

Letter to the Editor

microRNA in native and processed cow's milk and its implication for the farm milk effect on asthma

To the Editor:

Consumption of unprocessed milk reduces the risk of asthma and atopic sensitization by 30% to 50%.¹ Already in the first year of life, consumption of unprocessed milk protects against respiratory tract infections.² However, consumption of raw milk is no solution because it bears a risk of potentially life-threatening infections.² Hence there is a need to identify those components of native cow's milk that carry this asthma- and allergy-protective, as well as anti-infectious, effect. A candidate molecule class with relation to immune functions³ are the microRNAs (miRNAs), which have been described in both cow's milk and human breast milk.³

The aim of this study was to screen differently processed milk types originating from the same 3 batches of native cow's milk (see Table E1 in this article's Online Repository at www.jacionline.org) for miRNA expression and to investigate possible interference of differentially expressed miRNAs with genes and pathways implied in the pathogenesis of asthma and allergic diseases. To reflect the entire spectrum of milk treatment, we subjected the milk samples to 5 different procedures usually applied during industrial processing (see Table E2 in this article's

Online Repository at www.jacionline.org): separation/centrifugation resulting in a skim milk fraction (SKI) and a fat fraction (FAT), homogenization of whole milk (HOM), pasteurization (PAS), ultraheat treatment (UHT), and heat treatment for extended shelf life (ESL) of whole milk without homogenization. We then compared these samples to whole raw (RAW) or boiled (BOI) milk samples with respect to essentially all miRNA species detectable in milk by using next-generation sequencing (NGS). We focused on the human miRNA homologues because they are more completely covered in databases than their bovine counterparts and because we were ultimately interested in their effect on the human organism.

All samples yielded a total RNA content of more than 361.8 ng, with raw milk containing the highest amount (see Table E3 in this article's Online Repository at www.jacionline.org). NGS resulted in 303,106 reads for all 24 samples, including various types of small RNA reads, such as miRNA, Rfam filtered (rRNA, transfer RNA [tRNA], small nuclear [snRNA], and small nucleolar [snoRNA]), unaligned, and trimmed sequences. The distribution of these types of small RNA did not vary between farms (Fig 1, A) but with milk type (Fig 1, B). In high heat-treated milks, RNA reads with the length of 22 nucleotides typical for miRNA were strongly reduced (see Fig E1 in this article's Online Repository at www.jacionline.org), as illustrated for miR-148a-3p, the most abundant miRNA species in milk (Fig 1, C).

