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Title page

The Impact of Formula Choice for the Management of Pediatric Cow's Milk Allergy on the Occurrence of other Allergic Manifestations: The Atopic March Cohort Study

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Data statement

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

Keywords: atopic march, food allergy, gut microbiota, probiotics.

Abbreviations:

CMA: Cow's milk allergy
EHCF: Extensively hydrolyzed casein formula
EHWF: Extensively hydrolyzed whey formula
LGG: *Lactobacillus rhamnosus* GG
DBPCFC: Double-blind placebo-controlled food challenge
SPT: Skin prick test
CRF: Case report form
IQR: Interquartile range
BRM: Binomial regression model
RR: Risk ratio

Objectives. To compare the impact of different formulas on the occurrence of other atopic manifestations and the time of immune tolerance acquisition.

Study design. In a 36-month prospective cohort study the occurrence of other AMs (eczema, urticaria, asthma, and rhinoconjunctivitis) and the time of immune tolerance acquisition were comparatively evaluated in immunoglobulin E-mediated CMA children treated with: extensively hydrolyzed casein formula containing the probiotic *L. rhamnosus* GG(EHCF+LGG), rice hydrolyzed formula(RHF), soy formula(SF), extensively hydrolyzed whey formula(EHWF), or amino-acid based formula(AAF).

Results. 365 subjects were enrolled into the study, 73 per formula cohort. The incidence of AMs was: 0.22 (Bonferroni corrected 95% confidence interval(CI) 0.09 to 0.34) in the EHCF+LGG cohort; 0.52 (0.37 to 0.67) in the RHF cohort; 0.58 (0.43 to 0.72) in the SF cohort; 0.51 (0.36 to 0.66) in the EHWF cohort; and 0.77 (0.64 to 0.89) in the AAF cohort. The incidence of AMs in the RHF, SF, EHWF and AAF cohorts *vs.* the EHCF+LGG cohort was always higher than the pre-specified absolute difference of 0.25 at an alpha-level of 0.0125, with corresponding risk ratios of 2.37 (1.46 to 3.86, p <0.001) for RHF *vs.* EHCF+LGG; 2.62 (1.63 to 4.22, p<0.001) for SF *vs.* EHCF+LGG; 2.31(1.42 to 3.77, P < .001) for EHWF *vs.* EHCF+LGG; and 3.50(2.23 to 5.49, p<0.001) for AAF *vs.* EHCF+LGG. The 36-month immune tolerance acquisition rate was higher in the EHCF+LGG cohort.

Conclusion. The use of EHCF+LGG for CMA treatment is associated with lower incidence of AMs and higher rate of immune tolerance acquisition.

Cow's milk allergy (CMA) is the most widespread food allergy (FA) among young children, with a 2.0-7.5% global prevalence, which accounts for approximately one-fifth of childhood food allergies (1-7). During the previous two decades there has been an alteration in the natural history of CMA with a rise in prevalence, severity of clinical manifestations, higher risk of persistence into later ages with significant direct costs for the healthcare system and even larger costs for the families (4,8-10).

In addition, data suggest that early life CMA could be the first stage of the "allergic march", leading to the occurrence of other atopic manifestations (AMs) especially asthma, atopic eczema, urticaria and rhinoconjunctivitis later in life (11-13).

The current standard of care for CMA is strict dietary avoidance of cow's milk proteins, with use of substitute formulas in non-breastfed subjects (14-16). The formulas considered effective in the dietary management of CMA include extensively hydrolyzed whey formula (EHWF), extensively hydrolyzed casein formula (EHCF), rice hydrolyzed formula (RHF), soy formula (SF), or amino-acid based formula (AAF) (16,17).

Data suggest that in children with CMA, dietary intervention with EHCF supplemented with the probiotic *Lactobacillus rhamnosus* GG (LGG) has benefits in decreasing inflammation and gastrointestinal symptoms (18), in reducing disease duration (19-23), the occurrence of functional gastrointestinal disorders (24), and other AMs later in the life compared with standard EHCF without LGG (21). These findings are consistent with those of recent studies revealing that first-line management of newly diagnosed CMA infants treated with EHCF+LGG may slow down the allergic march compared with infants treated with other formulas (25,26).

Multiple mechanisms might be responsible for such effects, including a positive modulation of gut microbiome metagenomic and metabolomic features, and epigenetic regulation of genes involved in immune tolerance (27-29). Such mechanisms suggest a possible long-term effect on the immune system of CMA children treated with EHCF+LGG.

, The present study was designed to assess the incidence of AMs later in life in children with CMA treated with different substitute formulas.

METHODS

A prospective cohort study was conducted from December 2014 to June 2019 on non-breastfed infants (aged 1-12 months) with suspected immunoglobulin E (IgE)-mediated CMA. The infants had previously been placed on a hypoallergenic formula by their family pediatrician or physician and were referred to a tertiary center for pediatric allergy to undergo an oral food challenge to confirm the diagnosis of CMA. At enrollment all subjects were in stable clinical condition without CMA-related symptoms, following a strict cow milk protein elimination diet and on a substituted formula (EHCF+LGG, RHF, SF, EHWF, or AAF) for a period of 15-30 days prior to recruitment. The exclusion criteria were: treatment with pre- or probiotics in the previous 3 months; treatment with antibiotics in the previous 3 months; cow's milk protein-induced anaphylaxis; food protein induced enterocolitis syndrome; food allergies other than CMA; atopic eczema not related to CMA; eosinophilic disorders of the gastrointestinal tract; chronic systemic disease; genetic diseases; congenital cardiac defects; active tuberculosis; autoimmune diseases; primary or secondary immunodeficiencies; chronic intestinal bowel disease; celiac disease; inflammatory bowel disease; evidence of *Helicobacter pylori* infection; cystic fibrosis; lactose intolerance; obesity; autism or neuropsychiatric disorders; metabolic diseases; malignancy; chronic pulmonary disease; malformations of the gastrointestinal and/or respiratory tract; history of gastrointestinal tract surgery; participation in other studies; conditions that made compliance with the protocol unlikely.

Ethical approval

The study protocol, the patient information sheet, the informed consent form, and the clinical chart were reviewed and approved by the Ethical Committee of the University of Naples Federico II. The study was conducted in accordance with the Helsinki Declaration (Fortaleza revision, 2013), the Good Clinical Practice Standards (CPMP/ICH/135/95), the Italian Decree-Law 196/2003 regarding personal data, and the European regulations on this subject. The study is a part of a project and it was registered in the Clinical Trials Protocol Registration System with the ID number NCT03861910.

