Infant consumption of microRNA miR-375 in human milk lipids is associated with protection from atopy

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ABSTRACT

Background: Human milk 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 4 microRNAs (miR-146b-5p, miR-148b-3p, miR-21–5p, and miR-375–3p) in human milk would be associated with reduced atopy risk.

Methods: The Breast Milk Influence of the Microtranscriptome Profile on Atopy in Children over Time (IMPACT) study involved a cohort of mother-infant dyads who planned to breastfeed beyond 4 mo. Infant consumption of the 4 human milk microRNAs (miRNAs) in the first 6 mo was calculated as the product of milk miRNA concentration and the number of human milk feeds, across 3 lactation stages: early milk (0–4 wk), transitional milk (4–16 wk), and mature milk (16–24 wk). The primary outcome was infant atopy in the first year, defined as atopic dermatitis (AD), food allergies, or wheezing. The final analysis included 432 human milk samples and 7824 wk of longitudinal health data from 163 dyads.

Results: Seventy-three infants developed atopy. Forty-one were diagnosed with AD (25%), 33 developed food allergy (20%), and 10 had wheezing (6%). Eleven developed >1 condition (7%). Infants who did not develop atopy consumed higher concentrations of miR-375–3p (d = 0.18, $P = 0.022$, adj $P = 0.044$) and miR-148b-3p (d = 0.23, $P = 0.007$, adj $P = 0.028$). The consumption of miR-375–3p ($X^2 = 5.7$, $P = 0.017$, OR: 0.92, 95% CI: 0.86, 0.99) was associated with reduced atopy risk. Concentrations of miR-375–3p increased throughout lactation (r = 0.46, F = 132.3, $P = 8.4 \times 10^{-34}$) and were inversely associated with maternal body mass (r = –0.11, t = –2.1, $P = 0.032$).

Conclusions: This study provides evidence that infant consumption of miR-375–3p may reduce atopy risk. *Am J Clin Nutr* 2022;116:1654–1662.

Keywords: breastfeeding, breastmilk, microRNA, miR-375, allergies, risk reduction, miR-148b, body mass

Introduction

Atopic conditions, such as atopic dermatitis (AD), food allergies, and asthma, occur 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 3 mo may have a lower risk of certain atopic conditions (4–6). Human milk contains numerous immunomodulatory components that could convey atopy protection (7, 8). Bioactive factors in human milk, called microRNAs (miRNAs), may play a role (9). miRNAs are small, noncoding molecules that regulate gene expression across multiple tissues (10). There are nearly 1000 different miRNAs in human milk (11). The majority are found in the lipid or cellular fractions of milk (12), and evidence suggests that they regulate immune pathways (10, 13). Although human milk miRNA composition can vary based on maternal weight, diet, or genetics (14–17), human milk reliably contains miRNAs, whereas formula does not (15).

miRNAs are often packaged within protective vesicles (16), making them stable in human milk and transferable to the infant gut, where they may be absorbed and functionally incorporated by epithelial and immunologic cells in the oropharynx (17–22). For example, milk exosomes have been shown to induce immunomodulatory effects through regulation of *Forkhead box P3* (*FOXP3*), a transcription factor critical to T cell regulation

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Supplemental Figure 1 and Supplemental Tables 1–3 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: AD, atopic dermatitis; FOXP3, Forkhead box P3; IFP, infant feeding practices; IMPACT, Influence of the Microtranscriptome Profile on Atopy in Children over Time; ISAAC-WQ, International Study of Asthma and Allergies in Childhood – Wheezing Questionnaire; JAK2, Janus kinase 2; miRNA, microRNA; SCORAD, Scoring Atopic Dermatitis.

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(23). Animal studies suggest nutritional miRNA influences development of the immune system (18, 19), and this may occur through modulation of T cell populations that are implicated in atopic conditions (24, 25). This illustrates one important mechanism through which exposure to milk miRNAs in microvesicles may mitigate infant risk of atopic disease (26).

Circulating miRNA concentrations are dysregulated in atopic conditions (20–35). For example, concentrations of miR-21 are elevated in the skin of patients with AD and in the bronchial cells of patients with asthma (20). Upregulation of miR-375 may prevent inflammatory pathways associated with allergic rhinitis (22). Increases in miR-146 are associated with reduced inflammation in airway smooth muscle, and protection from asthma and AD (22, 27). Polymorphisms targeting miR-148 have also been implicated in asthma (28). Notably, all of these miRNAs have a robust presence in human milk (29).