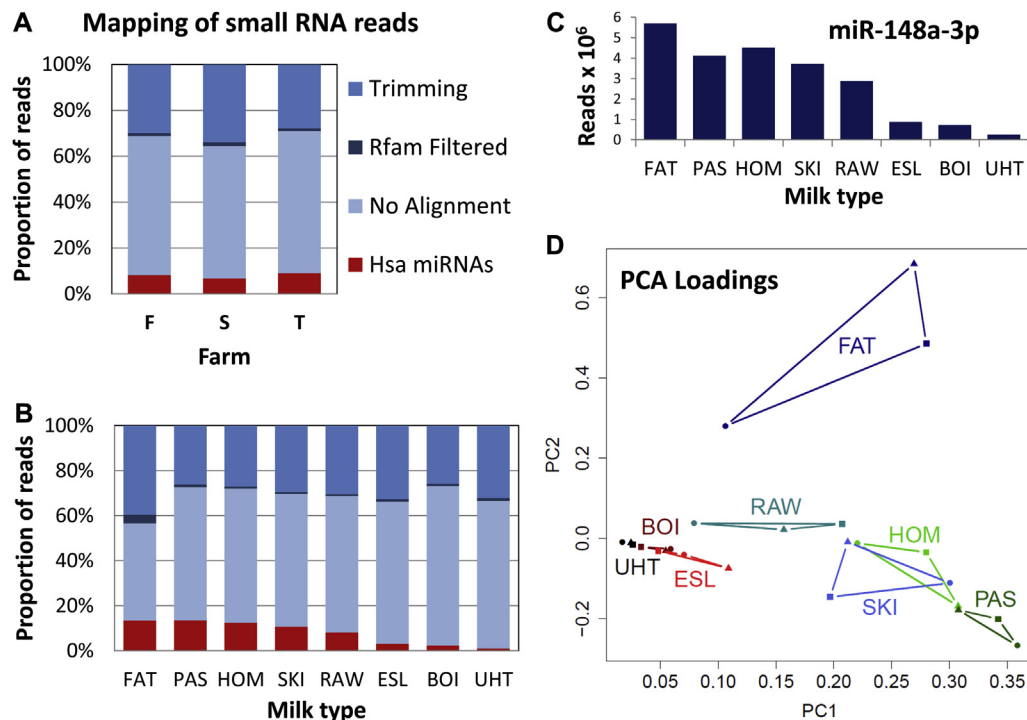


FIG 1. PCA. **A**, Mapping of small RNA reads across farms. Relative frequencies of reads are shown for the respective farms (F, S, and T). **B**, Mapping of small RNA reads across milk fractions. BOI, boiled milk; ESL, high-heat treatment for extended shelf life; FAT, fat fraction after separation; HOM, homogenized nonpasteurized milk; PAS, pasteurized nonhomogenized milk; RAW, native raw milk; SKI, skim milk after separation; UHT, ultra-high heat-treated milk. **C**, Distribution of reads of the most abundant miRNA species in milk (abbreviations as in Fig 1, B). **D**, Loading of the first 2 components of the analyzed milk samples. Rectangles, circles, and triangles represent samples from farms F, S, and T, respectively. Samples of the same milk type are linked by lines (abbreviations as in Fig 1, B).

TABLE I. Candidate genes targeted by differentially expressed miRNAs

Gene symbol	Gene name	Interfering miRNAs
<i>STAT3</i>	Signal transducer and activator of transcription 3	let-7e-5p miR-20a-5p* miR-21-5p miR-92a-5p miR-93-5p* miR-125b-5p* miR-155-5p*
<i>PTGS2</i>	Prostaglandin-endoperoxide synthase 2S	let-7b-5p miR-16-5p miR-26b-5p miR-101-3p miR-128-3p miR-181a-5p
<i>IL13</i>	IL-13	let-7d-5p let-7f-5p let-7g-5p let-7i-5p miR-98-5p
<i>IL6</i>	IL-6	let-7a-5p miR-26a-5p miR-365a-3p miR-98-5p miR-155-5p
<i>IFNG</i>	IFN- γ	miR-15a-5p miR-16-5p miR-26b-5p miR-29b-3p
<i>IFNGR1</i>	IFN- γ receptor 1	miR-98-5p miR-155-5p miR-181b miR-196a

Genes targeted by more than 3 miRNA species were differentially expressed with respect to high-heat treatment.

*These 4 miRNA species interfering with *STAT3* were also differentially expressed with respect to fat content.

For data reduction of the less abundant miRNA species (see Fig E2 in this article's Online Repository at www.jacionline.org), principal component analysis (PCA) was applied. The first component (PC1) explained 97.5% of total variance and differentiated well between high heat-treated milk samples (BOI, ESL, and UHT) and raw (RAW, SKI, and HOM) or pasteurized (PAS) samples, whereas the second component (PC2) explained 2.2% of the variance and separated the fat samples from all other samples (Fig 1, D, and see Fig E3 in this article's Online Repository at www.jacionline.org). Validation of NGS results by using RT quantitative PCR (qPCR) revealed high correlation and agreement between both methods (see Fig E4 and Table E4 in this article's Online Repository at www.jacionline.org). Differential expression of miRNAs was found for 52 miRNA species between pasteurized and UHT milk and for 25 species between pasteurized whole milk and raw milk fat (see Table E5 in this article's Online Repository at www.jacionline.org). Possible targets of the differentially expressed miRNA species were derived from the miRTarBase database, which relies on experimentally validated effects instead of *in silico* predictions. Table I shows a selection of the most relevant genes, which are targeted by more than 3 different miRNA species.

Among those is prostaglandin-endoperoxide synthase 2S (*PTGS2*), which is involved in the synthesis of proinflammatory eicosanoids, potent mediators of allergic inflammation.⁴ Eicosanoid metabolism is also influenced by intake of ω -3 polyunsaturated fatty acids, which we found to explain a substantial part of the effect of milk fat content on asthma (Brick et al, in this issue⁵).

Signal transducer and activator of transcription 3 (*STAT3*) transduces signals of receptors of innate immunity, contributes to regulation of T helper cell differentiation, and has been implied to be a factor in impaired lung function seen in asthmatic patients.⁶ In addition, it mediates the inhibitory effects of the proinflammatory cytokine IL-6 on dendritic cells.⁷ Like *STAT3*, *IL6* itself is regulated by the pleiotropic miRNA miR-155-5p, which also interferes with *IFNGR1* (Table I).

IL-13 induces all the cardinal features of T_H2 -mediated inflammation (ie, airway hyperresponsiveness, infiltration by inflammatory cells, increased mucus production, airway remodeling, and induction of IgE synthesis).⁷ Posttranscriptional regulation of *IL13* is tuned by members of the let-7 family, and intranasal administration of let-7 mimic in a murine asthma model reduced IL-13 levels in lungs and alleviated asthma features.⁸

IFNG and *IL13* have been found to be differentially expressed between acute virus-induced exacerbations of asthma and convalescence.⁹ Decreased activation of *IFNG* signaling during episodes of asthma exacerbation has been implied in chronic airway obstruction.⁹

The higher miRNA levels of homogenized and skimmed raw milk compared with native raw milk might be partially explained by a shorter storage time because we had deliberately frozen native raw milk on the last day of processing. However, pasteurized milk was also frozen on the same day (see Table E2) and had comparably high miRNA levels as well. Pasteurizing might not affect miRNAs themselves but possibly inactivate RNases. At the usual 72°C, microvesicles, such as exosomes, are still intact and protect packaged miRNAs from degradation and inactivation. This effect might be lost by high-temperature treatment and longer heat exposure, as applied in boiling and ESL/UHT milk.