Data collection

At baseline, after the first evaluation by the Research Team a Multidisciplinary Pediatric Allergy Team formed by pediatric allergists, dietitians and nurses unaware of study aims performed a full anamnestic and clinical evaluation with the collection of all demographic, anthropometric, and clinical data (including those related to CMA), skin prick test (SPT) to cow's milk proteins and fresh cow's milk and the oral food challenge (OFC) to confirm the diagnosis of IgE-mediated CMA, as previously described (21, 30). At the baseline, informed consent from the

parents/caregivers of each child was collected by the RT comprised of pediatric allergists and pediatric research nurses. Detailed information was collected on anamnestic and clinical features including sociodemographic factors, family and living conditions, parental history of allergic diseases, maternal smoking during pregnancy, environmental tobacco smoke exposure, number of siblings, pet ownership, and the use of formula.

Patients with a confirmed diagnosis of IgE-mediated CMA based upon the result of OFC were enrolled in the study and continued the exclusion diet using the same formula previously prescribed by the referring family pediatrician or physician when CMA was suspected. In addition, to check the compliance to the study formula, parents or caregivers were asked to keep a daily record of formula use. Then, according to the standard care procedures for patients with IgE-mediated CMA, the RT planned 3 visits every 12 months during a 3-year follow-up. During these visits, the MPAT assessed: clinical status, body growth, occurrence of allergic symptoms, the compliance to the cow milk proteins free diet, compliance to the formula previously prescribed (operationally defined as the consumption of at least 80% of the formula used), the SPT to cow's milk proteins and fresh milk. The MPAT also performed an OFC to evaluate the possible acquisition of immune tolerance to cow's milk proteins. In subjects with demonstration of immune tolerance acquisition by the results of OFC a cow milk protein containing diet was allowed for the remainder of the study period. Unscheduled visits were made if necessary because of allergic symptoms or other morbidities. Whenever allergic symptoms or other morbidities occurred, parents were instructed to contact the RT to have a medical examination of their child. At each visit, the MPAT performed a full physical examination, and then, using standardized criteria, decided on the AM diagnosis.

The occurrence of AM was investigated evaluating potential condition in differential diagnosis, the possible influence of non-strict cow milk protein exclusion diet and the results of allergy screening tests. In case of discordance about an AM diagnosis, further evaluation by another pediatric allergist, unaware of the study aims, was performed.

. 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 reported elsewhere (31). 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 (32). Allergic urticaria was diagnosed if at least 2 episodes of itching eruptions or swelling with typical appearance were observed by the parents or a physician and were caused by the same allergen. In the case of a single episode, immunologic evidence (SPT with the suspected undiluted native allergen causing a wheal reaction $\geq$ 3 mm or an allergen-specific IgE level $\geq$ 0.35 KU/L) or a

positive provocation response with the suspected allergen was performed for definitive diagnosis (33). 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 (34). 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 the 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, diarrhea, bloody stools, abdominal pain, rhinitis, nasal congestion, wheeze, cough, stridor, difficulty breathing during the challenge; and 4) results of SPT (wheal size > 3 mm) and/or serum IgE (> 0.1kU/L) (19, 35, 36).
All study teams, procedures and assessments were performed as shown in **Figure 1** (available at www.jpeds.com).

Data entry

All data were recorded anonymously. RT entered all collected data in the case report form (CRF). Two researchers performed separate checks of data completeness, clarity, consistency, and accuracy, and instructed the personnel to make any required corrections or additions. Using a single data-entry method, all data recorded in the CRF were entered in the study database by the same researcher. Then, Statistical Team unaware of study cohorts reviewed the study dataset and underwent data cleaning and verification according to standard procedures. Finally, ST locked the database once it was declared complete and accurate, and the statistical analysis was performed.

Study outcomes

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

Sample size

Under the assumption of an incidence rate of the main outcome equal to 0.20 in the EHCF+LGG cohort, a sample size of 73 subjects per cohort was needed to declare as statistically significant at a

Bonferroni-adjusted alpha-level of 0.0125 and with a power of 0.80 an absolute difference of 0.25 in any of the four prespecified comparisons RHF *vs* EHCF+LGG, SF *vs.* EHCF+LGG, EHWF *vs.* EHCF+LGG, and AAF *vs.* EHCF+LGG (37). Infants were allocated to cohorts based on the substituted formula they were receiving. Recruitment continued until there were 73 infants in each of the respective cohorts as per the sample size calculation.

Statistical Analyses

Descriptive statistics

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

Main outcome

We used a binomial regression model (BRM) to estimate the incidence of the main outcome, *i.e.* at least one AM at 36 months, in the RHF *vs.* EHCF+LGG, SF *vs.* EHCF+LGG, EHWF *vs.* EHCF+LGG and AAF *vs.* EHCF+LGG cohorts (38). The response variable of the BRM was the presence of at least one AM at 36 months (0 = no; 1 = yes), and the predictor was the treatment cohort (0 = EHCF+LGG; 1 = RHF; 2 = SF; 3 = EHWF; 4 = AAF). Because of the above pre-specified four comparisons, a p-value < 0.0125 was considered statistically significant (see *sample size*). To evaluate the effect of potential confounders on the main outcome, we added each of them separately to the above BRM and evaluated the changes in the estimated risk ratios (RR) (39). The evaluated potential confounders were sex (0 = female; 1 = male), age (months), cesarean delivery (0 = no; 1 = yes), born at term (0 = no; 1 = yes), breastfed for at least 2 months (0 = no; 1 = yes), weaning (months), siblings (number), familial risk of allergy (0 = no; 1 = yes), exposed to passive smoking (0 = no; 1 = yes), mother smoked during pregnancy (0 = no; 1 = yes), and exposed to pets (0 = no; 1 = yes).

Secondary outcome

We used a BRM with cluster confidence intervals to estimate the incidence of the acquisition of tolerance in the five cohorts at 36 months (40). The response variable of the BRM was the acquisition of tolerance at 36 months (0 = no; 1= yes) and the predictor was the treatment cohort (discrete: 0 =EHCF+LGG; 1 = RHF; 2 = SF; 3 = EHWF; 4 = AAF). For exploratory purposes only, we also calculated a BRM in which the response variable was the acquisition of tolerance (0 = no; 1= yes), and the predictors were the treatment cohort (discrete: 0 = EHCF+LGG; 1 = RHF; 2 = SF;

3 = EHWF; 4 = AAF), time (discrete: 0 = 12; 1 =24; 2 = 36 months), and a treatmentXtime (discreteXdiscrete) interaction.

Statistical analysis was performed using Stata 16.1 (Stata Corporation, College Station, TX, USA).

RESULTS

The flow of the subjects throughout the study is reported in **Figure 2** (available at www.jpeds.com). Of 390 consecutive potentially eligible children, 7 refused to participate and 3 presented exclusion criteria (treatment with probiotics in the previous 3 months; genetic disease; or metabolic disease). Of the remaining 387 children, 15 were excluded because of negative OFC leaving a total of 365 children, 73 per formula cohort. All the children were from families of middle socioeconomic status and lived in urban areas. The cohorts had similar demographic and anamnestic features at the enrollment (**Table I**). Age of subjects at last follow-up visit (months, median, IQR) was similar among cohorts (EHCF+LGG: 41, 39-43; RHF: 41, 40-44; SF: 41, 39-43; EHWF: 41, 39-44.5; AAF: 41, 41-44).

