

Letter to the Editor

Immune factors in breast milk related to infant milk allergy are independent of maternal atopy

To the Editor:

The first few months of life represent a window of opportunity for neonatal oral tolerance induction. During this critical time, breast milk is a rich source of immunomodulatory factors, including TGF- β , which has been shown to influence the maturation of the mucosal immune system of the neonates.¹ Reports on breast milk cytokines, chemokines, and growth factors in cow's milk allergy (CMA), including both IgE- and non-IgE-mediated mechanisms, are largely lacking, although IL-5 and IL-13 were identified as risk factors for asthma and eotaxin for atopic dermatitis.^{2,3} Because food allergy represents a failure in development of mucosal tolerance to foods, immune factors in human milk may have a more direct impact on tolerance to foods compared with environmental allergens. In the present study, we sought to assess the immunologic milieu of human milk received by non-CMA infants and those who develop CMA.

We used stored frozen human milk samples from a prospective birth cohort of 145 mother-infant pairs, designed to assess

immunologic factors in human milk and oversampled for newborns with high risk for food allergies.^{4,5} All available samples were assessed from 39 mothers of non-CMA infants and 35 mothers of CMA infants, verified by oral food challenges⁶ performed at median 5.5 months of age (interquartile range [IQR], 3.1-7.7 months). Clinical characteristics are shown in Table E1. Twenty-eight cytokines, chemokines, and growth factors were measured in milk samples after processing⁴ by multiplex kits (Milliplex, Billerica, Mass) and read on a Luminex 200 Multiplex analyzer (Luminex, Austin, Tex); TGF- β 1 was measured by ELISA (Genzyme, Cambridge, Mass) from acid activated samples. As a control, we diluted samples or spiked samples with known amounts of analytes to assess whether levels could be accurately recovered. Those that were not were excluded from further analysis, including TNF- α and - β , CCL1, CCL17, IL-31, eotaxin 3, and CXCL9. Total protein was measured by Pierce protein assay (Thermo Scientific, Rockford, Ill). Please see the supplementary text in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org for details of statistical analysis.

Immune markers were detectable in the majority of samples, except for IL-5, GM-CSF, and IL-12p70 detected in <10% of

