The protective effect of farm milk consumption on childhood asthma and atopy: The GABRIELA study

Georg Loss, MSc,[a,b] Silvia Apprich, PhD,[c] Marco Waser, PhD,[a,b] Wolfgang Kneifel, PhD,[c] Jon Genuneit, MD,[d] Gisela Büchele, PhD,[d] Juliane Weber, MD,[e] Barbara Sozanska, MD,[f] Hanna Danielewicz, MD,[f] Elisabeth Horak, MD,[g] R. J. Joost van Neerven, PhD,[h] Dick Heederik, PhD,[i] Peter C. Lorenzen, PhD,[j] Erika von Mutius, MD,[e] Charlotte Braun-Fahrländer, MD,[a,b] and the GABRIELA study group* *Basel, Switzerland, Vienna and Innsbruck, Austria, Ulm, Munich, and Kiel, Germany, Wroclaw, Poland, and Deventer and Utrecht, The Netherlands*

Background: Farm milk consumption has been identified as an exposure that might contribute to the protective effect of farm life on childhood asthma and allergies. The mechanism of action and the role of particular constituents of farm milk, however, are not yet clear.

Objective: We sought to investigate the farm milk effect and determine responsible milk constituents.

Methods: In rural regions of Germany, Austria, and Switzerland, a comprehensive questionnaire about farm milk consumption and other farm-related exposures was completed by parents of 8334 school-aged children, and 7606 of them provided serum samples to assess specific IgE levels. In 800 cow's milk samples collected at the participants' homes, viable bacterial counts, whey protein levels, and total fat content were analyzed. Asthma, atopy, and hay fever were associated to reported milk consumption and for the first time to objectively measured milk constituents by using multiple regression analyses.

Results: Reported raw milk consumption was inversely associated to asthma (adjusted odds ratio [aOR], 0.59; 95% CI, 0.46-0.74), atopy (aOR, 0.74; 95% CI, 0.61-0.90), and hay fever (aOR, 0.51; 95% CI, 0.37-0.69) independent of other farm exposures. Boiled farm milk did not show a protective effect. Total viable bacterial counts and total fat content of milk were not significantly related to asthma or atopy. Increased levels of the whey proteins BSA (aOR for highest vs lowest levels and asthma, 0.53; 95% CI, 0.30-0.97), α-lactalbumin (aOR for interquartile range and asthma, 0.71; 95% CI, 0.52-0.97), and β-lactoglobulin (aOR for interquartile range and asthma, 0.62; 95% CI, 0.39-0.97), however, were inversely associated with asthma but not with atopy.

Conclusions: The findings suggest that the protective effect of raw milk consumption on asthma might be associated with the whey protein fraction of milk. (J Allergy Clin Immunol 2011;128:766-73.)

Key words: *Allergic diseases, asthma, atopy, children, farming, hay fever, microorganism, farm milk, risk, whey protein*

Discuss this article on the JACI Journal Club blog: www.jaci-online.blogspot.com.

Childhood asthma and allergies remain a major health problem in industrialized countries and increasingly in developing countries.[1] Study populations with a similar genetic background but striking differences in environmental exposures have been especially informative to clarify environmental causes for the onset of asthma and atopy. Studies focusing on differences between rural farming and nonfarming communities have consistently shown that children growing up on a farm are at significantly lower risk of asthma, hay fever, and atopic sensitization than children living in the same rural area but not on a farm.[2]

Environmental factors that have been hypothesized to explain this protective effect of farm life are contact with animals,[3,4] the diversity of microbial exposure,[5] endotoxin levels in house dust,[6] and farm milk consumption.[7-9] Exposure to farm milk in early life[8] and consumption of raw farm milk[7] have been associated with a reduced asthma and atopy risk, and it has been suggested that this protection might be mediated through receptors of the innate immune system.[10]

All previous studies on the effect of farm milk consumption have been questionnaire based and lacked objective measurements of milk components. Hence determination of the biological components associated with a protective farm milk effect is warranted.

The GABRIEL (a multidisciplinary study to identify the genetic and environmental causes of asthma in the European Community) Advanced studies program,[11] comprising a large population of European children, was established to investigate the environmental

From [a]the Swiss Tropical and Public Health Institute, Basel; [b]the University of Basel; [c]BOKU Vienna, Department of Food Science and Technology, University of Natural Resources and Life Sciences, Vienna; [d]the Institute of Epidemiology and Medical Biometry, Ulm University; [e]LMU Munich, University Children's Hospital, Munich; [f]the 1st Department of Paediatrics, Allergology and Cardiology, Wroclaw Medical University; [g]the Department of Pediatrics and Adolescents, Division of Cardiology and Pulmonology, Innsbruck Medical University; [h]Friesland Campina Research, Deventer; [i]the Institute for Risk Assessment Sciences (IRAS), Division of Environmental Epidemiology, Utrecht University; and [j]Max Rubner-Institut, Federal Research Institute of Nutrition and Food, Department of Safety and Quality of Milk and Fish Products, Kiel.

*The members of the GABRIELA study group are shown in Appendix 1.

Supported by a European Union Research grant under the FP6-LifeSCIHEALTH Integrated Program LSH-2004-1.2.5-1 (contract no. 018996).

Disclosure of potential conflict of interest: J. Weber, B. Sozanska, H. Danielewicz, A Boznanski, A. Dębińska, M. Depner, A. Kosmęda, and C. Strunz-Lehner have received research support from the European Commission. D. Heederik and I. M. Wouters have received research support from the European Union. E. von Mutius is a consultant for Novartis, GlaxoSmithKline, ALK-Abelló, and Protectimmun; has received a speaker's fee from InfectoPharm; has received research support from Airsonett AB; is a member of the Expert Panel for UK Research Excellence Framework; and is an Associate Editor for the *Journal of Allergy and Clinical Immunology*. M. Ege has received research support from the European Commission and the Deutsche Forschungsgemeinschaft (DFG). M. Kabesch has financial interests in Roxall, GlaxoSmithKline, Novartis, Sanofi-Aventis, Allergopharma, and AstraZeneca GmbH and has received research support from DFG, BMBF, and the European Union. The rest of the authors have declared that they have no conflict of interest.

Received for publication February 2, 2011; revised July 14, 2011; accepted for publication July 16, 2011.

Available online August 27, 2011.

Corresponding author: Georg Loss, MSc, Swiss Tropical and Public Health Institute, Socinstrasse 57, PO Box, 4002 Basel, Switzerland. E-mail: georg.loss@unibas.ch.

0091-6749/$36.00

doi:10.1016/j.jaci.2011.07.048

Abbreviations used
ALP: Alkaline phosphatase
aOR: Adjusted odds ratio

causes of asthma and atopy and includes data on analytically determined milk constituents. The aim of the present analysis was to find biological components of cow's milk that might explain the protective effect of farm milk on childhood asthma and atopy.

METHODS

Study population and study design

The GABRIEL Advanced studies were conducted in 5 rural areas of southern Germany, Switzerland, Austria, and Poland. Because of differences in study design, the Polish data will be reported separately. In phase I a short recruitment questionnaire was distributed through elementary schools to parents of all 6- to 12-year-old school children in the selected study areas. Three strata were defined as follows: (1) farm children (ie, children living on a farm run by the family); (2) exposed nonfarm children (ie, children not living on a farm but regularly exposed to stables, barns, or cow's milk produced on a farm); and (3) nonexposed nonfarm children. For phase II analyses, a stratified random sample of 9,668 was taken from 34,491 eligible participants. Children whose parents had provided written informed consent for blood sampling, genetic analyses, and dust sampling were eligible (Table I). A comprehensive questionnaire (n = 8,334) provided information about the participants' farm-related exposures, and 7,606 also gave blood samples for IgE measurements.