Because of the strong analogy between human and bovine miRNAs, the presence of these molecules in raw milk and particularly the fat fraction might be accountable for a part of the beneficial effect of farm milk consumption on asthma and allergic diseases. A potential uptake of milk miRNAs has been shown in cell-culture experiments and after human consumption.¹⁰ However, raw milk is not an option for future population-wide prevention strategies because of its potential health hazard. Rather, less heat-intensive procedures in industrial milk processing might help preserve bovine miRNA from degradation and maintain its favorable properties for future prevention strategies.

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METHODS

Milk samples

Cow's milk samples from different breeds were collected from 3 farms located around Munich, Germany, and comprised milk from the morning and previous evening (see Table E1). The milk was stored at 1°C until further processing to minimize bacterial growth. The physical characteristics (ie, the parameters heat, pressure, and duration) of the different procedures applied to the 3 milk batches are shown in Table E2.

A total of 8 different milk fractions were obtained per farm. The high-heat treatment fractions were produced on the first day (ie, within 12 hours after milking). Separation, homogenization, and boiling were performed on the subsequent day, whereas pasteurization was completed on day 3. After processing, all milk fractions were immediately frozen at −20°C. The native raw milk samples were frozen on day 3 to avoid potential bias caused by reduced enzymatic degradation processes as a result of early freezing.

Purification of total RNA

Total RNA, including small RNA, was extracted in triplicate from all milk fraction samples by using the miRNeasy Mini Kit (Qiagen, Hilden, Germany). Milk samples were thawed on ice, chloroform was added at a ratio of 1:5, and isolation was continued according to the manufacturer's instructions. Total RNA was eluted in 30 µL of RNase-free water, and RNA concentrations were measured with the Qubit 2.0 Fluorometer (Life Technologies, Grand Island, NY). RNA purity was assessed by measuring absorbance levels on a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, Mass). The triplicates for each milk fraction and farm were pooled, resulting in 24 samples stored at −80°C.

Library preparation and sequencing of small RNAs

Of each milk sample, 500 ng of total RNA was converted into barcoded cDNA libraries with the NEBNext Multiplex Small RNA Library Prep Set for Illumina (New England Biolabs, Ipswich, Mass). After PCR preamplification, cDNA constructs were purified with the MinElute PCR Purification Kit (Qiagen), and PCR products were separated on a 4% agarose gel for size selection. Small RNAs of approximately 135 to 160 bp (miRNA transcript plus ligated sequencing adapters) were excised and extracted with the MinElute Gel Extraction Kit (Qiagen). Successful library preparation was verified with a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, Calif) by using the High Sensitivity DNA Kit (Agilent Technologies). The resulting cDNA libraries were subjected to the Illumina sequencing pipeline consisting of clonal cluster generation on a single-read flow cell (Illumina, San Diego, Calif), bridge amplification on the cBOT via TruSeq SR Cluster Kit v3-cBOT-HS (Illumina), and 50 cycles of sequencing-by-synthesis on the HiSeq2000 (Illumina). Sequenced reads were deposited in European Nucleotide Archive ENA under accession number PRJEB9597.

Alignment of sequencing reads

Raw read data were trimmed of 3' end adapters by using Btrim,^{E1} and reads without a detectable adapter sequence or with less than 16 nucleotides were excluded from the data set. Mean Phred quality scores per complete sequence and per sequencing position, as well as sequence length distribution, were assessed with the quality control software FastQC (Version 0.10.1; Babraham Bioinformatics, Cambridge, United Kingdom). Alignment was carried out by using the Bowtie short read aligner's "best alignment algorithm" of reads with a maximum of 1 mismatch within the first 15 nucleotides.^{E2} Read data mapping to bovine rRNA, tRNA, snRNA, and snoRNA sequences contained in the Rfam database were removed.^{E3} The remaining sequences were aligned to the most recent miRBase database (release 20) for mature human miRNAs.^{E4} Read counts were generated by sorting, indexing, and calling the sum of hits per miRNA using SAMtools.^{E5}

Validation by means of RT-qPCR

The presence of miRNA sequences discovered by means of small RNA sequencing (RNA-Seq) was verified by using RT-qPCR for 9 selected miRNAs. These miRNAs were chosen based on their frequency and relation to industrial milk processing. For cDNA synthesis, total RNA samples were polyadenylated and reverse transcribed with the miRCURY LNA universal cDNA Synthesis Kit II (Exiqon, Vedbæk, Denmark), and qPCR was done on a Rotor-Gene-Q (Qiagen) with ExiLENT SYBR Green master mix and the corresponding miRCURY LNA PCR primer sets (Exiqon). The potential effect of confirmed miRNAs on human health was evaluated by comparing them with experimentally verified miRNA targets of the miRtarbase v4.0 database,^{E6} with a focus on 139 candidate gene for asthma, atopy, and respiratory tract infections.^{E7-E10}

Statistical analysis

Raw read counts were standardized to a denominator of 10⁷ reads, thereby compensating for differences in library sizes. Data reduction of expression profiles was achieved by means of PCA with R software (www.R-project.org) and the packages pcaMethods^{E11} and NOISeq.^{E12} Correlation between sequencing and RT-qPCR results was assessed by using the Pearson correlation coefficient (*r*).

