

Accepted Manuscript

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

Roberto Berni Canani, MD, PhD, Margherita Di Costanzo, MD, Giorgio Bedogni, MD, Antonio Amoroso, BS, Linda Cosenza, MD, Carmen Di Scala, LDN, Viviana Granata, MD, Rita Nocerino, RN

PII: S0091-6749(16)32487-3

DOI: [10.1016/j.jaci.2016.10.050](https://doi.org/10.1016/j.jaci.2016.10.050)

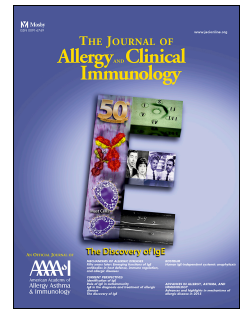
Reference: YMAI 12557

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 6 April 2016

Revised Date: 13 September 2016

Accepted Date: 17 October 2016



Please cite this article as: Berni Canani R, Di Costanzo M, Bedogni G, Amoroso A, Cosenza L, Di Scala C, Granata V, Nocerino R, Extensively hydrolysed casein formula containing *L. rhamnosus* GG reduces the occurrence of other allergic manifestations in children with cow's milk allergy: 3-year randomized controlled trial, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2016.10.050.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Title page

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

Roberto Berni Canani, MD, PhD^{1,2,3}, Margherita Di Costanzo, MD¹, Giorgio Bedogni, MD^{3,4}, Antonio Amoroso, BS^{1,3}, Linda Cosenza, MD¹, Carmen Di Scala, LDN¹, Viviana Granata, MD¹, Rita Nocerino, RN^{1,3}

¹Department of Translational Medical Science, ²European Laboratory for the Investigation of Food-Induced Diseases and ³CEINGE Advanced Biotechnologies, University of Naples "Federico II", Naples, Italy; ⁴Clinical Epidemiology Unit, Liver Research Center Basovizza, Trieste Italy.

Corresponding Author:

Prof. Roberto Berni Canani, MD, PhD
 Director, Pediatric Allergy Unit
 Department of Translational Medical Science – Pediatric Section
 European Laboratory for the Investigation of Food Induced Diseases
 CEINGE – Advanced Biotechnologies
 University of Naples "Federico II"
 Via S. Pansini 5 80131 Naples, Italy
 Tel.: +39-081-7462680
 E mail: berni@unina.it

Funding

This study was supported in part by the Italian Ministry of Health (Grant PE-2011-02348447) and by an unrestricted grant from Mead Johnson Nutrition (Evansville, Indiana, USA) devoted to the Department of Translational Medical Science of the University of Naples "Federico II". However, the Italian Ministry of Health and Mead Johnson Nutrition had no influence on: (1) the study design; (2) the collection, analysis, and interpretation of the data; (3) the writing of the manuscript; or (4) the decision to submit the manuscript for publication.

Clinical implications

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

Capsule summary

EHCF+LGG reduces the incidence of other AMs in children with IgE-mediated CMA and increases the acquisition of tolerance at 12 months, 24 months and 36 months.

Key words: eczema, urticaria, asthma, rhinoconjunctivitis, atopic march, food allergy, gut microbiota, probiotics.

Abbreviations:

FA: Food allergy
 CMA: Cow's milk allergy
 AMs: Allergic manifestations
 EHCF: Extensively hydrolysed casein formula
 LGG: *Lactobacillus rhamnosus* GG
 ARD: Absolute risk difference
 DBPCFC: Double-blind placebo-controlled food challenge
 FP: Family paediatrician
 SPT: Skin Prick Testing
 IQR: Interquartile range
 IRR: Incidence rate ratio
 NNT: Number needed to treat
 BRM: Binomial regression model
 RCT: Randomized controlled trial
 CCA: complete case analysis
 SA-EQS: sensitivity analysis - equal worst outcome scenario
 SA-BCS: sensitivity analysis - best case scenario
 SA-WCS: sensitivity analysis - worst case scenario

Abstract

Background: Children with cow's milk allergy (CMA) have an increased risk of developing other allergic manifestations (AMs).

Objective: We performed a parallel-arm randomized controlled trial (RCT) to test whether the administration of an extensively hydrolysed casein formula (EHCF) containing the probiotic *L. rhamnosus* GG (LGG) can reduce the occurrence of other AMs in CMA children.

Methods: Children with IgE-mediated CMA were randomly allocated to EHCF or EHCF+LGG groups and followed for 36 months. The main outcome was the occurrence of at least one AM (eczema, urticaria, asthma and rhinoconjunctivitis). The secondary outcome was tolerance acquisition, defined as the negativization of a double-blind food challenge at 12 months, 24 months and 36 months. AMs were diagnosed according to standardized criteria. Tolerance acquisition was evaluated every 12 months.

Results: A total of 220 children (147 males, 67%) with a median (IQR) age of 5.0 (3.0–8.0) months were randomized and 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 (ARD) for the occurrence of at least one AM over 36 months was -0.23 [95%CI -0.36 to -0.10, $p<0.001$] and the ARD for the acquisition of cow's milk tolerance was 0.20 (95%CI 0.05 to 0.35, $p<0.01$) at 12 months, 0.24 (95%CI 0.08 to 0.41, $p<0.01$) at 24 months and 0.27 (95%CI 0.11 to 0.43, $p<0.001$) at 36 months. In the sensitivity analysis, the effect size of the main outcome was virtually unchanged when the occurrence of AM 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.

Introduction

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 two 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 impact, with significant direct costs for the healthcare system and even larger costs for the families of food-allergic patients (4). In addition, children with FAs are at increased risk to develop other allergic manifestations (AMs) later in life. According to a recent CDC study, children with a FA are 2 to 4 times more likely to develop other AMs, such as asthma (4.0 times), atopic eczema (2.4 times), and respiratory allergies (3.6 times), compared to children without a 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 hydrolysed casein formula (EHCF) supplemented with the probiotic *Lactobacillus rhamnosus* GG (LGG) induced higher tolerance rates compared to EHCF without LGG and other formulas (8,9). These findings were consistent with those of a 1-year follow-up study performed in the US that showed better outcomes using an EHCF+LGG vs. an EHCF or amino acid-based formula for the first-line dietary management of CMA (10). Multiple mechanisms may 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 Th1 and Th2 cytokine genes expression (12). Such mechanisms suggest a possible long-term effect on the immune system of CMA children treated with EHCF+LGG. The present randomized controlled trial (RCT) was designed to test whether a dietary intervention with EHCF supplemented with LGG could influence the occurrence of other AMs in children with IgE-mediated CMA.

