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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Abstract

Background and aims. Cow's milk protein allergy (CMPA) is a significant health issue in the pediatric age, carrying lifelong health implications. To compare the impact of different formulas on the occurrence of other atopic manifestations(AMs), autoimmune disorders(ADs) and the time of immune tolerance acquisition in a population of children with immunoglobulin E (IgE)-mediated cow CMPA.

Methods. In a 72-month prospective cohort study the occurrence of other AMs(i.e., eczema, urticaria, asthma, and rhinoconjunctivitis), ADs(i.e., celiac disease, thyroiditis, type 1 diabetes, inflammatory bowel diseases, idiopathic juvenile arthritis) and the time of immune tolerance acquisition were comparatively evaluated in IgE-mediated CMPA children treated with different formulas: extensively hydrolyzed casein formula containing the probiotic *L. rhamnosus* G(EHCF+LGG), rice hydrolyzed formula(RHF), soy formula(SF), extensively hydrolyzed whey formula(EHWF), or amino-acid based formula(AAF).

Results.313 subjects were evaluated: EHCF+LGG(n=64), RHF(n=62), SF(n=63), EHWF(n=60) and AAF(n=64). The incidence of AMs was: 0.30(Bonferroni-corrected 95%CI 0.15 to 0.44) for EHCF+LGG cohort, 0.68(0.52 to 0.83) for RHF cohort, 0.73(0.59 to 0.87) for SF cohort, 0.70(0.55 to 0.85) for EHWF cohort and 0.83(0.71 to 0.95) for AAF cohort. The corresponding risk ratios are 2.28 (1.51 to 3.45) for RHF vs. EHCF+LGG ($p<0.001$), 2.46(1.64 to 3.69) for SF vs. EHCF+LGG($p < 0.001$), 2.36 (1.56 to 3.56) for EHWF vs. EHCF+LGG ($p<0.001$), and 2.79(1.88 to 4.13) for AAF vs. EHCF+LGG($p<0.001$). The 72-month immune tolerance acquisition rate was higher in the EHCF+LGG cohort. The incidence of celiac disease was 2/313(0.006, binomial exact 95%CI 0.0007 to 0.023). No cases of other ADs were reported.

Conclusion.The dietary treatment with EHCF+LGG is associated with lower incidence of AMs and higher rate of immune tolerance acquisition in children with CMPA.

Keywords: allergic march, food allergy, gut microbiota, probiotics, L.rhamnosus GG, immune tolerance

Abbreviation list

CMPA: cow's milk protein allergy

FA: food allergies

AMs: allergic manifestations

FGIDs: functional gastrointestinal disorders

EHWF: extensively hydrolyzed whey formula

EHCF: extensively hydrolyzed casein formula

RHF: rice hydrolyzed formula

SF: soy formula

AAF and amino-acid-based formula

LGG: *Lactobacillus rhamnosus* GG

ADs: autoimmune diseases

IgE: immunoglobulin E

RT: Research Team

MPAT: Multidisciplinary Pediatric Allergy Team

SPT: skin prick tests

OFC oral food challenge

BRM: binomial regression model

Introduction

Cow's milk protein allergy (CMPA) is a significant health issue in the pediatric age, carrying lifelong health implications (1-4). with a global prevalence of up to 3% in the first years of life, CMPA is one of the most common food allergies (FA) and of food-induced anaphylaxis (5-9).

Recent studies have highlighted a shift in the natural history of CMPA over the past two decades, characterized by increased prevalence, severity of clinical manifestations, and risk of persistence into later ages (9-11).

These trends significantly impact the quality of life of affected individuals and their families and incur higher individual and societal costs, being one of the most expensive allergic diseases to manage in the pediatric age (1, 12-14).

Additionally, children with CMPA are also at increased risk of developing other allergic manifestations (AMs) such as oculorhinitis, atopic eczema, asthma, and urticaria, known as the "Allergic March", such as other diseases including functional gastrointestinal disorders (FGIDs), inflammatory bowel diseases (IBD), celiac disease, eosinophilic esophagitis (EoE), and neuropsychiatric disorders (1, 15-20).

The current standard of care for CMPA involves strict and careful cow milk proteins dietary avoidance, complemented by the use of substitute formulas for non-breastfed infants. Established formulas for managing CMPA include extensively hydrolyzed whey formula (EHWF), extensively hydrolyzed casein formula (EHCF), rice hydrolyzed formula (RHF), soy formula (SF), and amino-acid-based formula (AAF) (21-26).

Research suggests that in children with CMPA, dietary intervention with EHCF supplemented with the probiotic *L. rhamnosus* GG (LGG) provides several benefits on gastrointestinal symptoms (27-28), disease duration (15, 16, 29-34), and incidence of functional gastrointestinal disorders (35).

In previous prospective studies analyzing children with CMPA, we demonstrated that during a follow-up period of up to 36 months, EHCF+LGG provided protection against the development of other AMs later in life compared to other formulas (15, 16).

Several mechanisms may contribute to these effects, including the positive modulation of the gut microbiome's metagenomic and metabolomic profiles and the epigenetic regulation of genes involved in immune tolerance (32, 36-40). These mechanisms suggest a potential long-term impact on the immune system of CMPA children treated with EHCF supplemented with LGG.

In this study, we extended the follow-up period of a cohort of children with CMPA treated with different formulas, previously evaluated in a 36-month observational study (16), with an additional 3-year period of investigation for evaluating the occurrence of AMs, the rate of immune tolerance

acquisition, and the incidence of autoimmune diseases (ADs, including celiac disease, thyroiditis, type 1 diabetes, inflammatory bowel diseases, and idiopathic juvenile arthritis).

Methods

Study design and study population

From December 2014 to June 2019, we conducted a prospective cohort study on non-breastfed infants (aged 1-12 months) with suspected immunoglobulin E (IgE)-mediated CMPA. This study expanded on a previous cohort by adding a 3-year follow-up period (16), extending the observation until June 2022.

Initially, these infants were placed on a hypoallergenic formula by their family pediatricians or physicians and referred to our tertiary center for pediatric allergy for the necessity of the oral food challenge to confirm the CMPA diagnosis. At the time of enrollment, all subjects were in stable clinical condition without CMPA-related symptoms. They had been on a strict cow's milk proteins elimination diet and substituted formulas (EHCF+LGG, RHF, SF, EHWF, or AAF) for 15-30 days before recruitment. The formulas were prescribed by the family pediatricians or physicians upon suspicion of CMPA.

Exclusion criteria at enrolment included: treatment with pre- or probiotics in the previous 3 months; antibiotic treatment in the previous 3 months; cow's milk protein-induced anaphylaxis; food protein-induced enterocolitis syndrome; food allergies other than CMPA; atopic eczema unrelated to CMPA; eosinophilic gastrointestinal disorders; chronic systemic diseases; genetic disorders; congenital heart defects; active tuberculosis; autoimmune diseases; primary or secondary immunodeficiencies; chronic intestinal bowel disease; celiac disease; inflammatory bowel disease; *Helicobacter pylori* infection; cystic fibrosis; lactose intolerance; obesity; autism or neuropsychiatric disorders; metabolic diseases; malignancies; chronic pulmonary diseases; gastrointestinal and/or respiratory tract malformations; history of gastrointestinal surgery; participation in other studies; and conditions that could impede protocol compliance.

