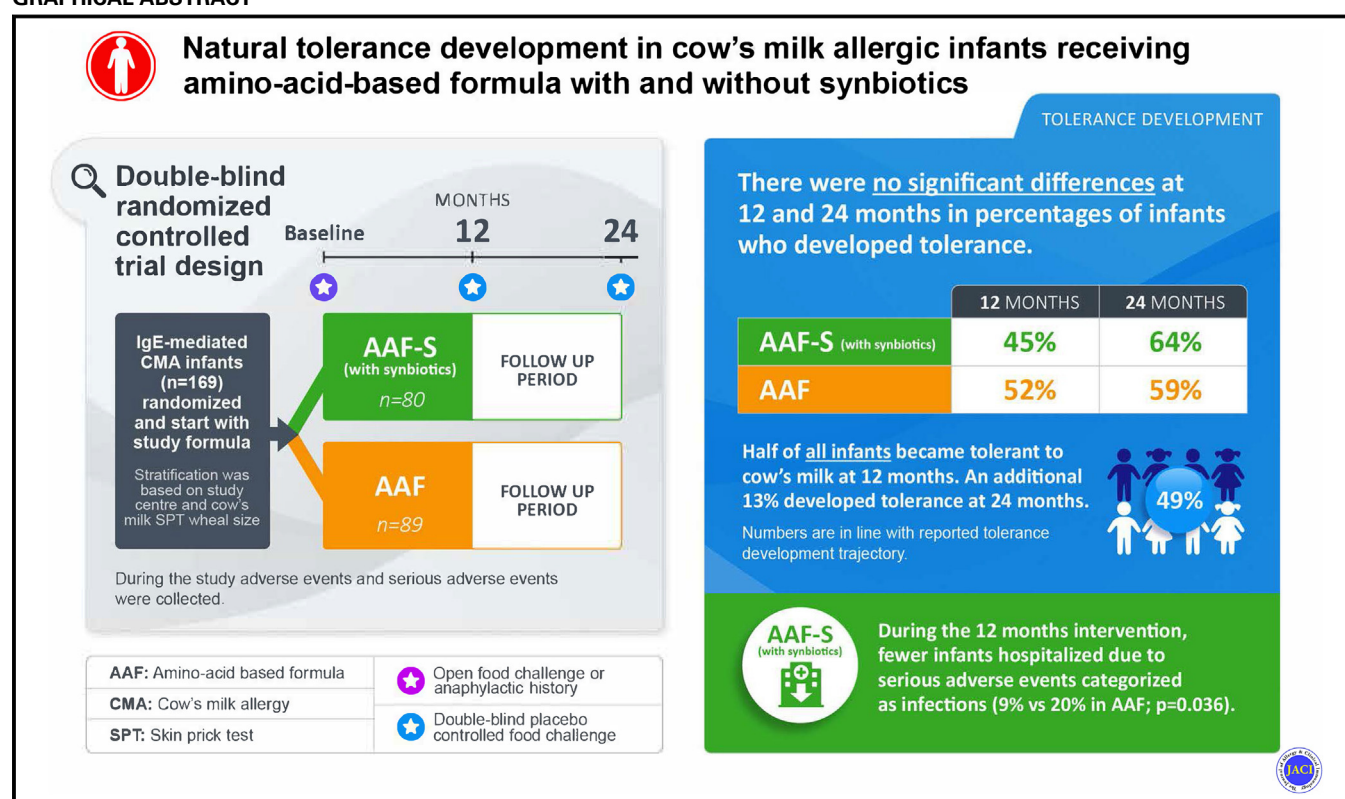


Tolerance development in cow's milk-allergic infants receiving amino acid-based formula: A randomized controlled trial

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GRAPHICAL ABSTRACT



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*Additional PRESTO study team members are listed in the Acknowledgments.

This study was funded by Danone Nutricia Research, Utrecht, The Netherlands.

Disclosure of potential conflict of interest: A. Nowak-Wegrzyn reports royalty payments from UpToDate; research grants from Nutricia, Nestlé, Astellas Pharma, NIAID, and DBV Technologies; personal fees from Nestlé, Nutricia/Danone, Novartis, and Regeneron; serves the American College of Allergy, Asthma, and Immunology as deputy editor of the *Annals of Allergy, Asthma, and Immunology* and is a member of the American Board of Allergy and Immunology outside the submitted work; and is on the medical advisory board (unpaid) of the International FPIES Association. L. Lange

Background: Tolerance development is an important clinical outcome for infants with cow's milk allergy.

Objective: This multicenter, prospective, randomized, double-blind, controlled clinical study (NTR3725) evaluated tolerance development to cow's milk (CM) and safety of an amino acid-based formula (AAF) including synbiotics (AAF-S) comprising prebiotic oligosaccharides (oligofructose, inulin) and probiotic *Bifidobacterium breve* M-16V in infants with confirmed IgE-mediated CM allergy.

Methods: Subjects aged ≤ 13 months with IgE-mediated CM allergy were randomized to receive AAF-S ($n = 80$) or AAF ($n = 89$) for 12 months. Stratification was based on CM skin prick test wheal size and study site. After 12 and 24 months, CM tolerance was evaluated by double-blind, placebo-controlled food challenge. A logistic regression model used the all-subjects randomized data set.

Results: At baseline, mean \pm SD age was 9.36 ± 2.53 months. At 12 and 24 months, respectively, 49% and 62% of subjects were CM tolerant (AAF-S 45% and 64%; AAF 52% and 59%), and not differ significantly between groups. During the 12-month intervention, the number of subjects reporting at least 1 adverse event did not significantly differ between groups; however, fewer subjects required hospitalization due to serious adverse events categorized as infections in the AAF-S versus AAF group (9% vs 20%; $P = .036$).

Conclusions: After 12 and 24 months, CM tolerance was not different between groups and was in line with natural outgrowth. Results suggest that during the intervention, fewer subjects receiving AAF-S required hospitalization due to infections. (J Allergy Clin Immunol 2021;■■■:■■■-■■■.)