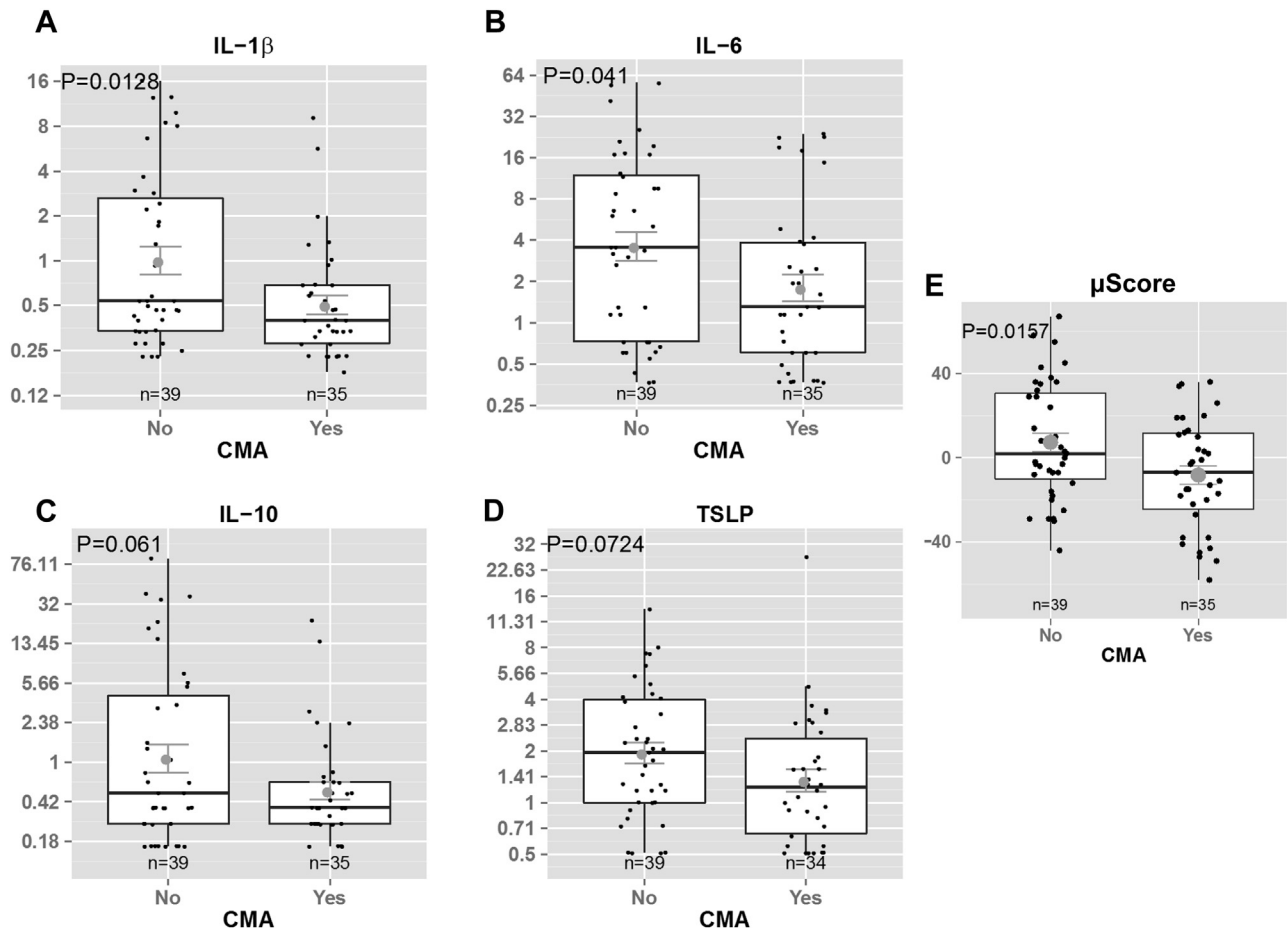


FIG 1. Human milk immune markers that were different between mothers with a CMA infant and those with a non-CMA infant. **A**, IL-1 β . **B**, IL-6. **C**, IL-10. **D**, TSLP (*t* test, shown after logarithmic transformation). **E**, Multivariate score combining all 4 cytokines A-D (multivariate U-statistics). On the box plots' horizontal line, median; box, 25th to 75th percentile; whiskers, 5th to 95th percentile; round symbol, geometric mean.

