)

Maternal atopy includes self-reported atopic dermatitis, food allergies, asthma, or allergic rhinitis. Daycare attendance includes any daycare in the first 12 months. Presence or absence of allergen mitigation techniques and household pet(s) was self-reported on the National Survey of Lead and Allergens in Housing. Atmospheric pollution was self-reported on the International Survey of Allergies and Asthma in Children – Wheezing Questionnaire. Abbreviations: Body mass index (BMI), Gestational (Gest.)

FIGURE LEGENDS

Figure 1. CONSORT Diagram

There were 2,487 mother-infant dyads screened for eligibility, and 359 eligible dyads were approached. 221 dyads consented to participate, and 163 completed the 12 month longitudinal study. There were 73 infants who developed atopy – 41 had atopic dermatitis (AD), 33 had food allergy, and 10 had wheezing. Eleven infants had more than one atopic condition. In total, 442 breastmilk samples were collected from the 163 participants and underwent RNA sequencing. Ten samples were excluded for insufficient microRNA read counts. This left 190 samples from mothers of atopic infants and 242 samples from mothers of non-atopic infants. Each mother provided up to three samples: at 0 weeks ("early milk"), 4 weeks ("transitional milk") and 16 weeks ("mature milk").

Figure 2. Increased consumption of breastmilk miR-375 is associated with reduced atopy risk

The boxplots display average consumption of breastmilk miR-375-3p, miR-146b-5p, and miR-21-5p for atopic (n = 73) and non-atopic (n = 90) infants (A). Mann-Whitney U testing revealed that infants without atopy consumed higher levels of miR-375-3p (d = -0.33, p = 0.007) and miR-146b-5p (d = -0.28, p = 0.037). Across atopy sub-groups, there was no difference (p > 0.05) in consumption of miR-375-3p, miR-146b-5p, or miR-21-5p (B). Sub-groups were defined as atopic dermatitis (AD), food allergy (FA), wheezing (W), or infants with more than one atopic condition (>1). A binomial regression controlling for medical, demographic, and environmental risk factors related to infant atopy revealed that consumption of miR-375-3p was associated with reduced atopy risk (Z = -2.0, p = 0.045, OR = 0.48, 95% CI: 0.23 – 0.98) (C). The marginal means plot is shown with 95% CI.

Figure 3. Breastmilk miRNA levels change throughout lactation

The spaghetti plots display maternal breastmilk levels of miR-375-3p (A), miR-146b-5p (B), and miR-21-5p (C) across three lactation stages: early milk (0 weeks), transitional milk (4 weeks), and mature milk (16 weeks). Repeated measures ANOVA revealed a significant effect of lactation stage on miR-375-3p (F = 132.3, p = 8.4 x 10-34), miR-146b-5p (F = 55.4, p = 4.5 x 10-14), and miR-21-5p (F = 23.7, p = 1.4 x 10-9). Only miR-375-3p displayed an interaction (F = 6.5, p = 0.002) between lactation stage and atopy status (D).

Figure 4. Breastmilk miRNA levels are impacted by maternal body mass, diet, and smoking

Linear mixed effects models were used to assess the relationship between breastmilk miRNA levels and modifiable maternal traits over time. The scatter plot and trend line display an inverse relationship between maternal body mass index (BMI, kg/m^2) and breastmilk miR-375-3p levels (F = 4.7, t = -2.1, p = 0.032) (A). Levels of miR-146b-5p were directly associated with maternal calcium intake (mg/day), as reported on the Dietary Screener Questionnaire (F = 5.3, t = 2.3, p = 0.021) (B). Whisker box plots display breastmilk miR-146b-5p levels for mothers who reported prior tobacco use and those who had never used tobacco (C). Milk miR-146b-5p level were higher among mothers who had never used tobacco (F = 6.8, t = -2.6, p =0.009).