Ethical approval

The study protocol, patient information sheet, informed consent form, and clinical chart were reviewed and approved by the Ethical Committee of the University of Naples Federico II. The study adhered to the Helsinki Declaration (Fortaleza revision, 2013), Good Clinical Practice standards (CPMP/ICH/135/95), and relevant European and Italian data protection regulations. This study is part

of a larger project and was registered in the Clinical Trials Protocol Registration System with the ID number NCT03861910.

Data collection

As previously described (16), at baseline, following an initial evaluation by the Research Team (RT) composed by pediatric allergists and pediatric research nurses, a Multidisciplinary Pediatric Allergy Team (MPAT) consisting of pediatric allergists, dietitians, and nurses (all blinded to the study aims) conducted a comprehensive anamnestic and clinical assessment. This included the collection of demographics, anthropometric, and clinical data (related to CMPA), skin prick tests (SPT) for cow's milk proteins and fresh cow's milk, and an oral food challenge (OFC) to confirm the diagnosis of IgE-mediated CMPA (15, 41, 42). Informed consent from the parents or guardians of each child was obtained by the RT. Detailed information was collected on sociodemographic factors, family and living conditions, including parental history of allergic diseases and the presence of allergic first-degree relatives, number of siblings, pet exposure, parental smoking habits, maternal smoking during pregnancy, exposure to environmental tobacco smoke, urban vs. rural living environments, birth mode, breastfeeding history, and early dietary patterns. Infants with a confirmed diagnosis of IgE-mediated CMPA based on OFC results were enrolled in the study and continued the exclusion diet using the same formula previously prescribed when CMPA was suspected. To ensure compliance with the study formula, parents or caregivers were asked to maintain a daily record of formula use. These records were systematically reviewed by certified dietitians during follow-up visits to verify adherence to the prescribed formula regimen and assess compliance. As the children transitioned to a more diversified diet, complementary feeding was introduced following standardized guidelines. Dietary diversification was monitored through structured interviews during follow-up visits, where parents reported on newly introduced food groups. A periodic structured 24-hour dietary recall was conducted to evaluate daily intake patterns, while a Food Frequency Questionnaire (FFQ) provided a broader overview of nutritional intake, including allergenic food exposure and micronutrient consumption.

The RT planned six visits every 12 months over a 6-year follow-up period, adhering to standard care procedures for patients with IgE-mediated CMPA. During these visits, the MPAT assessed clinical status, body growth, occurrence of allergic symptoms, compliance with the cow's milk protein-free diet, adherence to the prescribed formula (defined as consuming at least 80% of the formula), conducted SPT for cow's milk proteins and fresh milk, and performed OFC to evaluate possible acquisition of immune tolerance to cow's milk proteins. If immune tolerance was demonstrated through OFC results, a diet containing cow's milk proteins was allowed for the remainder of the study

period. Unscheduled visits were made as needed due to allergic symptoms or other morbidities, with parents instructed to contact the RT for medical examination if necessary.

At each visit, as previously described, the MPAT performed a full physical examination and, using standardized criteria and current guidelines, diagnosed any AMs (16, 42, 43-51) and/or ADs (52-55). In case of disagreement on an AMs and/or ADs diagnosis, a further evaluation by another pediatric allergist, unaware of the study aims, was conducted. All study teams, procedures, and assessments were conducted as illustrated in **Figure 1.**

Data entry

All data were recorded anonymously. The RT entered all collected data into the case report form (CRF). Two researchers independently checked the data for completeness, clarity, consistency, and accuracy, and instructed the staff to make any necessary corrections or additions. Using a standardized data-entry method, a single researcher entered all CRF data into the study database. Subsequently, the Statistical Team (ST), which was unaware of the study cohorts, reviewed the dataset and performed data cleaning and verification according to standard procedures. Once the dataset was declared complete and accurate, the ST locked the database, and statistical analysis was conducted.

Study outcomes

The main outcome was the occurrence of any AMs after 72 months from baseline.

The secondary outcomes the evaluation of immune tolerance acquisition to CMP after 72 months of follow-up.

Explorative outcomes were: the occurrence of any ADs after 72 months from baseline and the acquisition of immune tolerance acquisition to CMP at 48, 60 and 72 months of follow- up.

The occurrence of any other IgE-mediated FA alone or in combination with AMs and the results of skin prick test was also recorded.

Sample size

This study represents the long-term follow-up of a previously published study whose sample size was evaluated on the basis of the expected incidence of at least 1 AM at 36 months in the RHF, SF, EHWF, and AAF cohorts vs. the EHCF+LGG cohort (16). As such, no formal sample size calculation was performed for the present analysis.

Statistical analysis

232

233 *Descriptive statistics*

234 Most continuous variables were not Gaussian-distributed, and all are reported as median (50^{th}
235 percentile) and interquartile interval (IQI; 25^{th} and 75^{th} percentiles). Discrete variables are reported
236 as the number and proportion of subjects with the characteristic of interest.

237

238 *Main outcome*

239 We used a binomial regression model (BRM) to estimate the incidence of the main outcome (i.e., the
240 occurrence of at least 1 AM during the 72-month follow up period in the five dietary treatments
241 cohorts) (56). The response variable of the BRM was the presence of at least 1 AM at 72 months (0
242 = no; 1 = yes), and the predictor was the treatment cohort (0 = EHCF+LGG; 1 = RF; 2 = SF; 3 =
243 EHWF; 4 = AAF). To evaluate the effect of environmental and demographic factors as potential
244 confounders on the main outcome, we added each of them separately to the aforementioned BRM
245 and evaluated the changes in the estimated risk ratios (RR) (57). The evaluated potential confounders
246 were sex (0 = female; 1 = male), age (months), cesarean delivery (0 = no; 1 = yes), born at term (0 =
247 no; 1 = yes), breastfed for at least 2 months (0 = no; 1 = yes), weaning (months), siblings (number),
248 familial risk of allergy (0 = no; 1 = yes), exposed to passive smoking (0 = no; 1 = yes), mother smoked
249 during pregnancy (0 = no; 1 = yes), and exposed to pets (0 = no; 1 = yes). Bonferroni-corrected 95%
250 confidence intervals were calculated with a correction for five comparisons (cohorts) (16).

251

252 *Secondary outcome*

253 We used a BRM with cluster confidence intervals to estimate the incidence of tolerance acquisition
254 in the five treatment cohorts at 72 months (56). The response variable of the BRM was the acquisition
255 of immune tolerance at 72 months (0 = no; 1= yes) and the predictor was the treatment cohort
256 (discrete: 0 = EHCF+LGG; 1 = RF; 2 = SF; 3 = EHWF; 4 = AAF). Bonferroni-corrected 95%
257 confidence intervals were calculated with a correction for five comparisons (cohorts) (16).

258

259 *Other outcomes*

260 For exploratory purposes (16), we also calculated a BRM in which the response variable was the
261 acquisition of tolerance (0 = no; 1= yes), and the predictors were the treatment cohort (discrete: 0 =
262 EHCF+LGG; 1 = RF; 2 = SF; 3 = EHWF; 4 = AAF), time (discrete: 0 = 12; 1 = 24; 2 = 36; 3 = 48; 4
263 = 60; 5 = 72 months), and a treatment x time (discrete x discrete) interaction (56). No correction for
264 multiple comparisons (cohorts) was performed because of the exploratory nature of this analysis (16).