Patients and Methods

Study design

The present parallel-arm RCT was designed to test whether an EHCF+LGG formula can reduce the incidence of other AMs compared to EHCF in children with IgE-mediated CMA. The RCT was performed from October 2008 to December 2014 in collaboration with family paediatricians (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 where 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 www.clinicaltrials.gov (registration number: NCT01891916).

Study subjects

The design of the study is depicted in Figure 1.

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

The inclusion criteria were: age 1–12 months and suspected IgE-mediated CMA.

The exclusion criteria were: cow’s milk protein-induced anaphylaxis, food protein induced enterocolitis syndrome, other food allergies, 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 enrolment.

Only subjects who met the inclusion criteria were invited to participate to 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 socio-demographic 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 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 two groups of dietary intervention: group 1, which received EHCF (Nutramigen®, Mead Johnson Nutrition, Rome, Italy), or group 2, which received EHCF containing LGG (Nutramigen LGG®, Mead Johnson Nutrition, Rome, Italy).

From 2 weeks to 4 weeks after 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 pre-specified number of 110 subjects per group with DBPCFC-proven IgE-mediated CMA was achieved, and only these subjects continued the exclusion diet using the hypoallergenic formula prescribed at randomization and started the trial.

Intervention and data collection

Both study products were commercially available as formula powder for CMA dietary treatment in Italy. The composition of the two 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 two formulas were identical. The physicians responsible for enrolment 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 were not involved in the evaluation of the study outcomes. Parents or caregivers were asked to keep a daily record of formula use. The amount prepared (millimetres 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 to 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 (12 months, 24 months and 36 months) were performed. Skin prick tests 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 DBPCFC and clinical examinations were performed at the tertiary centre by two investigators blinded to the group assignments. In the case of discordance about an AM diagnosis, further evaluation by a third paediatrician experienced in paediatric allergy was performed. AMs were diagnosed using standardized criteria. Atopic eczema was diagnosed by 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 on the basis of 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 (a 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). The symptoms considered for the diagnosis of

asthma were: recurrent wheeze (more than once a month), difficulty in breathing and/or chest tightness; 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) 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 (*i.e.*, pruritus without skin lesions, urticaria, atopic eczema exacerbation, angioedema, vomiting, diarrhoea, bloody stools, abdominal pain, rhinitis, nasal congestion, wheeze, cough, stridor, difficulty breathing during the challenge); and 3) results of SPTs (wheal size >3 mm) and/or serum IgE (>0.1kU/L) (1,6,8).

Compliance was operationally defined as the 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, rhinoconjunctivitis) during the 36 months of the study. The secondary outcome was the acquisition of tolerance at 12 months, 24 months 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 in 20% in the incidence of AM in the EHCF+LGG group vs. the EHCF group with a power of 90% at an alpha level of 0.05 (Pearson's chi-squared test). Assuming a missingness rate of 10% at follow-up, this implies the enrolment 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 two treatments, and we designed the study to enrol up to 150 children per group until at least 110 children per group had a DBPCFC-confirmed CMA diagnosis.

Study tests

Skin Prick Testing (SPT). Skin prick tests were performed with fresh milk and allergens of cow 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. Skin prick tests were performed using a 1-mm single peak lancet (ALK, 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 millimetres) of the wheal and flare at 15 min. The SPT result was considered "positive" if the wheal was 3 mm or larger, without 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 centre involved in the study on two separate days with a one week interval. Parents of infants taking antihistamines were advised to withhold these medications for 72 h before and during the challenge. Randomization and preparation of the challenges were performed by experienced food allergy dietitians who were not directly involved in the procedures. In detail, every 20 minutes, increasing doses (0.1 mL, 0.3 mL, 1 mL, 3 mL, 10 mL, 30 mL and 100 mL) of fresh pasteurized CM 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 paediatric allergists. Study subjects were scored for 9 items divided into 4 main categories: *i.* General (lowered blood pressure plus tachycardia); *ii.* Skin (rash, urticaria/angioedema); *iii.* Gastrointestinal (nausea or repeated vomiting, crampy-like abdominal pain, diarrhea); and *iv.* Respiratory (sneezing or itching, nasal congestion or rhinorrhea, stridor deriving from upper airway obstruction or

wheezing) on a 0- to 3-point scale (0, none; 1, light; 2, moderate; and 3, severe). 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 h of administering the highest dose were defined as having “immediate reactions”. Infants were observed for 2 h after the final dose and then discharged. In the case of a positive DBPCFC 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 give one single feed of 100 ml of the tested formula (verum or placebo) every day at home for seven days. If any symptoms occurred during this period, the patients returned to the outpatient clinic on the same day. After seven days of verum or placebo administration, the patients were examined and the parents were interviewed at the centre. To rule out false-negative challenge results, parents were asked to contact the centre if any symptoms occurred in the 7 days after the DBPCFC procedures. The challenge 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. Children with a negative DBPCFC at any subsequent visit were re-evaluated after six 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 (IQR). Discrete variables are reported as counts and proportions or as medians and IQR.

The main outcome, *i.e.*, the effect of EHCF+LGG vs. EHCF on the occurrence of any AM during 36 months, was evaluated 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% confidence interval (95%CI) of the absolute risk difference (ARD) were obtained from the

BRM and inverted to calculate the number needed to treat (NNT) (19). We also assessed the degree to which the ARD changed after the inclusion of the following discrete covariables into the BRM: gender (0 = female, 1 = male), birth at term (discrete: 0 = no, 1 = yes), caesarean delivery (discrete: 0 = no, 1 = yes), breastfeeding 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, *i.e.*, the time-specific incidence of CMA tolerance, was evaluated 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 treatmentXtime interaction (discreteXdiscrete). Repeated measures were taken into account by using subject-specific cluster confidence intervals. Three pre-specified between-group (EHCF+LGG vs. EHCF) within-time (12 months, 24 months and 36 months) contrasts were used to quantify the time-specific ARDs in CMA tolerance. This corresponds to evaluating the EHCF+LGG vs. LGG difference separately at 12 months, 24 months and 36 months. The relative ARDs and p-values were corrected using a Bonferroni correction for the three comparisons (contrasts).