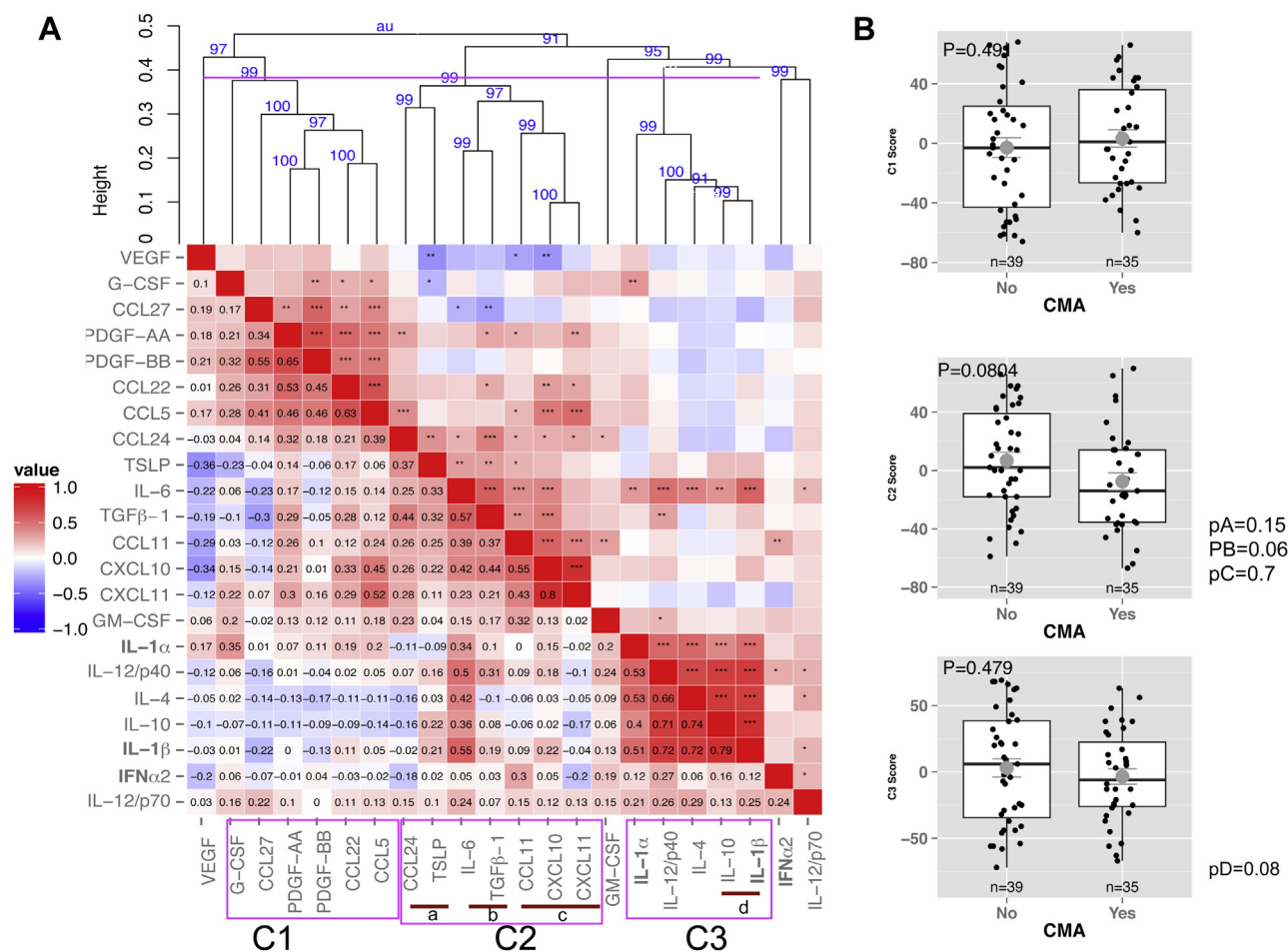


FIG 2. A, Hierarchical cluster analysis of human milk immune factors using Spearman correlation and average agglomeration algorithm. Correlation coefficient associated color coding shown; * $P < .05$, ** $P < .01$, *** $P < .001$. P values (as %) in blue in the resultant dendrogram indicate the degree of stability of each cluster. Height of ≤ 0.4 indicates clusters of significant correlation. Clusters C1, C2, and C3, and subclusters (a-d) were identified. **B,** Multivariate μ score combining all markers in the cluster (multivariate U-statistics).

samples (Table E2). Because mothers of CMA infants had more atopic dermatitis ($P = .002$) and a trend for longer duration of lactation at sampling ($P = .05$, Table E1), we assessed whether maternal covariates (age, duration of breast-feeding, atopic manifestations) had an impact on immune marker levels. Maternal age positively correlated with IL-1 α . Duration of lactation correlated positively with platelet-derived growth factor (PDGF)-BB, CCL27, and vascular endothelial growth factor (VEGF) and negatively with IL-6, TGF- β 1, thymic stromal lymphopoietin (TSLP), CCL11, CXCL10, and CXCL11 (Fig E1). Atopic mothers had lower levels of CCL22, TSLP, and total protein than non-atopic mothers ($P = .0002$, $P = .001$, and $P = .01$, respectively; data not shown). Further analyses were adjusted for maternal atopy and duration of lactation as appropriate.