This study, Breast Milk IMPACT (Influence of the Microtranscriptome Profile on Atopy in Children over Time), followed 221 mother-infant dyads from birth to 12 mo to test the hypothesis that infant consumption of 4 miRNAs (miR-146b-5p, miR-148b-3p, miR-21–5p, and miR-375–3p) in human milk would be associated with reduced atopy risk. These miRNAs were selected based on their biologic relation with atopic conditions (20–22, 27) and their bioavailability in breastmilk (29). To our knowledge, this is the first large-scale, prospective cohort study investigating the relation between longitudinal milk miRNA consumption and infant atopy (30).

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 participants at enrollment. The study was registered at clinicaltrials.gov as NCT04017520, and the study's hypothesis was published on 19 July, 2019 (prior to miRNA analysis).

Participants

This longitudinal cohort study involved a convenience sample of 221 mother-infant dyads followed from the age of 1 wk to 12 mo. This sample size was determined by a priori power analysis estimating that ∼33% of infants ($n = 73$) would develop atopy (31), providing >80% power to detect a 1.5-fold difference in miRNA consumption across atopy and nonatopy groups on Mann–Whitney U-testing ($\alpha = 0.05$). Inclusion criteria were mothers who delivered at term (>35 wk), and intended to feed their infant human milk beyond 4 mo. The sample was not enriched for atopic disease predisposition (based on family history). Exclusion criteria were: *1*) Maternal morbidities that could impact lactation success or influence human milk miRNA composition (e.g. cancer, drug addiction, HIV infection); *2*) plan for infant adoption; *3*) presence of neonatal conditions that could impact the ability to breastfeed (e.g. cleft lip, metabolic disease, NICU [Neonatal Intensive Care Unit] admission >7 d); *4*) plan for pediatric care outside the medical center, or *5*) non-English speaking. Between April 2018 and October 2020, research staff screened 2487 potential participants through the electronic medical record, approached 359 eligible participants (14%), and enrolled 221 participants (61% of those eligible). Recruitment occurred at 4 pediatric outpatient clinics affiliated with the academic medical center. All participants had access to on-site lactation support for the duration of the study. A total of 163 dyads completed the study (**Figure 1**). The primary medical outcome was presence or absence of atopic disease in the first 12 mo, defined by parent report of AD, food allergies, or wheezing on standardized questionnaires (32–36), and confirmed for all participants through review of the electronic medical record. This definition of atopy was developed in accordance with guidelines from the European Academy of Allergy, Asthma & Immunology (33). The 3 atopic conditions were chosen to represent a single allergic phenotype based upon their clinical presentation in the first year of life (1, 31), their association as part of the "atopic march" (1), and scientific literature supporting their respective associations with human milk consumption (4–6).

Survey collection

Medical and demographic characteristics were collected for all dyads through nurse-administered surveys at enrollment. Maternal age, tobacco use, BMI, and atopy history were recorded. Infant sex, gestational age, delivery route, birth weight, and race were recorded. Infant race was self-reported by mothers as American Indian or Alaska Native, Asian, Black or African American, Native Hawaiian or Other Pacific Islander, and White or Caucasian. The duration of lactation and proportion of feeds consisting of human milk were assessed at 4, 16, and 24 wk using the Infant Feeding Practices (IFP)-II survey (34). Infant AD was assessed by trained research nurses at 4, 16, 24, and 48 wk using the Scoring Atopic Dermatitis (SCORAD) tool (32). 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 reactions were assessed through the IFP-II survey at 4, 16, 24, and 48 wk (34). Infant wheezing during the first 12 mo was assessed through administration of a standardized wheezing questionnaire from the International Study of Asthma and Allergies in Childhood (ISAAC-WQ) at 48 wk (35). The ISAAC-WQ was developed to measure the prevalence of recurrent wheezing and related risk factors in infants during the first 12 mo. Allergens in the infant environment were reported at 4 wk using a modified form of the National Survey of Lead and Allergens in Housing (NSLAH) (36). This survey was developed by the NIEHS to assess environmental allergens within the home. In order to assess the relation between human milk miRNAs and maternal diet, the Dietary Screener Questionnaire (DSQ) was administered at each milk collection (0, 4, and 16 wk) (37). Missing survey data (42/2608, 1.6%) was imputed using the mean cohort value for downstream statistical analysis.