Besides doing a complete case analysis (CCA), we performed a sensitivity analysis (SA) using the following scenarios: 1) all missing values of EHCF+LGG and EHCF set to the worst outcome (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 (best case scenario, SA-BCS); 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 (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 pre-specified by

the study protocol. All other SAs were implemented post-hoc. Statistical analysis was performed using Stata 14.1 (Stata Corporation, College Station, TX, USA).

Results

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

We designed the study to enrol 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 gave 110 cases of DBPCFC-confirmed CMA in the ECHF group, and 131 of 150 planned children gave 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 males, 67%) with a median (IQR) age of 5.0 (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 1).

All children were compliant, i.e., they consumed at least 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 ECHF group and 12 in the ECHF+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 centre or by the FPs). Of the 15 children lost to follow-up in the ECHF 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 ECHF+LGG group, 2 were lost at 12 months, 5 at 24 months and 5 at 36 months. Supplementary table 1 compares the baseline features of the subjects who completed the study with those of the subjects who were lost to follow-up; see Online Repository Supplementary table 1.

Main outcome

Table 2 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 vs. EHCF was: 1) -0.23 [95%CI -0.36 to -0.10, $p < 0.001$] at CCA; 2) -0.22 [95%CI -0.35 to -0.09, $p < 0.001$] at SA-EQS; 3) -0.33 [95%CI -0.45 to -0.21, $p < 0.001$] at SA-BCS; and 4) -0.08 [95%CI -0.21 to 0.04, $p = 0.5$] 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 favour of EHCF+LGG was still present (8%). Using the CCA estimate of the ARD, the NNT was 4 (95%CI 3 to 10). This means that, compared to EHCF, 4 subjects needed to be treated with EHCF+LGG for 36 months to prevent at least one AM.

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

Supplementary Table 2 gives the effect of selected baseline covariables on the incidence of the main outcome; see Online Repository Supplementary table 2. The effect was negligible in all cases, being <2% in absolute terms and <10% in relative terms.

Supplementary Figure 1 plots the incidence of the components of the main outcome during the study as determined by CCA; see Online Repository Supplementary figure 1. 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 CM tolerance for EHCF+LGG vs. LGG was: 1) 0.20 (95%CI 0.05 to 0.35, $p < 0.01$) at 12 months, 0.24 (95%CI 0.08 to 0.41, $p < 0.01$) at 24 months and 0.27 (95%CI 0.11 to 0.43, $p < 0.001$) at 36 months using CCA; 2) 0.15 (95%CI 0.00 to 0.31, $p = 0.06$) at 12 months, 0.20 (95%CI 0.05 to 0.35, $p < 0.01$) at 24 months and 0.23 (95%CI 0.09 to 0.37, $p < 0.001$) at 36 months using SA-EQS; 3) 0.29 (95%CI 0.15 to 0.43, $p < 0.001$) at 12 months, 0.34 (95%CI 0.18 to 0.49, $p < 0.001$) at 24 months and 0.37 (95%CI 0.21 to 0.53, $p < 0.001$) at 36 months using SA-BCS.

0.001) at 24 months and 0.36 (95%CI 0.22 to 0.51, $p < 0.001$) at 36 months 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, $p = 0.5$) at 24 months, 0.12 (95%CI -0.03 to 0.27, $p = 0.2$) at 36 months using SA-WCS. Even under the worst case scenario (SA-WCS), there was a difference in favour of the ECHF+LGG group. This difference was 12% at 24 months. Figure 4 plots the incidence of CMA tolerance in the two study groups during the study. As plotted in supplementary Figure 2, the SPT negativization rate closely mirrored the tolerance acquisition rate; see Online Repository Supplementary figure 2.

Table 2 reports the overall frequency of other food allergies alone or in combination with AM.

Safety

No child was intolerant to the study formulas. No case of placebo refusal was observed during DBPCFC. 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 ECHF+LGG and ECHF 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 may benefit from preventive strategies (22). 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 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 (23).

The present study was aimed at evaluating whether ECHF+LGG is more effective than ECHF 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 effective than EHCF at preventing the occurrence of other AMs. As the primary outcome is concerned, the number of children needed to treat with EHCF+LGG to prevent the occurrence of at least one AM during the 36 month period was estimated to be 4 (95%CI 3 to 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 an allergy is supported by the results of the GINI study (16, 24-27), where 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 (27). 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 (2,28).

Another relevant aspect for clinical practice is that we did not observe any adverse reaction to the study formulas. These findings do not agree with those obtained from small case-series, showing that up to 10% of CMA children could react adversely to EHCF (29,30). 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 food allergies. 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 (6,31-33).

This study has several strengths. First, it is a RCT that was performed on a large sample of children with DBPCFC-proven CMA followed at a tertiary paediatric allergy centre 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 vs. 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 favours the development of oral tolerance in children with IgE-mediated CMA at 12 months, 24 months and 36 months, longer follow-up times are needed to test whether this effect persists in over the long term. Third, our results are limited by the lack of data on gut microbiota and Th1/Th2 cytokines, which would be useful to investigate the mechanisms by which the EHCF+LGG produces its effect.

The transitional gut microbiota in the first year of life is relevant to the development of allergy (34). 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 CMA infants (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 6 increases FoxP3 gene expression as well as the production and suppressive function of Tregs (35,36). We observed that CMA children treated with EHCF+LGG, who acquired oral tolerance, showed a different Th1/Th2 cytokine and FoxP3 gene DNA methylation pattern compared to CMA patients treated with other formulas. This pattern was more closely related to that observed in healthy controls. When we analysed the factors that

potentially influenced the cytokine DNA methylation rate in subjects who outgrew CMA, we found that the only variable was EHCF+LGG use (12, 37). These findings suggested that the positive action of EHCF+LGG observed in the present RCT could be at least partly due to a favourable modulation of gut microbiota and epigenetic mechanisms.