RESULTS

All samples yielded a total RNA content of more than 361.8 ng, with raw milk containing the highest amount of RNA (Table E3). Sequencing resulted in 303 × 10⁶ reads for all 24 samples. High sequencing accuracy was shown by a mean Phred score of 30 or more, corresponding to a probability of correct base call of 99.9% or greater for 99% of all reads.^{E13} The reads per sample ranged from 8.7 × 10⁶ to 15.7 × 10⁶, with an average of 12.6 × 10⁶ reads. Because of absent cDNA library adapter sequences or shorter read lengths than 16 nucleotides, 3.9 × 10⁶ ± 0.9 × 10⁶ reads (mean ± SD) per sample were excluded. Additionally, 0.17 × 10⁶ ± 0.15 × 10⁶ reads per sample were removed (Rfam filtered) from further analysis because they mapped to bovine rRNA, tRNA, snRNA, or snoRNA sequences. Of the remaining reads, 1.01 × 10⁶ ± 0.72 × 10⁶ aligned against 1796 of all 2578 known mature human miRNAs. Of those miRNAs, 155 were sufficiently abundant for subsequent analyses, with 1200 reads overall corresponding to an average of 50 reads per sample.

The distribution of types of small RNA reads, such as miRNA, Rfam filtered (rRNA, tRNA, snRNA, and snoRNA), unaligned, and trimmed sequences, did not vary between farms (Fig 1, A). However, milk processing affected the composition of small RNA sequences (Fig 1, B): high heat-treated milks (BOI, ESL, and UHT) had substantially reduced miRNA read counts (5.51-fold, *P* = .0012). A similar pattern was found by comparing read lengths across milk types (Fig E1): relative mean read counts clearly peaked at a nucleotide length of 22, corresponding to the typical size of miRNAs, in all samples except the high heat-treated samples (BOI, ESL, and UHT). The proportion of aligned miRNAs among all reads with a length of 22 nucleotides was much lower in high heat-treated samples (BOI, ESL, and UHT), indicating a large proportion of degraded RNA in milk samples.

The most common miRNA sequence (36% of all reads) aligned to miR-148a (Fig E2) and was expressed in high heat-treated milk samples with substantially reduced read counts (Fig 1, C). All other sequences were much less abundant (<10% of all reads) and were thus analyzed by using a PCA based on the transposed

matrix of miRNA species and milk types. The first component (PC1) explained 97.5% of the total variance and differentiated well between high heat-treated milk samples (BOI, ESL, and UHT) and raw (RAW, SHI, and HOM) or pasteurized (PAS) samples, whereas the second component (PC2) explained 2.2% of the variance and separated the fat samples from all other samples (Fig 1, D). PC1 was mainly determined by let-7a-5p, let-7b-5p, miR-200c-3p, miR-30a-5p, and miR-22-3p; PC2 was predominantly influenced by miR-22-3p and miR-30a-5p (Fig E3). For further analyses, we used the milk samples that were separated most by the first 2 components: UHT, PAS, and FAT.

The validation of small RNA-Seq results by using RT-qPCR revealed high correlation and agreement between both methods (Table E4), as shown in Fig E4 for high, medium, and low levels of miRNA species, respectively.

Differential expression of miRNAs was found for 52 miRNA species between pasteurized and UHT milk and for 25 species between pasteurized whole milk and raw milk fat (Table E5). The strongest effects by heat treatment ($\log_2$ fold change >4) were found for miR-125b-5p, miR-101-3p, miR-27b-3p, miR-365a-3p, miR-125a-5p, miR-30c-5p, miR-128-3p, miR-151a-3p, miR-26b-5p, miR-192-5p, let-7b-5p, and let-7a-5p, whereas the strongest effects by fat content ($\log_2$ fold change >2) were found for miR-30c-5p, miR-125a-5p, miR-200c-3p, and miR-125b-5p.

Genes targeted by 4 or more miRNA species differentially expressed with respect to high heat treatment and/or fat content comprised *STAT3* (7 times) and *PTGS2* (6 times), followed by *IL6* and *IL13* (each 5 times) and *IFNG* and IFN- γ receptor 1 (*IFNG* and *IFNGR1*; each 4 times; Table I). For differences between pasteurized whole milk and the fat fraction, *STAT3* also appeared most frequently, with 4 occurrences (Table I). Differential miRNA expression between the respective milk samples was also confirmed by using RT-qPCR for all miRNA species listed in Table E4 (data not shown).