Sample collection

Human milk (5 mL) was manually expressed into RNAse-free tubes from a sterilized nipple surface, as we have previously described (14, 38). Foremilk (prefeed) samples were collected in weeks 0, 4, and 16. These time points were chosen to reflect important changes in milk miRNA concentrations that occur during the course of lactation (29), and to capture the period when

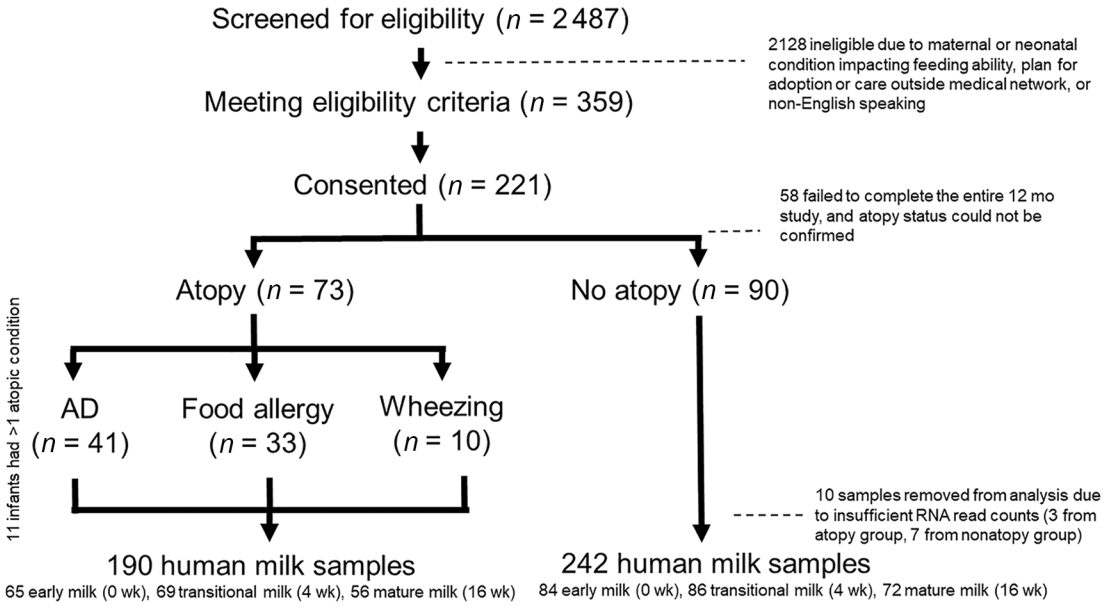

FIGURE 1 CONSORT diagram. There were 2487 mother–infant dyads screened for eligibility, and 359 eligible dyads were approached. A total of 221 dyads consented to participate, and 163 completed the 12-mo longitudinal study. There were 73 infants who developed atopy – 41 had atopic dermatitis (AD), 33 had food allergy, and 10 had wheezing. Eleven infants had >1 atopic condition. In total, 442 human milk samples were collected from the 163 participants and underwent RNA sequencing. Ten samples were excluded for insufficient microRNA (miRNA) read counts. This left 190 samples from mothers of infants with atopy and 242 samples from mothers of infants without atopy. Each mother provided ≤3 samples: at 0 wk ("early milk"), 4 wk ("transitional milk"), and 16 wk ("mature milk").

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 to ensure that daily variations in milk miRNA concentrations did not contribute to between-group differences. The 163 mothers who completed the study contributed 442 human milk samples: 153 "early milk" samples at 0 wk (4 ± 2 d postpartum), 155 "transitional milk" samples at 4 wk (39 ± 11 d postpartum), and 134 "mature milk" samples at 16 wk (128 ± 8 d postpartum). 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 4 wk, undergoing exactly 1 freeze-thaw cycle prior to RNA extraction.