To assess whether individual human immune markers were associated with protection or risk of CMA, we performed univariate analysis. Milk received by non-CMA infants contained higher levels of IL-1 β , IL-6, IL-10, and TSLP than that received by CMA infants, but only IL-1 β and IL-6 reached statistical significance ($P = .01$ and $P = .04$, respectively; Fig 1, A-D). The levels of these 4 cytokines combined were higher in milk received

by non-CMA infants, even after adjusted for duration of lactation ($P = .049$ muStat; Fig 1, E; $P = .047$ after TGF- β 1 was included in the analysis, decreasing the sample size to 39). IL-1 β and IL-6 positively correlated with milk macrophage/secretory epithelial cell numbers, suggesting these cells as their source (Fig E1). Logistic univariate regression was then used to assess the influence of multiple immunologic factors in human milk on CMA onset, including milk immune markers, leukocytes, eosinophilic cationic protein (ECP), and the levels of β -lactoglobulin (BLG)- and casein-specific IgA, which have been previously reported in this cohort.⁴⁻⁶ After adjusting for maternal covariates, including atopic diseases, age, and duration of lactation, the levels of IL-1 β ($P = .001$), IL-10 ($P = .04$), and BLG-specific IgA ($P = .005$) were lower in the milk of mothers with a CMA infant than in those with a non-CMA infant, and there was a trend for lower IL-6 ($P = .07$, Table E3).

Due to many interactions seen between immune markers, Spearman correlation and hierarchical cluster analysis were carried out to see whether CMA or tolerance would better correlate with clusters of immune markers rather than individual factors. Three main clusters and 4 subclusters were identified (Fig 2, A).

Although the *P* values are only marginally significant (when considering the established .05 cut-off), they show a trend for networks of cytokines, including IL-6 together with TGF- β 1, and IL-10 together with IL-1 β in protection against CMA (*P* = .06 and *P* = .08, respectively). Finally, we used classification and regression trees to classify CMA based on the immune markers, IgA, TGF- β 1, and maternal demographics. The best decision tree fitting the data showed that high levels of IL-6, IL-1 β , and specific IgA and low levels of IL-12p40 and CXCL10 correctly classified 80% of the samples between CMA and non-CMA infants, with sensitivity of 83% and specificity of 75% (Fig E2).

Here, we showed that networks of proinflammatory and regulatory cytokines, including IL-1 β , IL-6, IL-10, and TGF- β 1 in human milk, are associated with tolerance to cow's milk. In early life, intragastric protein digestion is underdeveloped, and high concentrations of anti-proteases such as α 1-antichymotrypsin and α 1-antitrypsin are present in human milk. With an exclusively breast-fed infant ingesting about 500 mL of human milk per day in early life, daily ingestion of cytokines in infants totals micrograms daily. The effects of IL-1 β on gut epithelial cells include stimulation of IL-6.⁷ IL-6 is involved in IgA synthesis, possibly induced by the follicular T helper cells in the Peyer patch germinal centers. Alternatively, these cytokines may provide stimulatory signals to the mammary epithelium to increase secretion of milk IgA, shown to be protective against CMA.⁴ Lastly, IL-1, IL-6, and TGF- β are known to promote Th17 differentiation, with some data suggesting a protective role for IL-17 in food allergy.⁸

We and others^{3,4} have found that human milk biomarker levels are largely, and surprisingly, not related to maternal atopy. Nevertheless, analyses were adjusted for maternal atopic diseases. Further strengths of this study include the prospective design and utilization of multiplex platform limiting variability. The limitations of the study include a relatively small sample size and a high-risk population, limiting applicability to the general population. TGF- β 1 was not measured in all samples, compromising the statistical analysis.

In summary, breast milk is a complex substance that contains a variety of immune factors capable of influencing the infant microbiome⁹ and cytokines such as IL-1 β , IL-6, and IL-10, which may facilitate development of neonatal oral tolerance to foods. It remains to be seen whether these cytokines are directly protective by acting on the infant gastrointestinal tract or are a biomarker of another protective mechanism vertically transmitted through breast milk.

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STATISTICAL ANALYSIS

Statistical comparison of clinical data between groups was done using a Mann-Whitney nonparametric test for 2 groups. Results are shown as median (IQR). Statistical significance for contingency tables was assessed using χ^2 tests (or Fisher exact test, if any cell had less than 5 counts) with GraphPad Prism 4 software (GraphPad Software, Inc, La Jolla, Calif).