In conclusion, our RCT, 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 proven by the present RCT, and to 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.

Figure legends

Figure 1.

Study design

Figure 2.

Flow of the children through the study.

Figure 3.

Incidence of the main study outcome at complete case analysis.

Figure 4.

Incidence of CMA tolerance at complete case analysis.

Acknowledgments

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

The study was supported in part by the Italian Ministry of Health (Grant PE-2011-02348447), and by an unrestricted grant from Mead Johnson Nutrition (Evansville, Indiana, USA) devoted to the Department of Translational Medical Science of the University of Naples “Federico II”. However, the Italian Ministry of Health and Mead Johnson Nutrition had no influence on: (1) the study design; (2) the collection, analysis, and interpretation of data; (3) the writing of the manuscript; and (4) the decision to submit the manuscript for publication.

Author contributions

RBC and RN conceptualized and designed the study, coordinated the research team, coordinated and supervised data collection, managed the study, interpreted the data, drafted the manuscript. GB performed statistical analysis, contributed to data interpretation, and drafted parts of the manuscript.

MDC, LC, AA, VG, and CDS contributed to data collection and clinical examination.

All of the authors approved the final manuscript for publication.

Conflict of Interest: The authors have no conflicts of interest that are directly relevant to the content of this paper, which remains their sole responsibility.

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 D1, Keil T, Grabenhenrich L, Dubakiene R, Drasutiene G, Fiocchi A, Dahdah L, 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, Wood RA, 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: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: 580–582.
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: 771–777.
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 Th1 and 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; Suppl. 92: 44-47.
 15. de Groot H, Brand PLP, Fokkens WF, Berger MY. Allergic rhinoconjunctivitis in children. *BMJ.* 2007; 335: 985–988.
 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(5 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;317:1309-1312.
 20. Rabe-Hesketh S, Skrondal A. Multilevel and Longitudinal Modeling Using Stata, 3rd Edition. College Station: Stata Press; 2012.
 21. Cordoba G, Schwartz L, Woloshin S, Bae H, Gøtzsche PC. Definition, reporting, and interpretation of composite outcomes in clinical trials: systematic review. *BMJ.* 2010;341:c3920.
 22. 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.
 23. 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.
 24. 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.
25. 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.
26. 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.
27. 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.
28. 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.
29. 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.
30. 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.
31. 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.
32. 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.
33. 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.

- 593 34. Liu AH. Revisiting the hygiene hypothesis for allergy and asthma. *J Allergy Clin Immunol.*
594 2015; 136: 860-5.
- 595 35. Shi Y, Xu LZ, Peng K, Wu W, Wu R, Liu ZQ, et al. Specific immunotherapy in combination
596 with *Clostridium butyricum* inhibits allergic inflammation in the mouse intestine. *Sci*
597 *Rep.* 2015; 2,5:17651.
- 598 36. Licciardi PV, Ververis K, Karagiannis TC. Histone deacetylase inhibition and dietary short-
599 chain Fatty acids. *ISRN Allergy.* 2011, 26;2011:869647.
- 600 37. Paparo L, Nocerino R, Cosenza L, Aitoro R, D'Argenio V, Del Monaco V, Di Scala C,
601 Amoroso A, Di Costanzo M, Salvatore F, Berni Canani R. Epigenetic features of FoxP3 in
602 children with cow's milk allergy. *Clin Epigenetics.* 2016 Aug 12;8:86, *in press.*
- 603
604
605
606
607
608
609

Table 1. Baseline features of the subjects enrolled into the study.

ACCEPTED MANUSCRIPT

	EHCF		EHCF+LGG	
	<i>n</i>		<i>n</i>	
Male	75/110	68.2%	72/110	65.5%
Cesarean delivery	69/110	62.7%	66/110	60.0%
Born at term	101/110	91.8%	98/110	89.1%
Weight at birth (kg) (median, IQR)	3.4	(3.0, 3.7)	3.2	(2.9, 3.5)
Breastfed for at least 2 months	84/110	76.4%	77/110	70.0%
Weaning (months) (median, IQR)	5.0	(5.0, 6.0)	5.0	(4.0, 6.0)
Siblings (count) (median, IQR)	1	(0.0, 1.0)	1	(0.0, 1.0)
Familial risk of allergy	79/110	71.8%	75/110	68.2%
Allergic first-degree relatives (count) (median, IQR)*	1	(1, 2)	1	(1, 2)
Exposure to passive smoking	39/110	35.5%	38/110	34.5%
Mother smoked during pregnancy	41/110	37.3%	35/110	31.8%
Exposure to pets	13/110	11.8%	18/110	16.4%
Age at CMA diagnosis (months) (median, IQR)	5.0	(3.0, 8.0)	5.0	(3.0, 8.0)
Weight at CMA diagnosis (kg) (median, IQR)	7.5	(6.1, 8.6)	7.4	(6.1, 8.7)
Weight at CMA diagnosis (SDS WHO) (median, IQR)	0.16	(-0.41, 0.69)	0.14	(-0.45, 0.79)
Length at CMA diagnosis (m) (median, IQR)	0.66	(0.61, 0.70)	0.66	(0.61, 0.70)
Length at CMA diagnosis (SDS WHO) (median, IQR)	0.05	(-0.76, 0.52)	-0.03	(-0.56, 0.94)
BMI at CMA diagnosis (kg/m²) (median, IQR)	17.3	(16.2, 18.1)	17.2	(16.2, 18.1)
BMI at CMA diagnosis (SDS WHO) (median, IQR)	0.29	(-0.45, 0.73)	0.26	(-0.48, 0.66)
Positive prick by prick test for fresh milk	110/110	100.0%	110/110	100.0%
Positive skin prick test for α-lactoalbumin	96/110	87.3%	94/110	85.5%
Positive skin test positive for β-lactoglobulin	60/110	54.5%	70/110	63.6%
Positive skin prick test positive for casein	49/110	44.5%	51/110	46.4%
Gastrointestinal symptoms at CMA onset	61/110	55.5%	65/110	59.1%
Cutaneous symptoms at CMA onset	75/110	68.2%	73/110	66.4%
Respiratory symptoms at CMA onset	17/110	15.5%	14/110	12.7%