Sample processing

Human milk RNA was purified from the lipid fraction using a Norgen Circulating and Exosomal RNA Purification Kit (Norgen Biotech), per manufacturer instructions (14, 29). 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 microvesicles) (11, 12, 14). Samples were processed in batches containing longitudinal samples from each participant, and matched ratios of atopic and nonatopic dyads. The yield and quality of RNA samples were assessed using the Agilent Bioanalyzer (Agilent Technologies) prior to library construction, which utilized 250 ng of RNA from each sample. RNA was sequenced at the SUNY (State University of New York) Molecular Analysis Core using the Illumina TruSeq Small RNA Prep protocol and a NextSeq500 instrument (Illumina) at a targeted depth of 10 million, 50 base, paired-end reads per sample. RNAseq was selected to permit estimation of miRNA concentrations as reads per million, rather than a relative value yielded by PCRs. FASTQ files were deposited into the Gene Expression Omnibus repository (GSE192543). Mature miRNA reads were aligned to the hg38 build of the human genome using miRbase 22 in Partek Flow (Partek), 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 4 miRNAs of interest (miR-146b-5p, miR-148b-3p, 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 of miR-146b-5p, miR-148b-3p, miR-21–5p, and miR-375–3p in the first 6 mo. Consumption, in mg, was calculated as the product of milk RNA concentration (mg/mL), infant milk consumption (mL/day), lactation duration (days), and the proportion of RNA sequencing reads accounted for by a specific miRNA across 3 lactation stages. For example, the consumption of miR-375–3p was calculated as follows: $[RNA_{T0}] \times V_{T0} \times D_{T0} \times [\text{miR-375}_{T0}] + [RNA_{T4}] \times V_{T4} \times D_{T4} \times [\text{miR-375}_{T4}] + [RNA_{T16}] \times V_{T16} \times D_{T16} \times [\text{miR-375}_{T16}]$; where T represents the time period (T0: 0–4 wk, T4: 4–16 wk, T16: 16–24 wk), RNA represents the small RNA concentration of the human milk sample (mg/mL), V represents the volume of human milk consumed (mL/day), D represents the number of days in

TABLE 1 Participant characteristics

	All ($n = 163$)	Atopy ($n = 73$)	No atopy ($n = 90$)	P value (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)
Biracial, *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 mo, *n* (%)	142 (87)	60 (82)	82 (91)	0.091 (2.8)
Breastfeeding ≥6 mo, *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 mo. 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. *P* values were determined using chi-square or student's t tests as appropriate. Gest., Gestational.

which human milk was consumed, and miR-375 represents the proportion of small RNA reads accounted for by miR-375–3p (**Supplementary Figure 1**). There were 24 missing samples (4% of all samples), due to failure to collect a milk sample (i.e. missed appointment or COVID-related interruptions). For these samples, miRNA concentration was imputed for the final analysis using the mean miRNA value for the specific lactation stage.

The primary medical outcome was the presence or absence of atopy in the 12 mo after delivery. Medical, demographic, and environmental characteristics were compared between infants with atopy and infants without atopy using 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 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. Colinearity was assessed. ORs with 95% CIs were reported. A Kruskal–Wallis test was used to investigate differences in milk miRNA consumption between atopy subgroups (e.g. AD, food allergy, wheezing). Multiple testing correction was performed using the Benjamini–Hochberg method.

The following secondary analyses were used to assess expression patterns for miRNAs associated with atopy risk reduction: *1*) A Friedman repeated measures ANOVA with posthoc Durbin Conover pairwise comparisons was used to determine whether the miRNA displayed significant changes across the 3 stages of lactation; *2*) a mixed effects model was fit by restricted maximum likelihood to examine the impact of modifiable maternal characteristics (i.e. dietary scores, BMI, and tobacco use) on milk miRNA concentrations over time. Human milk 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. Finally, Mann–Whitney U-tests were performed to compare the consumption of 85 additional human milk miRNAs (which did not form the basis for our a priori hypothesis) between atopic and nonatopic groups. All statistical analyses were performed using Jamovi v2.2.5 software.

Results

Participants

Participating infants were predominantly female (92/163, 56%), Caucasian (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 infants were introduced to solid foods prior to the age of 6 mo (142/163, 87%) and consumed human milk for ≥6 mo (132/163, 81%).