Key words: Cow's milk allergy, prebiotics, probiotics, amino acid-based formula, oral tolerance, infection, synbiotics

Infants and young children experiencing immune-mediated responses to cow's milk (CM) face immediate and long-term health issues.¹ CM allergy (CMA) is among the most common food allergies in early life,¹⁻³ and approximately 60% of patients present with IgE-mediated CMA.¹ The outcome of CMA is generally favorable; most children will gradually outgrow their allergy and develop tolerance to milk proteins over time.³⁻⁶ The development of CM tolerance is influenced by the type of CMA¹ and is notably slower for children with IgE-mediated CMA than those with non-IgE-mediated CMA.³

Abbreviations used

| | |
|---------|---|
| AAF: | Amino acid-based formula |
| AAF-S: | Amino acid-based formula with synbiotics |
| AE: | Adverse event |
| ASR: | All-subjects randomized |
| CM: | Cow's milk |
| CMA: | Cow's milk allergy |
| DBPCFC: | Double-blind, placebo-controlled food challenge |
| eHF: | Extensively hydrolyzed formula |
| ER/CC: | <i>Eubacterium rectale</i> / <i>Clostridium coccoides</i> |
| SAE: | Serious adverse event |
| SCORAD: | SCORing Atopic Dermatitis |
| SPT: | Skin prick test |

Greater understanding of the immune pathogenesis of CMA has been accompanied by a shift in dietary management of CMA from simple elimination diets to more active interventions designed to modulate the immune system and improve the development of tolerance.⁷⁻¹² Limited data from clinical studies in formula-fed infants implied better symptom resolution¹³ but slower tolerance development with amino acid-based formula (AAF) than extensively hydrolyzed formula (eHF).¹⁴

Gut microbiota can influence the development of immune responses and food allergies during early infancy.¹⁵⁻²² Clinical evidence has shown that infants with CMA typically have lower levels of bifidobacteria and increased levels of adult-like *Clostridia* group *Eubacterium rectale*/*Clostridium coccoides* (ER/CC), and this dysbiosis can be rebalanced by providing AAF containing specific synbiotics (a combination of prebiotics and probiotics).²³⁻²⁵

A hypoallergenic AAF can be used for the dietary management of CMA when breast-feeding with or without maternal exclusion diet is not possible and when an eHF is not tolerated or fails to resolve allergy symptoms, or for the subgroup of infants with more severe symptoms.^{26,27} An AAF containing a synbiotic blend of prebiotics and probiotics derived from milk-free ingredients was developed to maintain hypoallergenicity even when used in the most severe cases of CMA. Previous studies have shown that this specific AAF formula with synbiotics (AAF-S) is hypoallergenic, supports normal growth, and improves the gut microbiota when used as a sole source of nutrition in healthy, full-term infants and in infants with IgE- or non-IgE-mediated

received lecture fees from Nutricia, Nestlé Nutritional Institute, Allergopharma, Infectopharm, HAL Allergy, and DBV Technologies; and received consulting fees from Nestlé Nutritional Institute, DBV Technologies, Infectopharm, and Aimmune outside the submitted work. M. van Ampting, M. Oude Nijhuis, J. Langford, J. Knol, K. Knipping, and J. Garssen are employees of Danone Nutricia Research; the content of this article does not influence their salary. V. Trendelenburg received lecture fees from Danone/Nutricia. C. M. Davis receives research grant support from the National Institutes of Health/National Institute of Allergy and Infectious Disease, Food Allergy Research and Education, DBV Technologies, Aimmune Therapeutics, Nutricia North America, Regeneron Pharmaceuticals, and the Scurlock Foundation; and is a consultant for Moonlight Therapeutics. A. Muraro has received speaker's fees from DBV Technologies, Aimmune, Meda-Mylan, Nutricia/Danone, Nestlé Health Institute, Nestlé Purina, and Regeneron. M. Erlewyn-Lajeunesse has undertaken clinical research with Danone/Nutricia and Abbott Nutrition and was a member of the IMAP milk allergy guidelines group. A. T. Fox has received research funding from ALK-Abello and Danone and consultancy fees from Stallergenes Greer, DBV, Aimmune, Danone, and Mead Johnson; and is president of BSACI, previous director of KCL Allergy Academy, and trustee of Allergy UK, all of which receive commercial sponsorship. L. J. Michaelis reports consultant arrangements with advisory boards at Danone and Novartis, and speakers'

bureau participation for Danone, Mead Johnson, and Regeneron; involvement in commercial clinical trials at Danone and Regeneron; and membership in BSACI, EAACI, and BPAIIG. K. Beyer has received lecture fees from Aimmune, Allergopharma, Bencard, Danone/Nutricia, Infectopharm, Meda Pharma/Mylan, Nestlé, and ThermoFisher, and consulting fees from Aimmune, ALK, Bausch & Lomb, Bencard, Danone/Nutricia, DBV, Hipp, Hycor, Infectopharm, Mabylon, Meda Pharma/Mylan, Nestlé, and Novartis. L. Harthoorn is a former Danone Nutricia Research employee and has no conflict of interest. The rest of the authors declare that they have no relevant conflicts of interest. Received for publication October 10, 2020; revised June 7, 2021; accepted for publication June 16, 2021.

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0091-6749

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<https://doi.org/10.1016/j.jaci.2021.06.025>

CMA.^{23,28} These studies raised specific interest in reported adverse events (AE) and medication use related to infections. For example, Burks et al²⁵ described fewer infants reporting infection-related AE and/or systemic antibacterial use, and Fox et al²⁹ described fewer subjects reporting ear infection-related AE.

The effect of AAF and AAF-S on CM tolerance development trajectory is not known. PRESTO is the first randomized controlled clinical study to investigate the trajectory of CM tolerance development and safety of 12 months' receipt of an AAF containing synbiotics in infants with confirmed IgE-mediated CMA.

