Extensively hydrolyzed casein formula containing *Lactobacillus rhamnosus* GG reduces the occurrence of other allergic manifestations in children with cow's milk allergy: 3-year randomized controlled trial

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Background: Children with cow's milk allergy (CMA) have an increased risk of other allergic manifestations (AMs).
Objective: We performed a parallel-arm randomized controlled trial to test whether administration of an extensively hydrolyzed casein formula (EHCF) containing the probiotic *Lactobacillus rhamnosus* GG (LGG) can reduce the occurrence of other AMs in children with CMA.
Methods: Children with IgE-mediated CMA were randomly allocated to the EHCF or EHCF+LGG groups and followed for 36 months. The main outcome was occurrence of at least 1 AM (eczema, urticaria, asthma, and rhinoconjunctivitis). The secondary outcome was tolerance acquisition, which was defined as the negativization of a double-blind food challenge results at 12, 24, and 36 months. AMs were diagnosed according to standardized criteria. Tolerance acquisition was evaluated every 12 months.
Results: A total of 220 children (147 boys [67%]) with a median age of 5.0 months (interquartile range, 3.0-8.0 months) were randomized; 110 children were placed in the EHCF group, and 110 children were placed in the EHCF+LGG group. In the complete case analysis the absolute risk difference for the occurrence of at least 1 AM over 36 months was −0.23 (95% CI, −0.36 to −0.10; $P < .001$), and the absolute risk difference for the acquisition of cow's milk tolerance was 0.20 (95% CI, 0.05-0.35; $P < .01$) at 12 months, 0.24 (95% CI, 0.08-0.41; $P < .01$) at 24 months, and 0.27 (95% CI, 0.11-0.43; $P < .001$) at 36 months. In the sensitivity analysis the effect size of the main outcome was virtually unchanged when the occurrence of AMs was assigned to all 27 missing children.
Conclusions: EHCF+LGG reduces the incidence of other AMs and hastens the development of oral tolerance in children with IgE-mediated CMA. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

Key words: *Eczema, urticaria, asthma, rhinoconjunctivitis, atopic march, food allergy, gut microbiota, probiotics*

Food allergy (FA) is a common chronic condition in childhood.[1] Recent studies have suggested that the natural history of FA has changed during the last 2 decades, with an increased prevalence, severity of clinical manifestations, and risk of persistence into later ages.[2,3] The increased FA prevalence in children has an important economic effect, with significant direct costs for the health care system and even larger costs for the families.[4] In addition, children with FA are at increased risk of having other allergic manifestations (AMs) later in life. According to a recent US Centers for Disease Control and Prevention study, children with FA are 2 to 4 times more likely to have other AMs, such as asthma (4.0 times), atopic eczema (2.4 times), and respiratory allergies (3.6 times), compared with children without FA.[5,6]

Cow's milk allergy (CMA) is among the most common FA in early childhood, with an estimated prevalence of 2% to 3%.[7] We previously showed that in children with CMA, an extensively hydrolyzed casein formula (EHCF) supplemented with the probiotic *Lactobacillus rhamnosus* GG (LGG) induced higher tolerance rates compared with EHCF without LGG and other formulas.[8,9] These findings were consistent with those of a 1-year follow-up study performed in the United States that showed better outcomes with EHCF+LGG- versus EHCF- or amino acid–based formula for the first-line dietary management of CMA.[10] Multiple mechanisms might be responsible for the observed clinical effects of the EHCF+LGG formula. Preliminary data suggested that dietary intervention with EHCF+LGG has positive effects on gut dysbiosis, short-chain fatty acid production,[11] and epigenetic regulation of T_H1 and T_H2 cytokines gene expression.[12] Such mechanisms suggest a possible long-term effect on the immune system of children with CMA treated with EHCF+LGG. The present randomized controlled trial (RCT) was designed to test whether a dietary intervention with EHCF supplemented with

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Abbreviations used
AM: Allergic manifestation
ARD: Absolute risk difference
BRM: Binomial regression model
CCA: Complete case analysis
CMA: Cow's milk allergy
DBPCFC: Double-blind, placebo-controlled food challenge
EHCF: Extensively hydrolyzed casein formula
FA: Food allergy
FP: Family pediatrician
HDAC: Histone deacetylase
IQR: Interquartile range
LGG: *Lactobacillus rhamnosus* GG
RCT: Randomized controlled trial
SA-BCS: Sensitivity analysis–best-case scenario
SA-EQS: Sensitivity analysis–equal worst outcome scenario
SA-WCS: Sensitivity analysis–worst-case scenario
SPT: Skin prick test

LGG could influence the occurrence of other AMs in children with IgE-mediated CMA.

METHODS

Study design

The present parallel-arm RCT was designed to test whether EHCF+LGG can reduce the incidence of other AMs compared with EHCF in children with IgE-mediated CMA. The RCT was performed from October 2008 to December 2014 in collaboration with family pediatricians (FPs) who care for children up to 14 years of age in the Italian National Health System.

Before the start of the study, all of the involved FPs attended an investigator meeting in which the study protocol was illustrated and discussed, and all of the procedures and definitions were shared.

Ethics

The study was approved by the Ethics Committee of the University of Naples "Federico II" and was registered at at www.clinicaltrials.gov (registration no. NCT01891916).

Study subjects

The design of the study is depicted in Fig 1.

Children who were consecutively observed at FP's office because the recent occurrence of signs and symptoms possibly related to IgE-mediated CMA were sent to our tertiary center for pediatric allergy for possible inclusion in the study.

The inclusion criteria were age of 1 to 12 months and suspected IgE-mediated CMA. The exclusion criteria were cow's milk protein–induced anaphylaxis, food protein–induced enterocolitis syndrome, other FAs, other allergic diseases, non–CMA-related atopic eczema, eosinophilic disorders of the gastrointestinal tract, chronic systemic diseases, congenital cardiac defects, active tuberculosis, autoimmune diseases, immunodeficiency, chronic inflammatory bowel diseases, celiac disease, cystic fibrosis, metabolic diseases, malignancy, chronic pulmonary diseases, malformations of the gastrointestinal and/or respiratory tract, and administration of prebiotics or probiotics during the 4 weeks before enrollment.

Only subjects who met the inclusion criteria were invited to participate in the study. Written informed consent was obtained from the parents/tutors of each subject. Anamnestic, demographic, anthropometric, and clinical data, as well as information on sociodemographic factors, family and living conditions, parental history of allergic diseases, maternal smoking during pregnancy, environmental tobacco smoke exposure, number of siblings, and pet ownership, were obtained from the parents of each infant and recorded in a clinical database.

Then skin prick tests (SPTs) were performed, and according to a 1:1 randomization list prepared by a biostatistician who was not involved in the statistical analysis, infants were randomly allocated to one of 2 groups of dietary intervention: group 1, who received EHCF (Nutramigen; Mead Johnson Nutrition, Evansville, Ind), or group 2, who received EHCF containing LGG (Nutramigen LGG, Mead Johnson Nutrition).

From 2 to 4 weeks after the first assessment, when full and stable remission of CMA symptoms was achieved, a double-blind, placebo-controlled food challenge (DBPCFC) was performed. The recruitment continued until a prespecified number of 110 subjects per group with DBPCFC-proved IgE-mediated CMA was achieved, and only these subjects continued the exclusion diet using the hypoallergenic formula prescribed at randomization.