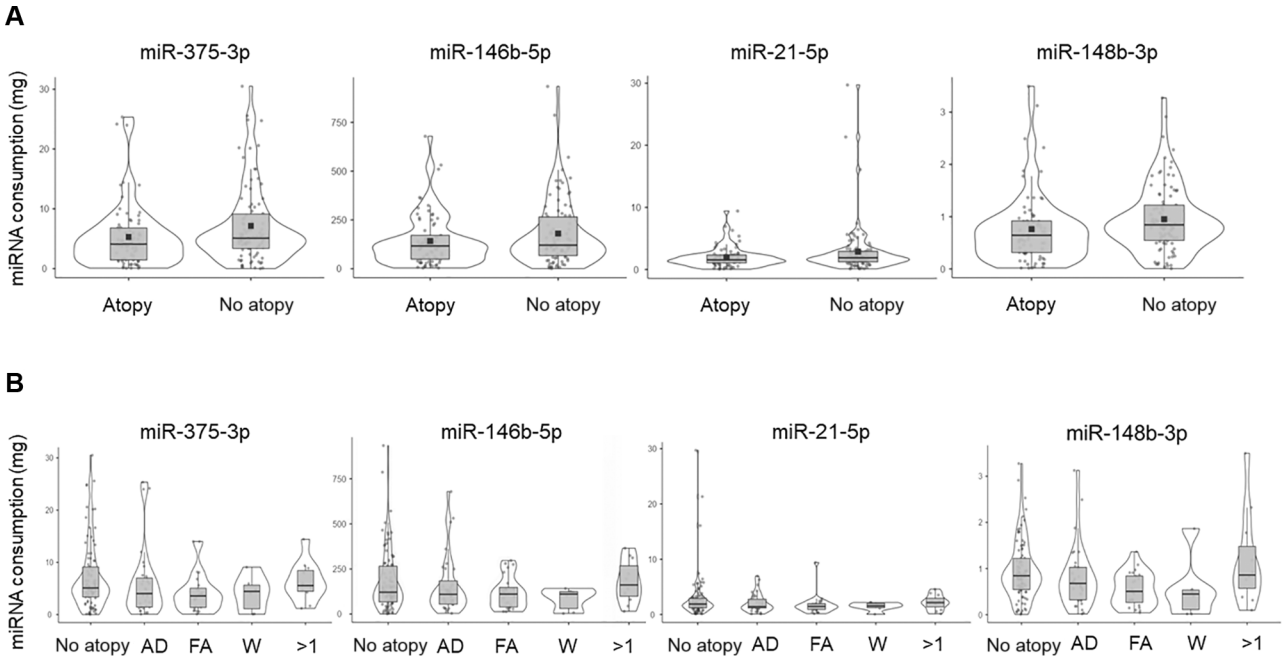

FIGURE 2 Infants with atopy consume lower concentrations of miR-375–3p and miR-148b-3p. Mann–Whitney U-testing revealed that infants without atopy consumed higher concentrations of miR-375–3p (d = 0.18, $P = 0.022$, adj $P = 0.044$) and miR-148b-3p (d = 0.23, $P = 0.007$, adj $P = 0.028$). The boxplots display consumption of human milk miR-375–3p, miR-146b-5p, and miR-21–5p for atopic ($n = 73$) and nonatopic ($n = 90$) infants (A). Mean (black bar) and median (diamond) values are displayed for each group. Across atopy subgroups, there was no difference (adj $P > 0.05$) in consumption of miR-375–3p, miR-146b-5p, or miR-21–5p on Kruskal–Wallis testing (B). Subgroups were defined as atopic dermatitis (AD; $n = 32$), food allergy (FA; $n = 24$), wheezing (W; $n = 6$), or infants with >1 atopic condition (>1; $n = 11$). miRNA, microRNA.

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 >1 atopic condition. The majority of infants with atopy (42/73, 57%) experienced symptom onset after the age of 6 mo. There were no differences (P <0.05) in maternal traits, infant traits, or environmental exposures between infants with atopy ($n = 73$) and infants without atopy ($n = 90$).

Milk miRNA consumption

Human milk samples collected from mothers of infants with atopy and mothers of infants without atopy did not differ (P >0.05) in collection time (12:39 ± 3:33 compared with 12:38 ± 3:07), total RNA counts ($6.4 \times 10^6 \pm 5.0 \times 10^5$ compared with $7.1 \times 10^6 \pm 6.0 \times 10^5$), or RNA quality (34.6 ± 0.1 compared with 34.6 ± 0.2). Infants who did not develop atopy consumed higher concentrations of miR-375–3p (d = 0.18, $P = 0.022$, adj $P = 0.044$) and miR-148b-3p (d = 0.23, $P = 0.007$, adj $P = 0.028$), but not miR-21–5p (d = 0.15, $P = 0.049$, adj $P = 0.65$), or miR-146b-5p (d = 0.11, $P = 0.10$, adj $P = 0.10$) (**Figure 2**A). Atopy subgroups displayed no difference in consumption of miR-148b-3p ($X^2 = 12.2$, $P = 0.015$, adj $P = 0.060$), miR-375–3p ($X^2 = 7.58$, $P = 0.10$, adj $P = 0.20$), miR-146b-5p ($X^2 = 4.59$, $P = 0.033$, adj $P = 0.33$), or miR-21–5p ($X^2 = 5.10$, $P = 0.27$, adj $P = 0.36$) (Figure 2B). Among the 85 additional miRNAs that did not constitute our a priori hypothesis, 15 displayed nominal differences in consumption between infants who developed atopy and peers who did not (raw $P < 0.05$), but none withstood multiple testing correction (**Supplementary Table 1**).