*Calculated only for the subjects with familial risk of allergy

CMA: cow's milk allergy; IQR: interquartile range; SDS: standard deviation score; WHO: World Health Organization

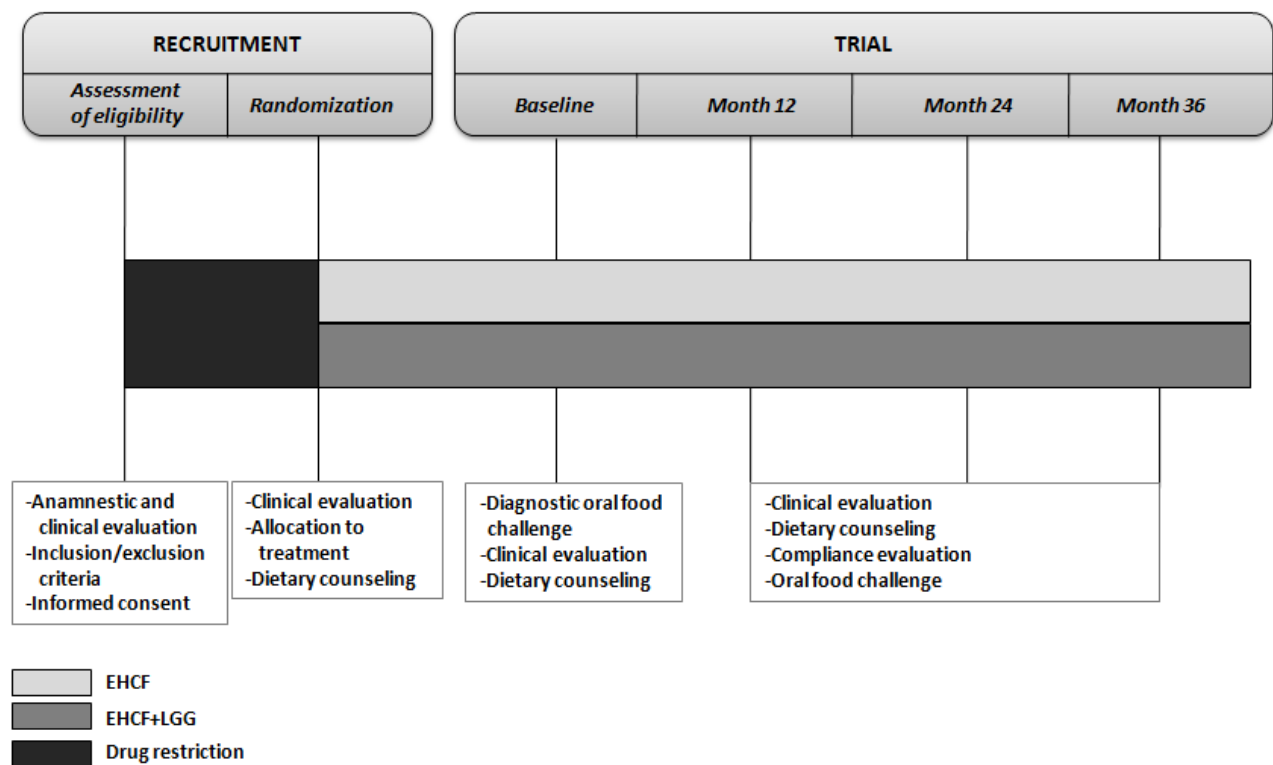
Table 2. Frequency of the main outcome, its components and other food allergies.

ACCEPTED MANUSCRIPT

	EHCF		EHCF+LGG	
	n	%	n	%
Eczema at 12 months				
No	78	82.1	84	85.7
Yes	17	17.9	14	14.3
Total	95	100.0	98	100.0
Eczema at 24 months				
No	92	96.8	98	100.0
Yes	3	3.2	0	0.0
Total	95	100.0	98	100.0
Eczema at 36 months				
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 months				
No	77	81.1	94	95.9
Yes	18	18.9	4	4.1
Total	95	100.0	98	100.0
Urticaria at 24 months				
No	95	100.0	96	98.0
Yes	0	0.0	2	2.0
Total	95	100.0	98	100.0
Urticaria at 36 months				
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 months				
No	93	97.9	92	93.9
Yes	2	2.1	6	6.1
Total	95	100.0	98	100.0
Asthma at 24 months				
No	87	91.6	96	98.0
Yes	8	8.4	2	2.0
Total	95	100.0	98	100.0
Asthma at 36 months				
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 months				
No	90	94.7	93	94.9
Yes	5	5.3	5	5.1
Total	95	100.0	98	100.0
Rhinoconjunctivitis at 24 months				
No	92	96.8	97	99.0
Yes	3	3.2	1	1.0
Total	95	100.0	98	100.0
Rhinoconjunctivitis at 36 months				
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 one allergic episodes 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 food allergies total episodes				
No	58	61.1	66	67.3
Yes	37	38.9	32	32.7
Total	95	100.0	98	100.0
Other food allergy plus allergic manifestations 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 dependent of food allerg

Figure 1.



Additional visit(s) were performed when necessary

Figure 2.