METHODS

Study design

This prospective, randomized, double-blind, controlled clinical study (registration NTR3725) was conducted at 20 sites in 6 countries (Germany, Italy, Singapore, Thailand, the United Kingdom, and the United States). Subjects were enrolled between August 7, 2013, and February 6, 2017. Eligible subjects were stratified by study site and CM skin prick test (SPT) wheal size (0–5 and ≥ 6 mm) at entry and randomly allocated in a double-blind manner to receive either AAF-S or AAF for 12 months, with follow-up for another 2 years. Further details of the randomization procedure are described in the Methods section in the Online Repository at www.jacionline.org.

Parents/guardians, investigators, and study staff from Danone Nutricia Research remained blinded to receipt of the study product. Unblinded information was available only to the statistician responsible for generating the randomization sequence and the supplies manager responsible for labeling the study products.

This study was designed and conducted in accordance with the World Medical Association Declaration of Helsinki and the International Conference on Harmonization guidelines for Good Clinical Practice. Relevant national ethics committees and regulatory authorities approved the study protocol and amendments. Written informed consent was obtained from each subject's parents/guardians before study screening and enrollment.

Study population

Infants aged ≤ 13 months with confirmed IgE-mediated CMA were eligible for the study. Sensitization to CM was demonstrated by CM-specific serum IgE >0.1 kU/L and/or CM-SPT wheal size ≥ 3 mm. IgE-mediated CMA was confirmed by either an open or double-blind, placebo-controlled food challenge (DBPCFC) for CM, or an objective clinical history within 6 months before study entry where isolated ingestion of CM had resulted in anaphylaxis. In the latter cases, diagnosis had to be confirmed by 2 independent physicians.

Infants were excluded for the following reasons: birth weight <2500 g (<2250 g in Asian countries), <37 weeks' gestation requiring specific premature infant formula at study entry, severe concurrent illness, receipt of antihistamines within 4 days before SPT and CM challenge, and receipt of systemic corticosteroids, systemic antibiotics, antimycotic drugs, probiotic bacteria, or probiotic-containing drinks/supplements within 4 weeks before study entry.

Dietary intervention

Both the AAF-S and AAF were nutritionally complete, powdered, elemental infant formulas. The AAF-S was a hypoallergenic AAF (produced by Nutricia, Liverpool, United Kingdom) containing synbiotics, comprising a prebiotic blend of chicory-derived neutral oligofructose and long-chain inulin (BENEO-Orafti SA, Oreye, Belgium; 9:1 ratio at a total concentration of 0.63 g/100 mL), and a probiotic strain *Bifidobacterium breve* M-16 V (Morinaga Milk Industry, Tokyo, Japan) at a concentration of 1.47×10^9 colony-forming units/100 mL formula. The AAF was a commercially available AAF (Neocate LCP, Nutricia). Caregivers were instructed to provide subjects with a minimum, age-specific, daily study product intake based on standard

dilution or equivalent amount of powder: 0 to 8 months of age, 450 mL; 9 to 18 months of age, 350 mL; and over 18 months of age, 250 mL. After 12 months of intervention, subjects continued an age-appropriate diet advised by their clinician. Receipt of probiotic bacteria or probiotic-containing drinks/supplements in the 4 weeks preceding study entry, and caregivers' being unwilling to exclude such drinks/supplements during the study intervention (12 months), was part of study exclusion criteria. During the study, a diet diary was completed at 6 and 12 months, and accidental probiotic intake was monitored via specific food-related questions.

End points and assessments

Clinical visits were scheduled after 6, 12, 24, and 36 months. The primary study end point was the proportion of the subjects developing tolerance to CM after 12 months of intervention as measured by DBPCFC with CM powder. Tolerance to CM by DBPCFC at 24 and 36 months was a secondary end point. Specific information about the DBPCFC is provided in the Methods section in the Online Repository. To support safe introduction of CM in the diet, subjects with a negative outcome to the DBPCFC with CM powder (absence of major or minor criteria) were given an oral, fresh milk challenge with locally purchased (pasteurized) milk. If the DBPCFC or fresh milk challenge was positive at 12 or 24 months, DBPCFC was repeated at 24 or 36 months, respectively, to evaluate CM tolerance. Other secondary end points included SPT results, SCORing Atopic Dermatitis (SCORAD) rating scale, clinical assessment of CMA-related symptoms using rating scales, subject weight and length/height, number of acquired infections, and medication (including antibiotic) receipt. Parents/guardians completed a diary including methods to record (1) infections during the 12-month intervention; and (2) skin, respiratory, gastrointestinal, and general symptoms as well as bowel habits during the full study period using a 4-point scale. A predetermined interest was indicated for infections; therefore, infection data were collected via an infection scorecard during the intervention period. Investigators were requested to add infections into the electronic case report form accordingly and to indicate if an AE was considered an infection (yes or no).

Stool samples were collected for fluorescence *in situ* hybridization analysis of fecal microbiota (bifidobacteria and ER/CC group) using 16S ribosomal RNA-targeted oligonucleotide probe, as described previously.^{23,29}

Statistical analysis

Sample size estimation and interim analyses are described in the Methods section in the Online Repository. Statistical analyses for primary and secondary parameters were performed on the all-subjects randomized (ASR) data set and safety analyses on the all-subjects treated data set; both data sets included all subjects who received the study product. All statistical inferential procedures were performed at a significance level of 5%.

The primary end point was analyzed using a logistic regression model including treatment, stratification factors, age at baseline, and CM IgE at baseline as fixed effects. Secondary categorical ordinal end points were analyzed using a proportional odds model (symptoms and bowel habits). Secondary continuous end points were analyzed using either repeated measures mixed model (microbiota and anthropometrics) or a van Elteren test (SCORAD) in case the distributional assumptions were not met. All models were corrected for the stratification factors. AEs were tabulated in frequency tables by using the System Organ Class and MedDRA preferred terms. Medications provided were tabulated according to the medication subcategory, and both were analyzed by the Miettinen-Nurminen score test. Statistical analyses were performed by SAS Enterprise Guide v4.3 or higher software for Windows (SAS Institute, Cary, NC).