Intervention and data collection

Both study products were commercially available as formula powder for CMA dietary treatment in Italy. The composition of the 2 formulas was identical except for the presence of LGG; the price was the same, and the packaging was very similar. The physical and organoleptic properties of the 2 formulas were identical. The physicians responsible for enrollment of patients allocated the next available number on entry into the trial.

The dietitians counselled parents about issues that could arise during the elimination diet and on how to replace cow's milk with an alternative source of nutrients to reach the daily recommended intake for Italian children.[13] The parents received a written prescription for an exclusion diet for CMA with the formula name, daily amount, and preparation instructions, according to the manufacturer's specifications. The randomized formulas were directly purchased by the parents of the children.

Effective use of the formula was evaluated during the study by dietitians counselling parents about issues that could arise during the elimination diet, but these dietitians were not involved in evaluation of study outcomes. Parents or caregivers were asked to keep a daily record of formula use. The amount prepared (millimeters of water and number of formula spoons) and amount left after each consumption were recorded in a diary to assess the amount consumed by the child. This allowed the study staff to evaluate compliance with the assigned formula and to ensure that the patients received an appropriate quantity of formula to meet their nutritional requirements.

Then, during a 3-year follow-up, 3 visits (at 12, 24, and 36 months) were planned. SPTs and oral food challenges were performed to explore tolerance acquisition to cow's milk every 12 months. Unscheduled visits were made when the FP noticed any allergic symptoms. At each visit, the children were physically examined, body growth was assessed, and a structured interview on health problems, including allergic symptoms, was carried out. Both DBPCFCs and clinical examinations were performed at the tertiary center by 2 investigators blinded to group assignments. In the case of discordance about an AM diagnosis, further evaluation by a third pediatrician experienced in pediatric allergy was performed. AMs were diagnosed by using standardized criteria.

Atopic eczema was diagnosed based on pruritus, typical morphology and distribution, a chronic or chronically relapsing course, and personal or family atopic history (3 of 4 criteria) in addition to 3 minor criteria among a list of 21, as previously reported.[14]

Allergic rhinoconjunctivitis was diagnosed based on the symptoms of rhinitis, such as nasal congestion, sneezing, itching, rhinorrhea, current use of medication for these symptoms, and/or conjunctivitis, after exclusion of infection.[15]

Allergic urticaria was diagnosed if at least 2 episodes of itching eruptions or swelling with typical appearance were observed by the parents or a physician and were caused by the same allergen. In the case of a single episode, immunologic evidence (SPT with the accused undiluted native allergen causing a wheal reaction of ≥3 mm or an allergen-specific IgE level of ≥0.35 KU/L) or a positive provocation response with the suspected allergen was performed for definitive diagnosis.[16]

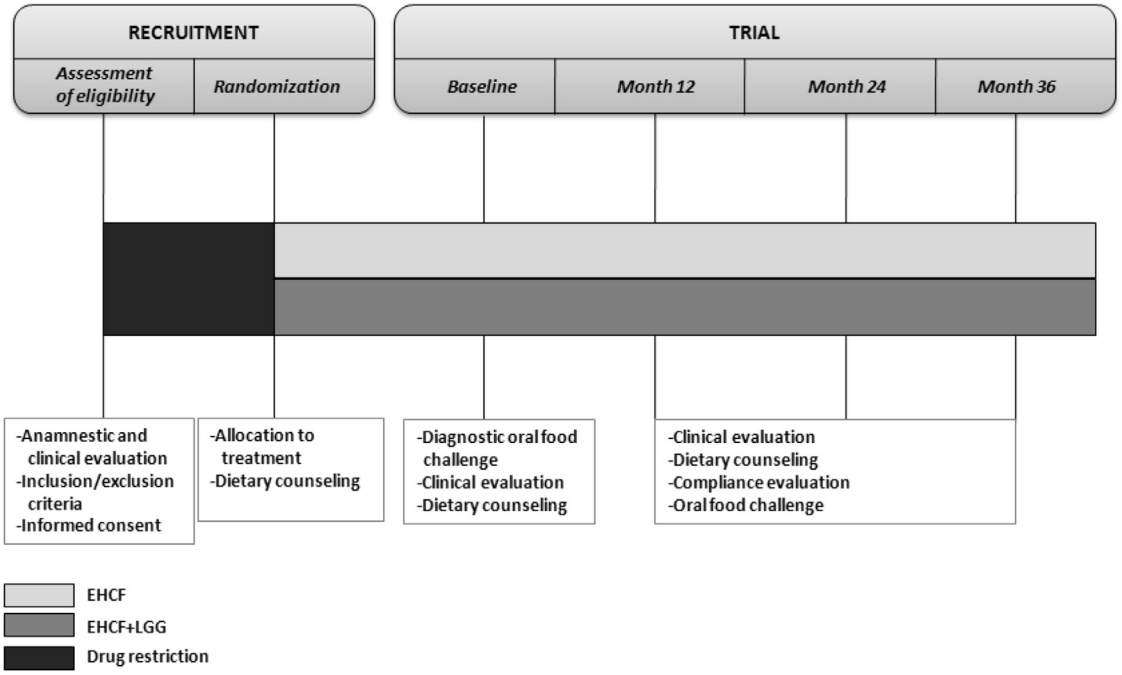

FIG 1. Study design.

The symptoms considered for the diagnosis of asthma were as follows: recurrent wheeze (more than once a month), difficulty in breathing, chest tightness, or both; cough (worse at night); clinical improvement during treatment with short-acting bronchodilators and inhaled steroids; and worsening when treatment was stopped. Alternative causes of recurrent wheezing were considered and excluded.[17]

IgE-mediated FA was defined as the presence of (1) a clinical history suggestive of an IgE-mediated mechanism (acute onset of symptoms after the ingestion of trigger food); (2) DBPCFC findings (occurrence of typical symptoms within 2 hours after the administration of the last dose); (3) occurrence of typical symptoms of IgE-mediated FA (ie, pruritus without skin lesions, urticaria, atopic eczema exacerbation, angioedema, vomiting, diarrhea, bloody stools, abdominal pain, rhinitis, nasal congestion, wheeze, cough, stridor, and difficulty breathing during the challenge); and (3) results of SPTs (wheal size >3 mm) and/or serum IgE measurements (>0.1 kU/L).[1,6,8]

Compliance was operationally defined as consumption of at least 80% of the assigned treatment and was evaluated as previously described at every follow-up visit by dietitians who were not involved in the evaluation of the study outcomes.

Study outcomes

The primary outcome was the occurrence of any AM (eczema, urticaria, asthma, or rhinoconjunctivitis) during the 36 months of the study. The secondary outcome was the acquisition of tolerance at 12, 24, and 36 months. The occurrence of any other IgE-mediated FA alone or in combination with AMs was also recorded.

Calculation of sample size

We aimed at detecting a difference of 20% in the incidence of AM in the EHCF+LGG group versus the EHCF group with a power of 90% at an α level of .05 (Pearson χ^2 test). Assuming a missingness rate of 10% at follow-up, this implies enrollment of 110 children per group. However, because the children had to have a DBPCFC-confirmed CMA diagnosis, we had to increase the pool of children allocated to the 2 treatments, and we designed the study to enroll up to 150 children per group until at least 110 children per group had a DBPCFC-confirmed CMA diagnosis.