265

Results

Study population

Of the 365 children who had been followed-up at 36 months (16), 313 (86%) were available at the 72-month follow-up. The flow of the subjects throughout the study is reported in **Figure Supplementary 1.**

The **Table 1** describes the main features at the enrollment of the children available and not available at follow-up. The vast majority (n=51) of the subjects lost to the follow-up had outgrown CMPA at 36 months from baseline, and 14 out of 52 were diagnosed with other AMs. The age and sex of the children available and not available to follow-up were similar, as were most of the baseline features. **Table 2** gives the baseline features of the children available at follow-up for the EHCF+LGG (n = 64), RHF (n = 62), SF (n = 63), EHWF (n = 60) and AAF (n = 64) cohorts.

All children were compliant, i.e., they consumed at least 80% of the assigned formula, as assessed by the evaluation of 3-day food diary analyzed by dietitians experienced in pediatric FA. No substantial differences in dietary composition beyond formula consumption were identified among study cohorts. No case of misunderstanding of formula use was reported during the study period.

Main outcome

The Figure 2 plots the incidence of the main outcome (i.e., the occurrence of ≥1 AM in a 72-month follow up period) observed in the five dietary treatment cohorts.

The incidence was 0.30 (Bonferroni-corrected 95%CI 0.15 to 0.44) for EHCF+LGG cohort, 0.68 (0.52 to 0.83) for RHF cohort, 0.73 (0.59 to 0.87) for SF cohort, 0.70 (0.55 to 0.85) for EHWF cohort and 0.83 (0.71 to 0.95) for AAF cohort. The corresponding risk ratios were 2.28 (1.51 to 3.45) for RHF *vs.* EHCF+LGG ($p < 0.001$), 2.46 (1.64 to 3.69) for SF *vs.* EHCF+LGG ($p < 0.001$), 2.36 (1.56 to 3.56) for EHWF *vs.* EHCF+LGG ($p < 0.001$), and 2.79 (1.88 to 4.13) for AAF *vs.* EHCF+LGG ($p < 0.001$). The point estimate of all risk ratios increased substantially after the environmental and demographic factors as potential confounders were entered into the model, but the corresponding 95%CI were wide, showing an increase in the imprecision of the estimate **(Supplementary Table 1).**

The **Table 3** reports the time-specific and cumulative incidence of the components of the main outcome (eczema, urticaria, asthma, and oculorhinitis) observed at 12, 24, 36, 48, 60 and 72-month follow up. This was an exploratory analysis, performed because the main outcome was a composite outcome, and as such it can be used only for hypothesis-generating purposes.

Secondary outcome

The **Figure 3** plots the incidence of the secondary outcome (i.e., the evaluation of immune tolerance acquisition to CMP after 72-month follow- up) in the five dietary treatment cohorts.

The incidence was 0.95 (Bonferroni-corrected 95%CI 0.88 to 1.00) for EHCF+LGG cohort, 0.84 (0.72 to 0.95) for RHF cohort, 0.70 (0.56 to 0.84) for SF cohort, 0.79 (0.67 to 0.92) for EHWF cohort and 0.60 (0.46 to 0.75) for AAF cohort. The corresponding risk ratios were 0.88 (0.79 to 0.99) for RHF vs. EHCF+LGG ($p < 0.05$), 0.74 (0.63 to 0.87) for SF *vs.* EHCF+LGG ($p < 0.001$), 0.84 (0.74 to 0.96) for EHWF *vs.* EHCF+LGG ($p < 0.01$) and 0.64 (0.53 to 0.77) for AAF *vs.* EHCF+LGG ($p < 0.001$).

Other outcomes

The **Table 4** reports the time-specific and cumulative incidence of immune tolerance acquisition at 12, 24, 36, 48, 60 and 72-month follow up in the five study cohorts.

In the **Supplementary Figure 2,** we reported the cumulative incidence of SPT negativization rate in the five study cohorts. The response closely mirrored the CMP immune tolerance acquisition rate.

Occurrence of autoimmune diseases

The incidence of ADs in the study population was notably low. Specifically, CD was diagnosed in only 2 out of 313 children (0.6%; binomial exact 95% CI: 0.07% to 2.3%), with one case in the RHF cohort at 70 months of age and another in the SF cohort at 45 months of age. Importantly, no cases of other ADs, such as thyroiditis, type 1 diabetes, inflammatory bowel disease, or idiopathic juvenile arthritis, were reported throughout the study period.

Safety

No child exhibited reactions or intolerance to any of the study formulas. Additionally, there were no adverse events linked to the consumption of these formulas, and no significant differences were observed in their daily intake (data not shown). Furthermore, the cohorts showed similar time-related changes in weight, length, and height (data not shown). These findings suggest that all formulas used in the study were well-tolerated by children with IgE-mediated CMPA.

Discussion

This study provides several important insights into the long-term management of children with IgE-mediated CMPA treated with different substitute formulas. Firstly, the study confirms a generally high rate of the allergic march across all formula types evaluated, consistent with previous findings also in similar populations (15, 16, 58-60). The incidence rates of AMs were similar across the different formula types, with the lowest rate observed in the EHCF+LGG cohort and the highest in the AAF cohort. This finding is consistent with prior research, underscoring the protective effect of EHCF+LGG against the development of other allergic conditions (15,16). In fact, regarding the main outcome, the incidence of other AMs at 72 months in the EHCF+LGG cohort was consistently lower if compared to the other cohorts, with RRs ranging from 2.28 to 2.79. In addition, the use of EHCF+LGG affected all of the components of the main study outcome; although these findings can be taken only as exploratory, the data are consistent with what has already been demonstrated, and additional studies are needed to explore the potential of this nutritional strategy against each specific allergic disease (15,16).

The differences in the allergic march rates between the formula types suggest possible hypotheses regarding disease modification, which could influence future therapeutic approaches and the development of targeted nutritional interventions. The lower incidence of AMs in the EHCF+LGG cohort may be attributed to the positive modulation of the gut microbiome, immune system priming, and epigenetic effects exerted by the probiotic LGG included in this formula. These mechanisms could play a role in modifying the natural course of CMPA, reducing the likelihood of progressing to other allergic diseases. The potential role of infant formulas in preventing AMs in infants with CMPA was first suggested approximately 20 years ago. The effectiveness of EHCF in preventing allergies is substantiated by the GINI study, which demonstrated that high-risk infants receiving EHCF were protected from AMs (47, 61-65).

Additionally, a notable reduction in asthma incidence was observed in children treated with EHCF at 15 years of age (64). These findings are consistent with a prospective cohort study on IgE-mediated CMPA revealing the use of EHCF significantly protected against other allergic diseases compared to other hypoallergenic or soy-based formulas (66).

These evidence align with recent studies indicating that EHCF+LGG as a first-line approach for CMPA infants may inhibit the occurrence of AMs compared to other formulas. A retrospective study found that the type of formula used could influence the natural history of CMPA children, with high-

grade EHF and EHF+LGG showing significant reductions in AMs occurrence (60). Similarly, in a retrospective cohort study, the first-line management of newly diagnosed CMPA infants with EHCF combined with LGG may mitigate the progression of atopic dermatitis and asthma compared to those treated with EHWF (67). To date, only one randomized controlled trial has tested the potential of formula-based dietary interventions on AMs prevention in CMPA pediatric patients in a 36-month follow-up, showing a beneficial effect of EHCF+LGG on the occurrence of AMs (15).