For more extensive environmental sampling, the study population was restricted to 1 center (Bavaria). Three exclusive disease strata were defined within each exposure stratum: (1) asthma, (2) atopy but no asthma, and (3) no asthma and no atopy. Of the 1903 eligible Bavarian children, 895 were selected by applying disproportionate stratified random sampling to create equally sized samples within each of the 9 strata (the study design is described in more detail elsewhere[11]). Milk samples of 800 subjects were analyzed. The ethics committees of the respective universities and the data protection authorities approved the study.

Atopy

Serum IgE levels against inhalant and food allergens were measured by using a fluorescence immunoassay. Atopy was defined as positive test results for specific IgE antibodies against *Dermatophagoides pteronyssinus*, cat, or birch (cutoff, 0.7 kU/L) or against a grass mix (cutoff, 0.35 kU/L). Food allergy was defined as a positive fx5 test (fish, cow's milk, hen's egg, peanut, soybean, and wheat flour).

Clinical outcomes

Health outcomes were assessed according to International Study of Asthma and Allergies in Childhood standards.[12] Childhood asthma was defined as either wheeze in the past 12 months, asthma inhaler use ever, or a doctor's diagnosis of asthma at least once or wheezy bronchitis more than once. Current asthma was defined as childhood asthma and wheeze in the past 12 months. Hay fever required occurrence of nasal symptoms with itchy or watery eyes in the past 12 months or a doctor's diagnosis of hay fever ever. Atopic dermatitis was defined as a doctor's diagnosis ever.

Milk exposure assessed by means of questionnaire

The phase II comprehensive questionnaire provided information about the child's farm-related exposures. Cow's milk consumption was determined by asking whether the child consumed milk purchased at a shop (shop milk) or directly from a farm (farm milk) and whether farm milk was boiled or skimmed. The heating status of shop milk was not assessed. The parents had to indicate the life period of milk exposure from pregnancy to school age and the corresponding amounts of milk consumption.

Children were grouped into the following categories: (1) exclusive shop milk exposure, (2) mixed milk exposure (exposure to both shop and farm milk), and (3) exclusive farm milk exposure. The information on milk boiling was used to subdivide the farm milk exposure into "only boiled farm milk drinkers" and "any unboiled farm milk drinkers." The latter included children consuming exclusively unboiled farm milk, as well as those consuming both unboiled and boiled farm milk. The "any unboiled farm milk" group was further subdivided by frequency of consumption (daily unboiled farm milk vs less than daily unboiled farm milk) and timing of first unboiled milk exposure (first exposure to unboiled farm milk in the first year of life or during pregnancy vs after 1 year of age).

Milk sample collection and analyses

In phase III trained field workers collected cow's milk that was consumed at the participants' homes on the day of the field visit. Parents were instructed to prepare the milk as they usually did and filled out standardized milk documentation sheets. All samples were analyzed by laboratory staff blinded to the milk type and the health and exposure status.

The heating status of milk samples was defined by the residual activity of the milk indigenous enzymes alkaline phosphatase (ALP) and lactoperoxidase, according to European Commission Council Directive 92/46/EC. Low levels of ALP (<80 mU/L) correspond to milk having been heated to greater than 72°C for at least 15 seconds (minimum for pasteurized milk), and low levels of lactoperoxidase (<20,000 mU/L) correspond to milk having been heated to greater than 85°C for at least 5 seconds (minimum for high heat–treated milk). The measurements and the milk type allowed to categorize the samples as (1) high heat–treated shop milk (≥85°C), (2) pasteurized shop milk (not heated to >85°C), (3) heated farm milk (≥72°C), and (4) raw farm milk (not heated to >72°C). Because 85% of the heat-treated farm milk samples were heated to greater than 85°C, all heated farm milk samples were combined for analysis. The total fat content and whey protein levels were determined for all available phase III samples. For detailed methods, see the Methods section in this article's Online Repository at www.jacionline.org.

Microbiological analyses

The total viable bacterial count was assessed in all 800 milk samples, and 222 samples were selected for advanced microbiological analyses by using stratified random sampling (strata based on milk type, heating status, and fat content). The following microbiological groups were determined by using selective plate count methods: pseudomonades, Enterobacteriaceae, micrococci plus staphylococci, lactobacilli, yeast plus mold, bacilli plus endospores, psychrotropic bacteria, and human pathogens. For detailed methods, see the Methods section in this article's Online Repository.

Statistical analyses

All statistical analyses were performed with STATA/SE 10.1 software for Windows (StataCorp, College Station, Tex). The stratification of the study sample was taken into account by using fixed weights (weighted up to the 34,491 participants eligible for phase II) and the linearized Taylor series method for variance estimation. First, associations between milk exposure and health outcomes were determined in phase II participants by using weighted multivariate logistic regression models adjusting for age, sex, farming status (farmers vs nonfarmers), number of siblings, familial history of asthma or hay fever, study center, and breast-feeding. In sensitivity analyses all final models were adjusted for food allergens (fx5), asthma models were adjusted for atopy, and atopy and hay fever models were adjusted for asthma. An additional adjustment for contact with farm animals or contact with stables and barns was performed to avoid confounding by concomitant farm exposures.

The phase III data were used to explore associations between the objectively assessed heating status of milk or measured milk components and asthma and atopy. These regression models were adjusted for the same set of confounders as the phase II data. Milk type and heating status were categorized into 4 groups, with highly heated shop milk as the reference category. To take into account the distribution of milk constituents with high proportions of nondetectable values (total viable bacterial count, lactoferrin,

TABLE I. GABRIEL study population and design

Study module	Study area	Study population	Total no.	Farmer	Exposed nonfarmer	Nonexposed nonfarmer
Phase I	Four centers*	General population	34,491†	n = 4,533	n = 8,666	n = 21,292
↓				↓	↓	↓
Phase II	Four centers*	Subsample stratified by farm exposure	9,668‡	n = 3,477	n = 3,236	n = 2,955
		Parental questionnaires with milk exposure information available	8,334	n = 3,067	n = 2,796	n = 2,471
		IgE measurements and milk exposure information available	7,606§	n = 2,806	n = 2,544	n = 2,256
↓				↓	↓	↓
Phase III	Bavaria	Subsample stratified by exposure and outcome	895‖	n = 298	n = 300	n = 297
		Milk samples available	800¶	n = 274	n = 263	n = 263

*Germany (Bavaria and Baden-Wuerttemberg), Austria (Tyrol), and Switzerland (9 cantons).
†Eligible for phase II: Complete questionnaire plus written informed consent to further analyses were available (Bavaria: n = 11,183: 1,797/2,708/6,678).
‡Selected for phase II: Random selection of stratified (by farm exposure) eligible subjects for phase II (Bavaria: n = 2,573: 1,014/814/745).
§Blood samples with IgE measurements and parental questionnaires with milk exposure information available.
‖Selected for phase III environmental studies: Random selection of stratified (by farm exposure and health outcome) phase III eligible subjects (2,573 Bavarian children).
¶Milk samples and standardized milk documentation sheets available.

total IgG, and BSA), samples within the detection range were split at the median representing low and high levels, whereas nondetects were used as the reference group. Milk constituents that were measurable in all samples (α-lactalbumin, β-lactoglobulin, TGF-β2, and fat content) were divided into tertiles, with the lowest tertile as a reference group to test for linearity of the association with health outcomes. α-Lactalbumin and β-lactoglobulin were subsequently entered as continuous variables into the regression models. A factor analysis with continuous variables and varimax rotation (extraction of eigenvalues of ≥1.5) was used to evaluate whether the different milk constituents could be separated into different factors. Results from weighted logistic regression models were expressed as adjusted odds ratios (aORs) with corresponding 95% CIs. For full methods, see the Methods section in this article's Online Repository.