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 dieticians experienced in pediatric FA. No case of misunderstanding of formula use was reported.

Main outcome

Figure 3 plots the incidence of the main outcome in the five cohorts. The incidence was as follows: 0.22 (Bonferroni corrected 95%CI 0.09 to 0.34) for the EHCF+LGG cohort; 0.52 (0.37 to 0.67) for the RHF cohort; 0.58 (0.43 to 0.72) for the SF cohort; 0.51 (0.36 to 0.66) for the EHWF cohort; 0.77 (0.64 to 0.89) for the AAF cohort.

The incidence of the main outcome in the RHF, SF, EHWF, and AAF cohorts *vs.* the EHCF+LGG cohort was always higher than the pre-specified absolute difference of 0.25 at the pre-specified alpha level of 0.0125 with corresponding RRs of 2.37 (1.46 to 3.86, $p < 0.001$) for RHF *vs.* EHCF+LGG; 2.62 (1.63 to 4.22, $p < 0.001$) for SF *vs.* EHCF+LGG; 2.31 (1.42 to 3.77, $p < 0.001$) for EHWF *vs.* EHCF+LGG; 3.50 (2.23 to 5.49, $p < 0.001$) for AAF *vs.* EHCF+LGG.

Figure 4 plots the time-related incidence of the components of the main outcome (eczema, urticaria, asthma, and rhinoconjunctivitis) during the study. This is an exploratory analysis, performed because the main outcome is a composite outcome, and as such it can be used only for hypothesis-generating purposes.

Table 2 (available at www.jpeds.com) reports the frequency of the main outcome (any AM during

36 months), its components (eczema, urticaria, asthma, and rhinoconjunctivitis), and other FAs alone and in combination with AMs.

Secondary outcome

Figure 5, A plots the incidence of immune tolerance acquisition to cow's milk proteins in the five cohorts at 36 months, which is the following: 0.81 (Bonferroni corrected 95%CI: 0.69 to 0.93) for the EHCF+LGG cohort; 0.41 (0.26 to 0.56) for the RHF cohort; 0.40 (0.25 to 0.54) for the SF cohort; 0.42 (0.28 to 0.57) for the EHWF cohort; 0.19 (0.07 to 0.31) for the AAF cohort, with corresponding RRs of: 0.51 (0.38 to 0.68, $p< 0.001$) for RHF *vs.* EHCF+LGG; 0.49 (0.36 to 0.67, $p < 0.001$) for SF *vs.* EHCF+LGG; 0.53 (0.39 to 0.70, $p < 0.001$) for EHWF *vs.* EHCF+LGG; 0.24 (0.15 to 0.39, $p < 0.001$) for AAF *vs.* EHCF+LGG.

Figure 5, B plots the time-specific acquisition rate of immune tolerance to cow's milk proteins. This is an exploratory analysis, because the pre-specified analysis of the secondary outcome was planned to be done at 36 months only (Figure 5, A). The figure shows a faster and higher increase in immune tolerance to cow's milk proteins in the EHCF+LGG cohort. Without any correction for multiple comparisons, the values for the EHCF+LGG cohort are: 0.41 (0.30 to 0.52) at 12 months; 0.64 (0.53 to 0.75) at 24 months; 0.81 (0.72 to 0.90) at 36 months. Note that the point-estimate of the incidence of the immune tolerance acquisition at 36 months is the same given in Figure 5, A, but the 95%confidence intervals (CIs) are narrower because multiple comparisons were not taken into account.

Table 3 (available at www.jpeds.com) shows that the effect of selected confounders on the incidence of the main outcome was virtually nil in every cohort.

Safety

No child was intolerant to the study formulas. No adverse event was attributed to the consumption of the formulas, and no difference was detected in their daily intake (data not shown). Moreover, the time-related changes in weight, length, and height were comparable among the cohorts (data not shown).

DISCUSSION

Regarding the primary outcome, the incidence of other AMs in the EHCF+LGG cohort was significantly lower as compared with the other cohorts, with corresponding RRs ranging from 2.31 to 3.50.

Although EHCF+LGG affected all the components of the main study outcome, these findings can be taken only as exploratory, and further studies are necessary to investigate the potential of this strategy against any single allergic disease.

The ability of EHCF to prevent allergy is supported by the results of the GINI study in which infants at high-risk of allergic diseases were protected from AMs when they received EHCF (40-45). Moreover, a significant reduction of asthma incidence was also observed in children treated with EHCF at 15 years of age (44). These data are well in keeping with those of a retrospective study revealing that the first-line management of newly diagnosed CMA infants treated with EHCF+LGG may slow down the allergic march if compared with infants treated with EHWF (25).

Some relevant insights were derived from our secondary outcomes. The results of this cohort study indicate that EHCF+LGG also has a greater potential in reducing disease duration. We provide additional evidence on the positive effect elicited by EHCF+LGG on immune tolerance acquisition in children with IgE-mediated CMA (18-23). In the present study, we confirmed that the effect of EHCF+LGG is sustained until 36 months of intervention also in comparison with other formulas. These data are relevant considering the most recent evidence suggesting that the natural history of CMA has changed over time, with slower rates of resolution and a higher proportion of children with disease persisting into school age and older (4,46,47).

The supportive evidence of the potential beneficial role of EHCF+LGG may be due to multiple mechanisms including a positive epigenetic regulation of forkhead box P3, Th1/Th2 cytokine genes and microRNAs expression. In addition, it has been demonstrated that EHCF+LGG exerts a positive modulation of gut microbiota structure and function increasing the number of bacteria strains involved in immune tolerance induction in CMA children. These effects paralleled with an increased production of the short chain fatty acid (SCFA) butyrate that is considered one of the most active gut microbiota-derived metabolites able to drive immune tolerance (27-29, 48-53).

This study has several strengths. First, it was performed on a large number of children with a challenge-proven CMA followed at a tertiary pediatric allergy center with a high follow-up rate. Second, the AM diagnosis and the immune tolerance acquisition evaluation was performed by a multidisciplinary pediatric allergy team unaware of study aims. Third, the effect sizes associated with both the primary and secondary outcomes were clinically relevant.

Nonetheless, the main limitation is that this was a cohort study and not a randomized controlled trial. Another limitation is that our data cannot be generalized to children with conditions that were reasons for exclusion from the study or children with non-IgE-mediated CMA. In addition, we compared only the impact of most commonly used products for the treatment of pediatric CMA. The effect other commercially available formulas should be explored in future studies. Fourth,

although our results showed that EHCF+LGG reduces the incidence of other AMs and favors the development of immune tolerance in children with IgE-mediated CMA at 12, 24, and 36 months, longer follow-up is required to test whether these effects could persist for a longer period of time. Lastly, our results are limited by the lack of data on gut microbiota and Th1/Th2 cytokines, which would be useful to further investigate the mechanisms by which the EHCF+LGG produces its effect, and future studies are advocated to elucidate the mechanisms of this beneficial effect.
In summary, this cohort study performed in a well-characterized population of children with CMA shows that EHCF+LGG could be effective in preventing the allergic march and in accelerating the time of immune tolerance acquisition.