RESULTS

A total of 169 subjects with confirmed IgE-mediated CMA were randomly allocated to AAF-S ($n = 80$) or AAF ($n = 89$). All subjects randomized to the study product received at least 1 sip of study product; therefore, all subjects were included in the ASR

TABLE I. Baseline characteristics

| Characteristic | AAF-S (n = 80) | AAF (n = 89) | Total (n = 169) |
|--|----------------|--------------|-----------------|
| CMA diagnosed by: | | | |
| Anaphylaxis history | 9 (11) | 9 (10) | 18 (11) |
| DBPCFC | 11 (14) | 12 (14) | 23 (14) |
| Open milk challenge | 60 (75) | 68 (76) | 128 (75) |
| SPT wheal size for CM (stratification factor) | | | |
| 0-5 mm | 43 (54) | 45 (51) | 88 (52) |
| ≥6 mm | 37 (46) | 44 (49) | 81 (48) |
| CM-specific IgE level at baseline (kU/L), mean ± SD | 28.7 ± 121.7 | 13.0 ± 34.2 | 20.4 ± 87.6 |
| Mode of delivery | | | |
| Cesarean section | 43 (54) | 41 (46) | 84 (50) |
| Vaginal | 37 (46) | 48 (54) | 85 (50) |
| Family history of atopy | | | |
| At least 1 parent | 64 (80) | 64 (72) | 128 (76) |
| Medical history of presenting allergy complaints of: | | | |
| Eczema | 65 (81) | 74 (83) | 139 (82) |
| Acute urticaria | 33 (41) | 32 (36) | 65 (39) |
| Wheezing | 5 (6) | 13 (15) | 18 (11) |
| Dyspnea | 3 (4) | 7 (8) | 10 (6) |
| Stridor | 4 (5) | 6 (7) | 10 (6) |
| Dysphonia | 1 (1) | 2 (2) | 3 (2) |
| Aphonia | 1 (1) | 1 (1) | 2 (1) |
| Sneezing/congestion | 17 (21) | 19 (21) | 36 (21) |
| Conjunctivitis | 14 (18) | 8 (9) | 22 (13) |
| Severe abdominal symptoms | 6 (8) | 13 (15) | 19 (11) |
| Change in behavior such as irritability | 15 (19) | 16 (18) | 31 (18) |
| Sensitized to multiple foods (based on SPT results) | 60 (75) | 52 (58) | 112 (66) |
| Subjects breast-fed at all | | | |
| Yes | 79 (99) | 85 (96) | 164 (97) |
| No | 1 (1) | 4 (5) | 5 (3) |
| Subjects exclusively breast-fed until study entry | 6 (18) | 8 (24) | 14 (21) |
| Type of bottle feeding | | | |
| Whole protein (milk/soy) | 37 (51) | 48 (59) | 85 (56) |
| Extensively hydrolyzed formula | 39 (54) | 47 (58) | 86 (56) |
| AAF | 51 (71) | 59 (73) | 110 (72) |
| Missing | 8 | 8 | 16 |

Denominator to calculate percentage is number of subjects in treatment group with nonmissing data. Data are presented as no. (%) unless otherwise indicated.

and all-subjects treated data set. Subject disposition is shown in [Fig E1](#) in this article's Online Repository at www.jacionline.org. There were 23 early withdrawals, 11 in the AAF-S group (14%) (5 protocol violations, 5 lost to follow-up, and 1 subject withdrawal) and 12 in the AAF group (13%) (2 protocol violations, 4 lost to follow-up, 3 subject withdrawal, and 3 for other reasons).

Subject demographics are shown in [Table E1](#) in the Online Repository available at www.jacionline.org. Mean ± SD age at baseline was 9.36 ± 2.53 months. In the total population, 72% of subjects were male, 49% Asian, and 44% White. Demographics were similar between treatment groups, except that in Italy all 5 subjects were randomized to AAF. Baseline clinical characteristics and medical history are listed in [Table I](#). In 89% of subjects, CMA diagnosis was confirmed by an oral milk challenge, and 11% of subjects had a history of anaphylaxis. In the AAF-S and AAF groups, 46% and 49% of subjects were stratified in the higher CM SPT wheal size stratum (≥6 mm), respectively. CM-specific IgE levels at baseline were higher, with a greater SD, in the AAF-S group than in the AAF group (28.7 ± 121.7 kU/L vs 13.0 ± 34.2 kU/L, respectively). More subjects in the AAF-S group than in the AAF group were born by cesarean section (54% vs 46%) and had a family history of atopy (80% vs 72%). The majority of infants had eczema (82%). Most reported allergy

symptoms were acute urticaria (39%), followed by respiratory symptoms (wheezing 11%, dyspnea 6%, stridor 6%) and gastrointestinal symptoms (11%). More subjects in the AAF-S group were sensitized to multiple foods based on SPT results (75% vs 58%, respectively). In both groups, most subjects had already received eHF (56%) or any AAF (72%) before study entry.

Mean ± SD daily study product intake was similar in AAF-S and AAF groups at 6 months (576.49 ± 303.38 mL vs 628.07 ± 249.75 mL) and at 12 months (546.67 ± 302.38 mL vs 530.42 ± 307.52 mL). Four and 8 subjects in group AAF-S and AAF, respectively, consumed <80% of the required amount of study product.