RESULTS

The distribution of milk consumption stratified by farm and nonfarm children is shown in Table II (the prevalence of health outcomes is shown in Table E1 in this article's Online Repository at www.jacionline.org). Among nonfarm children, 71.2% reported exclusive shop milk consumption, whereas 45.0% of the farm children indicated exclusive farm milk consumption. Consumption of both farm and shop milk (mixed milk exposure) was more or less comparable between farm and nonfarm children, respectively. The majority of farm milk consumers drank unboiled farm milk, and many were exposed to unboiled farm milk already during pregnancy, during the first year of life, or both. Phase II questionnaire reports of milk consumption showed high agreement with the analytically determined heating status of milk samples in phase III, which were collected at the participants' homes (see Table E2 in this article's Online Repository at www.jacionline.org).

Children exclusively drinking farm milk as reported in the phase II questionnaire had significantly lower odds ratios for asthma, current asthma, atopy, and hay fever compared with children exclusively drinking shop milk (Table III). The association with atopic dermatitis was of borderline significance. Mixed milk consumption (consumption of both shop and farm milk) was protective for hay fever and atopy. Consumption of any unboiled farm milk was consistently inversely associated with asthma, hay fever, and atopy in both exclusive and mixed farm milk drinkers. Early exposure and daily consumption of farm milk showed a stronger inverse association with health outcomes in mixed milk

TABLE II. Milk exposure of farmers and nonfarmers in phases II and III

	Total no.	Farmer (%)§	Nonfarmer (%)§
Reported milk exposure in phase II (n = 8334)			
Exclusively shop milk	3670	22.3	71.2†
Mixed milk	3010	32.7	26.4†
Only boiled farm milk	597	14.3	26.1†
Any unboiled farm milk	2413	85.7	73.9†
First unboiled farm milk <1 y	1628	68.2	42.3†
First unboiled farm milk >1 y	785	17.5	31.7†
Daily unboiled farm milk	1153	49.6	27.0†
Less than daily unboiled farm milk	1857	50.4	73.0†
Exclusively farm milk	1654	45.0	2.4†
Only boiled farm milk	174	10.7	10.6
Any unboiled farm milk	1480	89.3	89.4
First unboiled farm milk <1 y	1307	89.0	83.6*
First unboiled farm milk >1 y	173	11.0	16.4*
Daily unboiled farm milk	1051	71.6	69.3
Less than daily unboiled farm milk	429	28.4	30.7
Collected milk samples in phase III (n = 800)			
Shop milk: high heat treated§	531	42.3	78.9†
Shop milk: pasteurized‖	52	4.0	7.8†
Farm milk: heated¶	60	13.5	4.4†
Farm milk: raw#	157	40.2	8.9†

P values of the Pearson χ^2 test for farmer versus nonfarmer: *.01 ≤ *P* < .05 and †*P* < .001.
‡Percentages weighted to phase I: Differences in numbers occur because of varying proportions of missing values.
§Shop milk heated to at least 85°C.
‖Shop milk heated to at least 72°C and not more than 85°C.
¶Farm milk heated to at least 72°C (9 samples were 72°-85°C and 51 samples were >85°C).
#Farm milk not heated to greater than 72°C.

TABLE III. Adjusted association§ of reported milk exposure and asthma, atopy, hay fever, and atopic dermatitis (phase II, n = 8334)

Milk exposure reported in phase II	Asthma, aOR (95% CI)		Current asthma, aOR (95% CI)		Atopy,‖ aOR (95% CI)		Hay fever, aOR (95% CI)		Atopic dermatitis, aOR (95% CI)	
Exclusively shop milk	1.00		1.00		1.00		1.00		1.00	
Mixed milk	0.91	0.78-1.06	0.86	0.71-1.04	0.77	0.67-0.88‡	0.72	0.60-0.87‡	0.97	0.82-1.14
Only boiled farm milk	1.11	0.86-1.44	1.08	0.78-1.50	0.85	0.67-1.08	0.99	0.72-1.36	1.24	0.94-1.64
Any unboiled farm milk	0.84	0.71-1.00*	0.79	0.64-0.97*	0.74	0.64-0.86‡	0.64	0.52-0.78‡	0.88	0.74-1.05
First unboiled farm milk <1 y	0.69	0.57-0.84‡	0.66	0.52-0.84†	0.72	0.61-0.85‡	0.63	0.50-0.79‡	0.71	0.58-0.86†
First unboiled farm milk >1 y	1.08	0.85-1.37	0.98	0.73-1.30	0.78	0.63-0.97*	0.66	0.49-0.88†	1.16	0.90-1.48
Daily unboiled farm milk	0.76	0.61-0.96*	0.69	0.52-0.92*	0.68	0.57-0.82‡	0.60	0.45-0.79‡	0.81	0.65-1.02
Less than daily unboiled farm milk	0.97	0.82-1.15	0.93	0.75-1.14	0.81	0.69-0.94†	0.77	0.63-0.95*	1.03	0.86-1.24
Exclusively farm milk	0.65	0.52-0.81‡	0.64	0.48-0.84†	0.76	0.63-0.92†	0.58	0.44-0.77‡	0.78	0.61-1.00
Only boiled farm milk	1.24	0.82-1.87	1.59	0.98-2.58	0.90	0.60-1.35	1.17	0.68-1.99	1.04	0.54-2.01
Any unboiled farm milk	0.59	0.46-0.74‡	0.55	0.40-0.74‡	0.74	0.61-0.90†	0.51	0.37-0.69‡	0.75	0.59-0.96*
First unboiled farm milk <1 y	0.55	0.43-0.70‡	0.54	0.39-0.73‡	0.74	0.60-0.91†	0.51	0.37-0.71‡	0.72	0.56-0.94*
First unboiled farm milk >1 y	0.61	0.34-1.07	0.42	0.18-0.99*	0.67	0.43-1.07	0.46	0.21-1.01	0.65	0.37-1.12
Daily unboiled farm milk	0.56	0.43-0.73‡	0.51	0.36-0.72‡	0.76	0.61-0.94*	0.53	0.37-0.76‡	0.72	0.55-0.96*
Less than daily unboiled farm milk	0.61	0.43-0.86†	0.59	0.37-0.94*	0.68	0.50-0.92*	0.46	0.29-0.74†	0.77	0.53-1.11

*$P < .05$, †$P < .01$, and ‡$P < .001$.
§aORs with 95% CIs calculated by using weighted logistic regression models adjusted for age, sex, farming status, 2 or more siblings, familial history of asthma or hay fever, breast-feeding, and study center. All models weighted to phase I: n = 34,491.
‖n = 7,606.

drinkers. Because most exclusive farm milk drinkers were exposed to farm milk early in life with daily consumption, the power to detect the influence of frequency and age of first farm milk exposure was limited. Consumption of only boiled farm milk was not associated with any health outcome.