Moreover, this study provides robust confirmation of the beneficial effects of EHCF+LGG on immune tolerance acquisition. Children in the EHCF+LGG cohort demonstrated a higher rate of tolerance acquisition by 72 months compared to those in other formula cohorts. This supports the growing body of evidence that suggests specific dietary interventions, particularly those incorporating probiotics like LGG, can enhance the immune system's ability to develop tolerance to CM allergens over time. The results of this long-term cohort study suggest that EHCF+LGG is more effective in reducing the duration of the CMPA disease. According to previous observations, we provide further evidence supporting the positive impact of EHCF+LGG on the acquisition of immune tolerance in children with IgE-mediated CMPA (15, 16, 29-34). In this study, we confirmed that the beneficial effects of EHCF+LGG persist up to 72 months of intervention, even when compared to other formulas. These findings are significant in light of evidence indicating that the natural history of CMPA has evolved, showing slower resolution rates and a higher proportion of children whose disease persists into school age and beyond (9, 68-70).

In addition, the current study does not provide sufficient data to comprehensively evaluate the impact of these dietary interventions on autoimmunity, representing a possible limitation that warrants further investigation. While the occurrence of CD was observed in two cases, no other autoimmune disorders were reported. This result is in apparent discordance with the result of large cohort study (19), suggesting that CD is highly prevalent in FA patients and could affect the FA severity (19). As such, the study cannot draw definitive conclusions about the relationship between CMPA, its dietary management, and the development of autoimmune conditions. Given these findings, our study highlights the necessity for extended follow-up and more comprehensive datasets to assess potential long-term autoimmune risks. Future studies should incorporate immunological biomarkers and microbiome analyses to elucidate mechanisms underlying autoimmunity in CMPA children and the role of formula-based interventions in mitigating such risks.

Finally, the conclusions drawn from this study underscore the significant potential of active diet therapy in the modification of disease progression, positioning EHCF+LGG as a promising strategy not only for managing CMPA but also for altering its natural course and improving long-term outcomes. The use of EHCF supplemented with LGG in fact not only appears to mitigate the progression of the allergic march but also promotes the earlier acquisition of immune tolerance. The mechanisms by which LGG exerts its effects include epigenetic regulation of immune-related genes and microRNAs expression, modulation of the gut microbiota, enhancement of mucosal barrier function, and immunomodulatory actions. The probiotic LGG also exerts changes in DNA methylation patterns, that are crucial for developing immune tolerance (32). Furthermore, LGG interacts with intestinal epithelial and dendritic cells, promoting the production of anti-inflammatory cytokines like IL-10 and TGF-β, which are essential for tolerance and reducing allergic responses (71). EHCF+LGG also positively modulates gut microbiota structure and function, reducing permeability and promoting epithelial integrity, which is beneficial for managing CMPA and preventing other AMs. This effect is accompanied by increased short-chain fatty acids (SCFAs), such as butyrate, a vital metabolite which induces regulatory T cells (Tregs) crucial for immune tolerance and the prevention of allergic diseases driving immune tolerance (32, 36-38, 72-75).
These findings suggest that such formulas should be considered a preferred strategy in managing CMPA, with implications for both clinical outcomes and cost-effectiveness.

One of the strengths of this study is its extended follow-up period of six years, providing robust data on the persistence and evolution of AMs and the acquisition of immune tolerance. The large sample size and comprehensive assessment of AMs and ADs enhance the reliability and generalizability of the findings. Additionally, the study design and the use of a multidisciplinary team for assessments, combined with blinding of the study aims, minimize bias and improve the validity of the results. The rigorous methodology, including regular follow-ups and validated diagnostic criteria for CMPA, other AMs, and ADs, further strengthens the study.

Despite its strengths, the study has several limitations. The observational nature of the study design means causality cannot be definitively established. The exclusion of infants with severe CMPA manifestations or significant comorbidities limits the generalizability of the findings to all CMPA populations. Future randomized controlled trials are necessary to confirm these findings and establish a causal relationship between LGG supplementation and reduced AMs in CMPA patients. Lastly, our results are constrained by the absence of data on gut microbiota and Th1/Th2 cytokines,

439 which are essential for a deeper understanding of the mechanisms through which EHCF+LGG exerts
440 its effects. Future studies are recommended to elucidate these mechanisms more comprehensively.
441
442 In conclusion, this study highlights the significant role of formula choice in the management
443 of CMPA. EHCF supplemented with LGG not only reduces the incidence of other AMs but also
444 promotes the acquisition of immune tolerance, underscoring its potential as a preferred "active diet
445 therapy," also for its cost-effectiveness. Future research, particularly well-designed randomized
446 controlled trials, is needed to confirm these findings and to further explore the underlying mechanisms
447 and long-term benefits of probiotic-supplemented hypoallergenic formulas.
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Authors Contributors' Statement

Rita Nocerino: Conceptualization, Methodology, Investgation, Formal Analysis, Data Curation, Resources, Project Administration, Supervision, Writing - Original Draft; Giorgio Bedogni: Methodology, Formal Analysis, Data Curation, Writing - Original Draft; Laura Carucci: Validation, Investigation, Visualization; Greta Aquilone: Validation, Investigation; Franca Oglio: Validation; Serena Coppola: Validation, Investigation, Visualization; Antonio Masino: Validation, Investigation, Visualization; Roberto Berni Canani: Conceptualization, Methodology, Investigation, Visualization, Funding Acquisition, Resources, Project Administration, Supervision, Writing - Original Draft.

Conflict of interest statement

Roberto Berni Canani have had the following relevant financial relationships with the following manufacturers: Biostime (research grant), Ch. Hansen (research grant, speaker), DBV (research grant), Dr. Schar (research grant), Humana (research grant), iHealth (research grant), Kraft-Heinz (research grant, speaker), Mead Johnson Nutrition (research grant, speaker), Nestlè (research grant, speaker), Novalac (research grant, speaker), Nutricia (research grant, speaker), Sanofi (research grant, speaker) as part of publicly funded research projects with the support of the Italian Ministry of Health, the Italian Ministry of the University and Research, and the EU. The other authors declared that they have no conflicts of interest.

Ethical Approval

The study was approved by Ethics Committee of the University Federico II of Naples and was performed in accordance with the Helsinki Declaration (Fortaleza revision, 2013), the Good Clinical Practice Standards (CPMP/ICH/135/95), and with the pertinent European and Italian regulations about privacy. Written informed consent was obtained from the parents/caregivers of each subject.

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Figure legends

Figure 1.

The design of the study.

Figure 2. Incidence of at least 1 AM at 72 months in the five treatment cohorts.

Values are means and 95% estimated from binomial regression with cluster confidence intervals. Abbreviations: AAF = amino-acid formula; EHCF+LGG = extensively hydrolyzed casein formula; EHWF = extensively hydrolyzed whey formula; RHF = rice hydrolyzed formula; SF = soy formula.

Figure 3. Cumulative incidence of immune tolerance acquisition in the five treatment cohorts at 72 months.

Values are means and 95% estimated from binomial regression with cluster confidence intervals Abbreviations: AAF = amino-acid formula; EHCF+LGG = extensively hydrolyzed casein formula; EHWF = extensively hydrolyzed whey formula; RHF = rice hydrolyzed formula; SF = soy formula.

Supplementary Figure 1. The flow of the subjects throughout the study.

Supplementary Figure 2. Cumulative incidence of skin prick test negativization in the five study cohorts.

Values are means and 95% estimated from binomial regression with cluster confidence intervals (exploratory analysis, see statistical analysis for details). Abbreviations: SPT = skin prick test; AAF = amino-acid formula; EHCF+LGG = extensively hydrolyzed casein formula; EHWF = extensively hydrolyzed whey formula; RHF = rice hydrolyzed formula; SF = soy formula.