Study tests

SPTs. SPTs were performed with fresh milk and allergens of cow's milk protein, such as α-lactalbumin, β-lactoglobulin, and casein (Lofarma S.p.A, Milan, Italy). The allergen was applied to the patient's volar forearm. SPTs were performed with a 1-mm single peak lancet (ALK-Abelló, Copenhagen, Denmark) with histamine dihydrochloride (10 mg/mL) and an isotonic saline solution (NaCl 0.9%) as positive and negative controls, respectively. Reactions were recorded on the basis of the largest diameter (in millimeters) of the wheal and flare at 15 minutes. The SPT response was considered "positive" if the wheal was 3 mm or larger without a reaction to the negative control.

Oral food challenge. All food challenges were performed in a double-blind, placebo-controlled manner in the outpatient clinic of the tertiary center involved in the study on 2 separate days with a 1-week interval. Parents of infants taking antihistamines were advised to withhold these medications for 72 hours before and during the challenge. Randomization and preparation of the challenges were performed by experienced FA dietitians who were not directly involved in the procedures. In detail, every 20 minutes, increasing doses (0.1, 0.3, 1, 3, 10, 30, and 100 mL) of fresh pasteurized cow's milk containing 3.5% of fat or an amino acid formula were administered. Full emergency equipment and medications (epinephrine, antihistamines, and steroids) were available. The results were assessed simultaneously by experienced pediatric allergists. Study subjects were scored for 9 items divided into 4 main categories on a 0- to 3-point scale (0, none; 1, light; 2, moderate; and 3, severe): (1) general (decreased blood pressure plus tachycardia); (2) skin (rash and urticaria/angioedema); (3) gastrointestinal (nausea or repeated vomiting, crampy-like abdominal pain, and diarrhea); and (4) respiratory (sneezing or itching, nasal congestion or rhinorrhea, and stridor deriving from upper airway obstruction or wheezing). If at least 2 of the 3 physicians independently scored any item at level 3 or 2 (or more) items at level 2, the test result was considered positive. Clinical symptoms occurring within 2 hours of administering the highest dose were defined as having "immediate reactions." Infants were observed for 2 hours after the final dose and then discharged. In case of a positive DBPCFC result at any testing dose, the patient remained under observation until symptom resolution. If the patient did not show any symptoms within the first 24 hours, parents were advised to provide a single feed of 100 mL of the tested formula (verum or placebo) every

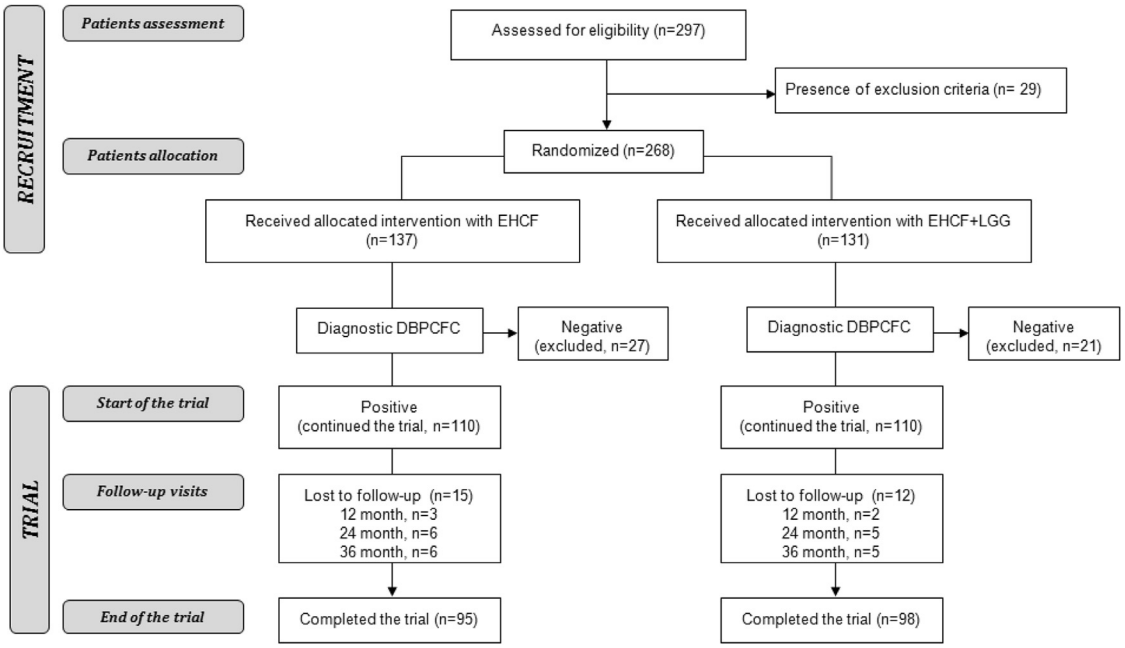

FIG 2. Flow of the children through the study.

day at home for 7 days. If any symptoms occurred during this period, the patients returned to the outpatient clinic on the same day. After 7 days of verum or placebo administration, the patients were examined, and the parents were interviewed at the center. Parents were asked to contact the center if any symptoms occurred in the 7 days after the DBPCFC procedures to rule out false-negative challenge results. The challenge result was considered negative if the patient tolerated the entire challenge, including the observation period. Clinical tolerance acquisition was defined by the presence of a negative DBPCFC result. Children with a negative DBPCFC result at any subsequent visit were re-evaluated after 6 months to check for the persistence of tolerance acquisition.

Statistical analysis

Most continuous variables had non-Gaussian distributions, and all are reported as medians and interquartile ranges (IQRs). Discrete variables are reported as counts and proportions or as medians and IQRs.

The main outcome (ie, the effect of EHCF+LGG vs EHCF on the occurrence of any AM during 36 months) was evaluated by using a binomial regression model (BRM).[18] The response variable of the BRM was the occurrence of any AM during 36 months (discrete: 0 = no, 1 = yes), and the predictor variable was treatment (discrete: 0 = EHCF, 1 = EHCF+LGG). The point estimate and 95% CI of the absolute risk difference (ARD) were obtained from the BRM and inverted to calculate the number needed to treat.[19] We also assessed the degree to which the ARD changed after the inclusion of the following discrete covariables into the BRM: sex (0 = female, 1 = male), birth at term (discrete: 0 = no, 1 = yes), cesarean delivery (discrete: 0 = no, 1 = yes), breast-feeding for at least 2 months (discrete: 0 = no, 1 = yes), familial risk of allergy (discrete: 0 = no, 1 = yes), exposure to passive smoking (discrete: 0 = no, 1 = yes), mother smoking during pregnancy (discrete: 0 = no, 1 = yes), and exposure to pets (discrete: 0 = no, 1 = yes).

The secondary outcome (ie, the time-specific incidence of CMA tolerance) was evaluated by using a BRM for repeated measures.[20] The response variable of the BRM was the development of CMA tolerance (discrete: 0 = no, 1 = yes), and the predictors were treatment (discrete: 0 = EHCF, 1 = EHCF+LGG), time (discrete: 0 = 12 months, 1 = 24 months, 2 = 36 months), and a treatment-by-time interaction (discrete-by-discrete). Repeated measures were taken into account by using subject-specific cluster CIs. Three prespecified between-group (EHCF+LGG vs EHCF) within-time (12, 24, and 36 months) contrasts were used to quantify the time-specific ARDs in CMA tolerance. This corresponds to evaluating the EHCF+LGG versus LGG difference separately at 12, 24, and 36 months. The relative ARDs and *P* values were corrected by using a Bonferroni correction for the 3 comparisons (contrasts).