Consumption of farm milk was also inversely related to food allergen sensitization (fx5). Compared with exclusive shop milk drinking, the association between a positive fx5 test result and mixed milk consumption and exclusive farm milk drinking was an aOR of 0.85 (95% CI, 0.73-0.99) and 0.84 (95% CI, 0.69-1.03), respectively. The associations of milk consumption and asthma were robust to adjustment for atopy and food allergen sensitization.

In Table IV total fat content, total viable bacterial count, and whey protein levels are depicted and stratified by milk type and milk heating status. Highly heated shop milk showed much lower levels of all parameters compared with raw farm milk. Heated farm milk samples had a similar fat content as raw samples but significantly lower total viable bacterial counts and lower whey protein levels (not significant for α-lactalbumin). Pasteurized shop milk showed higher whey protein levels than highly heated shop or heated farm milk.

Fig 1 shows the results of the advanced microbiological analyses. Microorganisms could be detected in few shop milk and heated farm milk samples (<15% for all groups except micrococci and staphylococci [25%]). In many raw farm milk samples, micrococci and staphylococci (85.2%), lactobacilli (94.1%), bacilli and bacterial endospores (63.4%), and psychrotrophic bacteria (58.4%) could be detected. Pathogenic *Listeria innocua* and *Listeria ivanovii* strains were found in only 3 unboiled farm milk samples.

Analyses of the phase III samples (Table V) showed consumption of objectively assessed raw farm milk to be inversely associated with asthma ($P = .04$) and current asthma ($P = .03$) but not with atopy when compared with high heat–treated shop milk. A similar risk reduction, although not significant, was observed for consumption of pasteurized shop milk and asthma. Heated farm milk was not associated with asthma outcomes. Total fat content and total viable bacterial counts had no clear association with any of the analyzed health outcomes. No association was further found between these health outcomes and total protein content, somatic cell count, lactose levels, or microbiological subgroups (analyses not shown). Yet increased levels of the whey proteins tended to be inversely associated with asthma but not with atopy. Statistically significant inverse associations with asthma and current asthma were found for α-lactalbumin (asthma, $P = .03$; current asthma, $P = .03$), β-lactoglobulin (asthma, $P = .03$), and high levels of BSA (asthma, $P = .04$; current asthma, $P = .04$). Lactoferrin and total IgG levels showed a nonsignificant inverse association with asthma indicative of a dose-response relation. TGF-β2 was not significantly associated with asthma or atopy, although the highest tertile compared with the lowest tertile tended to be associated with a reduced asthma risk. In 2 exposure models including total viable bacterial counts or total fat content and individual whey proteins, the results were essentially unchanged. Applying factor analysis, the different whey proteins could not be separated from each other or from milk heating status because all were loading on the same factor.

DISCUSSION

The results of this large epidemiologic study add to the increasing body of evidence identifying consumption of farm milk (early in life) to be associated with a reduced risk of childhood asthma and allergies independently of concomitant farm exposures.[7-10] The results indicate that the effect is due to the consumption of unheated farm milk. For the first time, associations between objectively measured milk constituents and asthma and atopy could be demonstrated. Neither total viable bacterial counts nor the total fat content of the milk were related to asthma or atopy. However, some whey proteins (BSA, α-lactalbumin, and β-lactoglobulin) were associated with a significantly reduced risk of asthma but not with atopy. Prospective analyses need to confirm the results of this cross-sectional study, and further analyses are needed to determine the specific compounds underlying the

TABLE IV. Levels and percentage of detectable values of all milk constituents stratified by milk type and milk heating status

Milk parameter*	Shop milk: high heat–treated		Shop milk: pasteurized	
	Observations (% detectable)	Geometric mean (95% CI)	Observations (% detectable)	Geometric mean (95% CI)
Fat content (%)	529 (100.0)	2.01 (1.94-2.09)	52 (100.0)	2.66 (2.37-2.97)
Total viable bacteria (CFU/mL)	509 (38.3)	4.55 (3.60-5.76)	51 (94.1)	70.35 (31.89-155.24)
TGF-β2 (ng/mL)	519 (99.6)	2.97 (2.81-3.14)	47 (100.0)	8.63 (7.58-9.83)
Lactoferrin (μg/mL)	530 (14.5)	0.010 (0.008-0.012)	52 (100.0)	58.89 (45.44-76.32)
Total IgG (μg/mL)	496 (1.2)	0.016 (0.014-0.019)	52 (100.0)	29.31 (13.93-61.66)
BSA (μg/mL)	479 (13.6)	0.019 (0.015-0.024)	52 (100.0)	54.56 (42.07-70.75)
α-Lactalbumin (μg/mL)	475 (97.3)	353.92 (305.83-409.58)	52 (100.0)	1111.24 (1054.16-1171.42)
β-Lactoglobulin (μg/mL)	484 (100.0)	257.03 (242.17-272.82)	52 (100.0)	3704.11 (3524.64-3892.73)

CFU, Colony-forming unit.
*Levels are expressed as geometric means with 95% CIs. Values of less than the detection limit were set to the value of the detection limit.

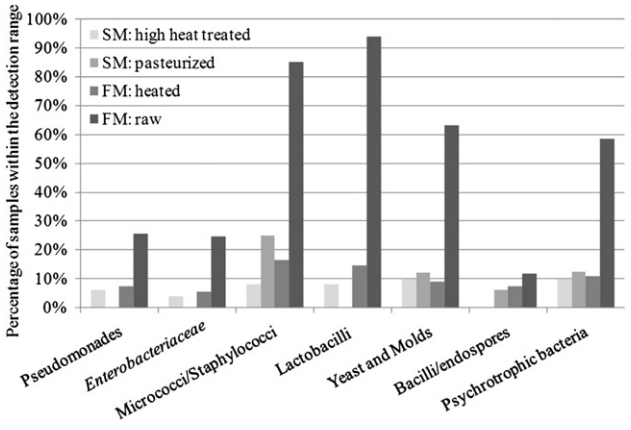

FIG 1. Proportion of samples greater than the detection limit in the advanced microbiological analyses (n = 222) shown for each microbiological group stratified by milk type and milk heating status (SM: high heat treated [n = 50], SM: pasteurized [n = 16], FM: heated [n = 55], and FM: raw [n = 101]). *FM*, Farm milk; *SM*, shop milk.

epidemiologically observed inverse association of farm milk consumption with atopy and hay fever.