749 **Figure 1.**

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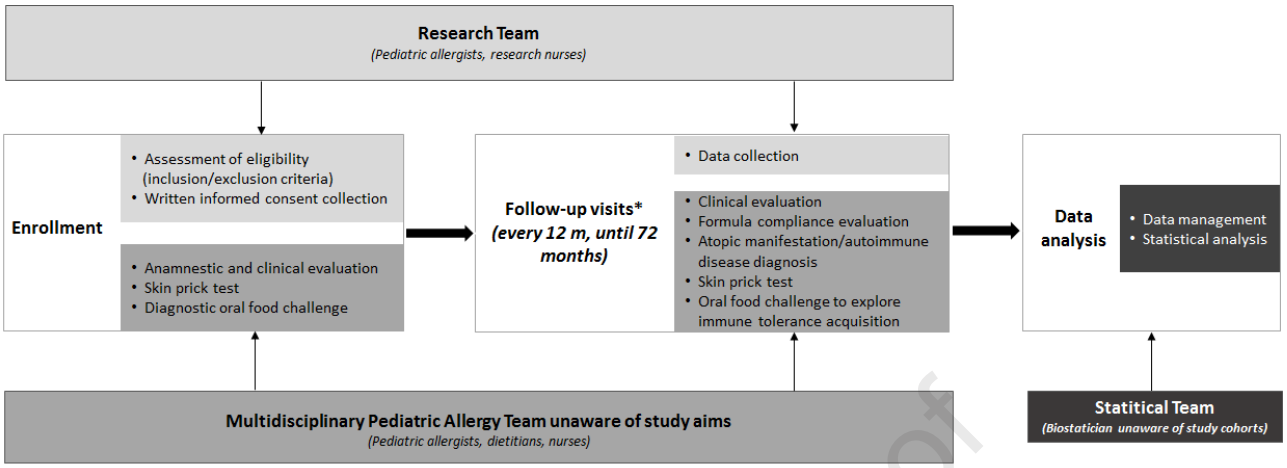

Unscheduled visits were made if necessary because of allergic symptoms/autoimmune disorders or other morbidities. Whenever allergic symptoms or other morbidities occurred, parents were instructed to contact the Center to have a medical examination of their child. At these medical examinations, the pediatric allergist team unaware of study cohorts performed a full physical examination, and then, using standardized criteria, decided on the atopic manifestation/autoimmune disorders diagnosis.

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Figure 2.

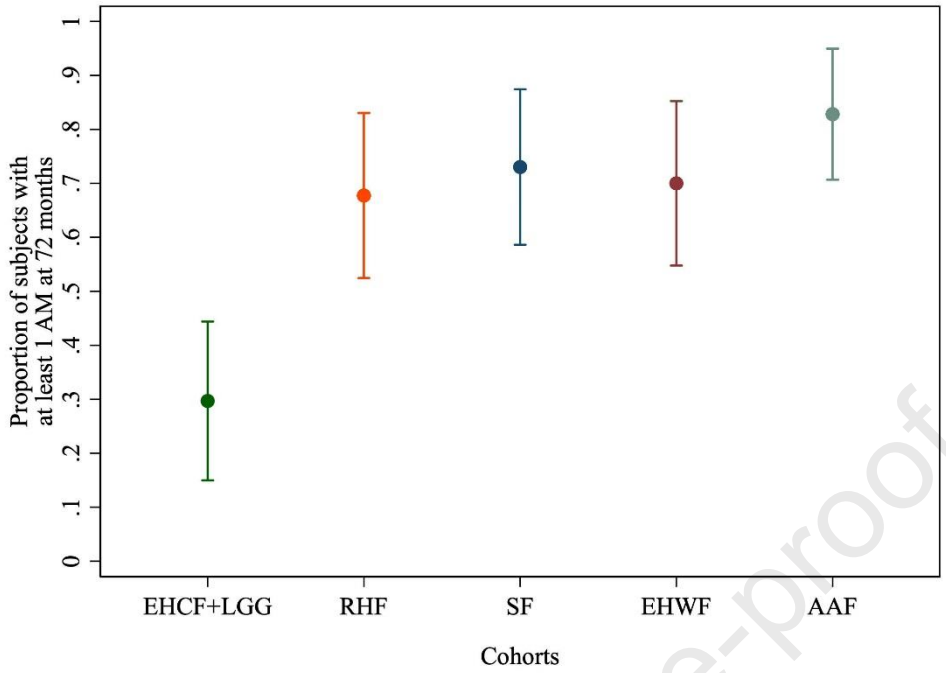
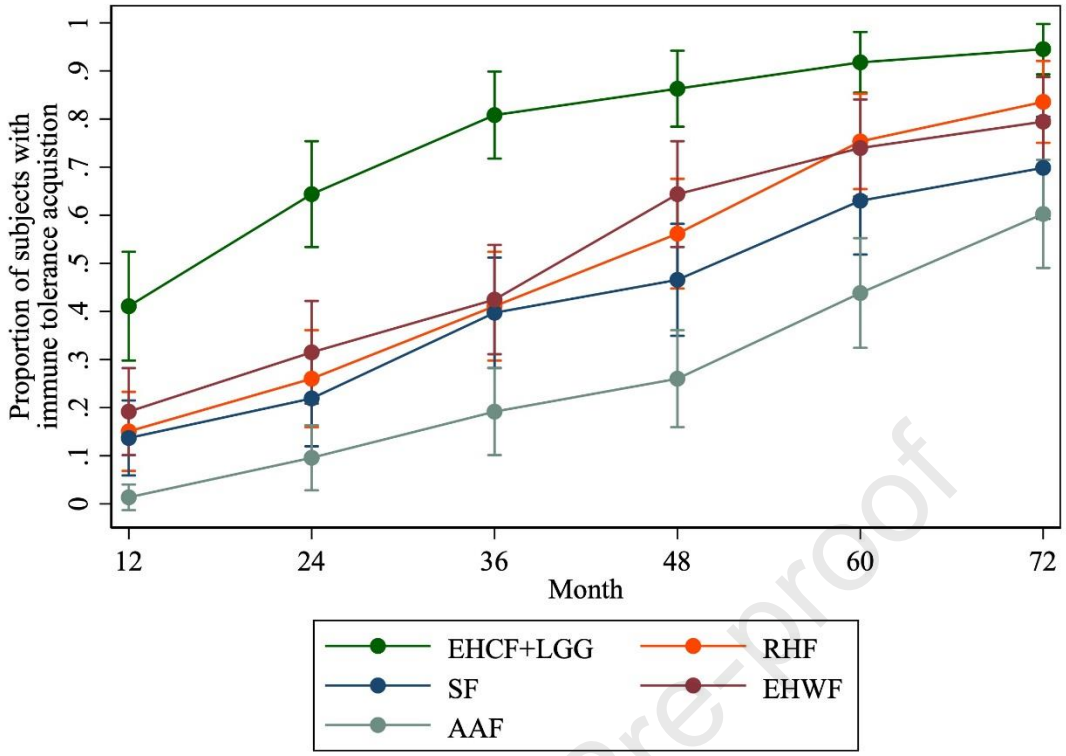


Figure 3.