DISCUSSION

Batches of milk samples from 3 different farms were processed by using different procedures, resulting in 8 milk fractions. From all 24 samples, total RNA was extracted, selected for size, and sequenced for short reads (small RNA-Seq). After removal of noninformative reads, the remaining miRNA sequences were equally distributed across farms but occurred much less frequently in all high heat-treated milk samples. Most of the variance in the data set was explained by the discrepancy between high heat-treated and raw or pasteurized samples, particularly in the expression of let-7a-5p, let-7b-5p, and miR-200c-3p. Some variance was also attributable to the difference between whole milk and the fat fraction, particularly by miR-22-3p. High-heat treatment affected miRNAs interfering with the genes *STAT3*, *PTGS2*, *IL6*, *IL13*, *IFNG*, *IFNGR1*, and others. Also, fat separation reduced expression of miRNAs interfering with *STAT3*.

In a related manuscript (Brick et al, in this issue^{E14}) we demonstrate that the protective effect of raw milk consumption on asthma can partially be attributed to a higher fat content of farm milk and particularly to higher levels of ω -3 polyunsaturated fatty acids. The effect of fat content seems to protect predominantly against mild and reversible forms of asthma. However, a similar or even stronger effect is clearly related to the contrast between

raw milk and high heat-treated milk, particularly UHT milk, which is often used as a reference category in epidemiologic studies.^{E15-E17} Here we also included ESL milk because it increasingly replaces traditionally pasteurized milk for the convenience of longer shelf life but has been studied rarely in epidemiologic studies.

Mild heat treatment, such as pasteurization, still maintains some but not all beneficial properties, as demonstrated in the GABRIELA study, in which pasteurized milk halved the risk of asthma similarly to raw farm milk, although it narrowly missed statistical significance.^{E18} In contrast, boiled farm milk had no effect on asthma insofar as it was comparable with UHT milk. On atopic sensitization, hay fever, and eczema, only unboiled farm milk exerted a protective effect. In infancy respiratory tract infections were prevented by farm milk irrespective of boiling, whereas fever episodes were additionally prevented by pasteurized milk.^{E17}

Superficially, the described pattern seems to be inconsistent. On a closer look, however, it reflects a multitude of protective effects on closely related but distinct disease entities. In addition, milk is a rather complex mixture of organic molecules, with rather specific functions reflecting the entire evolution of mammals. Consequently, unravelling the underlying variants of the “milk effect” is rather challenging. For a first approach, we examined well-known and clearly defined molecule classes, such as whey proteins,^{E18} fatty acids,^{E14} and miRNAs.

Thus far, miRNAs have been described in human breast milk^{E19} and cow's milk.^{E20} These small regulatory molecules circulate in body fluids and regulate physiologic and pathologic processes by interfering with mRNA. In milk and other body fluids they are stored and transported in microvesicles,^{E20} which renders them resistant to RNase and acidic conditions, such as those found in gastric fluid. The majority of miRNA species are related to immune functions^{E19}; are differentially expressed in T_H1, T_H2, and regulatory T cells^{E21}; and have even been implicated in mediating suppressive effects of regulatory T cells on T helper cells.^{E22}

The primary finding of this analysis was a substantial reduction of miRNA levels in high heat-treated milk samples. Infant formula, which is strongly heated, has been reported to contain only minimal miRNA residues.^{E23} Rather unexpectedly, pasteurized, homogenized, and skimmed milk contained higher levels compared with raw milk. For homogenized and skimmed milk, this might be explained in part by a shorter storage time until freezing because we had deliberately frozen raw milk on the last day of milk processing to avoid any bias caused by early freezing of raw milk. However, pasteurized milk was also frozen on the same day (Table E2), thereby suggesting an individual effect of pasteurization. A potential explanation might be found in the rather mild temperature of 72°C not affecting miRNAs themselves but possibly inactivating RNases. Moreover, microvesicles, such as exosomes, which contain and protect miRNA from degradation and inactivation, are still intact at 72°C.^{E13} In contrast, high-temperature treatment and longer heat exposure as applied in boiled and ESL/UHT milk might destroy protective microvesicles. Nevertheless, we have to acknowledge that the raw milk samples of farm S almost clustered with the high heat-treated samples in the PCA, and therefore we cannot exclude artificial degradation of miRNA in this sample caused by contamination during storage. Yet the most abundant miRNA in milk, miR-148a-3p, was found at higher RT-qPCR

levels in pasteurized compared with raw milk in the other 2 farms (Fig E4, A); ultimately, this argues in favor of some preservation of miRNAs by pasteurization. Obviously, miRNA levels vary with the circadian rhythm and lactation status^{E23}; however, we assessed milk samples pooled over morning and evening milk of more than a dozen cows per farm, thereby leveling out such potentially confounding effects.