[Fig 1](#) shows the proportions of subjects tolerant to CM at 12 months (primary outcome) and 24 months (secondary outcome). Test results were missing in 17 subjects (10%) (9 AAF-S, 8 AAF) at 12 months and in 34 subjects (20%) (16 AAF-S, 18 AAF) at 24 months because of early termination (the majority of cases; see [Fig E1](#)), because the DBPCFC was not performed (at the request of parents or investigator), or because the subject refused to drink during the DBPCFC. Overall, 49% (74/152) subjects developed tolerance to CM after 12 months' intervention, rising to 62% (83/135) at 24 months. There were no statistically significant differences in the proportions of subjects who developed tolerance to

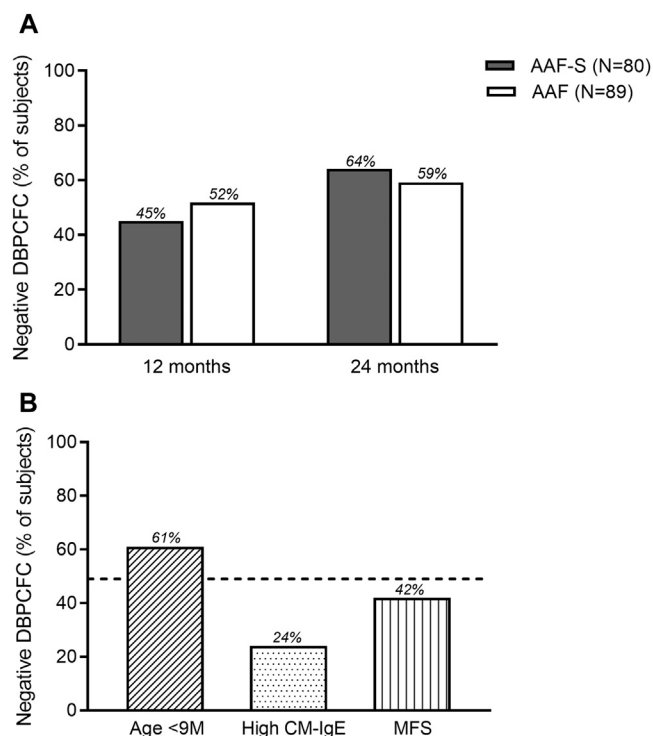


FIG 1. CM tolerance. Percentage of subjects tolerant to CM as defined by a negative response to an oral DBPCFC with CM powder at 12 and 24 months (A). Percentage of subjects tolerant at 12 months in overall study population (dotted horizontal line) and subgroups defined by baseline characteristics: age <9 months, high CM-specific IgE, and multiple-food sensitization (MFS) (B).

CM (negative DBPCFC with CM powder) between the AAF-S and AAF groups at 12 months (32/71 [45%] vs 42/81 [52%]; odds ratio 0.689, 95% confidence interval 0.289-1.644; $P = .401$) and at 24 months (41/64 [64%] vs 42/71 [59%]; odds ratio 1.331, 95% confidence interval 0.546-3.246; $P = .530$). An oral, fresh CM challenge for subjects tolerant to CM powder (DBPCFC) was not done for 2 subjects in both treatment groups at 12 months. There was no statistically significant difference between the AAF-S and AAF groups in the development of tolerance on the basis of a negative fresh CM challenge (among subjects with negative DBPCFC with powder CM) tested at 12 months (77% [23/30] vs 80% [32/40], respectively). At 24 months, for the subjects who were provided fresh CM (14 in the AAF-S group and 11 in the AAF group), there was also no statistically significant difference in tolerance development (86% [12/14] vs 64% [7/11], respectively).

There were no statistically significant differences in the development of tolerance between the AAF-S and AAF groups when the DBPCFC data were analyzed adjusting for predefined covariates/factors associated with delayed CMA outgrowth. After 12 months, the overall rates of CM tolerance in predefined subgroups related to these factors (Fig 1, B) were 61% (37/61) for age <9 months at baseline subgroup versus 41% (37/91) for the complementary group, with age ≥ 9 months at baseline, 71% (52/73) for those with CM-specific IgE ≤ 3.5 kU/L at baseline versus 24% (14/59) for infants with CM-specific IgE > 3.5 kU/L at baseline, and 63% (29/46) for infants who were sensitized only to CM versus 42% (42/101) for those with multiple food sensitization based on positive SPT at baseline.

The proportions of subjects who were CM tolerant at 12 months according to their level of study product intake (above vs below median, respectively) were 60% (21/35) versus 31% (11/35) in the AAF-S group and 52% (24/46) versus 51% (18/35) in the AAF group. Corresponding numbers at 24 months were 77% (27/35) versus 50% (14/28) in the AAF-S group and 63% (25/40) versus 55% (17/31) in the AAF group.

Clinical symptoms decreased over time with AAF-S and AAF, and there were no differences between the groups at 6 and 12 months (data not shown).

Anthropometric data showed weight-for-age, length-for-age, and weight-for-length mean z scores were in the range +0.5 to -0.5 over 12 months (see Fig E2 in this article's Online Repository at www.jacionline.org). There were no differences in growth parameters between groups.

AE and serious AE (SAE), reported irrespective of possible relationship to study product, are shown in Table II. As a result of previously reported clinical study outcomes,^{25,29} a predetermined interest was indicated for AE or SAE related to infection, and investigators were requested to indicate if an AE was considered an infection (yes or no). AEs were mostly categorized as gastrointestinal disorders and infections/infestations. There was no statistically significant difference in the total cluster of AEs reported between treatment groups. During the 12-month intervention period, more than 80% of the subjects reported AEs related to infection, with no difference between the groups. However, there were fewer subjects in the AAF-S group reporting SAEs (which were all documented as hospitalization) categorized as infections compared to the AAF group (7/80 [9%] vs 18/89 [20%], respectively; $P = .036$), driven by the difference in gastrointestinal infections/diarrhea and respiratory infections (Table III). The ratio of Asian versus non-Asian numbers of subjects reporting SAE related to infections were 6:5 and 13:8 in AAF-S and AAF, respectively. Further details on reporting of infection-related SAEs by country are provided in Table III.