817 **Supplementary Figure 1.**

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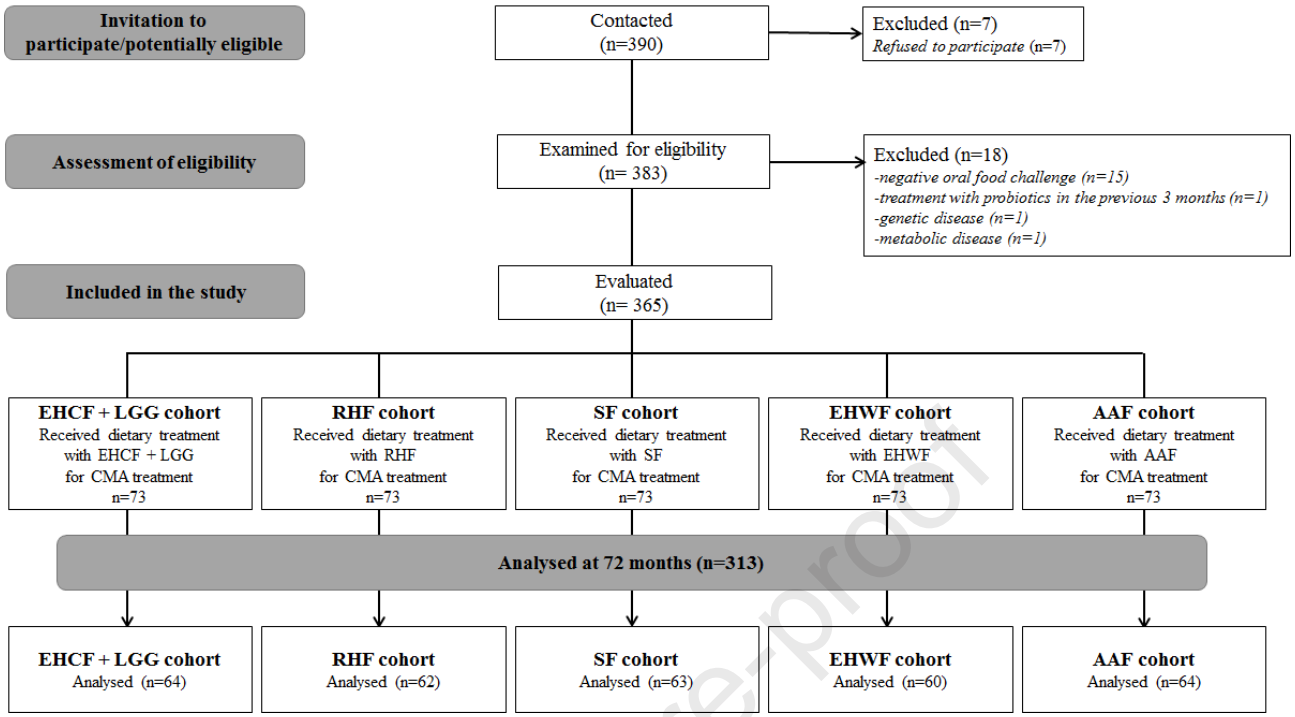

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820 **Supplementary Figure 2.**
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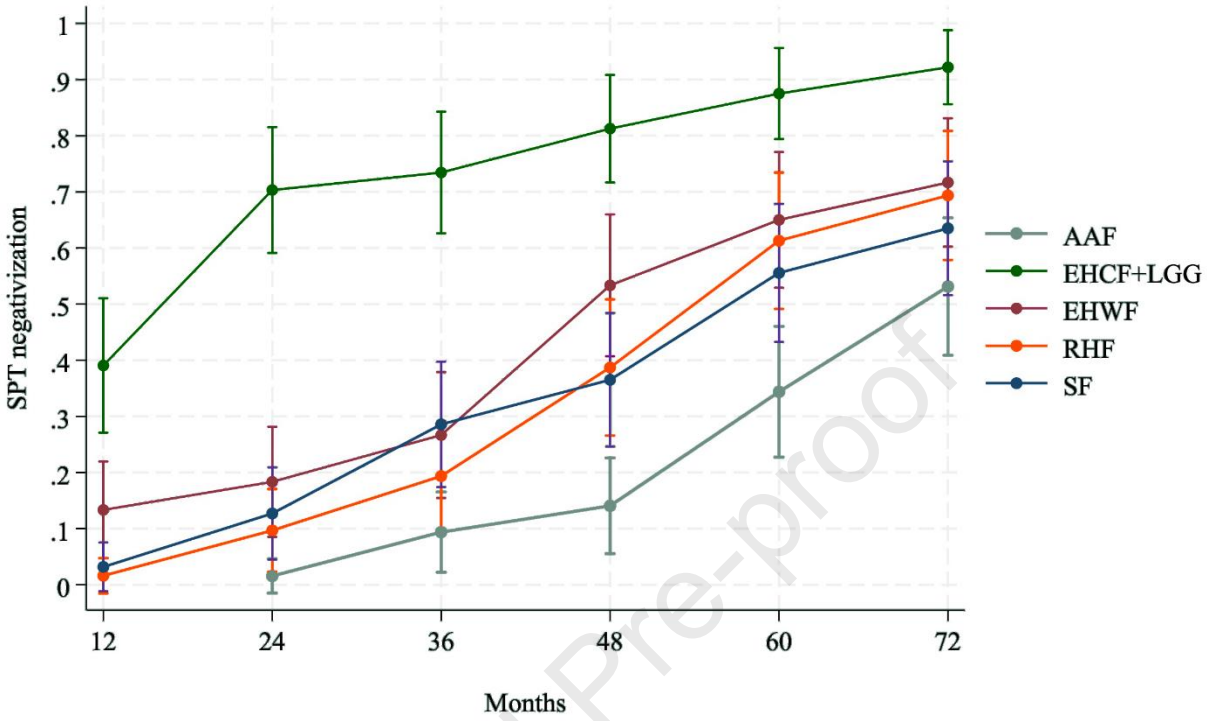

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Table 1. Baseline features of the children available and not available at follow-up.

	Total N=365	Available at follow-up N=313	Lost to follow-up N=52
Cohort			
EHCF+LGG	73 (20%)	64 (20.4%)	9 (17.3%)
RHF	73 (20.0%)	62 (19.8%)	11 (21.2%)
SF	73 (20.0%)	63 (20.1%)	10 (19.2%)
EHWF	73 (20.0%)	60 (19.2%)	13 (25.0%)
AAF	73 (20.0%)	64 (20.4%)	9 (17.3%)
Male sex	240 (65.8%)	202 (64.5%)	38 (73.1%)
Age (months)	5.0 (4.0; 8.0)	5.0 (4.0; 8.0)	5.0 (3.0; 7.0)
Cesarean delivery	214 (58.6%)	185 (59.1%)	29 (55.8%)
Born at term	339 (92.9%)	290 (92.7%)	49 (94.2%)
Birth weight (kg)	3.2 (3.0; 3.5)	3.2 (3.0; 3.5)	3.3 (3.0; 3.6)
Breastfed for at least 2 months	267 (73.2%)	228 (72.8%)	39 (75.0%)
Weaning (month)	5 (4; 6)	5 (4; 6)	5 (4; 6)
Siblings	0 (0; 1)	0 (0; 1)	1 (0; 1)
Familial risk of allergy	237 (64.9%)	204 (65.2%)	33 (63.5%)
Allergic first-degree relatives	1 (1; 2)	1 (1; 2)	1 (1; 1)
Exposed to passive smoking	139 (38.1%)	117 (37.4%)	22 (42.3%)
Mother smoked during pregnancy	118 (32.3%)	95 (30.4%)	23 (44.2%)
Exposed to pets	62 (17.0%)	58 (18.5%)	4 (7.7%)
Age at diagnosis (months)	5 (4; 8)	5 (4; 8)	5 (3; 7)
Weight at diagnosis (kg)	7.5 (6.1; 8.9)	7.5 (6.1; 9.0)	7.1 (6.0; 8.1)
Length at diagnosis (cm)	66 (61; 70)	66 (61; 70)	65 (60; 69)
Prick test positive for fresh milk	365 (100.0%)	313 (100.0%)	52 (100.0%)
Prick test positive for α-lactoalbumin	295 (80.8%)	248 (79.2%)	47 (90.4%)
Prick test positive for β-lactoglobulin	243 (66.6%)	207 (66.1%)	36 (69.2%)
Prick test positive for casein	167 (45.8%)	147 (47.0%)	20 (38.5%)
Gastrointestinal symptoms at diagnosis	223 (61.1%)	192 (61.3%)	31 (59.6%)
Cutaneous symptoms at diagnosis	246 (67.4%)	210 (67.1%)	36 (69.2%)
Respiratory symptoms at diagnosis	58 (15.9%)	51 (16.3%)	7 (13.5%)