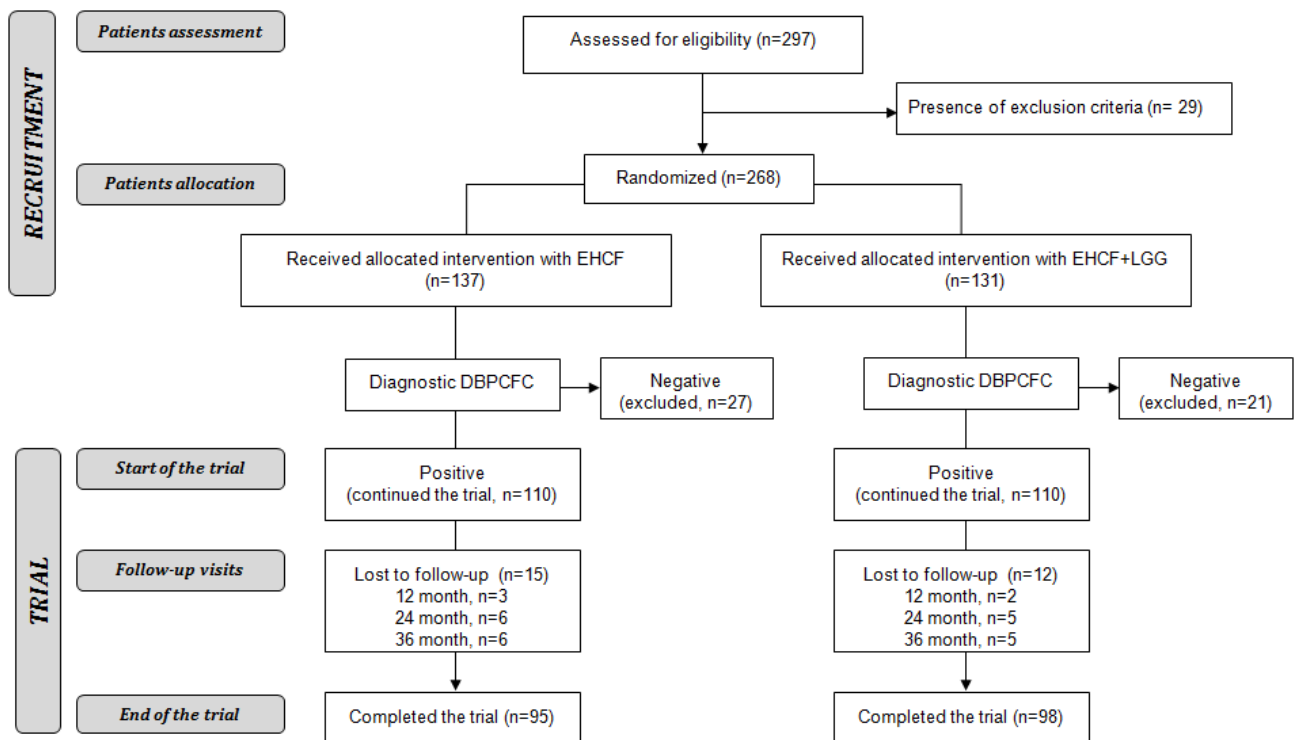


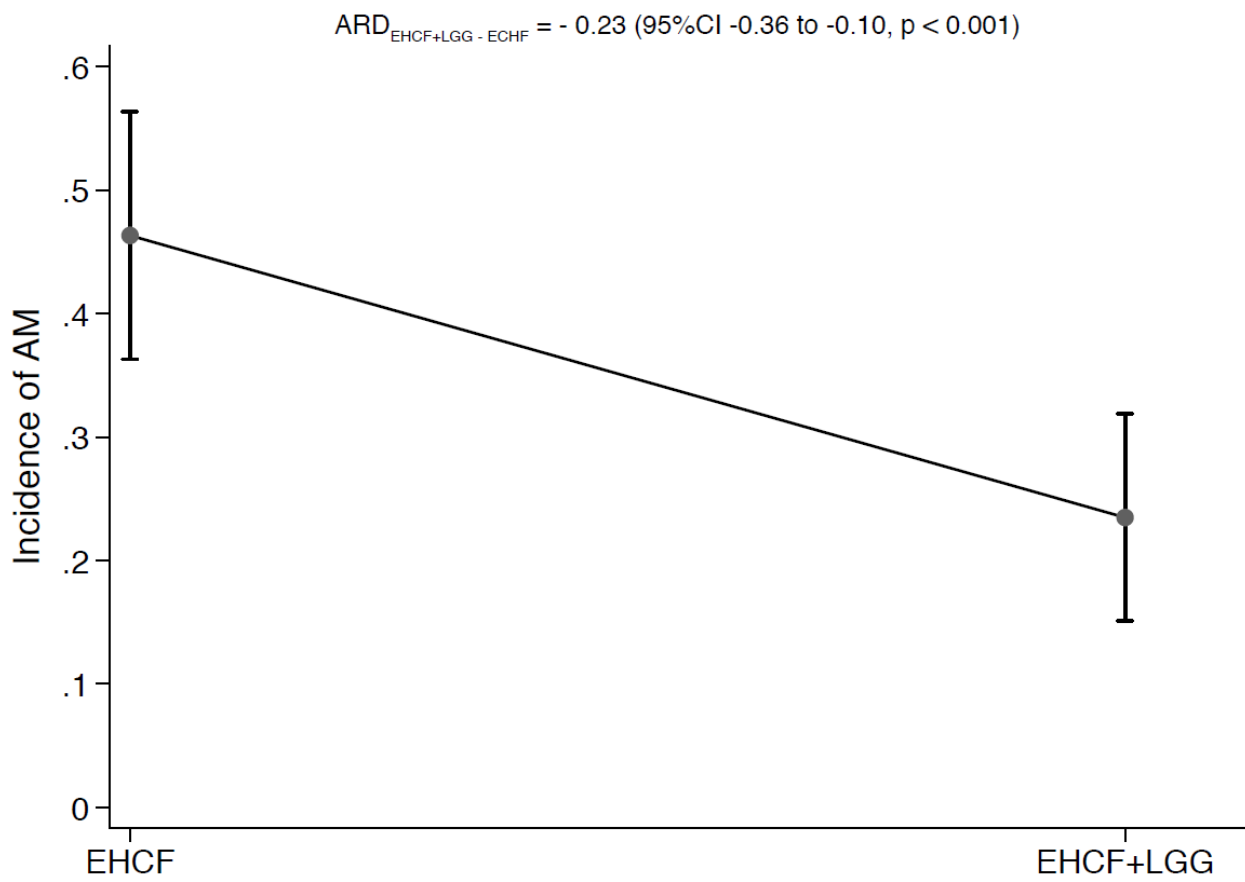
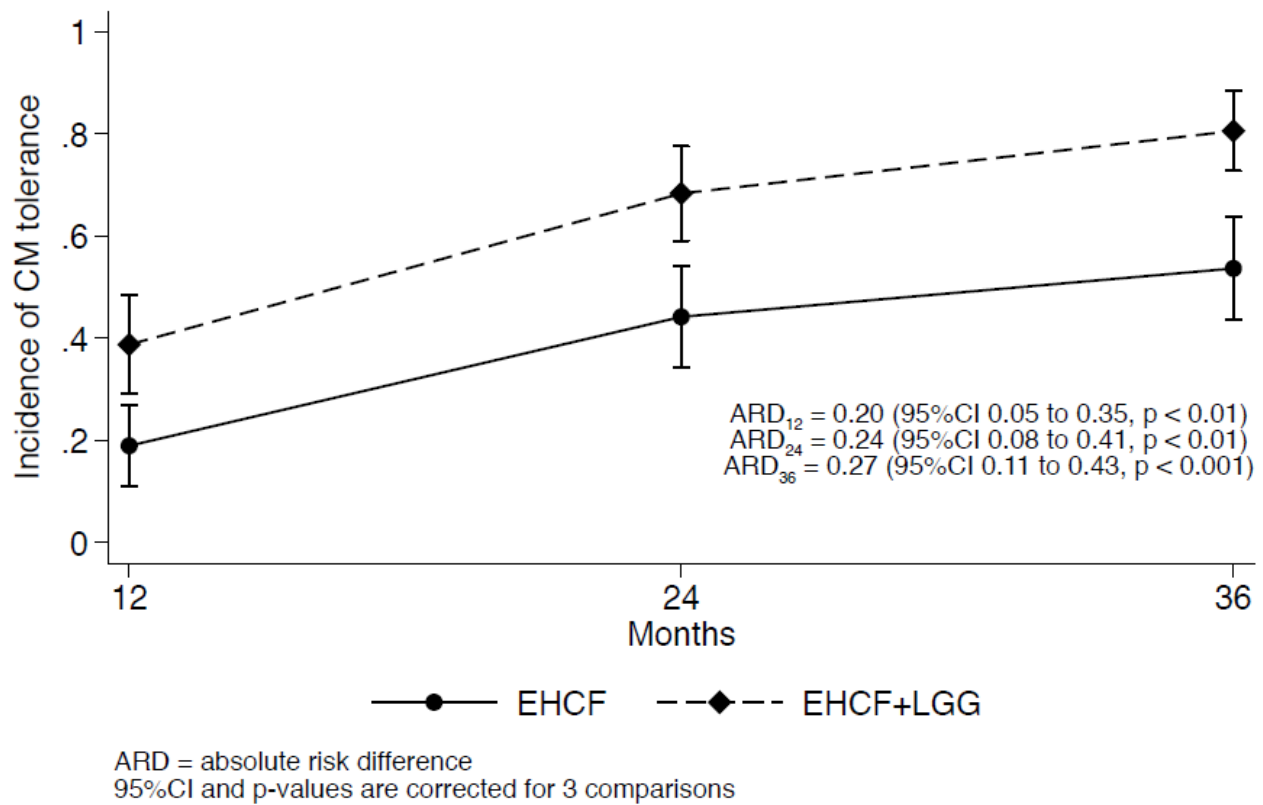
Figure 3.