The study allowed validation of parental reports of raw milk consumption against objective measurements of milk heating status and showed very good agreement. Obviously, parental reports of the raw status of the milk are reliable and not biased by social desirability, as previously speculated.[9] Under the hygiene hypothesis and given the role of microbial diversity in house dust to explain farm-related reduction of asthma risk,[5] one might assume that a higher microbial load of unboiled farm milk might be responsible for the protective farm milk effect. Milk is an excellent growth medium, allowing rapid proliferation of microbes. Indeed, the present results showed much higher counts of viable microbes in raw farm milk samples compared with heated farm milk and pasteurized and highly heated shop milk samples, as has been reported by others.[13,14] Contrary to our expectations, we did not observe an association between total viable bacterial counts in milk and investigated health outcomes. Given the cross-sectional design of the study and the restriction to viable microbe determination, the results need to be interpreted with caution. We cannot determine how representative current levels of microbes are for the long-term exposure of children, and we cannot preclude that repeated consumption of raw milk since infancy might influence the developing gut flora and interact with the immune system of the host.[15] Microbiological subgroups were measured in only 222 samples, with a high number of samples at less than the detection limit. Individual subgroups were not associated with asthma or atopy, but given the small sample size, inferences are limited. For future (prospective) analyses, new culture-independent methods to better characterize the microbial diversity of milk samples are warranted. We recently reported that the exposure to a wider range of microbes measured in house dust explained a substantial fraction of the inverse relation between asthma and growing up on a farm.[5] The association between farm milk consumption and asthma presented here was independent of and adjusted for farming and only partially attenuated the farming effect on asthma, as previously observed.[9]

Certain whey proteins were the only assessed milk components inversely associated with asthma, but the effect could not be ascribed to a single whey protein because of their high intercorrelation. Milk processing, such as heating, does not affect heat-stable caseins, whereas whey proteins, accounting for 18% of the total protein in cow's milk, are more sensitive to heat treatment[16] and might influence the bioavailability of the proteins. Bovine whey contains proteins secreted by the mammary gland, such as β-lactoglobulin, α-lactalbumin, and lactoferrin, and from serum, such as IgG, serum albumin, and TGF-β.[17] Whey proteins from bovine milk seem to play an important role in host defense against infection and excessive inflammation, yet the mechanism of action remains poorly understood.[17,18] Recent reviews have shown that lactoferrin has marked effects on immune cells in culture, being an immunostimulator and immunoregulator,[18] and that TGF-β, a multifunctional cytokine, inhibits the immunopathology to self without compromising immune responses to pathogens.[19] Higher levels of TGF-β were found in unpasteurized farm milk[20] and in human breast milk of mothers exposed to a farming environment.[21] Furthermore, TGF-β in human breast milk has been associated with reduced allergy-related outcomes in infancy and early childhood.[22] In the present study TGF-β2 was not significantly associated with asthma. Whey also contains the major milk allergens β-lactoglobulin and α-lactalbumin, and it remains perplexing that early consumption of raw cow's milk decreases the risk of asthma. Immunomodulatory effects have been ascribed to α-lactalbumin[23] and conjugates of β-lactoglobulin.[24] In addition, one might speculate that milk processing, such as homogenization, might alter the context in which potentially allergenic structures are presented to the immune system.

TABLE IV. *(Continued)*

Farm milk: heated		Farm milk: raw	
Observations (% detectable)	Geometric mean (95% CI)	Observations (% detectable)	Geometric mean (95% CI)
59 (100.0)	3.39 (3.11-3.70)	154 (100.0)	3.87 (3.66-4.11)
58 (89.7)	114.47 (58.54-223.84)	153 (98.0)	9533.94 (6206.20-14645.99)
48 (83.3)	1.52 (1.06-2.19)	125 (100.0)	5.71 (5.23-6.25)
60 (31.7)	0.028 (0.011-0.068)	157 (98.1)	80.26 (60.33-106.79)
56 (21.7)	0.095 (0.034-0.265)	154 (100.0)	224.96 (208.90-242.25)
55 (40.0)	0.15 (0.05-0.43)	154 (100.0)	84.94 (77.69-92.86)
23 (87.0)	307.59 (85.45-1107.24)	154 (100.0)	1113.08 (1075.33-1152.15)
23 (100.0)	663.96 (340.02-1296.52)	154 (100.0)	4025.40 (3892.53-4162.81)

TABLE V. Adjusted association† of asthma or atopy and milk heating status, total fat content, total viable bacterial count, or whey protein levels (phase III)

Milk parameter	No.	Asthma, aOR (95% CI)		Current asthma, aOR (95% CI)		Atopy, aOR (95% CI)	
Milk type and heating status							
Shop milk: high heat–treated	531	1.00		1.00		1.00	
Shop milk: pasteurized	52	0.50	(0.22-1.12)	0.49	(0.19-1.28)	1.28	(0.59-2.75)
Farm milk: heated	60	0.97	(0.49-1.91)	0.90	(0.38-2.16)	0.74	(0.38-1.44)
Farm milk: raw	157	0.58	(0.34-0.99)*	0.45	(0.22-0.93)*	0.90	(0.56-1.45)
Fat content (%)‡							
Lowest tertile	267	1.00		1.00		1.00	
Medium tertile	269	1.13	(0.73-1.75)	1.37	(0.83-2.26)	0.88	(0.57-1.36)
Highest tertile	258	0.98	(0.60-1.59)	0.92	(0.51-1.65)	1.39	(0.88-2.19)
Total viable bacteria (CFU/mL)							
Less than detection limit	326	1.00		1.00		1.00	
Low levels	223	0.94	(0.60-1.48)	0.88	(0.52-1.50)	0.85	(0.55-1.31)
High levels	222	1.02	(0.62-1.69)	0.85	(0.46-1.60)	0.94	(0.58-1.53)
TGF-β2 (ng/mL)‡							
Lowest tertile	247	1.00		1.00		1.00	
Medium tertile	246	1.36	(0.86-2.15)	1.23	(0.72-2.11)	1.07	(0.68-1.67)
Highest tertile	246	0.75	(0.46-1.22)	0.75	(0.42-1.32)	0.98	(0.62-1.55)
Lactoferrin (μg/mL)							
Less than detection limit	497	1.00		1.00		1.00	
Low levels	151	0.83	(0.50-1.37)	0.83	(0.46-1.52)	1.26	(0.78-2.03)
High levels	151	0.72	(0.41-1.26)	0.64	(0.31-1.32)	1.01	(0.62-1.65)
Total IgG (μg/mL)							
Less than detection limit	449	1.00		1.00		1.00	
Low levels	155	0.85	(0.52-1.40)	0.77	(0.42-1.40)	1.08	(0.68-1.73)
High levels	154	0.61	(0.34-1.07)	0.71	(0.35-1.45)	1.32	(0.81-2.17)
BSA (μg/mL)							
Less than detection limit	447	1.00		1.00		1.00	
Low levels	147	0.76	(0.46-1.26)	0.77	(0.42-1.41)	0.95	(0.58-1.55)
High levels	146	0.53	(0.30-0.97)*	0.45	(0.21-0.98)*	0.90	(0.54-1.51)
α-Lactalbumin (μg/mL)§	704	0.71	(0.52-0.97)*	0.67	(0.47-0.97)*	1.07	(0.78-1.48)
β-Lactoglobulin (μg/mL)§	713	0.62	(0.39-0.97)*	0.62	(0.39-1.06)	1.12	(0.74-1.68)

CFU, Colony-forming unit.
*$P < .05$.
†Weighted logistic regression models adjusted for age, sex, farming status, 2 or more siblings, and familial history of asthma or hay fever.
‡Divided into tertiles because requirements of linearity were not met.
§aORs for interquartile range.

Presentation of the allergenic epitopes might also be influenced by complexing the allergen with immunoglobulins, as recently proposed in an animal model.[25]

Phase III analyses allowed us to differentiate shop milk samples according to heat treatment and found pasteurized shop milk consumption to be associated with less asthma and to have higher whey protein levels than high heat–treated shop milk. Yet the association between pasteurized milk consumption and asthma was not statistically significant and needs to be confirmed in larger studies.