Continuous variables are reported as median (50th percentile) and interquartile interval (25th and 75th percentiles). Discrete variables are reported as the number and proportion of subjects with the characteristic of interest.

Table 2. Baseline features of the children available at 72 m-follow-up in the five treatment cohorts.

	EHCF+LGG N=64	RHF N=62	SF N=63	EHWF N=60	AAF N=64
Male sex	42 (65.6%)	40 (64.5%)	40 (63.5%)	39 (65.0%)	41 (64.1%)
Age (months)	6.0 (3.0; 7.0)	5.5 (4.0; 8.0)	5.0 (3.0; 8.0)	5.0 (3.0; 8.2)	5.5 (5.0; 9.0)
Cesarean delivery	40 (62.5%)	35 (56.5%)	37 (58.7%)	37 (61.7%)	36 (56.2%)
Born at term	59 (92.2%)	57 (91.9%)	59 (93.7%)	56 (93.3%)	59 (92.2%)
Birth weight (kg)	3.1 (2.8; 3.5)	3.1 (3.0; 3.7)	3.5 (3.1; 3.7)	3.2 (3.0; 3.5)	3.1 (3.0; 3.2)
Breastfed for at least 2 months	43 (67.2%)	47 (75.8%)	46 (73.0%)	44 (73.3%)	48 (75.0%)
Weaning (month)	5 (5; 6)	5 (4; 5)	5 (4; 6)	5 (4; 6)	5 (4; 6)
Siblings	1 (0; 1)	0 (0; 1)	1 (0; 1)	1 (0; 1)	0 (0; 1)
Familial risk of allergy	41 (64.1%)	41 (66.1%)	44 (69.8%)	40 (66.7%)	38 (59.4%)
Allergic first-degree relatives	1 (1; 2)	1 (1; 1)	1 (1; 2)	1 (1; 1)	1 (1; 1)
Exposed to passive smoking	26 (40.6%)	23 (37.1%)	23 (36.5%)	18 (30.0%)	27 (42.2%)
Mother smoked during pregnancy	24 (37.5%)	19 (30.6%)	19 (30.2%)	14 (23.3%)	19 (29.7%)
Exposed to pets	13 (20.3%)	7 (11.3%)	11 (17.5%)	12 (20.0%)	15 (23.4%)
Age at diagnosis (months)	6 (3; 7)	6 (4; 8)	5 (3; 8)	5 (3; 8)	6 (5; 9)
Weight at diagnosis (kg)	7.4 (6.3; 9.0)	7.8 (6.1; 9.0)	7.5 (5.9; 8.7)	7.4 (5.8; 8.7)	8.1 (6.6; 9.0)
Length at diagnosis (cm)	66 (62; 70)	66 (60; 69)	65 (60; 70)	65 (60; 70)	66 (63; 70)
Prick test positive for fresh milk	64 (100.0%)	62 (100.0%)	63 (100.0%)	60 (100.0%)	64 (100.0%)
Prick test positive for α-lactoalbumin	50 (78.1%)	50 (80.6%)	51 (81.0%)	48 (80.0%)	49 (76.6%)
Prick test positive for β-lactoglobulin	44 (68.8%)	41 (66.1%)	39 (61.9%)	40 (66.7%)	43 (67.2%)
Prick test positive for casein	31 (48.4%)	31 (50.0%)	28 (44.4%)	26 (43.3%)	31 (48.4%)
Gastrointestinal symptoms at diagnosis	38 (59.4%)	40 (64.5%)	38 (60.3%)	36 (60.0%)	40 (62.5%)
Cutaneous symptoms at diagnosis	41 (64.1%)	43 (69.4%)	44 (69.8%)	39 (65.0%)	43 (67.2%)
Respiratory symptoms at diagnosis	12 (18.8%)	8 (12.9%)	11 (17.5%)	8 (13.3%)	12 (18.8%)

Continuous variables are reported as median (50th percentile) and interquartile interval (25th and 75th percentiles). Discrete variables are reported as the number and proportion of subjects with the characteristic of interest.

Table 3. Time-specific and cumulative incidence of the components of the main outcome (eczema, urticaria, asthma, and oculorhinitis) at 12, 24, 36, 48, 60 and 72 months.