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Figure legends

Figure 1 online only.

The design of the study.

Figure 2 online only.

The flow of the children through the study.

Figure 3.

Incidence of the main study outcome in the five study cohorts.

The incidence of subjects with ≥1 atopic manifestation at 36 months was: 0.22 (Bonferroni corrected 95%CI 0.09 to 0.34) for the EHCF+LGG cohort; 0.52 (0.37 to 0.67) for the RHF cohort; 0.58 (0.43 to 0.72) for the SF cohort; 0.51 (0.36 to 0.66) for the EHWF cohort; 0.77 (0.64 to 0.89) for the AAF cohort.

Figure 4.

Exploratory analysis of the incidence of the components of the main outcome (panel a, asthma; panel b, eczema; panel c, rhinoconjunctivitis; panel d, urticaria) during the study period.

Figure 5. Results of the secondary study outcome: incidence of immune tolerance to cow milk protein in the study cohorts.

Panel A.: Point-estimate of the incidence of the immune tolerance acquisition at 36 months.

Panel B. Time-specific of rate of immune tolerance acquisition to cow milk protein.

Table 2 online only. Frequency of the main study outcome, its components and other food allergies at 36 months.

	EHCF+LGG	RHF	SF	EHWF	AAF
	N=73	N=73	N=73	N=73	N=73
At least one atopic manifestation	16 (22%)	38 (52%)	42 (58%)	37 (51%)	56 (77%)
Occurrence of eczema	10 (14%)	23 (32%)	27 (37%)	21 (29%)	30 (41%)
Occurrence of urticaria	9 (12%)	20 (27%)	18 (25%)	16 (22%)	21 (29%)
Occurrence of asthma	9 (12%)	19 (26%)	21 (29%)	20 (27%)	19 (26%)
Occurrence of rhinoconjunctivitis	8 (11%)	25 (34%)	24 (33%)	22 (30%)	26 (36%)
Other food allergies + atopic manifestation	24 (33%)	32 (44%)	36 (49%)	30 (41%)	34 (47%)
Other food allergies	14 (19%)	26 (36%)	26 (36%)	26 (36%)	34 (47%)

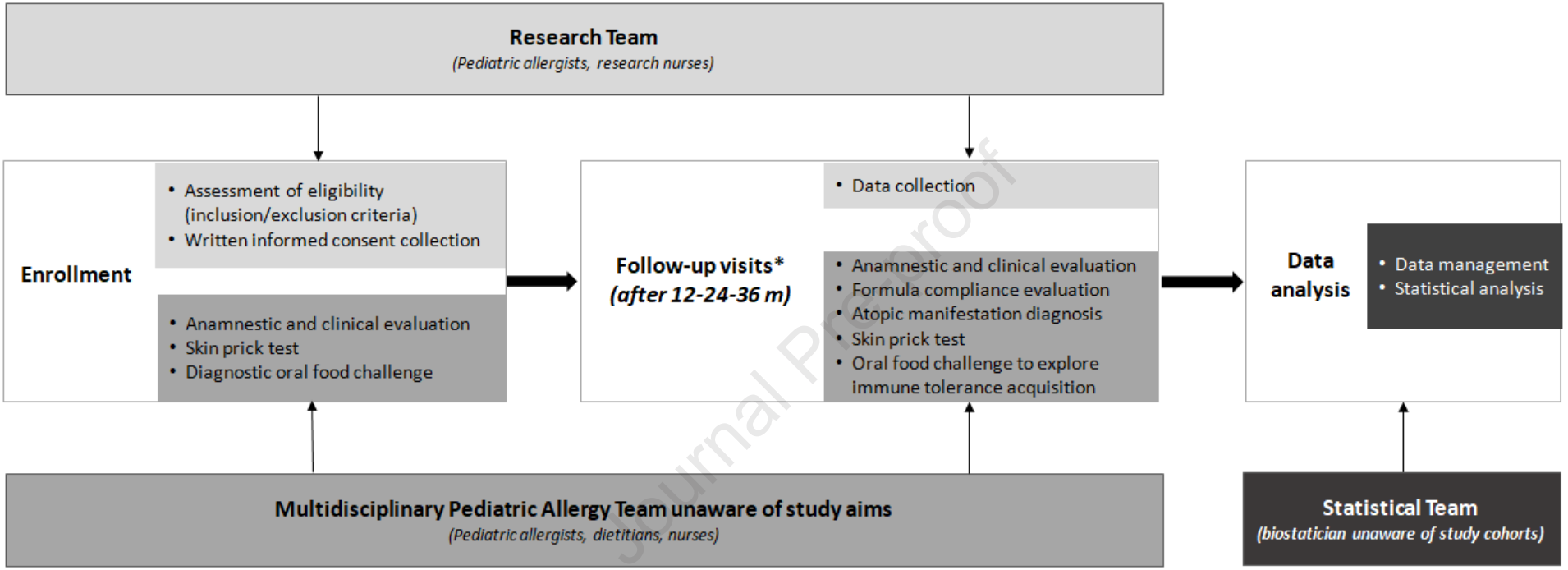
Discrete variables are reported as the number and proportion of subjects with the characteristic of interest.