In this study BSA, α-lactalbumin, and β-lactoglobulin levels were found to be inversely associated with asthma but not with

atopy. It is thus conceivable that milk components not measured in the present study underlie the epidemiologically observed inverse association between farm milk consumption and atopy. The fatty acid composition of farm milk might be one such factor, which has been hypothesized before.[26,27] In the present analysis the total fat content of the milk samples was not associated with asthma or atopy, which is in contrast to other epidemiologic studies reporting a reduced risk of asthma associated with consumption of milk fat–containing products, such as full cream milk and butter,[28] or modulation of cytokine production in cord blood associated with farm-produced butter consumed by the pregnant mother.[29]

The main strength of the present study is the objective determination of several milk compounds and the enzymatic classification of the heat treatment of a comparatively large number of milk samples consumed by study participants, thus expanding questionnaire-based analysis. The cross-sectional design of the study, the lack of fatty acid measurements, and the limitations of the microbial analyses represent the main limitations of the present study.

The long-term solution to the asthma epidemic is thought to be prevention and not treatment of established disease,[30] and nutritional interventions might represent an interesting avenue. However, on the basis of current knowledge, raw milk consumption cannot be recommended because it might contain pathogens. Once the mechanisms underlying the protective farm milk effect are better understood, ways of processing and preserving a safe and preventive milk can be developed.

We thank Dr Wulf Thierfelder and Michael Thamm from the Robert-Koch-Institute, Berlin, Germany, for their cooperation and the measurement of total and specific IgE levels that was used in the definition of atopic subjects. Furthermore we thank the participating children, their families, and the fieldworkers of the GABRIEL Advanced studies.

Key messages

- **Questionnaire-reported consumption of unboiled but not boiled farm milk was inversely associated with asthma, hay fever, and atopy.**
- **Higher levels of the whey proteins BSA, α-lactalbumin, and β-lactoglobulin in milk samples were associated with a reduced risk of asthma but not atopy.**
- **Neither total viable bacterial counts nor the total fat content of the milk were related to asthma or atopy.**

REFERENCES

1. Eder W, Ege MJ, von Mutius E. The asthma epidemic. N Engl J Med 2006;355:2226-35.
2. von Mutius E, Vercelli D. Farm living: effects on childhood asthma and allergy. Nat Rev Immunol 2010;10:861-8.
3. Remes ST, Iivanainen K, Koskela H, Pekkanen J. Which factors explain the lower prevalence of atopy amongst farmers' children? Clin Exp Allergy 2003;33:427-34.
4. Ege MJ, Bieli C, Frei R, van Strien RT, Riedler J, Ublagger E, et al. Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children. J Allergy Clin Immunol 2006;117:817-23.
5. Ege MJ, Mayer M, Normand A-C, Genuneit J, Cookson WOCM, Braun-Fahrländer C, et al. Exposure to environmental microorganisms and childhood asthma. N Engl J Med 2011;364:701-9.
6. Braun-Fahrlander C, Riedler J, Herz U, Eder W, Waser M, Grize L, et al. Environmental exposure to endotoxin and its relation to asthma in school-age children. N Engl J Med 2002;347:869-77.
7. Perkin MR, Strachan DP. Which aspects of the farming lifestyle explain the inverse association with childhood allergy? J Allergy Clin Immunol 2006;117:1374-81.
8. Riedler J, Braun-Fahrlander C, Eder W, Schreuer M, Waser M, Maisch S, et al. Exposure to farming in early life and development of asthma and allergy: a cross-sectional survey. Lancet 2001;358:1129-33.
9. Waser M, Michels KB, Bieli C, Floistrup H, Pershagen G, von Mutius E, et al. Inverse association of farm milk consumption with asthma and allergy in rural and suburban populations across Europe. Clin Exp Allergy 2007;37:661-70.
10. Bieli C, Eder W, Frei R, Braun-Fahrlander C, Klimecki W, Waser M, et al. A polymorphism in CD14 modifies the effect of farm milk consumption on allergic diseases and CD14 gene expression. J Allergy Clin Immunol 2007;120:1308-15.
11. Genuneit J, Büchele G, Waser M, Kovacs K, Debinska A, Boznanski A, et al. The GABRIEL Advanced Surveys: study design, participation, and evaluation of bias. Paediatr Perinat Epidemiol 2011;25:436-47.
12. Beasley R, The International Study of Asthma and Allergies in Childhood (ISAAC) Steering Committee. Worldwide variation in prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and atopic eczema: ISAAC. Lancet 1998;351:1225-32.
13. Desmasures N, Bazin F, Gueguen M. Microbiological composition of raw milk from selected farms in the Camembert region of Normandy. J Appl Microbiol 1997;83:53-8.
14. Perkin MR. Unpasteurized milk: health or hazard? Clin Exp Allergy 2007;37:627-30.
15. Conroy ME, Shi HN, Walker WA. The long-term health effects of neonatal microbial flora. Curr Opin Allergy Clin Immunol 2009;9:197-201.
16. Lorenzen PC, Clawin-Raedecker I, Einhoff K, Hammer P, Hartmann R, Hoffmann W, et al. A survey of the quality of extended shelf life (ESL) milk in relation to HTST and UHT milk. Int J Dairy Tech 2011;64:166-78.
17. Rusu D, Drouin R, Pouliot Y, Gauthier S, Poubelle PE. A bovine whey protein extract can enhance innate immunity by priming normal human blood neutrophils. J Nutr 2009;139:386-93.
18. Puddu P, Valenti P, Gessani S. Immunomodulatory effects of lactoferrin on antigen presenting cells. Biochimie 2009;91:11-8.
19. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA. Transforming growth factor-beta regulation of immune responses. Annu Rev Immunol 2006;24:99-146.
20. Peroni DG, Piacentini GL, Bodini A, Pigozzi R, Boner AL. Transforming growth factor-beta is elevated in unpasteurized cow's milk. Pediatr Allergy Immunol 2009;20:42-4.
21. Peroni DG, Pescollderungg L, Piacentini GL, Rigotti E, Maselli M, Watschinger K, et al. Immune regulatory cytokines in the milk of lactating women from farming and urban environments. Pediatr Allergy Immunol 2010;21:977-82.
22. Oddy WH, Rosales F. A systematic review of the importance of milk TGF-beta on immunological outcomes in the infant and young child. Pediatr Allergy Immunol 2010;21:47-59.
23. Krissansen GW. Emerging health properties of whey proteins and their clinical implications. J Am Coll Nutr 2007;26(suppl):713S-23S.
24. Yoshida T, Sasahara Y, Miyakawa S, Hattori M. Reduced T cell response to beta-lactoglobulin by conjugation with acidic oligosaccharides. J Agricultural Food Chem 2005;53:6851-7.
25. Mosconi E, Rekima A, Seitz-Polski B, Kanda A, Fleury S, Tissandie E, et al. Breast milk immune complexes are potent inducers of oral tolerance in neonates and prevent asthma development. Mucosal Immunol 2010;3:461-74.
26. Black PN, Sharpe S. Dietary fat and asthma: is there a connection? Eur Respir J 1997;10:6-12.
27. Thijs C, Muller A, Rist L, Kummeling I, Snijders BE, Huber M, et al. Fatty acids in breast milk and development of atopic eczema and allergic sensitisation in infancy. Allergy 2010;66:58-67.
28. Wijga AH, Smit HA, Kerkhof M, de Jongste JC, Gerritsen J, Neijens HJ, et al. Association of consumption of products containing milk fat with reduced asthma risk in pre-school children: the PIAMA birth cohort study. Thorax 2003;58:567-72.
29. Pfefferle PI, Buchele G, Blumer N, Roponen M, Ege MJ, Krauss-Etschmann S, et al. Cord blood cytokines are modulated by maternal farming activities and consumption of farm dairy products during pregnancy: the PASTURE Study. J Allergy Clin Immunol 2010;125:108-15, e1-3.
30. Sly PD, Boner AL, Bjorksten B, Bush A, Custovic A, Eigenmann PA, et al. Early identification of atopy in the prediction of persistent asthma in children. Lancet 2008;372:1100-6.