	EHCF+LGG	RHF	SF	EHWF	AAF
Eczema at 12 months					
No	64 (100.0%)	56 (90.3%)	45 (71.4%)	45 (75.0%)	45 (70.3%)
Yes	0 (0.0%)	6 (9.7%)	18 (28.6%)	15 (25.0%)	19 (29.7%)
Eczema at 24 months					
No	57 (89.1%)	54 (87.1%)	60 (95.2%)	59 (98.3%)	58 (90.6%)
Yes	7 (10.9%)	8 (12.9%)	3 (4.8%)	1 (1.7%)	6 (9.4%)
Eczema at 36 months					
No	61 (95.3%)	56 (90.3%)	58 (92.1%)	56 (93.3%)	61 (95.3%)
Yes	3 (4.7%)	6 (9.7%)	5 (7.9%)	4 (6.7%)	3 (4.7%)
Eczema at 48 months					
No	64 (100.0%)	58 (93.5%)	56 (88.9%)	59 (98.3%)	60 (93.8%)
Yes	0 (0.0%)	4 (6.5%)	7 (11.1%)	1 (1.7%)	4 (6.2%)
Eczema at 60 months					
No	63 (98.4%)	62 (100.0%)	60 (95.2%)	60 (100.0%)	63 (98.4%)
Yes	1 (1.6%)	0 (0.0%)	3 (4.8%)	0 (0.0%)	1 (1.6%)
Eczema at 72 months					
No	64 (100.0%)	61 (98.4%)	63 (100.0%)	59 (98.3%)	64 (100.0%)
Yes	0 (0.0%)	1 (1.6%)	0 (0.0%)	1 (1.7%)	0 (0.0%)
Total eczema					
No	53 (82.8%)	37 (59.7%)	27 (42.9%)	38 (63.3%)	31 (48.4%)
Yes	11 (17.2%)	25 (40.3%)	36 (57.1%)	22 (36.7%)	33 (51.6%)
Urticaria at 12 months					
No	62 (96.9%)	56 (90.3%)	54 (85.7%)	54 (90.0%)	54 (84.4%)
Yes	2 (3.1%)	6 (9.7%)	9 (14.3%)	6 (10.0%)	10 (15.6%)
Urticaria at 24 months					
No	60 (93.8%)	51 (82.3%)	56 (88.9%)	56 (93.3%)	58 (90.6%)
Yes	4 (6.2%)	11 (17.7%)	7 (11.1%)	4 (6.7%)	6 (9.4%)
Urticaria at 36 months					
No	61 (95.3%)	59 (95.2%)	61 (96.8%)	54 (90.0%)	61 (95.3%)
Yes	3 (4.7%)	3 (4.8%)	2 (3.2%)	6 (10.0%)	3 (4.7%)
Urticaria at 48 months					
No	63 (98.4%)	61 (98.4%)	58 (92.1%)	59 (98.3%)	61 (95.3%)
Yes	1 (1.6%)	1 (1.6%)	5 (7.9%)	1 (1.7%)	3 (4.7%)
Urticaria at 60 months					
No	64 (100.0%)	60 (96.8%)	61 (96.8%)	60 (100.0%)	62 (96.9%)
Yes	0 (0.0%)	2 (3.2%)	2 (3.2%)	0 (0.0%)	2 (3.1%)
Urticaria at 72 months					
No	64 (100.0%)	62 (100.0%)	63 (100.0%)	59 (98.3%)	64 (100.0%)
Yes	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (1.7%)	0 (0.0%)
Total urticaria					
No	54 (84.4%)	39 (62.9%)	38 (60.3%)	42 (70.0%)	40 (62.5%)
Yes	10 (15.6%)	23 (37.1%)	25 (39.7%)	18 (30.0%)	24 (37.5%)
Asthma at 12 months					
No	64 (100.0%)	62 (100.0%)	62 (98.4%)	54 (90.0%)	64 (100.0%)
Yes	0 (0.0%)	0 (0.0%)	1 (1.6%)	6 (10.0%)	0 (0.0%)
Asthma at 24 months					
No	63 (98.4%)	55 (88.7%)	57 (90.5%)	56 (93.3%)	59 (92.2%)
Yes	1 (1.6%)	7 (11.3%)	6 (9.5%)	4 (6.7%)	5 (7.8%)
Asthma at 36 months					
No	56 (87.5%)	52 (83.9%)	51 (81.0%)	51 (85.0%)	51 (79.7%)
Yes	8 (12.5%)	10 (16.1%)	12 (19.0%)	9 (15.0%)	13 (20.3%)

Asthma at 48 months					
No	64 (100.0%)	62 (100.0%)	62 (98.4%)	57 (95.0%)	63 (98.4%)
Yes	0 (0.0%)	0 (0.0%)	1 (1.6%)	3 (5.0%)	1 (1.6%)
Asthma at 60 months					
No	63 (98.4%)	57 (91.9%)	57 (90.5%)	57 (95.0%)	57 (89.1%)
Yes	1 (1.6%)	5 (8.1%)	6 (9.5%)	3 (5.0%)	7 (10.9%)
Asthma at 72 months					
No	63 (98.4%)	56 (90.3%)	60 (95.2%)	59 (98.3%)	61 (95.3%)
Yes	1 (1.6%)	6 (9.7%)	3 (4.8%)	1 (1.7%)	3 (4.7%)
Total asthma					
No	53 (82.8%)	34 (54.8%)	34 (54.0%)	34 (56.7%)	35 (54.7%)
Yes	11 (17.2%)	28 (45.2%)	29 (46.0%)	26 (43.3%)	29 (45.3%)
Oculorhinitis at 12 months					
No	64 (100.0%)	56 (90.3%)	57 (90.5%)	54 (90.0%)	54 (84.4%)
Yes	0 (0.0%)	6 (9.7%)	6 (9.5%)	6 (10.0%)	10 (15.6%)
Oculorhinitis at 24 months					
No	60 (93.8%)	54 (87.1%)	57 (90.5%)	55 (91.7%)	55 (85.9%)
Yes	4 (6.2%)	8 (12.9%)	6 (9.5%)	5 (8.3%)	9 (14.1%)
Oculorhinitis at 36 months					
No	60 (93.8%)	53 (85.5%)	52 (82.5%)	49 (81.7%)	61 (95.3%)
Yes	4 (6.2%)	9 (14.5%)	11 (17.5%)	11 (18.3%)	3 (4.7%)
Oculorhinitis at 48 months					
No	63 (98.4%)	57 (91.9%)	60 (95.2%)	55 (91.7%)	58 (90.6%)
Yes	1 (1.6%)	5 (8.1%)	3 (4.8%)	5 (8.3%)	6 (9.4%)
Oculorhinitis at 60 months					
No	61 (95.3%)	61 (98.4%)	58 (92.1%)	57 (95.0%)	61 (95.3%)
Yes	3 (4.7%)	1 (1.6%)	5 (7.9%)	3 (5.0%)	3 (4.7%)
Oculorhinitis at 72 months					
No	62 (96.9%)	62 (100.0%)	63 (100.0%)	59 (98.3%)	62 (96.9%)
Yes	2 (3.1%)	0 (0.0%)	0 (0.0%)	1 (1.7%)	2 (3.1%)
Total oculorhinitis					
No	50 (78.1%)	33 (53.2%)	32 (50.8%)	29 (48.3%)	31 (48.4%)
Yes	14 (21.9%)	29 (46.8%)	31 (49.2%)	31 (51.7%)	33 (51.6%)

Table 4. Time-specific and cumulative incidence of immune tolerance acquisition at 12, 24, 36, 48, 60 and 72 months.

	EHCF+LGG	RHF	SF	EHWF	AAF
N	64 (20.4%)	62 (19.8%)	63 (20.1%)	60 (19.2%)	64 (20.4%)
Tolerance at 12 months					
No	38 (59.4%)	56 (90.3%)	60 (95.2%)	50 (83.3%)	64 (100.0%)
Yes	26 (40.6%)	6 (9.7%)	3 (4.8%)	10 (16.7%)	0 (0.0%)
Tolerance at 24 months					
No	50 (78.1%)	56 (90.3%)	57 (90.5%)	56 (93.3%)	62 (96.9%)
Yes	14 (21.9%)	6 (9.7%)	6 (9.5%)	4 (6.7%)	2 (3.1%)
Tolerance at 36 months					
No	54 (84.4%)	55 (88.7%)	53 (84.1%)	56 (93.3%)	60 (93.8%)
Yes	10 (15.6%)	7 (11.3%)	10 (15.9%)	4 (6.7%)	4 (6.2%)
Tolerance at 48 months					
No	60 (93.8%)	51 (82.3%)	58 (92.1%)	44 (73.3%)	59 (92.2%)
Yes	4 (6.2%)	11 (17.7%)	5 (7.9%)	16 (26.7%)	5 (7.8%)
Tolerance at 60 months					
No	60 (93.8%)	48 (77.4%)	51 (81.0%)	53 (88.3%)	51 (79.7%)
Yes	4 (6.2%)	14 (22.6%)	12 (19.0%)	7 (11.7%)	13 (20.3%)
Total at 72 months					
No	62 (96.9%)	56 (90.3%)	58 (92.1%)	56 (93.3%)	52 (81.2%)
Yes	2 (3.1%)	6 (9.7%)	5 (7.9%)	4 (6.7%)	12 (18.8%)
Total tolerance					
No	4 (6.2%)	12 (19.4%)	22 (34.9%)	15 (25.0%)	28 (43.8%)
Yes	60 (93.8%)	50 (80.6%)	41 (65.1%)	45 (75.0%)	36 (56.2%)

Values are numbers and proportions.