Fig E3 in this article's Online Repository at www.jacionline.org shows forest plots of confidence limit for the risk difference, calculated using the Miettinen-Nurminen method of all AE as well as SAE sorted by body system.

Fig 2 shows the observed percentages of fecal microbiota at different time points. In the AAF-S group, the mean percentages of bifidobacteria were significantly higher at 6 and 12 months compared to those in the AAF group (37.1% vs 6.5%, $P = .001$; and 23.9% vs 6.5%, $P = .026$). The mean percentages of ER/CC were significantly lower in the AAF-S group than the AAF group at 6 months (14.6% vs 32.6%, $P = .007$) but not at 12 months (21.2% vs 35.7%, $P = .058$).

DISCUSSION

To our knowledge, this is the first randomized controlled study to investigate the natural tolerance development determined by DBPCFC in infants with confirmed IgE-mediated CMA receiving an AAF with or without synbiotics. The study demonstrated that 49% and 62% of infants receiving AAF developed tolerance to CM at 12 and 24 months, respectively, which is in line with clinical expectations for the outgrowth trajectory of CMA.^{3,30}

The EuroPrevall study showed that the development of tolerance is slower in children with IgE-mediated CMA than in those with non-IgE-mediated CMA (57% and 100% within 1 year, respectively),³ while other studies have shown that

TABLE II. Number of subjects with at least 1 adverse events or serious adverse event after 12 months

| Characteristic | AAF-S (n = 80), no. (%) | AAF (n = 89), no. (%) | Estimate (95% CI) | P value (Miettinen-Nurminen method) |
|-----------------------------------|-------------------------|-----------------------|-------------------|-------------------------------------|
| Any adverse event | 70 (88) | 75 (84) | 3% (−8, 14) | .549 |
| Gastrointestinal disorders* | 20 (25) | 20 (23) | 3% (−10, 16) | .700 |
| Infections and infestations* | 65 (81) | 68 (76) | 5% (−8, 17) | .444 |
| Any serious adverse event† | 11 (14) | 21 (24) | −10% (−22, 2) | .104 |
| Infections and infestations‡ | 7 (9) | 18 (20) | −12% (−22, −1) | .036 |

*The 2 most frequent reported body systems (of 16 total) are listed.

†All documented as hospitalization.

‡The most frequent reported body system (of 9 total) is shown.

TABLE III. Numbers of subjects with at least 1 serious adverse event related to gastrointestinal, respiratory, ear, or other infection reported in the body-system category “infections and infestations” by country of residence

| Country | Gastrointestinal | | Respiratory | | Ear | | Other | |
|---|------------------|--------|-------------|---------|-------|-----|-------|--------|
| | AAF-S | AAF | AAF-S | AAF | AAF-S | AAF | AAF-S | AAF |
| Total (N ₁ = 80, N ₂ = 89) | 0 | 5 (6) | 5 (6) | 14 (16) | 1 (1) | 0 | 1 (1) | 2 (2) |
| Germany (N ₁ = 17, N ₂ = 15) | 0 | 1 (7) | 2 (12) | 1 (7) | 0 | 0 | 0 | 0 |
| Italy (N ₁ = 0, N ₂ = 5) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Singapore (N ₁ = 8, N ₂ = 7) | 0 | 0 | 1 (13) | 2 (29) | 0 | 0 | 0 | 0 |
| Thailand (N ₁ = 27, N ₂ = 31) | 0 | 4 (13) | 2 (7) | 7 (23) | 0 | 0 | 1 (4) | 0 |
| United Kingdom (N ₁ = 22, N ₂ = 19) | 0 | 0 | 0 | 3 (16) | 1 (5) | 0 | 0 | 2 (11) |
| United States (N ₁ = 6, N ₂ = 12) | 0 | 0 | 0 | 1 (8) | 0 | 0 | 0 | 0 |

N₁ and N₂ indicate the number of subjects in the amino acid–based formula with synbiotics group (AAF-S) and the amino acid–based formula (AAF) group, respectively.

Other infections include hand, foot, and mouth disease (Thailand n = 1, United Kingdom n = 1) and tonsillitis (United Kingdom n = 1).

resolution of IgE-mediated CMA in more than 50% of children took 5 years or more.³¹ Comparison of CMA resolution rates reported in the literature is hard because of the heterogeneous study populations and different methodologies.^{1,31} Several factors affect the resolution of CMA, including age, severity of initial reactions, and presence of multiple food allergies or other comorbid atopic conditions.³² The magnitude of a food-specific IgE response predicts the development of tolerance,^{6,33,34} where low level of CM-specific IgE significantly correlates with the individual likelihood of growing out of CMA.³⁵ An analysis of subjects with CMA in the CoFAR study showed that slower CMA resolution rates were significantly associated with higher baseline IgE, larger SPT size, and more severe eczema.^{6,34} In the PRESTO study, we corrected for age at baseline and for CM-specific IgE level at baseline, and the subjects were stratified according to SPT wheal size for CM. The statistical models showed that age ≥ 9 months, high levels of CM-specific IgE (>3.5 kU/L), and presence of multiple food sensitizations were baseline factors associated with the delayed development of tolerance, which is consistent with previous findings and which indicates that while consuming AAF, the selected study population follows the natural trajectory for IgE-mediated CMA.

Tolerance to CM at 12 and 24 months was not statistically significantly modified by the addition of a synbiotic mixture to AAF. It should be noted that more subjects in the AAF-S group had higher CM-specific IgE levels and were sensitized to multiple foods, which are both factors associated with a delayed outgrowth of CMA. Despite these factors' individually not influencing the treatment effect in our statistical model, our study was not powered to investigate these elements—and especially not when combined as present in one group.