Table 3 online only. Binomial regression model

At least one allergic manifestation at 36 months												
EHCF+LGG	1.00 [1.00,1.00]	1.00 [1.00,1.00]	1.00 [1.00,1.00]	1.00 [1.00,1.00]	1.00 [1.00,1.00]	1.00 [1.00,1.00]	1.00 [1.00,1.00]	1.00 [1.00,1.00]	1.00 [1.00,1.00]	1.00 [1.00,1.00]	1.00 [1.00,1.00]	1.00 [1.00,1.00]
RHF	3.87*** [1.88,7.95]	3.86*** [1.88,7.93]	3.85*** [1.87,7.92]	3.92*** [1.90,8.07]	3.87*** [1.88,7.96]	3.94*** [1.91,8.10]	3.88*** [1.87,8.07]	3.93*** [1.90,8.10]	3.81*** [1.85,7.84]	3.88*** [1.88,7.97]	3.96*** [1.92,8.17]	3.91*** [1.90,8.05]
SF	4.83*** [2.34,9.96]	4.82*** [2.34,9.94]	4.82*** [2.34,9.94]	4.86*** [2.35,10.03]	4.82*** [2.34,9.94]	4.88*** [2.37,10.08]	4.82*** [2.34,9.94]	4.79*** [2.32,9.88]	4.73*** [2.29,9.78]	4.86*** [2.35,10.03]	4.82*** [2.34,9.95]	4.87*** [2.36,10.05]
EHWF	3.66*** [1.78,7.53]	3.66*** [1.78,7.53]	3.65*** [1.78,7.51]	3.65*** [1.78,7.51]	3.67*** [1.78,7.53]	3.73*** [1.81,7.67]	3.66*** [1.78,7.53]	3.67*** [1.78,7.53]	3.60*** [1.75,7.42]	3.75*** [1.82,7.73]	3.75*** [1.82,7.73]	3.67*** [1.79,7.54]
AAF	11.74*** [5.40,25.52]	11.72*** [5.39,25.46]	11.67*** [5.36,25.40]	11.91*** [5.47,25.93]	11.74*** [5.40,25.50]	11.90*** [5.47,25.90]	11.75*** [5.40,25.59]	11.88*** [5.45,25.91]	11.89*** [5.46,25.92]	11.72*** [5.39,25.50]	12.02*** [5.52,26.21]	11.71*** [5.39,25.46]
Male sex		0.91 [0.57,1.44]										
Age (months)			1.01 [0.93,1.09]									
Cesarean delivery				1.33 [0.85,2.08]								
Born at term					1.08 [0.46,2.52]							
Breastfed for at least 2 months						0.78 [0.47,1.29]						
Weaning (month)							1.01 [0.81,1.25]					
Siblings								1.07 [0.76,1.51]				
Familial risk of allergy									1.45 [0.91,2.31]			
Exposed to passive smoking										1.32 [0.84,2.09]		
Mother smoked during pregnancy											1.32 [0.82,2.12]	
Exposed to pets												1.26 [0.69,2.28]
Observations	365	365	365	365	365	365	365	365	365	365	365	365

Exponentiated coefficients; 95% confidence intervals in brackets
** p<0.05, ** p<0.01, *** p<0.001*

Figure 1 online only. The design of the study.



**Unscheduled visits were made if necessary because of allergic symptoms or other morbidities. Whenever allergic symptoms or other morbidities occurred, parents were instructed to contact the Research Team to have a medical examination of their child. At these medical examinations, the Multidisciplinary Pediatric Allergy Team performed a full physical examination, and then, using standardized criteria, decided on the atopic manifestation diagnosis.*

Figure 2 online only. The flow of the children through the study.