Figure 4.

Supplementary Table 1. Comparison of the features of the subjects who completed the study with those of the subjects who were lost to follow-up.

	Study completers		Lost to follow up	
	<i>n</i>		<i>n</i>	
Male	130/193	67.4%	17/27	63.0%
Cesarean delivery	118/193	61.1%	17/27	63.0%
Born at term	172/193	89.1%	27/27	100.0%
Weight at birth (kg) (median, IQR)	3.3	(3.0, 3.5)	3.2	(2.8, 3.5)
Breastfed for at least 2 months	146/193	75.6%	15/27	55.6%
Weaning (months) (median, IQR)	5.0	(4.0, 6.0)	5.0	(5.0, 6.0)
Siblings (count) (median, IQR)	1	(0, 1)	0	(0, 1)
Familial risk of allergy	140/193	72.5%	14/27	51.9%
Allergic first-degree relatives (count) (median, IQR)*	1	(1, 2)	1	(1, 1)
Exposure to passive smoking	69/193	35.8%	8/27	29.6%
Mother smoked during pregnancy	61/193	31.6%	15/27	55.6%
Exposure to pets	26/193	13.5%	5/27	18.5%
Age at CMA diagnosis (months) (median, IQR)	5.0	(3.0, 7.0)	7.0	(6.0, 8.0)
Weight at CMA diagnosis (kg) (median, IQR)	7.2	(5.9, 8.6)	8.1	(7.7, 9.4)
Weight at CMA diagnosis (SDS WHO) (median, IQR)	1.92	(1.28, 2.47)	2.24	(1.72, 2.64)
Length at CMA diagnosis (m) (median, IQR)	0.65	(0.60, 0.69)	0.69	(0.67, 0.71)
Length at CMA diagnosis (SDS WHO) (median, IQR)	2.69	(1.68, 3.77)	3.05	(2.73, 3.42)
BMI at CMA diagnosis (kg/m²) (median, IQR)	17.2	(16.1, 18.0)	18.0	(16.9, 18.9)
BMI at CMA diagnosis (SDS WHO) (median, IQR)	0.58	(0.07, 1.07)	0.74	(-0.03, 1.25)
Positive prick by prick test for fresh milk	193/193	100.0%	27/27	100.0%
Positive skin prick test for α-lactoalbumin	163/193	84.5%	27/27	100.0%
Positive skin test positive for β-lactoglobulin	103/193	53.4%	27/27	100.0%
Positive skin prick test positive 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%

*Calculated only for the subjects with familial risk of allergy

CMA: cow's milk allergy; IQR: interquartile range; SDS: standard deviation score; WHO: World Health Organization

Supplementary Table 2. Effect of baseline covariables on the absolute risk difference between the EHCF+LGG and EHCF groups. Values are absolute risk differences and 95% confidence intervals.