In addition to doing a complete case analysis (CCA), we performed a sensitivity analysis using the following scenarios: (1) all missing values of EHCF+LGG and EHCF set to the worst outcome (sensitivity analysis–equal worst-outcome scenario [SA-EQS]); (2) missing values of the EHCF+LGG group set to the best outcome and missing values of the EHCF group set to the worst outcome (sensitivity analysis–best-case scenario [SA-BCS]); and (3) missing values of the EHCF+LGG group set to the worst outcome and missing values of the EHCF group set to the best outcome (sensitivity analysis–worst-case scenario [SA-WCS]). The worst outcome was defined as the occurrence of any AM for the main outcome and as the absence of tolerance at all time points for the secondary outcome. The SA-EQS for the main outcome was prespecified by the study protocol. All other sensitivity analyses were implemented *post hoc*. Statistical analysis was performed with Stata 14.1 software (StataCorp, College Station, Tex).

RESULTS

The flow of the children during the study is reported in Fig 2.

We designed the study to enroll up to 150 children per group until at least 110 children per group had a DBPCFC-confirmed CMA diagnosis (see "Calculation of sample size"). A total of 137 of 150 planned children provided 110 cases of DBPCFC-confirmed CMA in the ECHF group, and 131 of 150 planned children provided 110 cases of DBPCFC-confirmed CMA in the ECHF+LGG group. A total of 220 subjects with a DBPCFC-confirmed diagnosis of IgE-mediated CMA (147 male subjects, 67%) with a median age of 5.0 months (IQR, 3.0-8.0 months) were enrolled.

All children were from families of middle socioeconomic status and lived in urban areas. At baseline, the main features of the study groups were similar (Table I).

TABLE I. Baseline features of the subjects enrolled in the study

Feature	EHCF	EHCF+LGG
Male sex, no. (%)	75/110 (68.2)	72/110 (65.5)
Cesarean delivery, no. (%)	69/110 (62.7)	66/110 (60.0)
Born at term, no. (%)	101/110 (91.8)	98/110 (89.1)
Weight at birth (kg), median (IQR)	3.4 (3.0 to 3.7)	3.2 (2.9 to 3.5)
Breast-fed for ≥2 mo, no. (%)	84/110 (76.4)	77/110 (70.0)
Weaning (mo), median, (IQR)	5.0 (5.0 to 6.0)	5.0 (4.0 to 6.0)
Siblings (count), median (IQR)	1 (0.0 to 1.0)	1 (0.0 to 1.0)
Familial risk of allergy, no. (%)	79/110 (71.8)	75/110 (68.2)
Allergic first-degree relatives (count), median (IQR)*	1 (1 to 2)	1 (1 to 2)
Exposure to passive smoking, no. (%)	39/110 (35.5)	38/110 (34.5)
Mother smoked during pregnancy, no. (%)	41/110 (37.3)	35/110 (31.8)
Exposure to pets, no. (%)	13/110 (11.8)	18/110 (16.4)
Age at CMA diagnosis (mo), median (IQR)	5.0 (3.0 to 8.0)	5.0 (3.0 to 8.0)
Weight at CMA diagnosis (kg), median (IQR)	7.5 (6.1 to 8.6)	7.4 (6.1 to 8.7)
Weight at CMA diagnosis (SDS WHO), median (IQR)	0.16 (−0.41 to 0.69)	0.14 (−0.45 to 0.79)
Length at CMA diagnosis (m), median (IQR)	0.66 (0.61 to 0.70)	0.66 (0.61 to 0.70)
Length at CMA diagnosis (SDS WHO), median (IQR)	0.05 (−0.76 to 0.52)	−0.03 (−0.56 to 0.94)
BMI at CMA diagnosis (kg/m^2), median (IQR)	17.3 (16.2 to 18.1)	17.2 (16.2 to 18.1)
BMI at CMA diagnosis (SDS WHO), median (IQR)	0.29 (−0.45 to 0.73)	0.26 (−0.48 to 0.66)
Positive prick by prick test result for fresh milk, no. (%)	110/110 (100.0)	110/110 (100.0)
Positive SPT response for α-lactalbumin, no. (%)	96/110 (87.3)	94/110 (85.5)
Positive skin test response for β-lactoglobulin, no. (%)	60/110 (54.5)	70/110 (63.6)
Positive SPT response for casein, no. (%)	49/110 (44.5)	51/110 (46.4)
Gastrointestinal symptoms at CMA onset, no. (%)	61/110 (55.5)	65/110 (59.1)
Cutaneous symptoms at CMA onset, no. (%)	75/110 (68.2)	73/110 (66.4)
Respiratory symptoms at CMA onset, no. (%)	17/110 (15.5)	14/110 (12.7)

SDS, SD score; *WHO*, World Health Organization.
*Calculated only for the subjects with familial risk of allergy.

All children were compliant (ie, they consumed ≥80% of the assigned formula). No case of misunderstanding of formula use was reported. A total of 27 (12%) children were lost during follow-up: 15 in the EHCF group and 12 in the EHCF+LGG group. Fourteen children withdrew from the study because their family changed their city of residence, and 13 withdrew for unknown reasons (they could not be contacted by the study center or by the FPs). Of the 15 children lost to follow-up in the EHCF group, 3 were lost at 12 months, 6 at 24 months, and 6 at 36 months. Of the 12 children lost to follow-up in the EHCF+LGG group, 2 were lost at 12 months, 5 at 24 months, and 5 at 36 months. Table E1 in this article's Online Repository at www.jacionline.org compares the baseline features of the subjects who completed the study with those of the subjects who were lost to follow-up.

Main outcome

Table II reports the frequency of the main outcome (any AM during 36 months), of the overall and time-specific frequency of its components (eczema, urticaria, asthma, and rhinoconjunctivitis), and of the overall frequency of other FAs alone or in combination with AMs.

The ARD of any AM for EHCF+LGG versus EHCF was (1) −0.23 (95% CI, −0.36 to −0.10; $P < .001$) at CCA; (2) −0.22 (95% CI, −0.35 to −0.09; $P < .001$) at SA-EQS; (3) −0.33 (95% CI, −0.45 to −0.21; $P < .001$) at SA-BCS; and (4) −0.08 (95% CI, −0.21 to 0.04; $P = .5$) at SA-WCS. The SA-EQS estimate was very similar to the CCA estimate. On absolute grounds, the SA-BCS was 10% higher and the SA-WCS was 15% lower than the CCA estimate. Even under the worst-case scenario, a difference in favor of EHCF+LGG was still present (8%). By using the CCA estimate of the ARD, the number needed to treat was 4 (95% CI, 3-10). This means that, compared with EHCF, 4 subjects needed to be treated with EHCF+LGG for 36 months to prevent at least 1 AM.

Fig 3 plots the incidence of the main outcomes under CCA.

Table E2 in this article's Online Repository at www.jacionline.org shows the effect of selected baseline covariables on the incidence of the main outcome. The effect was negligible in all cases, being less than 2% in absolute terms and less than 10% in relative terms.

Fig E1 in this article's Online Repository at www.jacionline.org plots the incidence of the components of the main outcome during the study, as determined by CCA. This is an exploratory analysis performed because the main outcome is a composite outcome and should therefore only be used for hypothesis-generating purposes.