Cytokine variables were log2 transformed prior to analysis. To compare the level of cytokines between CMA groups, an ANOVA model was used (similar results are obtained by nonparametric methods; data not shown). Multivariate analysis of variance (MANOVA) and multivariate nonparametric U-statistics^{E1} using R package muStat were used to assess the combined effect of the cytokine panel (IL-1 β , IL-6, IL-10, and TSLP) with CMA. Results are shown as multivariate score obtained for every sample across CMA status.

Hierarchical cluster analysis was carried out using Spearman correlation and average agglomeration algorithm. Order among variables represents their final position after average clustering based on the Euclidean distance of the Spearman correlation. Stability of the clusters was assessed via multiscale bootstrap resampling using *pvclust* library in R. *P* values are represented (as %) in blue in the resultant dendrogram, indicating the degree of stability of each cluster (values >95% indicate stable clusters). A hierarchical, unsupervised clustering is a bottom-up approach, in which each observation starts in its own cluster, and pairs of clusters are merged based on their distance as 1 moves up the hierarchy. Each agglomeration occurs at a greater distance between clusters than the previous agglomeration (represented by the height in the dendrogram).

Logistic univariate regression analysis was conducted to assess the influence of each variable on the risk of CMA using *glm* package in R, before and after adjusting for the mother's

covariates (age of the baby, duration of lactation, age of the mother, and the mother's atopic variables). Multivariate analysis was not performed, due to multiple interactions interfering with the analysis.

We first attempted to identify the best predictors of CMA using the standard multivariate logistic regression model, including all cytokine measurements as independent predictors, along with age of the baby and the mother's variables in an additive model (ie, no interaction). However, upon examination of the best (Akaike's Information Criterion-based) additive model, we realized that the interpretation of the estimated coefficients conflicted with those of the univariate model (ie, odds ratio with opposite directions). In the interpretation of the coefficients (say β_1 , associated with the variable X_1) in the multivariate logistic model, it is essential that none of the remaining effects ($X_2, X_3, \dots, X_k$) can be an interaction effect with x_1 . Upon close examination, we indeed identified quite a number of significant interactions among the cytokines. Modeling such a complex pattern of interactions through a logistic model is virtually impossible with the sample size at hand, and the interpretation of the model would become obscure. For this reason, we used the classification and regression trees (CART) to classify CMA based on the cytokine profiles. CART are nonparametric and nonlinear models, and, therefore, they do not rely on the implicit assumption that the underlying relationships between the predictor variables and the dependent variable are linear, follow some specific nonlinear link function, or are even monotonic in nature. CART decision trees are often able to uncover interactions between predictors in a more efficient and simple way than logistic models.