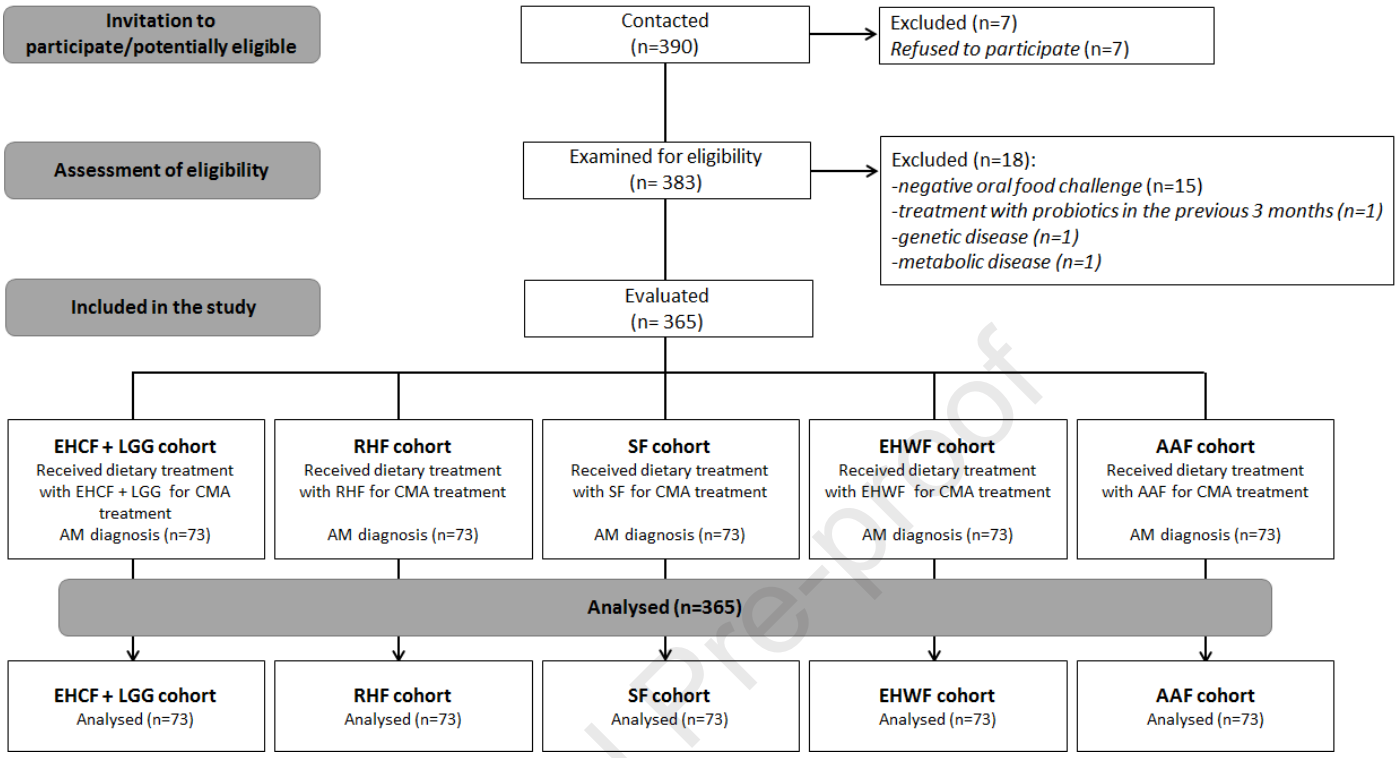


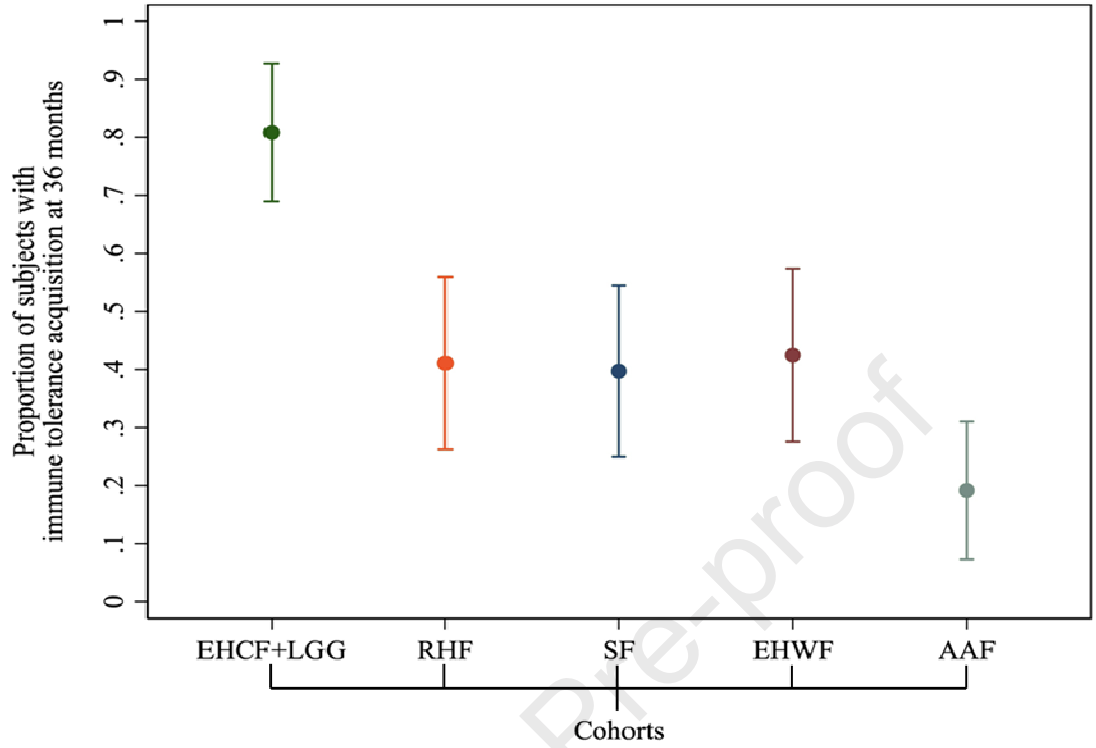
Table 1. Demographic and anamnestic features of the subjects enrolled into the study.

	EHCF+LGG	RHF	SF	EHWF	AAF
	N=73	N=73	N=73	N=73	N=73
Male	49 (67%)	47 (64%)	48 (66%)	49 (67%)	47 (64%)
Cesarean delivery	43 (59%)	41 (56%)	43 (59%)	45 (62%)	42 (58%)
Born at term	68 (93%)	67 (92%)	69 (95%)	67 (92%)	68 (93%)
Weight at birth (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	51 (70%)	55 (75%)	53 (73%)	55 (75%)	53 (73%)
Weaning (months)	5 (5; 6)	5 (4; 5)	5 (5; 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	44 (60%)	49 (67%)	50 (68%)	49 (67%)	45 (62%)
Allergic first-degree relatives	1 (1; 2)	1 (1; 1)	1 (1; 2)	1 (1; 1)	1 (1; 1)
Exposure to passive smoking	28 (38%)	29 (40%)	28 (38%)	23 (32%)	31 (42%)
Mother smoked during pregnancy	26 (36%)	21 (29%)	28 (38%)	21 (29%)	22 (30%)
Exposure to pets	13 (18%)	10 (14%)	11 (15%)	13 (18%)	15 (21%)
Age at CMA diagnosis (months)	5 (3; 7)	5 (4; 8)	5 (3; 7)	5 (3; 8)	5 (5; 8)
Weight at CMA diagnosis (kg)	7.3 (6.1; 8.6)	7.7 (6.1; 9.0)	7.5 (6.1; 8.5)	7.4 (5.8; 8.8)	7.9 (6.7; 9.0)
Length at CMA diagnosis (cm)	66 (61; 69)	65 (60; 69)	65 (61; 70)	65 (60; 70)	66 (64; 70)
Positive prick by prick test for fresh milk	73 (100%)	73 (100%)	73 (100%)	73 (100%)	73 (100%)
Positive skin prick test for α-lactalbumin	58 (79%)	60 (82%)	59 (81%)	61 (84%)	57 (78%)
Positive skin test positive for β-lactoglobulin	48 (66%)	51 (70%)	47 (64%)	48 (66%)	49 (67%)
Positive skin prick test positive for casein	36 (49%)	33 (45%)	31 (42%)	33 (45%)	34 (47%)
Gastrointestinal symptoms at CMA onset	45 (62%)	48 (66%)	43 (59%)	43 (59%)	44 (60%)
Cutaneous symptoms at CMA onset	47 (64%)	50 (68%)	51 (70%)	49 (67%)	49 (67%)
Respiratory symptoms at CMA onset	13 (18%)	9 (12%)	12 (16%)	11 (15%)	13 (18%)

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

Figure 5. Results of the secondary study outcome: incidence of immune tolerance to cow milk protein in the study cohorts.

Panel A. Point-estimate of the incidence of the immune tolerance acquisition at 36 months.



Panel B. Time-specific of rate of immune tolerance acquisition to cow milk protein.

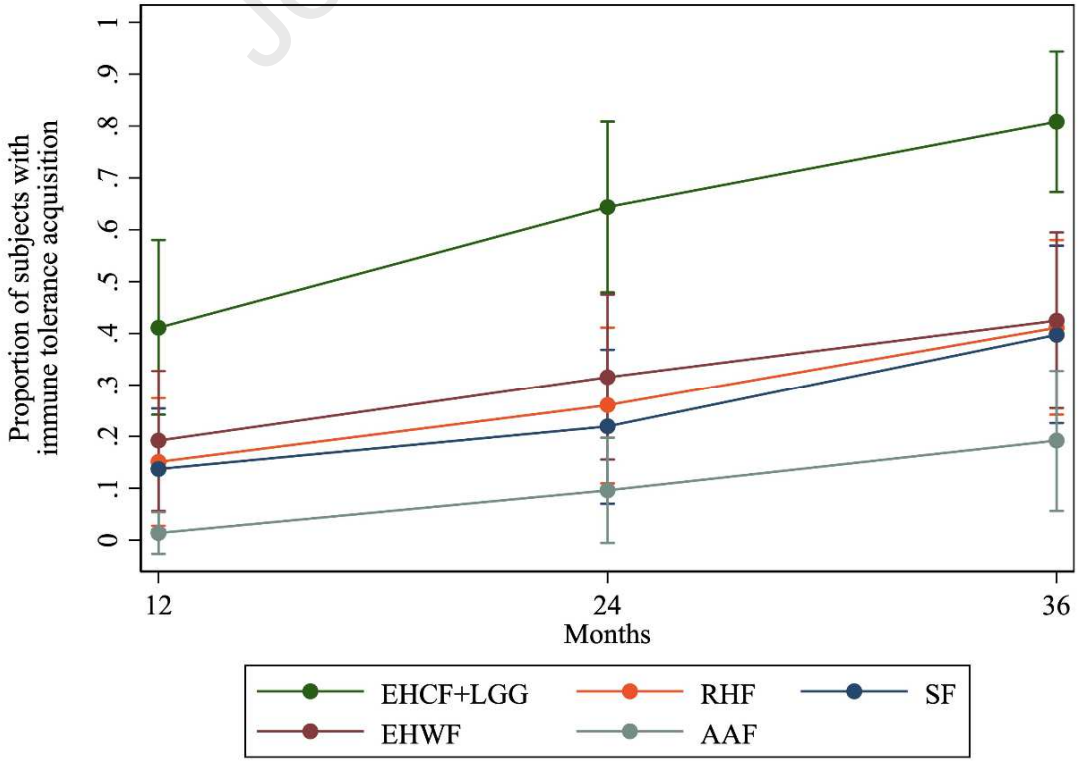


Figure 3. Incidence of the main study outcome.