Secondary outcome

The ARD of cow's milk tolerance for EHCF+LGG versus LGG was (1) 0.20 (95% CI, 0.05-0.35; $P < .01$) at 12 months, 0.24 (95% CI, 0.08-0.41; $P < .01$) at 24 months, and 0.27 (95% CI, 0.11-0.43; $P < .001$) at 36 months by using CCA; (2) 0.15 (95% CI, 0.00-0.31; $P = .06$) at 12 months, 0.20 (95% CI, 0.05-0.35; $P < .01$) at 24 months, and 0.23 (95% CI, 0.09-0.37; $P < .001$) at 36 months by using SA-EQS; (3) 0.29 (95% CI 0.15 to 0.43, $P < .001$) at 12 months, 0.34 (95% CI, 0.18-0.49; $P < .001$) at 24 months and 0.36 (95% CI, 0.22-0.51; $P < .001$) at 36 months by using SA-BCS; and (4) 0.05 (95% CI, −0.11 to 0.20; $P = 1.0$) at 12 months, 0.09 (95% CI, −0.07 to 0.25; $P = .5$) at

TABLE II. Frequency of the main outcome, its components, and other FAs

	EHCF		EHCF+LGG	
	No.	Percent	No.	Percent
Eczema at 12 mo				
No	78	82.1	84	85.7
Yes	17	17.9	14	14.3
Total	95	100.0	98	100.0
Eczema at 24 mo				
No	92	96.8	98	100.0
Yes	3	3.2	0	0.0
Total	95	100.0	98	100.0
Eczema at 36 mo				
No	89	93.7	97	99.0
Yes	6	6.3	1	1.0
Total	95	100.0	98	100.0
Eczema total episodes				
No	69	72.6	83	84.7
Yes	26	27.4	15	15.3
Total	95	100.0	98	100.0
Urticaria at 12 mo				
No	77	81.1	94	95.9
Yes	18	18.9	4	4.1
Total	95	100.0	98	100.0
Urticaria at 24 mo				
No	95	100.0	96	98.0
Yes	0	0.0	2	2.0
Total	95	100.0	98	100.0
Urticaria at 36 mo				
No	93	97.9	96	98.0
Yes	2	2.1	2	2.0
Total	95	100.0	98	100.0
Urticaria total episodes*				
No	75	78.9	90	91.8
Yes	20	21.1	8	8.2
Total	95	100.0	98	100.0
Asthma at 12 mo				
No	93	97.9	92	93.9
Yes	2	2.1	6	6.1
Total	95	100.0	98	100.0
Asthma at 24 mo				
No	87	91.6	96	98.0
Yes	8	8.4	2	2.0
Total	95	100.0	98	100.0
Asthma at 36 mo				
No	87	91.6	97	99.0
Yes	8	8.4	1	1.0
Total	95	100.0	98	100.0
Asthma total episodes				
No	77	81.1	89	90.8
Yes	18	18.9	9	9.2
Total	95	100.0	98	100.0
Rhinoconjunctivitis at 12 mo				
No	90	94.7	93	94.9
Yes	5	5.3	5	5.1
Total	95	100.0	98	100.0
Rhinoconjunctivitis at 24 mo				
No	92	96.8	97	99.0
Yes	3	3.2	1	1.0
Total	95	100.0	98	100.0
Rhinoconjunctivitis at 36 mo				
No	79	83.2	96	98.0
Yes	16	16.8	2	2.0
Total	95	100.0	98	100.0
Rhinoconjunctivitis total episodes				
No	71	74.7	90	91.8
Yes	24	25.3	8	8.2
Total	95	100.0	98	100.0
At least 1 allergic episode during the study				
No	51	53.7	75	76.5
Yes	44	46.3	23	23.5
Total	95	100.0	98	100.0
Other FAs, total episodes				
No	58	61.1	66	67.3
Yes	37	38.9	32	32.7
Total	95	100.0	98	100.0
Other FA plus AMs during the study				
No	34	35.8	50	51.0
Yes	61	64.2	48	49.0
Total	95	100.0	98	100.0

*All cases were related to FA.

24 months, and 0.12 (95% CI, −0.03 to 0.27; $P = .2$) at 36 months by using SA-WCS. Even under the worst-case scenario (SA-WCS), there was a difference in favor of the ECHF+LGG group. This difference was 12% at 24 months. Fig 4 plots the incidence of CMA tolerance in the 2 study groups during the study. As plotted in Fig E2 in this article's Online Repository at www.jacionline.org, the SPT response negativization rate closely mirrored the tolerance acquisition rate.

Table II reports the overall frequency of other FAs alone or in combination with AM.

Safety

No child was intolerant to the study formulas. No case of placebo refusal was observed during DBPCFCs. No adverse event was attributed to the consumption of the formulas, and no difference was detected in their daily intake. Moreover, the time-related changes in weight, length, and height were comparable between the EHCF+LGG and EHCF groups (data not shown).

DISCUSSION

A recent systematic review confirmed that early-life food sensitization leads to other AMs, especially asthma, atopic eczema, and rhinoconjunctivitis. The authors of this review concluded that early-life food sensitization should be used as a marker for developing subsequent allergic diseases that might benefit from preventive strategies.[21] Current knowledge suggests that allergic diseases are partly determined by an interaction between genetic and environmental factors during early life, with a major role played by the gut microbiota and epigenetics. Among the epigenetic mechanisms potentially responsible for the development of allergic diseases, DNA methylation is the most prominent and extensively investigated.[22]

The present study was aimed at evaluating whether EHCF+LGG is more effective than EHCF alone at reducing the occurrence of other AMs in children with IgE-mediated CMA. The results of the present RCT shows that EHCF+LGG is more

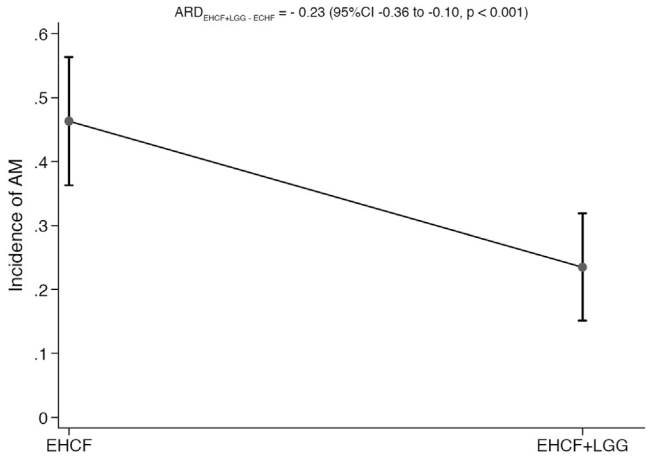

FIG 3. Incidence of the main study outcome at CCA.