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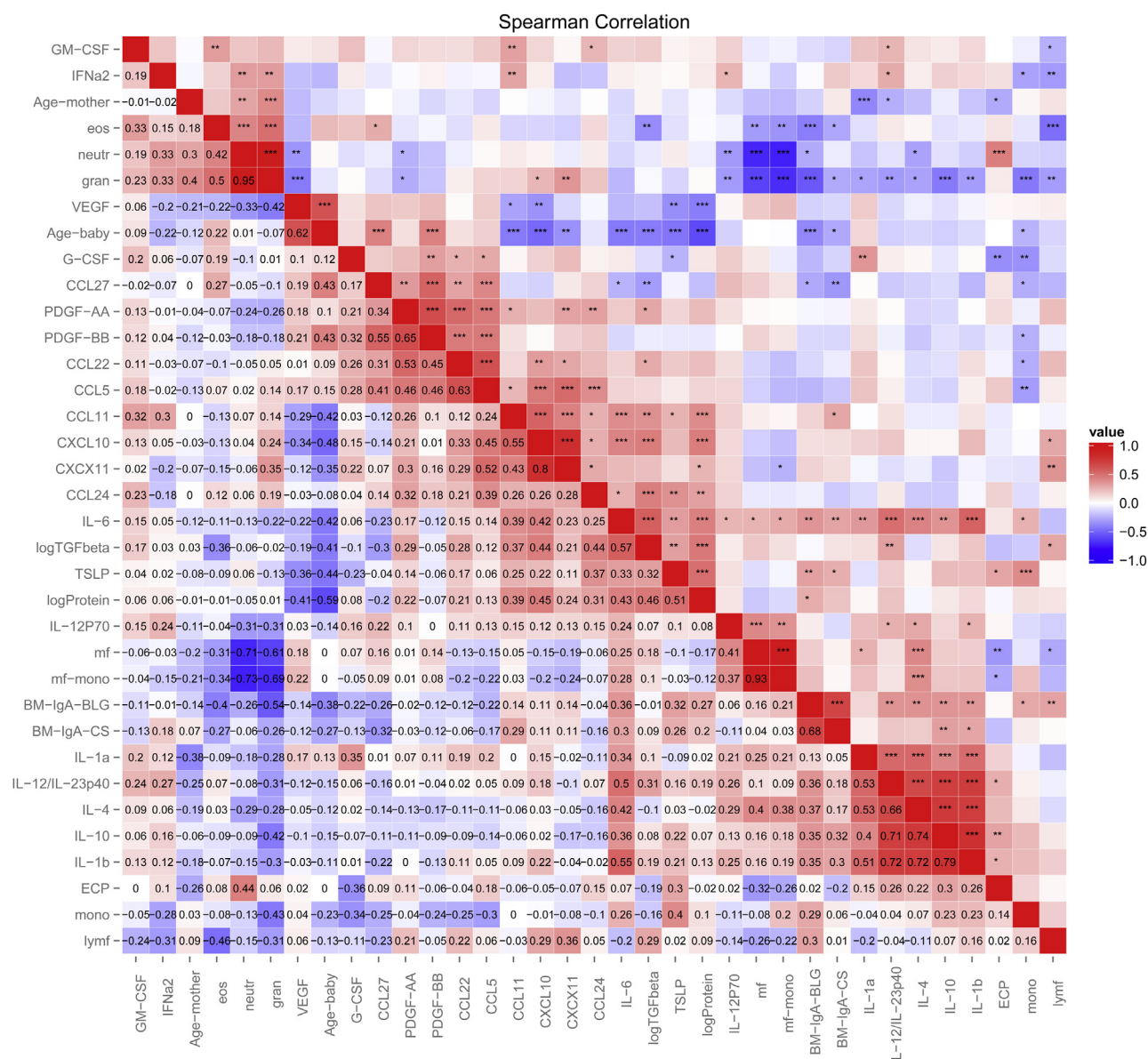


FIG E1. Spearman rank correlation of human milk factors, including cytokines, chemokines, growth factors, specific IgA, leukocyte proportion, age of baby (ie, duration of lactation), and age of mother. Shown are the correlation coefficient and color coding corresponding to values between -1.0 and 1.0 , indicating negative and positive correlation, respectively. $*P < .05$, $**P < .01$, $***P < .001$. BLG, Beta-lactoglobulin; CS, casein; ECP, eosinophil cationic protein; eos, eosinophil; gran, granulocyte; lymf, lymphocyte; mf, macrophage/secretory epithelial cell; mono, monocyte; neutr, neutrophil.

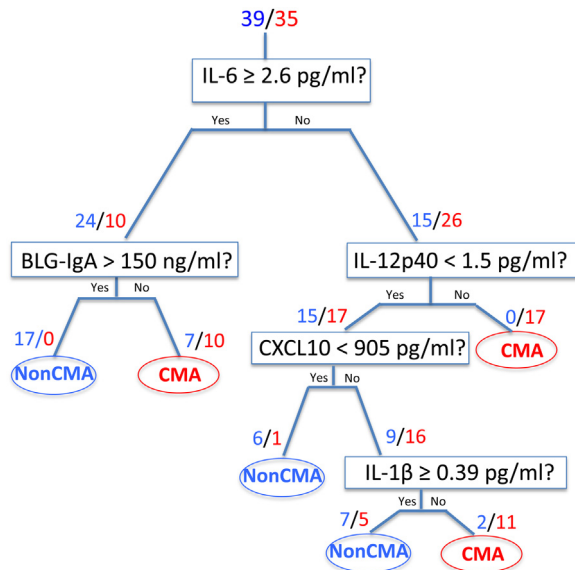


FIG E2. Classification and regression tree with input from human milk levels of cytokines, chemokines, growth factors, specific IgA, and maternal demographics. This model correctly classified CMA outcome in 80% of the samples. Numbers in *blue* indicate healthy infants, and numbers in *red* indicate CMA infants. There are 15 misclassified patients: 9 healthy subjects were misclassified as CMA (7+2+0; see the blue numbers arriving to the red nodes), and 6 CMA patients were misclassified as healthy (0+1+5; see red numbers arriving to the blue nodes).