Comparison with a previous randomized dietary intervention study in children with IgE-mediated CMA is relevant because of

discussion regarding the rate of CM tolerance development in infants receiving eHF or AAF.³⁶ The previous study suggested that adding a specific probiotic to eHF improved the development of tolerance to CM; approximate tolerance rates for eHF and eHF with probiotics were 19% versus 39% at 12 months after study entry, and 44% versus 68% at 24 months after study entry.³⁶ The current study shows percentages in a similar range of 49% and 62% tolerance after 12 and 24 months, respectively. An earlier nonrandomized study by the same group suggested that development of tolerance was faster when providing eHF than when providing other formulas, including AAF.^{14,36} Importantly, these studies did not stratify according to baseline factors associated with tolerance acquisition, and they excluded subjects with CM-induced anaphylaxis or other food allergies.^{14,36} The PRESTO randomized controlled study included subjects with CM-induced anaphylaxis and/or other food allergies, and stratified patients by CM SPT wheal size, thus reflecting the severity of sensitization. While it is not possible to make direct comparisons between studies, it is relevant to note that our results showed that the time to resolution of CMA with an AAF was comparable to rates reported for eHF in a population that was not stratified for severity of CMA. Also, the current study provides important evidence that development of tolerance to CM appears to follow similar trajectories for AAF and eHF with or without probiotics,^{3,30,36} contrary to popular belief as well as findings from a nonrandomized study,¹⁴ which suggests that receipt of AAF may delay tolerance acquisition by avoiding exposure to CM peptides. The current confirmation of CMA natural outgrowth rates is especially important for infants with more severe CMA and multiple food allergies who cannot tolerate eHF and therefore require an AAF.

The observed between-group difference in infectious AEs requiring hospitalization is an intriguing finding. There was no

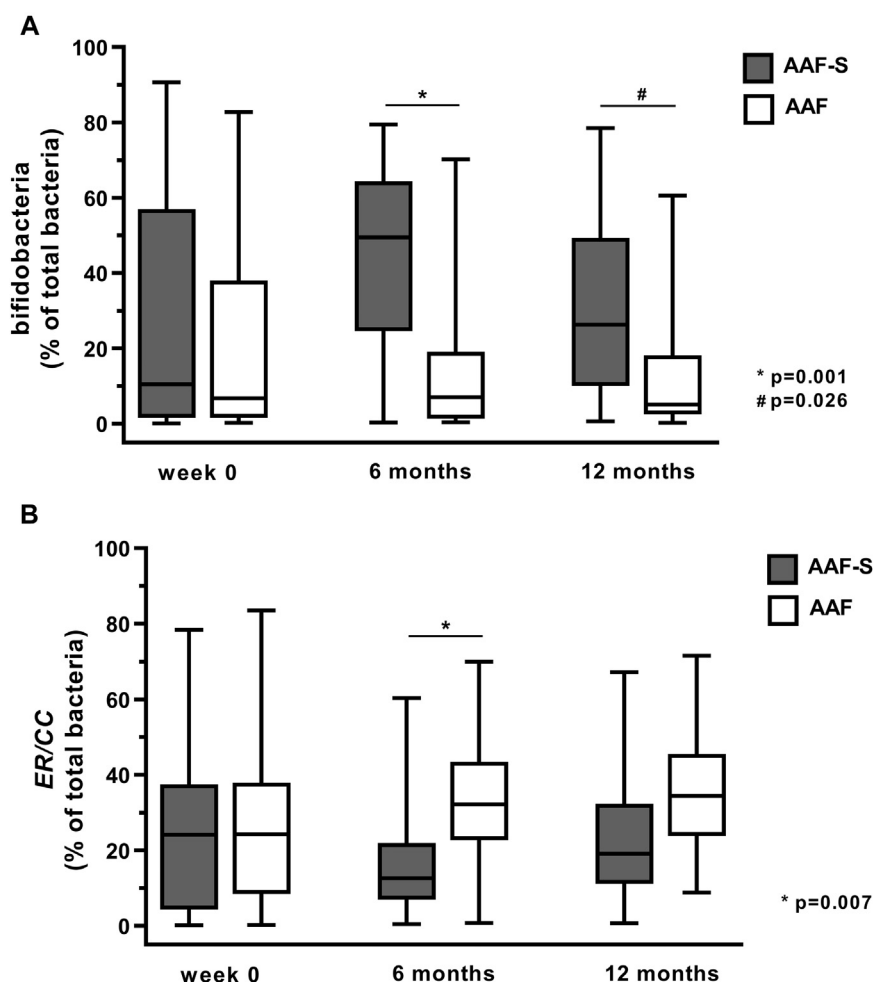


FIG 2. Fecal gut microbiota. Percentages of bifidobacteria (**A**) and ER/CC (**B**) at 0, 6, and 12 months in the ASR group. *Bottom and top edges of the box are located at the sample 25th and 75th percentiles. The center horizontal line is drawn at the 50th percentile (median). Whiskers indicate the minimum and maximum values.*

difference between general AEs related to infection. Because almost all (>80%) subjects experienced at least 1 AE categorized as infection, these results are difficult to interpret. Results from reported AEs in general should always be interpreted with caution and were not a primary outcome of the study. However, infections were a subject of predetermined interest, and a reduction in the incidence of infections requiring hospitalization is a clinically relevant finding in a population of infants. Our study population included subjects from Asian and non-Asian sites, whose approaches to treating infections may differ. However, our results show that subjects from both geographies are included in the observed SAE reports. Infections are prevalent in early life,³⁷ and the risk may be higher for infants for whom breast-feeding is not possible.³⁸ There is compelling evidence that synbiotics may improve defenses against infections—for example, by binding to pathogens or modulating the microbiota to promote colonization by beneficial bacteria and inhibiting pathogenic species.³⁹ An additional study, which reported a lower incidence of ear infections in non-IgE-mediated CMA infants receiving AAF-S, discussed potential systemic effects of the specific synbiotics beyond modification of gut microbiota.²⁹ Overall, our findings further build the hypothesis that AAF-S could reduce the risk of

early-life infections in CMA infants by improving the gut microbiota and rebalancing dysbiosis.