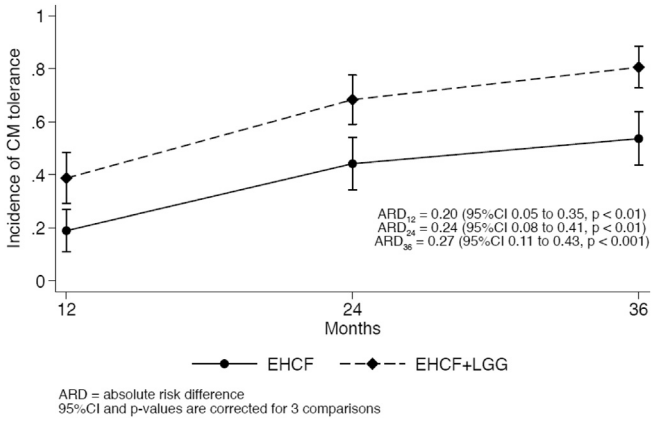

FIG 4. Incidence of CMA tolerance at CCA.

effective than EHCF at preventing the occurrence of other AMs. As far as the primary outcome is concerned, the number of children needed to treat with EHCF+LGG to prevent the occurrence of at least 1 AM during the 36-month period was estimated to be 4 (95% CI, 3-10) by the present study. Although it is of interest to observe that EHCF+LGG affected all of the components of the main study outcome, these findings can be taken only as exploratory, and further studies are necessary to investigate the potential of this strategy against any single allergic disease.

The ability of EHCF to prevent allergy is supported by the results of the German Infant Nutritional Intervention study,[16,23-26] in which infants at high risk of allergic diseases were protected from AMs when they received EHCF. Moreover, EHCF was the only formula that reduced the incidence of asthma at 15 years of age.[26] Our results show that in a population at higher risk for other allergies, because of the underlying IgE-mediated CMA, the addition of LGG to EHCF further enhances the protective effect of EHCF.

Some relevant insights were derived from our secondary outcomes. In keeping with previous observations, we provide additional evidence on the positive effect elicited by EHCF+LGG on oral tolerance acquisition in children with IgE-mediated CMA.[8-10] In the present study we also showed that this effect is sustained until 36 months of intervention. These data are relevant considering the most recent evidence suggesting that the natural history of CMA has changed over time, with a higher proportion of children with disease persistence through 5 years and subsequent ages.[2,27]

Another relevant aspect for clinical practice is that we did not observe any adverse reactions to the study formulas. These findings do not agree with those obtained from small case series, showing that up to 10% of children with CMA could react adversely to EHCF.[28,29] A possible reason for this discrepancy is that in the present RCT we adopted strict exclusion criteria, including CMA-related anaphylaxis, eosinophilic gastrointestinal disorders, and multiple FAs. Our findings support recent guidelines suggesting the use of EHCF as a first-line treatment for CMA, with the exception of patients with CMA-related anaphylaxis.[6,30-32]

This study has several strengths. First, it is an RCT that was performed on a large sample of children with DBPCFC-proved CMA followed at a tertiary pediatric allergy center with a high follow-up rate. Second, the effect sizes associated with both the primary and secondary outcomes were clinically relevant. Third, such effect sizes maintained a clear trend toward benefit under the worst-case scenario sensitivity analysis.

Nonetheless, this study has some limitations. First, our data cannot be generalized to children with conditions that were reasons for exclusion from the study. The effect of EHCF+LGG versus EHCF in these children will have to be addressed by further studies. Second, although our results showed that EHCF+LGG reduces the incidence of other AMs and favors the development of oral tolerance in children with IgE-mediated CMA at 12, 24, and 36 months, longer follow-up times are needed to test whether this effect persists over the long term. Third, our results are limited by the lack of data obtained in the study population on the gut microbiota and T_H1/T_H2 cytokines, which would be useful to investigate the mechanisms through which EHCF+LGG produces its effect.

The transitional gut microbiota in the first year of life is relevant to the development of allergy.[33] Evidence obtained by our group suggests that EHCF+LGG, but not EHCF alone, is able to increase the abundance of selected genera with the potential to produce butyrate in infants with CMA.[11] Strain-level demarcations for butyrate-producing genera (including *Roseburia*, *Coprococcus*, and *Blautia*) identified in infants that acquired tolerance to cow's milk suggest that LGG treatment contributes to acquisition of tolerance by altering the strain-level community structure of taxa with the potential to produce butyrate.[11] The mechanisms of action of butyrate against allergy are multiple, but many involve an epigenetic regulation of gene expression through inhibition of histone deacetylase (HDAC). Inhibition of HDAC9 and HDAC6 increases forkhead box P3 gene expression, as well as the production and suppressive function of regulatory T cells.[34,35] We observed that children with CMA treated with EHCF+LGG, who acquired oral tolerance, showed a different T_H1/T_H2 cytokine and forkhead box P3 gene DNA methylation pattern compared with patients with CMA treated with other formulas. This pattern was more closely related to that observed in healthy control subjects. When we analyzed the factors that potentially influenced the cytokine DNA methylation rate in patients who outgrew CMA, we found that the only variable was EHCF+LGG use.[12,36] These findings suggested that the positive action of EHCF+LGG observed in the present RCT could be at least in part due to a favorable modulation of gut microbiota and epigenetic mechanisms.

In conclusion, our RCT, which was performed in a well-characterized population of children with IgE-mediated CMA, shows that EHCF+LGG is superior to EHCF for the prevention of AMs during a period of 36 months. Further studies are needed to test whether EHCF+LGG can prevent a single AM, something that is suggested but cannot be proved by the present RCT, and to better elucidate the mechanisms of this beneficial effect. Our RCT also confirmed that the addition of LGG to EHCF speeds up the time to development of tolerance in children with IgE-mediated CMA.

We thank the children and families for their participation in the study, as well as the FPs team for the excellent work.

Clinical implications: EHCF+LGG is superior to EHCF for the prevention of AMs in children with IgE-mediated CMA. The addition of LGG to EHCF speeds the development of oral tolerance.