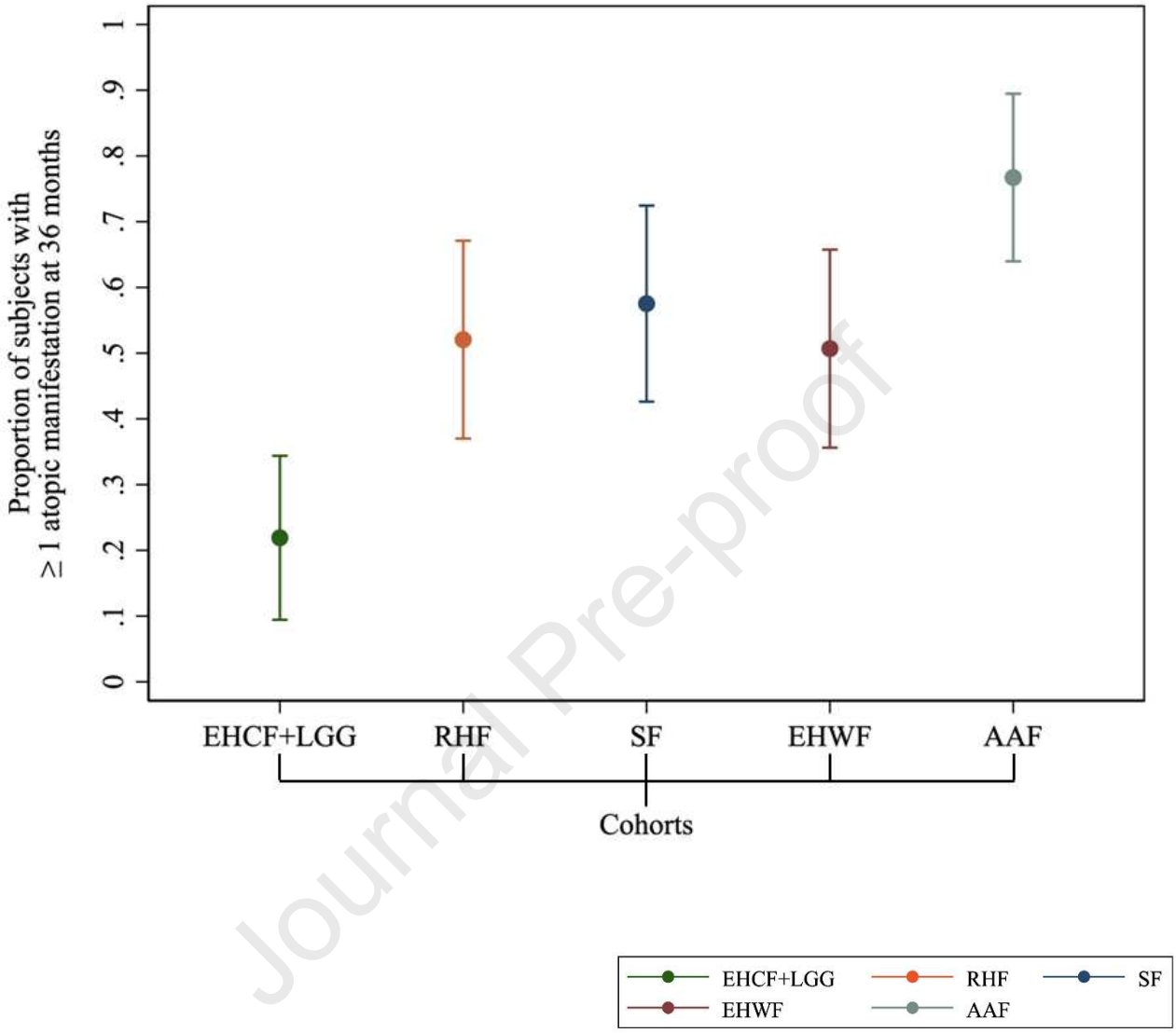
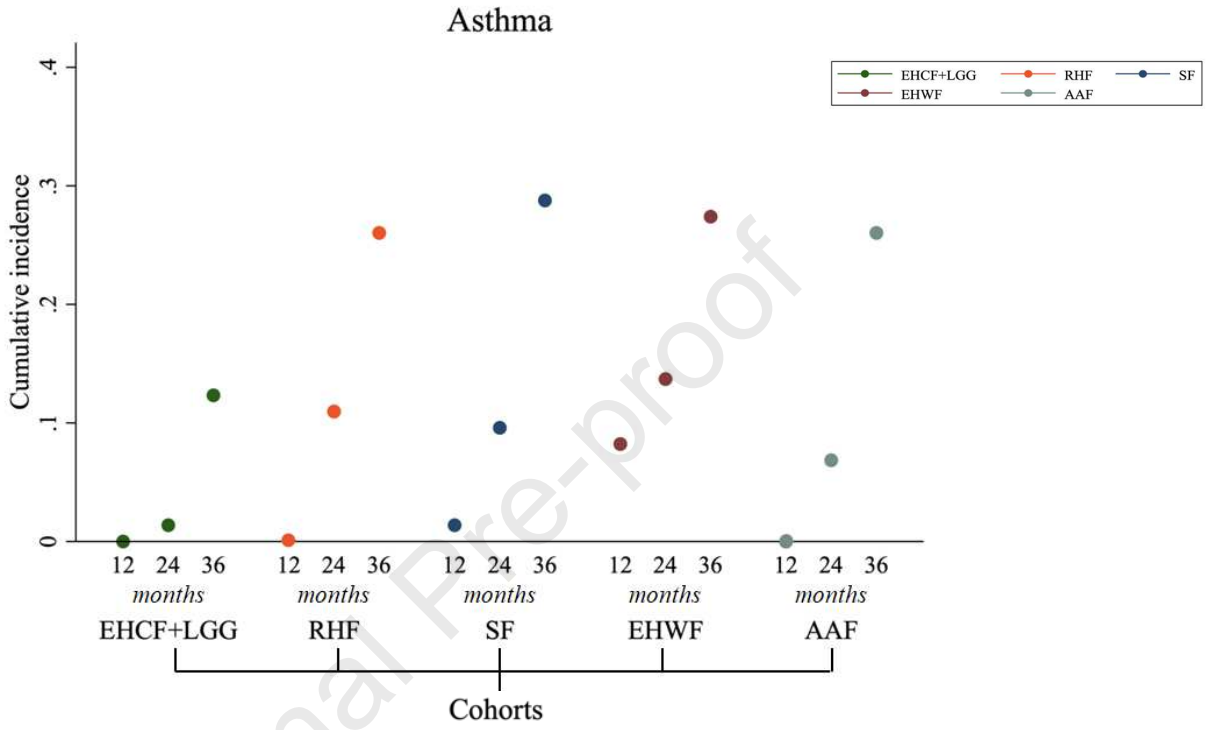
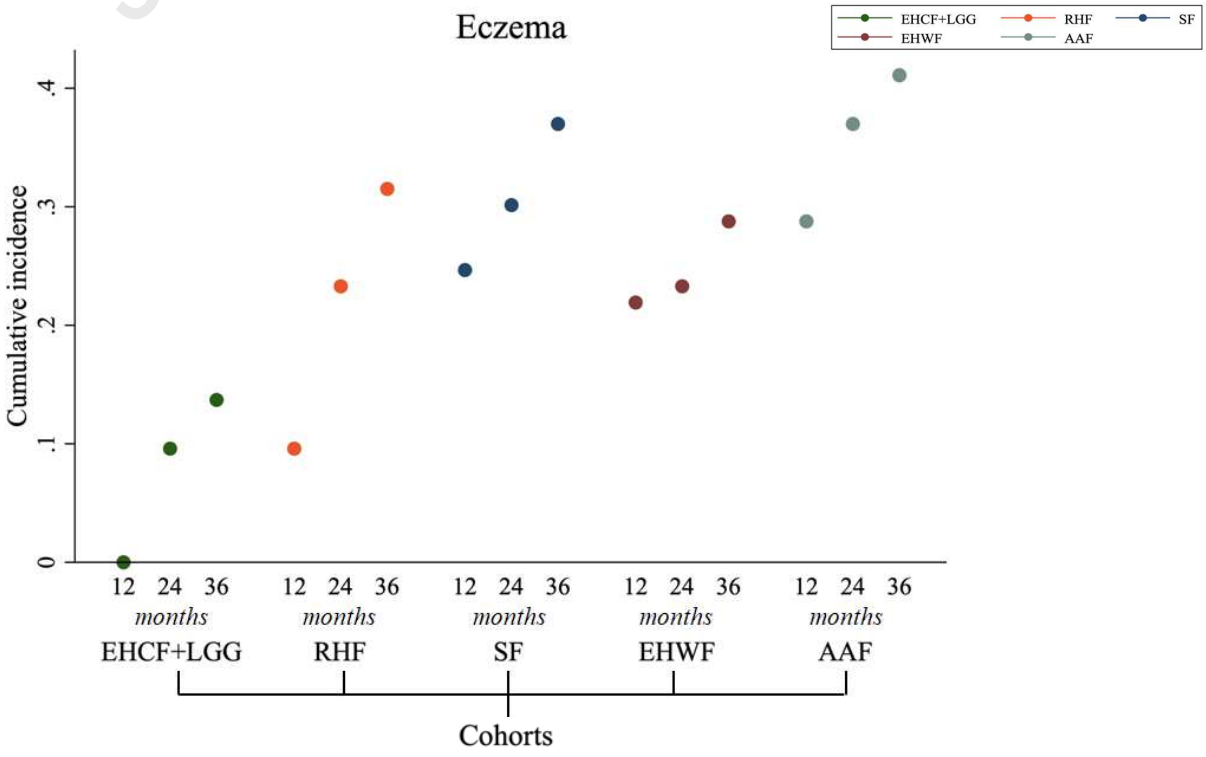