Modeling atopy risk

The consumption of miR-375–3p in human milk was associated with reduced atopy risk ($X^2 = 5.7$, $P = 0.017$, OR: 0.92, 95% CI: 0.86, 0.99) (**Figure 3**A). Addition of miR-148b-3p consumption improved the model ($X^2 = 3.9$, $P = 0.046$), but miR-148b-3p consumption was not significantly associated with atopy risk reduction (OR: 0.40, 95% CI: 0.15, 1.03; Figure 3B). Maternal age ($X^2 = 4.1$, $P = 0.047$, OR: 1.09, 95% CI: 1.001, 1.20) was the only medical, demographic, or environmental factor associated with atopy risk (**Supplementary Table 2**).

Factors impacting miR-375–3p concentrations in human milk

Concentrations of miR-375–3p in human milk displayed significant changes over the course of lactation (**Figure 4**A). Concentrations of miR-375–3p increased from early milk (4 ± 2 d postpartum) to transitional milk (39 ± 11 d postpartum), and remained elevated in mature milk (128 ± 8 d postpartum; 0.46, F = 132.3, $P = 8.4 \times 10^{-34}$). Concentrations of miR-375–3p also displayed an interaction between lactation stage and atopy status (F = 6.5, $P = 0.002$). Concentrations of miR-375–3p were highest in the mature milk from mothers of infants without atopy,

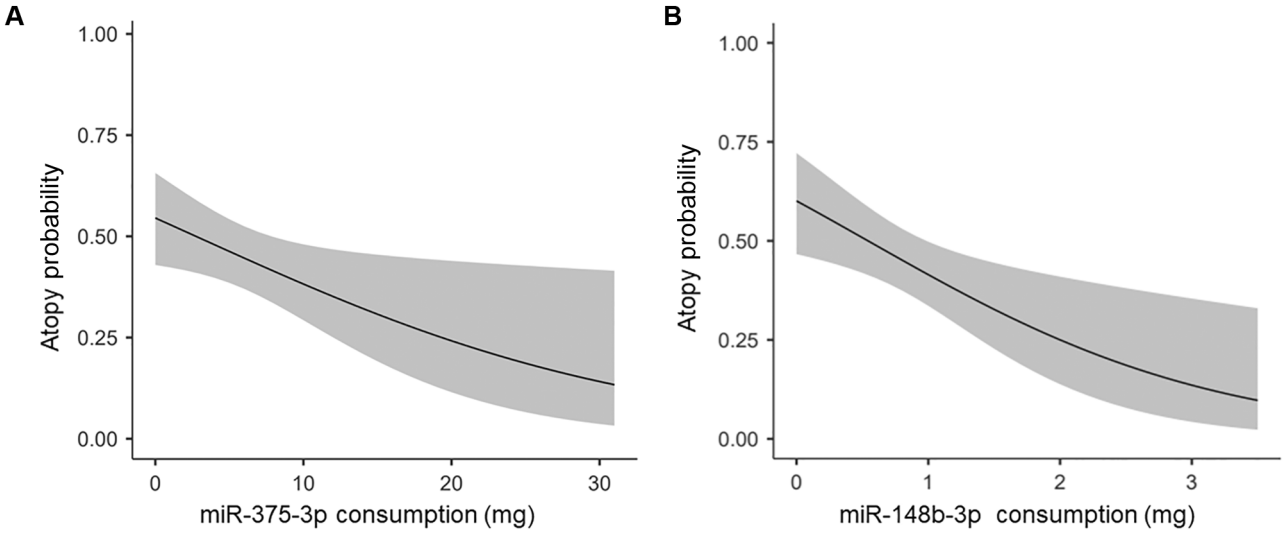

FIGURE 3 Consumption of miR-375–3p in human milk is associated with reduced atopy risk. A binomial regression controlling for medical, demographic, and environmental risk factors related to infant atopy revealed that consumption of miR-375–3p was associated with reduced atopy risk ($X^2 = 5.7$, $P = 0.017$, OR: 0.92, 95% CI: 0.86, 0.99) (A). Addition of miR-148b-3p consumption improved the model ($X^2 = 3.9$, $P = 0.046$), but miR-148b-3p consumption was not significantly associated with atopy risk reduction (OR: 0.40, 95% CI: 0.15, 1.03; B). This analysis was based on 73 infants with atopy and 90 infants without atopy. Marginal means plots with 95% CIs are shown.

and lowest in the early milk of mothers of infants with atopy (mean difference = 1.3 ± 0.15, t = 8.53, $P = 0.001$).