TABLE E1. Clinical characteristics of mothers and their infants who either had CMA or did not

	Infant with CMA N = 35	Infant with no CMA N = 39	P value
Mother			
Age, y (median, IQR)	31.7 (29.3-35.0)	32.0 (29.9-34.5)	.91
Atopic diseases	25 (71%)	19 (48%)	.047
Asthma	5 (14%)	3 (8%)	.36
Allergic rhinitis	19 (54%)	16 (41%)	.25
Atopic dermatitis	14 (40%)	4 (10%)	.002
Food allergy	7 (20%)	2 (5%)	.051
Infant			
Age at sample, mo (median, IQR)	1.6 (0.9-3.2)	1.0 (0.12-1.9)	.053
Breast-feeding, mo (median, IQR)			
Exclusive	3.0 (1-4)	3.0 (1.5-4.5)	.39
Partial	7.0 (4.6-9.5)	7.0 (5.3-8.6)	.91
CM formula			
Received in nursery	3 (9%)	3 (8%)	
Introduction, mo (median, IQR)	4.0 (1.5-7)	4.0 (1.5-6)	.70
Symptom onset, mo (median, IQR)	2.0 (0.6-3.9)	4.0 (3.5-5)	.07
Symptoms			
Atopic dermatitis	22 (63%)	13 (33%)	.011
Gastrointestinal*	20 (57%)	12 (31%)	.022
Asthma	6 (17%)	2 (3%)	.097
Immediate type CMA†	16		
Delayed type CMA‡	19		
Other food allergy§			
Hen's egg allergy	2 (6%)	2 (3%)	.9
Wheat allergy	6 (17%)	1 (3%)	.032
Multiple food allergy	13 (37%)	1 (3%)	.0001

Comparisons are done with the Mann-Whitney test.

CM, Cow's milk; CMA, cow's milk allergy.

*Regurgitation, vomiting, diarrhea, abdominal pain/colic.

†Onset within 2 hours of last dose (hives n = 11, maculopapular rash n = 3, vomiting n = 1; specific IgE present in 8 subjects).

‡Eczema exacerbation n = 3, diarrhea/regurgitation n = 7, eczema and diarrhea/regurgitation n = 9.

§Numbers are not mutually exclusive.

||P < .05.

TABLE E2. Cytokines, chemokines, and growth factors measured in human milk of mothers with a healthy child and in those with an infant with CMA