REFERENCES

1. Sicherer SH, Sampson HA. Food allergy: epidemiology, pathogenesis, diagnosis, and treatment. J Allergy Clin Immunol 2014;133:291-307.
2. Skripak JM, Matsui EC, Mudd K, Wood RA. The natural history of IgE-mediated cow's milk allergy. J Allergy Clin Immunol 2007;120:1172-7.
3. McBride D, Keil T, Grabenhenrich L, Dubakiene R, Drasutiene G, Fiocchi A, et al. The EuroPrevall birth cohort study on food allergy: baseline characteristics of 12,000 newborns and their families from nine European countries. Pediatr Allergy Immunol 2012;23:230-9.
4. Gupta R, Holdford D, Bilaver L, Dyer A, Holl JL, Meltzer D. The economic impact of childhood food allergy in the United States. JAMA Pediatr 2013;167:1026-31.
5. Branum AM, Lukacs SL. Food allergy among children in the United States. Pediatrics 2009;124:1549-55.
6. NIAID-Sponsored Expert Panel, Boyce JA, Assa'ad A, Burks AW, Jones SM, Sampson HA, et al. Guidelines for the diagnosis and management of food allergy in the United States: report of the NIAID-sponsored expert panel. J Allergy Clin Immunol 2010;126(suppl):S1-58.
7. Sicherer SH. Epidemiology of food allergy. J Allergy Clin Immunol 2011;127:594-602.
8. Berni Canani R, Nocerino R, Terrin G, Coruzzo A, Cosenza L, Leone L, et al. Effect of Lactobacillus GG on tolerance acquisition in infants with cow's milk allergy: a randomized trial. J Allergy Clin Immunol 2012;129:580-2.
9. Berni Canani R, Nocerino R, Terrin G, Frediani T, Lucarelli S, Cosenza L, et al. Formula selection for management of children with cow milk allergy influences the rate of acquisition of tolerance: a prospective multicenter study. J Pediatr 2013;163:771-7.
10. Ovcinnikova O, Panca M, Guest JF. Cost-effectiveness of using an extensively hydrolyzed casein formula plus the probiotic *Lactobacillus rhamnosus* GG compared to an extensively hydrolyzed formula alone or an amino acid formula as first-line dietary management for cow's milk allergy in the US. Clinicoecon Outcomes Res 2015;7:145-52.
11. Berni Canani R, Sangwan N, Stefka AT, Nocerino R, Paparo L, Aitoro R, et al. *Lactobacillus rhamnosus* GG supplemented formula expands butyrate producing bacterial strains in food allergic infants. ISME J 2016;10:742-50.
12. Berni Canani R, Paparo L, Nocerino R, Cosenza L, Pezzella V, Di Costanzo M, et al. Differences in DNA methylation profile of Th1and Th2 cytokine genes are associated with tolerance acquisition in children with IgE-mediated cow's milk allergy. Clin Epigenet 2015;7:38.
13. Berni Canani R, Leone L, D'Auria E, Riva E, Nocerino R, Ruotolo S, et al. The effects of dietary counseling on children with food allergy: a prospective, multicenter intervention study. J Acad Nutr Diet 2014;114:1432-9.
14. Hanifin JM, Rajka G. Diagnostic features of atopic dermatitis. Acta Derm Venereol 1980;60(suppl 92):44-7.
15. de Groot H, Brand PLP, Fokkens WF, Berger MY. Allergic rhinoconjunctivitis in children. BMJ 2007;335:985-8.
16. von Berg A, Koletzko S, Grübl A, Filipiak-Pittroff B, Wichmann HE, Bauer CP, et al. The effect of hydrolyzed cow's milk formula for allergy prevention in the first year of life: the German Infant Nutritional Intervention Study, a randomized double-blind trial. J Allergy Clin Immunol 2003;111:533-40.
17. National Asthma Education and Prevention Program. Expert Panel Report 3 (EPR-3): guidelines for the diagnosis and management of asthma–summary report 2007. J Allergy Clin Immunol 2007;120(suppl):S94-138.
18. Hardin JW, Hilbe JM. Generalized linear models and extensions. College Station (TX): Stata Press; 2012.
19. Altman DG. Confidence intervals for the number needed to treat. BMJ 1998;317:1309-12.
20. Rabe-Hesketh S, Skrondal A. Multilevel and longitudinal modeling using Stata. 3rd ed. College Station (TX): Stata Press; 2012.
21. Alduraywish SA, Lodge CJ, Campbell B, Allen KJ, Erbas B, Lowe AJ, et al. The march from early life food sensitization to allergic disease: a systematic review and meta-analyses of birth cohort studies. Allergy 2016;71:77-89.
22. Weinmann T, Gerlich J, Heinrich S, Nowak D, Gerdes J, Schlichtiger J, et al. Establishing a birth cohort to investigate the course and aetiology of asthma and allergies across three generations—rationale, design, and methods of the ACROSSOLAR study. BMC Public Health 2015;15:1210.
23. von Berg A, Koletzko S, Filipiak-Pittroff B, Laubereau B, Grübl A, Wichmann HE, et al. German Infant Nutritional Intervention Study Group. Certain hydrolyzed formulas reduce the incidence of atopic dermatitis but not that of asthma: three-year results of the German Infant Nutritional Intervention Study. J Allergy Clin Immunol 2007;119:718-25.
24. von Berg A, Filipiak-Pittroff B, Krämer U, Link E, Bollrath C, Brockow I, et al. GINI plus study group. Preventive effect of hydrolyzed infant formulas persists until age 6 years: long-term results from the German Infant Nutritional Intervention Study (GINI). J Allergy Clin Immunol 2008;121:1442-7.
25. von Berg A, Filipiak-Pittroff B, Krämer U, Hoffmann B, Link E, Beckmann C, et al, GINI plus study group. Allergies in high-risk schoolchildren after early intervention with cow's milk protein hydrolysates: 10-year results from the German Infant Nutritional Intervention (GINI) study. J Allergy Clin Immunol 2013;131:1565-73.
26. von Berg A, Filipiak-Pittroff B, Schulz H, Hoffmann U, Link E, Sußmann M, et al, GINI plus study group. Allergic manifestation 15 years after early intervention with hydrolyzed formulas—the GINI study. Allergy 2016;71:210-9.
27. Wood RA, Sicherer SH, Vickery BP, Jones SM, Liu AH, Fleischer DM, et al. The natural history of milk allergy in an observational cohort. J Allergy Clin Immunol 2013;131:805-12.
28. Caffarelli C, Plebani A, Poiesi C, Petroccione T, Spattini A, Cavagni G. Determination of allergenicity to three cow's milk hydrolysates and an amino acid-derived formula in children with cow's milk allergy. Clin Exp Allergy 2002;32:74-9.
29. Ragno V, Giampietro PG, Bruno G, Businco L. Allergenicity of milk protein hydrolysate formulae in children with cow's milk allergy. Eur J Pediatr 1993;152:760-2.
30. Koletzko S, Niggemann B, Arato A, Dias JA, Heuschkel R, Husby S, et al. European Society of Pediatric Gastroenterology, Hepatology, and Nutrition. Diagnostic approach and management of cow's-milk protein allergy in infants and children: ESPGHAN GI Committee practical guidelines. J Pediatr Gastroenterol Nutr 2012;55:221-9.
31. Luyt D, Ball H, Makwana N, Green MR, Bravin K, Nasser SM, et al, Standards of Care Committee (SOCC) of the British Society for Allergy and Clinical Immunology (BSACI). BSACI guideline for the diagnosis and management of cow's milk allergy. Clin Exp Allergy 2014;44:642-72.
32. Fiocchi A, Brozek J, Schünemann H, Bahna SL, von Berg A, Beyer K, et al, World Allergy Organization (WAO) Special Committee on Food Allergy. World Allergy Organization (WAO) Diagnosis and Rationale for Action against Cow's Milk Allergy (DRACMA) Guidelines. Pediatr Allergy Immunol 2010;21(suppl 21):1-125.
33. Liu AH. Revisiting the hygiene hypothesis for allergy and asthma. J Allergy Clin Immunol 2015;136:860-5.
34. Shi Y, Xu LZ, Peng K, Wu W, Wu R, Liu ZQ, et al. Specific immunotherapy in combination with *Clostridium butyricum* inhibits allergic inflammation in the mouse intestine. Sci Rep 2015;5:17651.
35. Licciardi PV, Ververis K, Karagiannis TC. Histone deacetylase inhibition and dietary short-chain fatty acids. ISRN Allergy 2011;2011:869647.
36. Paparo L, Nocerino R, Cosenza L, Aitoro R, D'Argenio V, Del Monaco V, et al. Epigenetic features of FoxP3 in children with cow's milk allergy. Clin Epigenetics 2016;8:86.