The relation between miR-375–3p concentrations and modifiable maternal characteristics (i.e. tobacco use, diet, BMI) were assessed with a linear mixed effects models (**Supplementary Table 3**). Concentrations of miR-375–3p in human milk were inversely associated with maternal BMI (r = –0.11, F = 4.7, t = –2.1, $P = 0.032$) (Figure 4B). No other modifiable maternal characteristics were associated with miR-375–3p concentrations.

Discussion

This study is the first to demonstrate that infant consumption of human milk miRNAs may provide protection against atopic

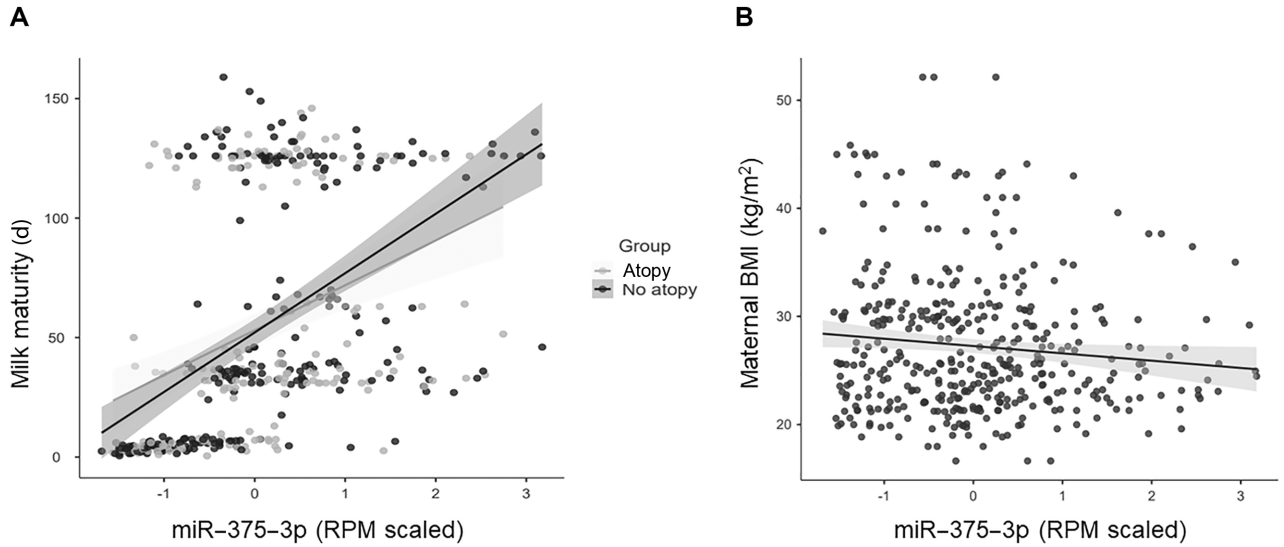

FIGURE 4 Concentrations of miR-375–3p in human milk are impacted by lactation stage and maternal body mass. A repeated measures ANOVA revealed a significant effect of lactation stage on miR-375–3p concentrations (F = 132.3, $P = 8.4 \times 10^{-34}$). A scatter plot with linear trend line and 95% CIs are shown for infants with atopy (gray; $n = 73$) and infants without atopy (black; $n = 90$; A). Concentrations of miR-375–3p displayed a significant interaction (F = 6.5, $P = 0.002$) between lactation stage and atopy status. Concentrations of miR-375–3p were higher in the mature milk consumed by infants without atopy. Linear mixed effects models were used to assess the longitudinal relation between miRNA concentrations in 432 human milk samples and modifiable maternal traits. The scatter plot and trend line display an inverse relation (r = –0.11) between maternal BMI (kg/m^2) and milk miR-375–3p concentrations (F = 4.7, t = –2.1, $P = 0.032$) on fixed omnibus testing (B). RPM, reads per million.

disease development. Specifically, the consumption of miR-375–3p over the first 6 mo was associated with reduced risk of AD, food allergies, and wheezing in the first year of life. The consumption of miR-375–3p was more strongly associated with atopic conditions 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 human milk.