Figure 4. Exploratory analysis of the incidence of the components of the main outcome (panel a, asthma; panel b, eczema; panel c, rhinoconjunctivitis; panel d, urticaria) during the study period.

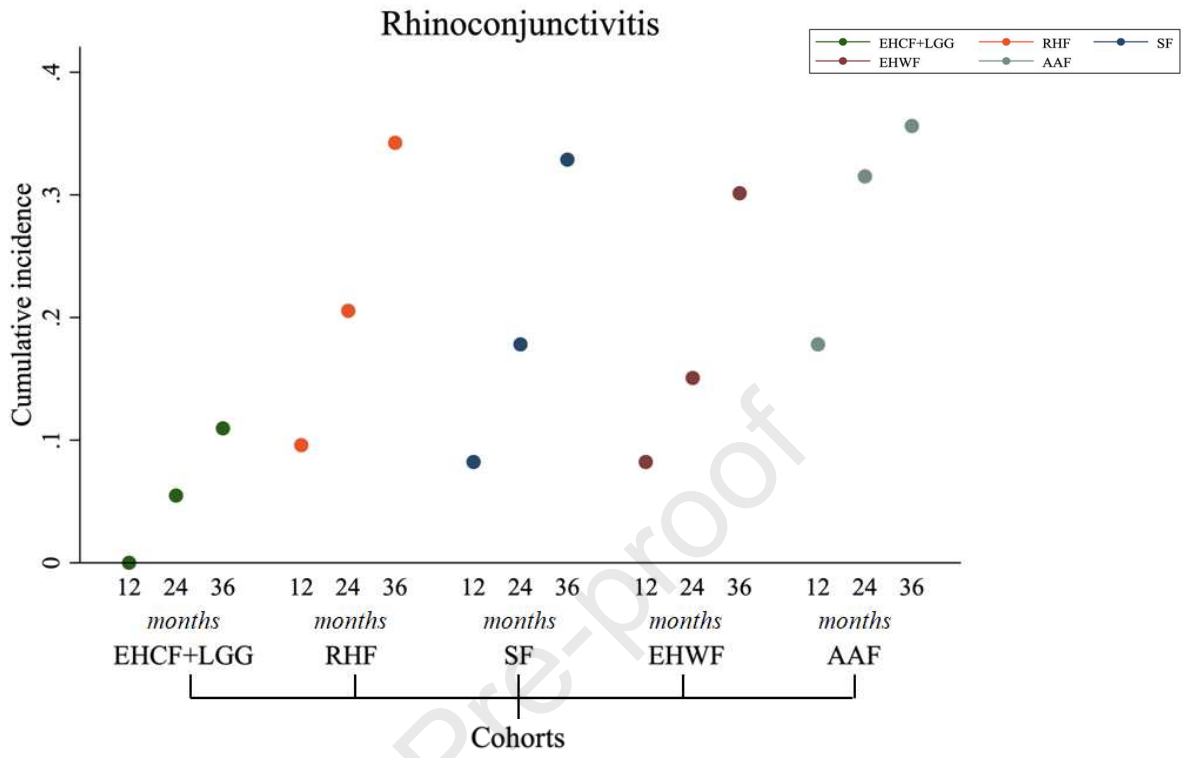
Panel A



Panel B



Panel C



Panel D