NGS with small RNA-Seq was chosen as an approach to screen for essentially all miRNA species present in milk. We focused on the human miRNA homologues because they are more completely covered in databases than their bovine counterparts and because we were ultimately interested in their effect on the human organism. The use of NGS as a fully quantitative method is questionable, and therefore we validated it against RT-qPCRs targeting mRNA species with low, medium, and high abundance. The strong agreement of both methods justified the approximation by read counts in the quantitative models. For data reduction, we performed a PCA and used the strongest contrasts (ie, the milk types at the extremes of the first 2 dimensions [PAS, UHT, and FAT]) for the subsequent analyses. In addition, we deliberately avoided raw milk as a comparator because it is not an option for future population-wide prevention strategies because of its potential health hazard. In light of the high number of detected miRNAs ($n = 155$) and our limited sample size ($n = 24$), we performed association analyses on the transposed data matrix, which enabled us to correct for multiple testing.

For the prediction of candidate genes targeted by the detected miRNAs, we used the mirTarbase v4.0 database,^{E6} which relies on experimentally validated effects instead of *in silico* predictions. Our list of detected gene targets is obviously influenced by the choice of database and the selective focus of the scientific community on immunity genes. Nevertheless, we consider the resulting pool of miRNAs and their target genes as a helpful starting point for further confirmatory analyses in population-based samples.

The detection of *PTGS2* as a major target of several differentially expressed miRNAs points to an interference with the synthesis of proinflammatory eicosanoids, such as prostaglandin D₂, a potent mediator of allergic inflammation.^{E24} The metabolism of eicosanoids is also influenced by the intake of ω -3 polyunsaturated fatty acids, which we found to explain a substantial part of the effect of milk fat content on asthma.^{E14}

STAT3 transduces signals of receptors of innate immunity, contributes to regulation of T_H cell differentiation, and has been implicated in impaired lung functions in asthmatic patients.^{E25} In addition, it mediates the inhibitory effects of the proinflammatory cytokine IL-6 on dendritic cells.^{E8} IL-6 itself is a key cytokine of inflammation, is involved in airway obstruction in neonates,^{E26} and triggers C-reactive protein release in the liver.^{E27} In the PASTURE birth cohort we found high-sensitivity C-reactive protein as a marker of low-grade inflammation to be inversely related with the consumption of raw milk in infancy^{E17} and with the ω -6/ ω -3 polyunsaturated fatty acid ratio of cow's milk consumed at preschool age.^{E14} Like *STAT3*, *IL6* itself is regulated by the pleiotropic miRNA miR-155-5p, which also interferes with *IFNGR1* (Table E5). The role of miR-155-5p in this context is possibly ambivalent because it has been described to rather enhance type 2 immunity.^{E28} Furthermore, *IL6* shares effects of miR-98-5p with *IL13* and *IFNGR1*, thereby suggesting some redundancy in the effect of milk-born miRNAs.

IL13 has a similarly important role but on the effector side of allergic inflammation; it induces all the cardinal features of T_H2-mediated inflammation (ie, airway hyperresponsiveness, infiltration by inflammatory cells, increased mucus production, airway remodeling, and induction of IgE synthesis).^{E8} Polymorphisms in the *IL13* gene are associated with both childhood-onset asthma and serum IgE levels.^{E9} Posttranscriptional regulation of *IL13* is tuned by members of the let-7 family, and intranasal administration of let-7 mimic in a murine asthma model reduced *IL13* expression in the lungs and alleviated asthma features, such as airway hyperresponsiveness, airway inflammation, and goblet cell metaplasia.^{E29}

Finally, the primary T_H1 cytokine *IFNG* and its receptor *IFNGR1* appear on the list of target genes. Both, *IFNG* and the T_H2-related cytokine *IL13* have been found to be differentially expressed between acute virus-induced exacerbations of asthma and convalescence.^{E10} Decreased activation of *IFNG* signaling during episodes of asthma exacerbation has been implicated in chronic airway obstruction.^{E10} In experimental rhinovirus infection in human subjects, *IFNG* production by T cells was associated with lower viral load and less severe common cold symptoms.^{E30} Respiratory syncytial virus infection resulted in increased *IFNG* levels, thereby prompting activation of natural killer and T_H cells.^{E31} A possible mechanism might be found in miR-15b-induced upregulation of *IFNG* production by human natural killer cells.^{E32}

Altogether, the common denominator of the miRNA target genes seems to be their involvement in the inflammatory response rather than other features of asthma. This notion is in line with our observation that consumption of unprocessed milk is less likely to affect severe forms of asthma; rather, it might temper airway inflammation and alleviate features of asthma possibly even before they manifest clinically.^{E14}

In summary, we found the contents and composition of miRNA in cow's milk to be substantially altered by high-heat treatment. A distinct set of miRNAs was enriched in the fat fraction. Several of the differentially expressed miRNA species are known to interfere with mRNAs coded by genes previously implicated in the development of asthma and allergies. Because of the strong analogy between human and bovine miRNAs, the presence of these molecules in native milk and particularly the fat fraction might be responsible for part of the beneficial effect of farm milk consumption on asthma and allergies. Less heat-intensive procedures in industrial milk processing might help preserve bovine miRNAs against degradation and maintain their favorable properties for future prevention strategies.