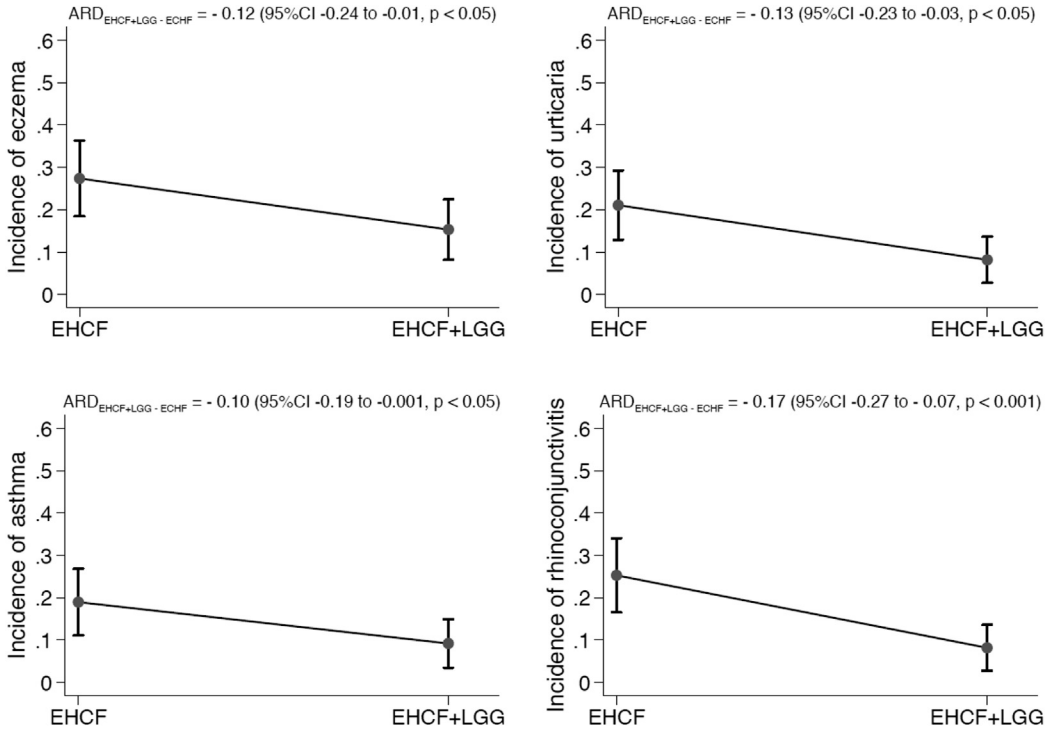

FIG E1. Exploratory analysis of the incidence of components of the main outcome (eczema, urticaria, asthma, and rhinoconjunctivitis) during the study period.

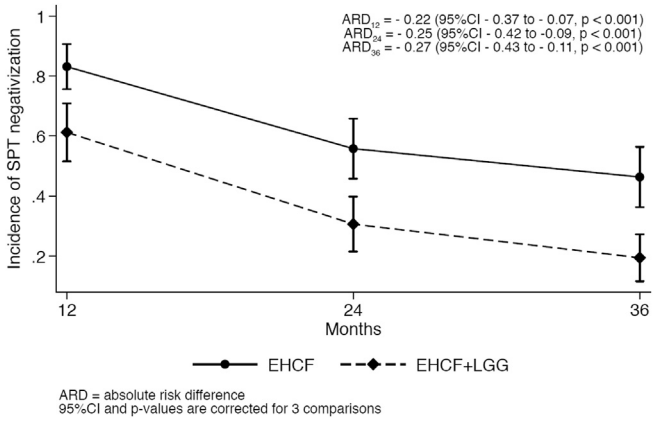

FIG E2. Incidence of SPT response negativization during the study period.

TABLE E1. Comparison of the features of subjects who completed the study with those of subjects lost to follow-up

	Study completers	Lost to follow-up
Male sex, no. (%)	130/193 (67.4)	17/27 (63.0)
Cesarean delivery, no. (%)	118/193 (61.1)	17/27 (63.0)
Born at term, no. (%)	172/193 (89.1)	27/27 (100.0)
Weight at birth (kg), median (IQR)	3.3 (3.0 to 3.5)	3.2 (2.8 to 3.5)
Breast-fed for ≥2 mo	146/193 (75.6)	15/27 (55.6)
Weaning (mo), median (IQR)	5.0 (4.0 to 6.0)	5.0 (5.0 to 6.0)
Siblings (count), median (IQR)	1 (0 to 1)	0 (0 to 1)
Familial risk of allergy	140/193 (72.5)	14/27 (51.9)
Allergic first-degree relatives (count), median (IQR)*	1 (1 to 2)	1 (1 to 1)
Exposure to passive smoking, no. (%)	69/193 (35.8)	8/27 (29.6)
Mother smoked during pregnancy, no. (%)	61/193 (31.6)	15/27 (55.6)
Exposure to pets, no. (%)	26/193 (13.5)	5/27 (18.5)
Age at CMA diagnosis (mo), median (IQR)	5.0 (3.0 to 7.0)	7.0 (6.0 to 8.0)
Weight at CMA diagnosis (kg), median (IQR)	7.2 (5.9 to 8.6)	8.1 (7.7 to 9.4)
Weight at CMA diagnosis (SDS WHO), median (IQR)	1.92 (1.28 to 2.47)	2.24 (1.72 to 2.64)
Length at CMA diagnosis (m), median (IQR)	0.65 (0.60 to 0.69)	0.69 (0.67 to 0.71)
Length at CMA diagnosis (SDS WHO), median (IQR)	2.69 (1.68 to 3.77)	3.05 (2.73 to 3.42)
BMI at CMA diagnosis (kg/m^2), median (IQR)	17.2 (16.1 to 18.0)	18.0 (16.9 to 18.9)
BMI at CMA diagnosis (SDS WHO), median (IQR)	0.58 (0.07 to 1.07)	0.74 (−0.03 to 1.25)
Positive prick by prick test result for fresh milk	193/193 (100.0)	27/27 (100.0)
Positive SPT response for α-lactalbumin	163/193 (84.5)	27/27 (100.0)
Positive skin test response for β-lactoglobulin	103/193 (53.4)	27/27 (100.0)
Positive SPT response for casein	73/193 (37.8)	27/27 (100.0)
Gastrointestinal symptoms at CMA onset	109/193 (56.5)	17/27 (63)
Cutaneous symptoms at CMA onset	135/193 (69.9)	13/27 (48.1)
Respiratory symptoms at CMA onset	22/193 (11.4)	9/27 (33.3)

SDS, SD score; *WHO*, World Health Organization.
*Calculated only for the subjects with familial risk of allergy.

TABLE E2. Effect of baseline covariables on the ARD between the EHCF+LGG and EHCF groups

EHCF+LGG vs EHCF	−0.21† (−0.34 to −0.08)	−0.22‡ (−0.35 to −0.09)	−0.24‡ (−0.37 to −0.11)	−0.23‡ (−0.36 to −0.10)	−0.23‡ (−0.35 to −0.10)	−0.23‡ (−0.36 to −0.10)	−0.22† (−0.35 to −0.09)	−0.24‡ (−0.37 to −0.11)
Male sex	0.09 (−0.04 to 0.23)							
Born at term		0.06 (−0.14 to 0.25)						
Cesarean delivery			0.15* (0.03 to 0.28)					
Breast-fed for at least 2 mo				−0.02 (−0.17 to 0.13)				
Familial risk of allergy					0.22‡ (0.10 to 0.34)			
Exposure to passive smoking						0.01 (−0.13 to 0.14)		
Mother smoked during pregnancy							0.05 (−0.09 to 0.20)	
Exposed to pets								0.15 (−0.05 to 0.35)
Observations	193	193	193	193	193	193	193	193

Values are ARDs, with 95% CIs in brackets.
*$P < .05$.
†$P < .01$.
‡$P < .001$.