APPENDIX 1

The members of the GABRIELA study group are listed in alphabetical order: Silvia Apprich, PhD,[g] Andrzej Boznanski MD, PhD,[k] Charlotte Braun-Fahrländer, MD,[d,e] Gisela Büchele, PhD,[c] William Cookson, MD, DPhil,[a] Paul Cullinan, MD,[a] Hanna Danielewicz, MD,[k] Anna Dębińska,[k] Martin Depner, PhD,[b] Markus Ege, MD,[b] Urs Frey, MD, PhD,[l] Oliver Fuchs, MD,[l] Jon Genuneit MD,[c] Dick Heederik, PhD,[f] Elisabeth Horak, MD,[m] Anne Hyvärinen, PhD,[h] Sabina Illi, PhD,[b] Michael Kabesch, MD,[n] Katalin Kovacs,[m] Aleksandra Kosmęda, PhD,[k] Wolfgang Kneifel, PhD,[g] Philipp Latzin, MD, PhD,[l] Roger Lauener, MD,[p] Georg Loss, MSc,[d,e] Stephanie MacNeill, MSc,[a] Bernhard Morass, MD,[m] Anne-Cécile Normand, PhD,[q] Renaud Piarroux, MD, PhD,[q] Helena Rintala, PhD,[h] Mascha K. Rochat, MD,[b] Nikolaos Sitaridis,[c] Barbara Sozanska, MD,[k] David Strachan, MD,[o] Christine Strunz-Lehner, MPH,[b] Bertrand Sudre, MD, PhD,[i] Erika von Mutius, MD, MSc,[b] Marco Waser, PhD,[d,e] Juliane Weber, MD,[b] and Inge M. Wouters, PhD.[f]

From [a]Imperial College London, National Heart and Lung Institute, South Kensington Campus, London, United Kingdom; [b]LMU Munich, University Children's Hospital, Munich, Germany; [c]Ulm University, Institute of Epidemiology and Medical Biometry, Ulm, Germany; [d]Swiss Tropical and Public Health Institute, Basel, Switzerland; [e]the University of Basel, Basel, Switzerland; [f]Utrecht University, Institute for Risk Assessment Sciences (IRAS), Division of Environmental Epidemiology, Utrecht, The Netherlands; [g]BOKU Vienna, University of Natural Resources and Life Sciences, Department of Food Science and Technology, Vienna, Austria; [h]THL Kuopio, National Institute for Health and Welfare, Kuopio, Finland; [i]Université de Franche-Comté, UMR 6249 Chrono-Environnement, Département de Parasitologie/Mycologie, Besançon, France; [k]Wroclaw Medical University, 1st Department of Paediatrics, Allergology and Cardiology, Wroclaw, Poland; [l]the Division of Pulmonology, Department of Paediatrics, Bern University Hospital, Bern, Switzerland; [m]the Department of Pediatrics and Adolescents, Division of Cardiology and Pulmonology, Innsbruck, Austria; [n]Hannover Medical School, Clinic for Paediatric Pneumology and Neonatology, Hannover, Germany; [o]St George's, University of London, Cranmer Terrace, London, United Kingdom; [p]High Mountain Hospital Davos, Herman-Davos-Wolfgang, Switzerland; and [q]the Department of Parasitology and Mycology, Hôpital de la Timone, Assistance Publique-Hôpitaux de Marseille, Marseille, France.

METHODS

Atopy

Serum IgE levels against inhalant (birch, cat, *D pteronyssinus*, and grass mix) and food (fx5 test: fish, cow's milk, hen's egg, peanut, soybean, and wheat flour) allergens were measured at a central laboratory (Robert-Koch Institute, Berlin, Germany) by means of fluorescence immunoassay (UNICAP 1000; Phadia AB, Uppsala, Sweden). Atopy was defined as positive test results for specific IgE antibodies against *D pteronyssinus*, cat, or birch (cutoff, 0.7 kU/L) or against a grass mix (cutoff, 0.35 kU/L). Food allergy was defined as a positive fx5 test result.

Milk sample collection and analyses

In phase III trained field workers collected 9 aliquots of milk (total of 300 mL) that were consumed at the participants' homes on the day of the field visit. Parents were instructed to prepare the milk as they usually do. Samples were transported on ice and frozen at −18°C immediately after arriving at the laboratory. During the field visit, standardized documentation sheets were filled in, including information about milk type (shop or farm purchased), storage conditions, and preparation of the milk before consumption. All milk analyses refer only to cow's milk. All samples were analyzed by laboratory staff blinded to milk type and health and exposure status.

Heating status of all milk samples was defined by residual activity of the milk-indigenous enzymes ALP and lactoperoxidase according to European Commission Council Directive 92/46/EC. ALP (fluorimetric method according to EN ISO 11816-1 [2000]; lower detection limit, 10 mU/L) and lactoperoxidase (Reflectoquant; MERCK KGaA, Darmstadt, Germany; lower detection limit, 5000 mU/L) levels were measured at the Max Rubner Institute, Kiel, Germany.

Low levels of ALP (<80 mU/L) correspond to milk having been heated to greater than 72°C for at least 15 seconds (minimum for pasteurized drinking milk [shop milk]), and low levels of lactoperoxidase (<20,000 mU/L) correspond to milk having been heated to greater than 85°C for at least 5 seconds (minimum for highly pasteurized drinking milk [shop milk]). The measurements allowed us to categorize the samples as (1) high heat–treated shop milk (≥85°C), (2) pasteurized shop milk (not heated to >85°C), (3) heated farm milk (≥72°C), and (4) raw farm milk (not heated to >72°C). The majority (85%) of heated farm milk samples were heated to greater than 85°C. The total fat content and all whey protein levels were determined for all available phase III samples.

The total fat content, total protein content, and lactose levels (infrared method), as well as the somatic cell count (flow cytometry with Fossomatic; FOSS, Hillerød, Denmark), were determined for all 800 milk samples at the Qualitaetslabor Lower Austria, Gmuend, Austria.