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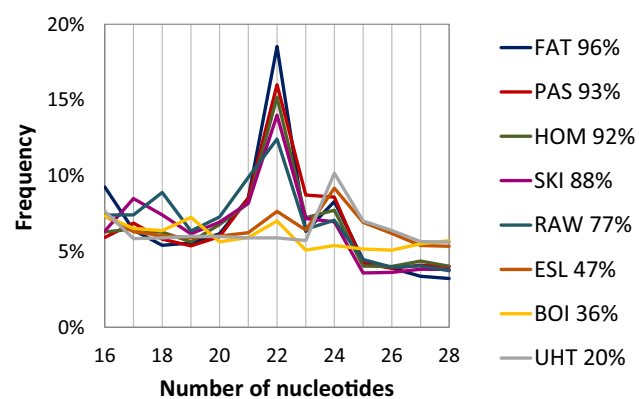


FIG E1. Distribution of RNA read length across milk types. Distributions of RNA read length are shown for the respective milk types. Percentages refer to proportion of read counts mapping to mature human miRNA sequences.

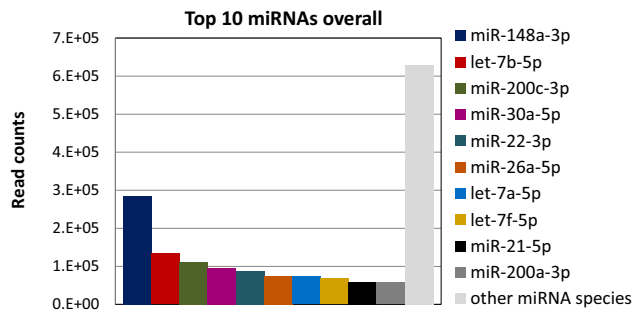


FIG E2. The most abundant miRNA species.

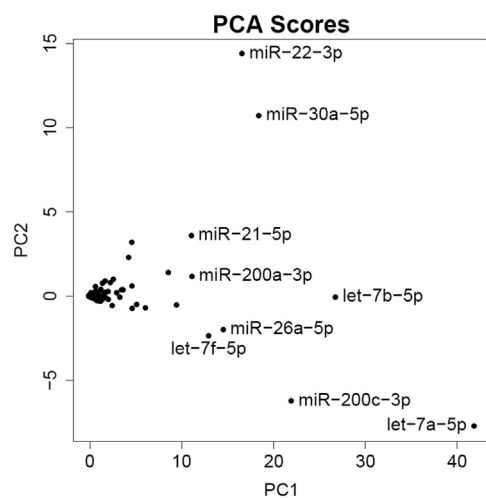


FIG E3. PCA scores. Scores per 104 reads of the first 2 components (PC1 and PC2) represent the detected miRNA species. Names are given only for species scoring high on either component.

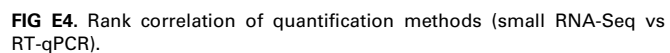


TABLE E1. Sources of raw milk

	Farm		
	T	F	S
Farming type	Conventional	Conventional	Organic
Cattle breed	Fleckvieh	Brown Swiss	Fleckvieh
Feeding	Corn silage, grass silage, concentrate	Corn silage, concentrate	Grass, hay, wheat
No. of cows	13	60	30
Time point of milking for pooled samples	Morning and evening	Morning and evening	Morning and evening
Microbial contamination (CFU/mL), mean $\pm$ SD	18,500 $\pm$ 7,800	7,300 $\pm$ 3,200	4,600 $\pm$ 3,800

CFU, Colony-forming unit.

TABLE E2. Treatment of milk samples

Milk fraction		Processing conditions	Day of processing
UHT	Ultra-high heat treated	Preheating at 93°C for 23 s, direct steam injection at 142°C for 5 s	1
ESL	Extended shelf life	Preheating at 95°C for 20 s, direct steam injection at 127°C for 5 s	1
BOI	Boiled milk	Preheating at >80°C for >300 s, boiling at 100°C for 30 s	2
HOM	Homogenized milk	Preheating to 55°C, 2-stage homogenization at 250/50 bar	2
SKI	Skim milk	Separation at 50°C	2
FAT	Fat fraction	Separation at 50°C	2
PAS	Pasteurized milk	72°C for 20 s	3
RAW	Native raw milk	—	3