Original name		Median (IQR), pg/mL*	Range of detection, pg/mL*	N above limit of detection/N measured
Proinflammatory				
IL-1 α	Interleukin-1 α	9.1 (0.2-12.7)	0.64-10,000	65/74
IL-1 β	Interleukin-1 β	0.47 (0.34-1.3)	0.64-10,000	74/74
IL-6	Interleukin-6	7.4 (0.69-9.4)	0.64-10,000	69/74
IFN- α 2	Interferon- α 2	1.5 (2.28-1.8)	0.64-10,000	50/74
T _H 1 cytokines				
IL-12p40	Interleukin-12 subunit p40	1.3 (0.06-8.0)	0.64-10,000	39/74
IL-12p70	Interleukin-12 subunit p70	0.34 (0.34-0.34)	0.64-10,000	6/74
T _H 2 cytokines				
IL-4	Interleukin-4	1.1 (0.63-4.2)	0.64-10,000	69/74
IL-5	Interleukin-5	1.4 (1.4-1.4)	6-25,000	2/66
TSLP	Thymic stromal lymphopoietin	2.6 (0.9-3.0)	2.45-10,000	63/73
Regulatory				
IL-10	Interleukin-10	4.7 (0.26-1.4)	0.64-10,000	62/74
TGF- β 1	Transforming growth factor- β 1	766 (426-935)	60-4,000	39/39
Chemokines				
CCL5 (RANTES)	Regulated on activation, normal T cell expressed and secreted	20.9 (5.3-26.9)	0.64-10,000	74/74
CCL11 (Eotaxin-1)	Eosinophil chemotactic protein-1	0.74 (0.74-7.4)	0.74-10,000	34/74
CCL22 (MDC)	Macrophage-derived chemokine	153 (25.3-182)	0.64-10,000	74/74
CCL24 (Eotaxin-2)	Eosinophil chemotactic protein-2	16.1 (5.9-18.8)	2.45-10,000	73/73
CCL27 (CTACK)	Cutaneous T-cell-attracting chemokine	9.7 (1.6-15.1)	1.22-5,000	70/73
CXCL10 (IP-10)	Interferon- γ -induced protein 10	3,261 (980-4,399)	0.64-10,000	74/74
CXCL11 (I-TAC)	Interferon-inducible T cell α chemoattractant	100 (25.2-120)	0.49-2,000	66/66
Growth factors				
G-CSF	Granulocyte-colony stimulating factor	2.4 (1.1-5.9)	0.64-10,000	74/74
GM-CSF	Granulocyte-macrophage colony stimulating factor	0.09 (0.09-0.09)	0.64-10,000	4/74
PDGF-AA	Platelet-derived growth factor-AA	87.9 (44.1-111)	0.64-10,000	74/74
PDGF-BB	Platelet-derived growth factor-BB	75.4 (12.3-103)	0.64-10,000	73/74
VEGF	Vascular endothelial growth factor	1,309.9 (1,051.5-1,549.8)	0.64-10,000	74/74

*Levels were measured by multiplex platform, except for TGF- β 1, which was measured by ELISA.

TABLE E3. Predictive models for CMA analyzed by univariate logistic regression analysis

	No covariates			Mother's covariates		
	RR	SEM	P value	RR	SEM	P value
BLG-IgA	−0.67	0.28	.014*	−1.14	0.41	.005*1
IL-1β	−0.38	0.16	.019*	−0.62	0.24	.011*1
IL-10	−0.20	0.11	.068	−0.32	0.16	.041
IL-4	−0.17	0.11	.113	−0.26	0.14	.060
Protein	−1.45	0.68	.033	−1.64	0.89	.066
IL-6	−0.24	0.12	.044	−0.24	0.13	.074
PDGF-BB	0.17	0.12	.148	0.24	0.14	.087
TSLP	−0.36	0.20	.077	−0.39	0.24	.113
IL-12p40	−0.06	0.07	.335	−0.12	0.08	.162
PDGF-AA	0.21	0.24	.386	0.35	0.28	.203
CXCL10	−0.18	0.16	.255	−0.22	0.17	.206
VEGF	0.74	0.47	.113	0.54	0.49	.270
CS-IgA	−0.24	0.14	.082	−0.16	0.16	.318
CCL24	−0.05	0.15	.761	−0.16	0.19	.390
IL-12p70	−0.58	0.71	.420	−0.67	0.85	.425
IFN-α2	0.15	0.16	.338	0.14	0.19	.456
G-CSF	0.05	0.14	.740	0.11	0.16	.501
CCL22	0.00	0.10	.974	0.08	0.13	.526
IL-5	−3.63	5.83	.533	−2.88	6.00	.632
CCL27	−0.02	0.12	.884	0.04	0.15	.793
CCL11	0.03	0.11	.791	0.03	0.12	.813
IL-1α	−0.02	0.07	.772	−0.02	0.09	.835
CCL5	−0.02	0.13	.852	0.02	0.15	.877
TGF-β1	−0.11	0.30	.708	0.04	0.37	.907
CXCL11	−0.02	0.15	.876	0.02	0.16	.923

Results are presented before and after adjusting for the mother's covariates (age, atopic diseases, current atopic symptoms, and duration of lactation).

RR, Risk ratio.