TGF-β2 levels (ELISA) were measured by Friesland CAMPINA Research, Deventer, The Netherlands, and all other whey proteins were measured at the University of Natural Resources and Life Sciences, Vienna, Austria. The following whey proteins were measured in all available phase III milk samples: lactoferrin (Bovine lactoferrin ELISA quantitation kit E10-126, Bethyl Laboratories, Montgomery, Tex; detection limit, 4 ng/mL), TGF-β2, total IgG (Bovine IgG ELISA quantitation kit E10-118, Bethyl; lower detection limit, 7.8 ng/mL), BSA (Bovine albumin ELISA quantitation kit E10-113, Bethyl; lower detection limit, 6.25 ng/mL), α-lactalbumin (Bovine α-La ELISA quantitation kit E10-128, Bethyl; lower detection limit, 0.78 ng/mL), and β-lactoglobulin (bovine β-Lg E10-125, Bethyl; lower detection limit, 1.95 ng/mL).

Microbiological analyses

The total viable bacterial count was assessed in all 800 milk samples, and 222 samples were selected for advanced microbiological analyses by using stratified random sampling (strata based on milk type, heating status, and fat content). Their total viable bacterial count was determined by using the standard plate count method according to Koch[E1] with a standard method agar (PCA; MERCK KGaA, Darmstadt, Germany; detection limit, 10 colony-forming units/mL). Colony-forming units of the following microbiological groups were determined by using selective plate count methods (detection limit, 10 colony-forming units/mL): pseudomonades, Enterobacteriaceae, micrococci plus staphylococci, lactobacilli, yeast plus molds, bacilli plus endospores, psychrotropic bacteria, and human pathogens. The total viable bacterial count of the remaining milk samples was assessed by using the automated most-probable-number method (TEMPO; bioMérieux, Marcy l'Etoile, France; detection limit, 1 colony-forming unit/mL) with corresponding total viable count broth. All microbiological measurements were performed at the University of Natural Resources and Life Sciences, Vienna, Austria.

For validation of TEMPO results, the viable count of every tenth milk sample was also assessed by using the standard plate count method according to Koch with standard methods agar (Plate Count Agar; Merck KGaA, Darmstadt, Germany). For 37 samples, both measurements were available and showed high agreement (Spearman rho = 0.81).

Samples selected for advanced microbiological analyses were thawed at room temperature, diluted, and analyzed with plate count methods according to Koch. The total bacterial count was determined with a standard agar method (Plate count agar, MERCK KGaA). The following microbiological groups were assessed by using the respective media and recommended incubation duration and temperature: pseudomonades (LAB 108: *Pseudomonas* Agar plus X107 C.N. selective supplement; LAB M Ltd, Bury, United Kingdom), Enterobacteriaceae (110275 Violet Red Bile Dextrose Agar according to Mossel, MERCK KGaA), micrococci and staphylococci (LAB 285: Baird Parker Media plus X085 egg yolk tellurit-supplement, LAB M Ltd), lactobacilli (110660 MRS Agar according to de Man, Rogosa and Sharpe, MERCK KGaA), yeast and molds (LAB 200: Yeast & Mould Agar, LAB M Ltd), bacilli and endospores (107324 Tryptic Soy Agar plus Polysorbate 80 und Lecithin, MERCK KGaA), and psychrotropic bacteria (1.10878 Plate count agar sugar free FIL-IDF, MERCK KGaA). The detection limit for all analyses was 10 colony-forming units/mL. Furthermore, human pathogenic bacteria (*Salmonella* species and *Listeria* species) were determined and identified.

Statistical analyses

All statistical analyses were performed with STATA/SE 10.1 software for Windows. The stratification of the study sample was taken into account by using fixed weights (weighted up to the participants eligible for phase II [34491]) and the linearized Taylor series method for variance estimation. First, the association of milk exposure and health outcomes was determined based on the phase II dataset by using weighted multivariate logistic regression models. Point estimate changes of at least 10% in bivariate models were a criterion for a covariate to be added to the final regression models. All models were adjusted for age, sex, farming status (exposed nonfarmers and nonexposed nonfarmers were combined for analyses), number of siblings, familial history of asthma or hay fever, study center, and breast-feeding. Other factors that were tested but not included in the final models were body mass index, milk avoidance caused by allergies, parental smoking, parental education, and milk storage time and location. In sensitivity analyses all final models were adjusted for food allergens (fx5), asthma models were adjusted for atopy, and atopy and hay fever models were adjusted for asthma. An additional adjustment for contact with farm animals or contact with stables and barns was performed to avoid confounding by concomitant farm exposures.

The phase III data were used to explore associations between the objectively assessed heating status of milk or measured milk components and asthma and atopy. These regression models were adjusted for the same set of confounders as the phase II data. Milk type and heating status were categorized into 4 groups, with highly heated shop milk as the reference category. To take into account the distribution of milk constituents with high proportions of nondetects (total viable bacterial count, lactoferrin, total IgG, and BSA), samples within the detection range were split at the median representing low and high levels, whereas nondetects were used as the reference group. Milk constituents that were measurable in all samples (α-lactalbumin, β-lactoglobulin, TGF-β2, and fat content) were divided into tertiles, with the lowest tertile as a reference group to test for linearity of the association with health outcomes. α-Lactalbumin and β-lactoglobulin were subsequently entered as continuous variables in the regression

models. A factor analysis with continuous variables and varimax rotation (extraction of eigenvalues of ≥1.5) was used to evaluate whether the different milk constituents could be separated into different factors. Results from weighted logistic regression models were expressed as aORs with corresponding 95% CIs.

REFERENCE

E1. International Dairy Federation. IDF Standard 100B. Milk and milk products, enumeration of microorganisms, colony count technique at 30°C. Brussels, Belgium: International Dairy Federation; 1991.

TABLE E1. Weighted prevalence of childhood asthma, atopy, hay fever, and atopic dermatitis by farming status (phases II and III)

	Phase II		Phase III	
Prevalence (%)§	**Farmer**	**Nonfarmer**	**Farmer**	**Nonfarmer**
Asthma	14.0	21.1‡	12.9	18.3†
Current asthma	9.2	15.2‡	8.7	12.8*
Atopic	4.5	8.1‡	5.2	8.6*
Nonatopic	3.5	5.7‡	3.8	5.0
Atopy	24.7	40.8‡	24.1	40.3‡
Hay fever	6.2	16.3‡	7.4	13.5†
Atopic dermatitis	12.9	17.8‡	11.2	18.0*

Differences in numbers occur because of varying proportions of missing values.
*P value of the Pearson χ^2 test for farmer versus nonfarmer < .05.
†P value of the Pearson χ^2 test for farmer versus nonfarmer < .01.
‡P value of the Pearson χ^2 test for farmer versus nonfarmer < .001.
§Weighted number in phase II = 34,491; weighted number in phase III = 11,183.

TABLE E2. Agreement of the reported milk consumption in phase II and the milk samples collected in phase III at the participants' homes (agreement tested for n = 796)

	Reported milk consumption in phase II		
Milk samples collected in phase III	**Exclusively shop milk (n = 419)***	**Shop and farm milk (n = 257)**	**Exclusively farm milk (n = 120)***
Shop milk	98.3%	64.2%	2.0%
Farm milk	1.7%	35.8%	98.0%
	Exclusively farm milk (n = 120)		
	Any unboiled farm milk (n = 102)	Only boiled farm milk (n = 18)	
Shop milk	2.0%	0.0%	
Farm milk >72°C	12.7%	66.7%	
Farm milk <72°C	85.3%	33.3%	

*κ Value of exclusive shop/farm milk consumption in phase II and collected shop/farm milk in phase III = 0.95 (95% CI, 0.92-0.98).