TABLE E3. RNA yields stratified for milk type

Milk type	RNA concentration (ng/ μ L)	Coefficient of variation
UHT	12.00	8%
ESL	8.89	50%
PAS	14.57	18%
HOM	17.77	20%
FAT	24.30	50%
SKI	17.10	11%
BOI	12.30	25%
RAW	46.33	11%

TABLE E4. Correlation of small RNA-Seq and RT-qPCR results

miRNA	Spearman correlation
let-7a-5p	0.88
let-7i-5p	0.83
miR-21-5p	0.79
miR-22-3p	0.92
miR-30a-5p	0.89
miR-92a-3p	0.83
miR-140-3p	0.81
miR-148a-3p	0.88
miR-200c-3p	0.84

TABLE E5. Differentially preserved miRNA levels between PAS, UHT, and FAT milk

miRNA	PAS vs UHT	PAS vs FAT	Targeted candidate genes
let-7a-5p	4.01	1.54	<i>IL6, ITGB3, VDR</i>
let-7b-5p	4.03		<i>IFNB1, PTGS2</i>
let-7d-5p	3.67	1.80	<i>IL13</i>
let-7e-5p	3.59		<i>STAT3</i>
let-7f-5p	3.81	1.64	<i>IL13</i>
let-7g-5p	3.88		<i>IL13</i>
let-7i-5p	3.48		<i>IL13, TLR4</i>
miR-15a-5p	2.67	−2.40	<i>IFNG</i>
miR-16-5p	2.84		<i>DAP3, IFNG, PTGS2</i>
miR-17-5p	3.65	1.23	<i>SMAD3</i>
miR-19b-3p	2.48		<i>TLR2</i>
miR-20a-5p	3.59	1.68	<i>STAT3</i>
miR-21-5p	3.90		<i>IL1B, STAT3, TGFB1, TLR4</i>
miR-24-3p	3.57		<i>TGFB1</i>
miR-25-3p	3.18	1.20	<i>ACPI, CCL26</i>
miR-26a-5p	3.94		<i>IFNB1, IL6</i>
miR-26b-5p	4.11		<i>AGT, C3, GSTP1, HLA-DQB2, IFNG, IFNGR2, IL3, MUC7, PTGS2, STAT6, TIMP1</i>
miR-27b-3p	4.48		<i>EDNRA, VDR</i>
miR-29b-3p	3.33		<i>IFNG, TBX21</i>
miR-30c-5p	4.34	2.50	<i>ITGB3</i>
miR-30d-5p	3.25		<i>IRF2</i>
miR-34a-5p	3.50		<i>IFNB1</i>
miR-92a-3p	3.57		<i>CTLA4, GATA3, GSTP1, RORA, STAT3, TLR1</i>
miR-93-5p	3.08	1.15	<i>IRF1, STAT3</i>
miR-98-5p	3.58		<i>C3, CCL5, IFNGR1, IL13, IL6, ITGB3</i>
miR-101-3p	4.65		<i>PTGS2</i>
miR-103a-3p	3.48		<i>IFNGR2</i>
miR-106a-5p	3.45		<i>IL1</i>
miR-106b-5p	3.64		<i>DAP3, RORA, TLR2</i>
miR-125a-5p	4.41	2.40	<i>CCL5</i>
miR-125b-5p	4.76	2.05	<i>STAT3, VDR</i>
miR-128-3p	4.22		<i>PTGS2</i>
miR-148a-3p	3.99		<i>HLA-G</i>
miR-148b-3p	3.83		<i>ADAM33, CCL11, HAVCR1, HLA-G, PTGER2, SLC22A5</i>
miR-151a-3p	4.15		<i>ACPI</i>
miR-152-3p	3.90		<i>HLA-G</i>
miR-155-5p	3.36	1.48	<i>CTLA4, EDN1, IFNGR1, IL13RA1, IL6, IL8, NOS3, SMAD3, STAT3, VCAM1</i>
miR-181a-5p	3.65		<i>PTGS2</i>
miR-181b-5p	3.90		<i>IFNGR1</i>
miR-191-5p	3.09	1.77	<i>IL1A</i>
miR-192-5p	4.08		<i>IL15</i>
miR-196a-5p	3.53		<i>IFNGR1</i>
miR-200a-3p	3.97		<i>SMAD3, VCAM1</i>
miR-200c-3p	3.74	2.29	<i>EDNRA</i>
miR-203a	3.04	1.01	<i>EDNRA, TNF</i>
miR-215-5p	3.83		<i>IL15</i>
miR-296-3p		−1.98	<i>CHRM3</i>
miR-320a	3.33	−1.42	<i>MIF</i>
miR-365a-3p	4.44		<i>IL6</i>
miR-423-3p	3.04	1.90	<i>CYFIP2</i>
miR-744-5p	2.45	1.71	<i>MIF, TGFB1</i>
miR-769-5p	3.30		<i>MIF</i>

NOISeq analyses were done with the transposed milk sample $\times$ miRNA matrix. Changes are presented as log₂ fold change, and only significant associations corrected for multiple testing are shown.