Currently, infant formula contains no human miRNA (39), and what little bovine miRNA survives the pasteurization process is unlikely to have a bioactive impact on the developing infant (40). The addition of synthetic miR-375–3p to formula might ameliorate the disparities in atopic outcomes reported between formula-fed and breastfed infants (4, 8), In contrast to formula, miR-375–3p is present in over 99% of all human milk samples and constitutes just under 1% of all miRNA in breastmilk (29).

This study shows that concentrations of miR-375–3p increase over the course of lactation, which may explain why sustained breastfeeding has displayed an association with reduced atopic conditions in some studies (5, 6). Intriguingly, miR-375–3p concentrations were lower in mothers with elevated BMI. This could explain previous findings linking maternal weight with infant AD and wheezing outcomes (41). It could also provide an opportunity to enhance the atopic benefits associated with breastfeeding through targeted interventions aimed at increasing miR-375–3p concentrations via maternal weight control (42).

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 (9, 17, 22, 38, 43). 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 (22, 44–51). A study of cultured esophageal tissue from children with eosinophilic esophagitis found that miR-375 concentrations were repressed by IL-13, and that miR-375 was inversely related to the concentration of eosinophils and the expression of mast-cell-specific genes (44). 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 (22).

A number of studies have examined the mechanism of action for miR-375, and collectively, these studies provide compelling evidence for miR-375 as a regulator of the developing immune system. For example, miR-375 binds directly to *Janus kinase 2* (*JAK2*) (52) and represses translation, which has been implicated in atopy-associated signal transduction by human thymic stromal lymphopoietin receptors (53, 54), and asthma-related signal transduction by platelet-derived growth factors (55). In addition, pharmacologic manipulation of the miR-375/*JAK2* interaction has been shown to impact inflammatory signaling and gastrointestinal pathology (56–58). In fact, inhibition of JAK pathways is a novel approach being explored for treatment of AD (59). The results of this study suggest that nutritional miR-375 may provide some of the same protective benefits to developing infants. We note that infants without atopy also consumed higher concentrations of miR-148b-3p. Given that tolerance acquisition in children with allergies is related to epigenetic control of *FOXP3* (60), and miR-148b-3p is known to target *forkhead box* transcripts and *DNA methyltransferase 1*, this milk miRNA may also play an important role in atopy protection.

To our knowledge, only one prior study has investigated the relation between human milk miRNAs and child atopy (45). A retrospective study by Simpson and colleagues (2015) used RNA sequencing to measure miRNA concentrations in mature milk from 54 females, and found no association with infant AD at the age of 2 y. However, this important study established that atopy-related miRNAs (e.g. miR-375–3p) were highly present in human milk and formed the basis for our hypothesis-driven investigation. Our ability to detect a relation between milk 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 6 mo. This approach controls for changes in miRNA concentration over the course of lactation, the small RNA concentration within each milk sample, and the quantity of milk consumed during each lactation stage. However, it assumes that the proportion of miRNAs within the small RNA fraction is stable across samples and does not assess the influence of total miRNA quantity. Both assumptions may introduce variability to the calculation of miRNA consumption.

There are several additional limitations of the study design. 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 mo 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 by regressing group differences toward the mean. Despite the importance of exosomes in miRNA transport (39, 46), we did not specifically isolate exosomal RNA. However, we note that the majority of miRNAs in human milk are contained within exosomes (40) and that lipid fractions are likely to contain additional sources of encapsulated miRNAs that may survive the digestive tract.

In conclusion, this study demonstrates that infant consumption of miR-375–3p in human milk 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 breastfeeding enhances atopy protection. An indirect relation between milk miR-375 concentrations and maternal BMI suggests that maternal weight reduction could potentially enhance the protective influence of human milk on infant atopy. Additional studies are necessary to confirm this important relation and determine if oral administration of synthetic miR-375 conveys atopy protection in translational models.

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The authors' responsibilities were as follows—SDH: designed the research plan and was responsible for study oversight; DC, KW, and AC: conducted the research through data collection and sample processing; SDH: analyzed the data; SDH, DC, and RB: wrote the manuscript; and all authors: read and approved the final manuscript.

Data Availability

Data described in the manuscript has been made publicly and freely available without restriction through the Gene Expression Omnibus repository (GSE192543).

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