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

95% confidence intervals in brackets

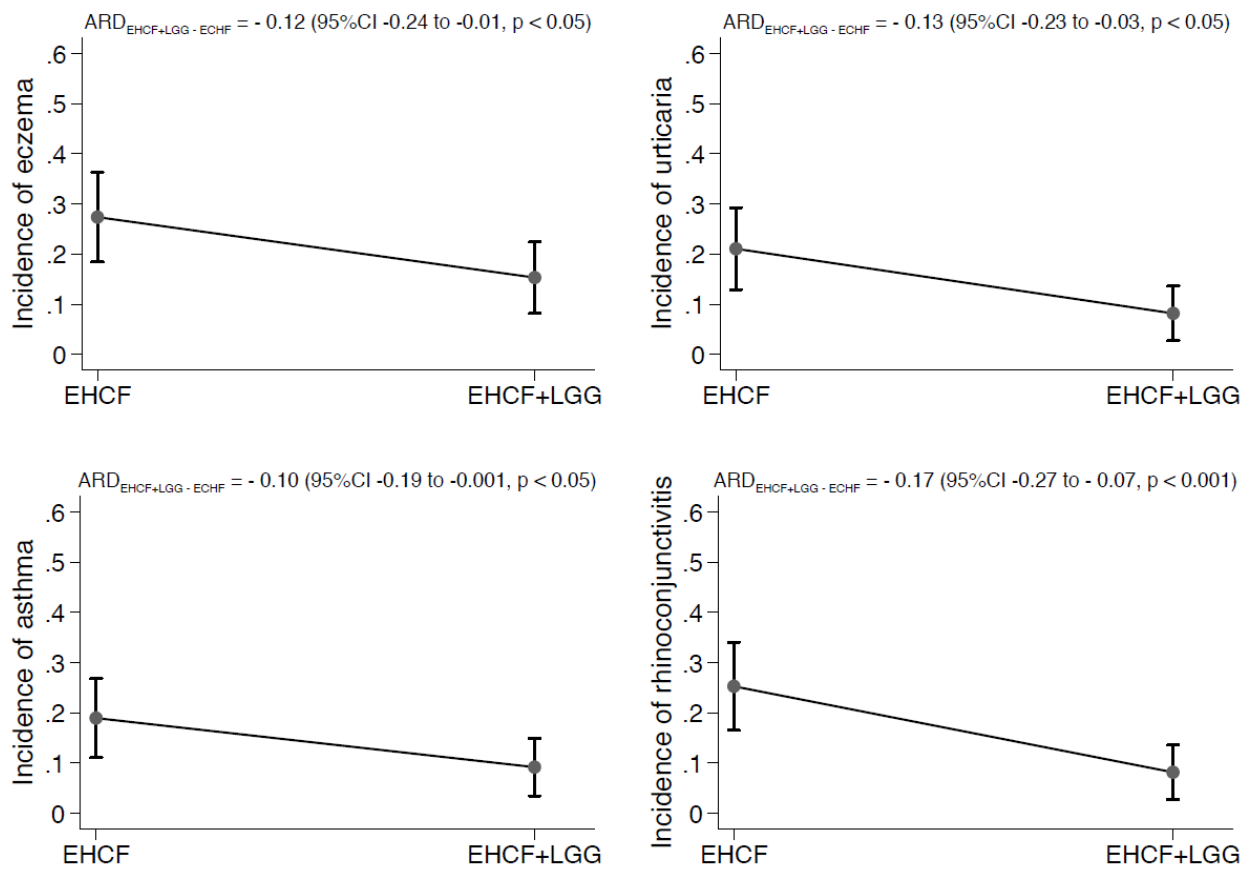
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

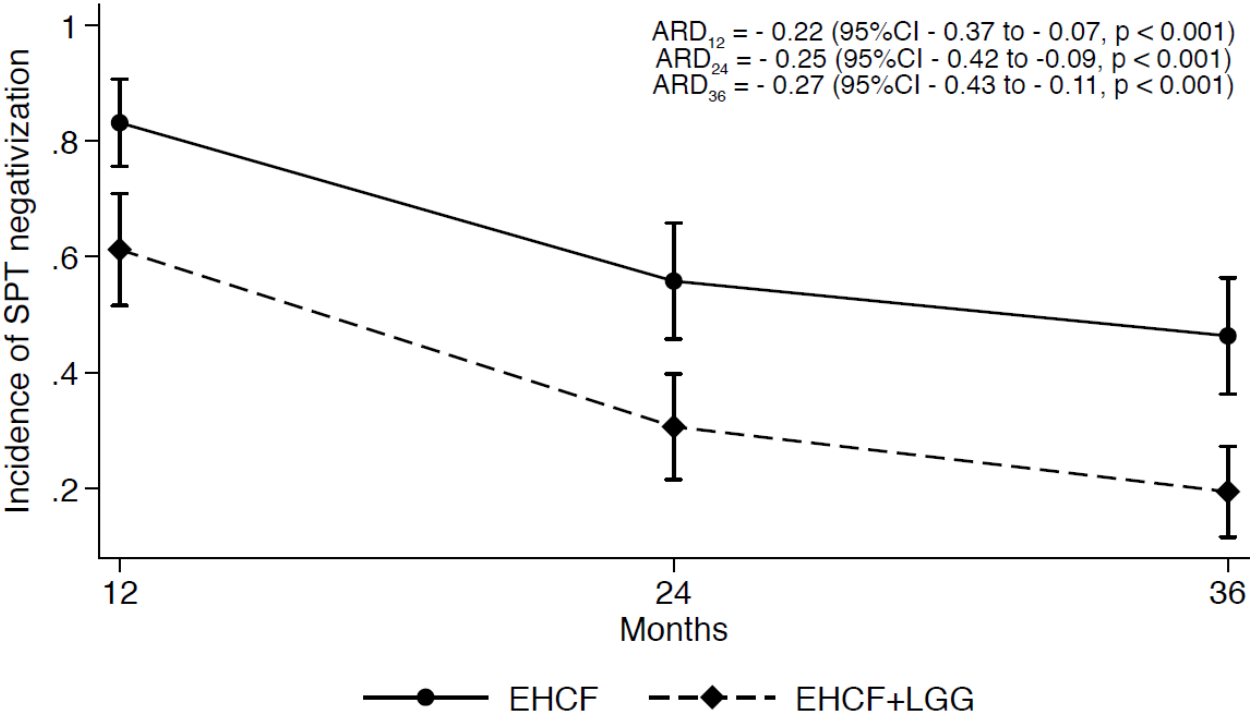
Supplementary Figure 1.

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

Supplementary Figure 2.

Incidence of SPT negativization during the study period.

Supplementary Figure 1.



ARD = absolute risk difference
95%CI and p-values are corrected for 3 comparisons