Several studies have reported dysbiosis in infants and children with CMA, generally showing lower levels of bifidobacteria and increased levels of members of the ER/CC group.^{24,40} Wopereis et al²⁴ demonstrated that AAF-S restored the gut microbiota and produced a more gradual increment in bacterial diversity compared to AAF. Companion translational research also showed that combining prebiotics and probiotics had a greater effect than the components individually on reducing allergic symptoms after allergen challenge.⁴¹ Similarly, the PRESTO study showed that AAF-S resulted in an increase in fecal bifidobacteria and a decrease in the ER/CC group, suggesting an overall composition closer to the profile of healthy, breast-fed infants, as shown in a previous study of AAF-S.^{23,24}

This study has several limitations. The study design aimed to include typical subjects for whom AAF is indicated, so the enrolled subjects had a relatively advanced median age at baseline (9 months), and multiple CM elimination diets had been used frequently before enrollment. Consequently, subjects had relatively mild but persistent symptoms at baseline. Furthermore, a prior CM elimination diet may have resulted in modification of

microbiome and changes in immunological markers compared to individuals with early diagnosed severe CMA for whom AAF is recommended as first-line treatment. Consumption of study product was lower in infants aged ≥ 9 months than in younger subjects because the older subjects received AAF or AAF-S as part of a diversified diet. As a result, the consumption of synbiotics in the AAF-S group was reduced among these older subjects. It is therefore possible that the reduced dose of synbiotics received could have diminished its effect and affected the reported outcomes. Also, introduction of additional foods, which was allowed during the study, in a diversified diet greatly affects intestinal microbiota composition.

This multicenter randomized controlled study showed that a hypoallergenic AAF with a specific synbiotic blend is safe and suitable for dietary management of infants with IgE-mediated CMA, including those with a history of anaphylaxis and suspected multiple food sensitizations. Although there were no statistically significant differences in CM tolerance development observed between the groups, the overall resolution in children is a clinically relevant result. During the 12-month intervention, fewer infants receiving AAF-S required hospitalization due to infections.

Additional PRESTO study team members include Lee Noimark (Barts/Royal London Hospital, London, England, United Kingdom), Gary Stiefel (Leicester Royal Infirmary, Leicester, England, United Kingdom), Uwe Schauer and Hamelman (Ruhr-Universität Bochum im St Josef-Hospital, Bochum, Germany), and Diego Peroni and Attilio Boner (University Hospital Verona, Verona, Italy).

We thank all infants, children, and caregivers for their participation in the PRESTO study. We thank Harm Wopereis and Rob Slump of Danone Nutricia Research, the Netherlands, for the analysis of the gut microbiota data, as well as the Clinical Study and Data Sciences teams of Danone Nutricia Research. We would also like to thank Graham Roberts and medical writer Tim Kelly for suggestions and critical review. Finally, we thank all involved physicians, dietitians, and research nurses at the study centers from the PRESTO study team for their great work.

Clinical implications: Infants with IgE-mediated CMA receiving an amino acid–based formula including synbiotics develop CM tolerance in line with natural tolerance development and may be at lower risk for hospitalization-requiring infections.

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METHODS

Randomization was performed during the screening visit or as soon as the diagnosis of cow's milk allergy (CMA) was confirmed, within 4 weeks of the screening visit.

The randomized allocation sequence was generated by Nutricia Research using block randomization. Randomization was stratified by study site and CM skin prick test (SPT) wheal size (0-5 and ≥ 6 mm) at entry. Stratification by study site was done so that an approximately equal number of subjects were included in both study groups at every site. Stratification by SPT was done because it is known that tolerance development depends on early sensitization.

Both study products were packaged in identical 400 g tins and labeled with a 1-letter code so that parents/guardians, those administering the interventions, and those assessing the outcomes remained unaware of the group assignment.

The study sample size was based on a 50% improvement in the proportion of subjects who develop tolerance in the amino acid-based formula with synbiotics (AAF-S) group compared to the amino acid-based formula (AAF) group (75% vs 50%, respectively). A sample size of 58 subjects per group was required to detect a difference in the proportion of subjects who develop tolerance. Following a semiblinded interim analysis by an external independent data monitoring committee and an independent expert committee, it was decided to keep the sample size unchanged. The external data monitoring committee consisted of 3 pediatric clinical experts within the allergy field and a statistician; the independent committee consisted of a clinical studies expert,

an allergy expert, an immune expert, and a statistician. All experts were not involved in any discussion or decision regarding the conduct of the study or study results after they evaluated semiblinded data.

Participating centers performed the double-blind, placebo-controlled food challenge according to standard practice with the following specific requirements: active and control challenges were done separately in a blinded, random order using a standard skimmed CM powder (provided by Nutricia) as the active product for the challenge, and antihistamine receipt was not permitted within the previous 4 days. Up to 7 graded doses of the blinded control (Neocate) or active (CM protein) formulas were provided to the subject to drink via a syringe, bottle, or cup at least 20 minutes apart. Responses to the active and control arms of the challenge were assessed by a nurse and clinician unaware of the challenge material; the investigating clinician decided whether to move to the next dose level on the basis of predefined major criteria (≥ 3 hives; ≥ 1 site of angioedema; wheezing, dyspnea, stridor, dysphonia, or aphonia; severe persistent abdominal symptoms for ≥ 30 minutes; and hypotension for age) and minor criteria (eczematous pruritic rash worsening in ≥ 10 SCORing Atopic Dermatitis [SCORAD] points; 1 or 2 hives; ≥ 1 episodes of sneezing, congestion, or rhinorrhea; conjunctivitis; ≥ 1 episode of nausea, vomiting, or diarrhea for < 20 minutes; and a change in behavior such as irritability, drowsiness, decreased activity, anxiety, or distress). The outcome of the food challenge was considered positive if ≥ 1 major criteria or ≥ 2 minor criteria occurred, and negative in the absence of a major or minor criterion.

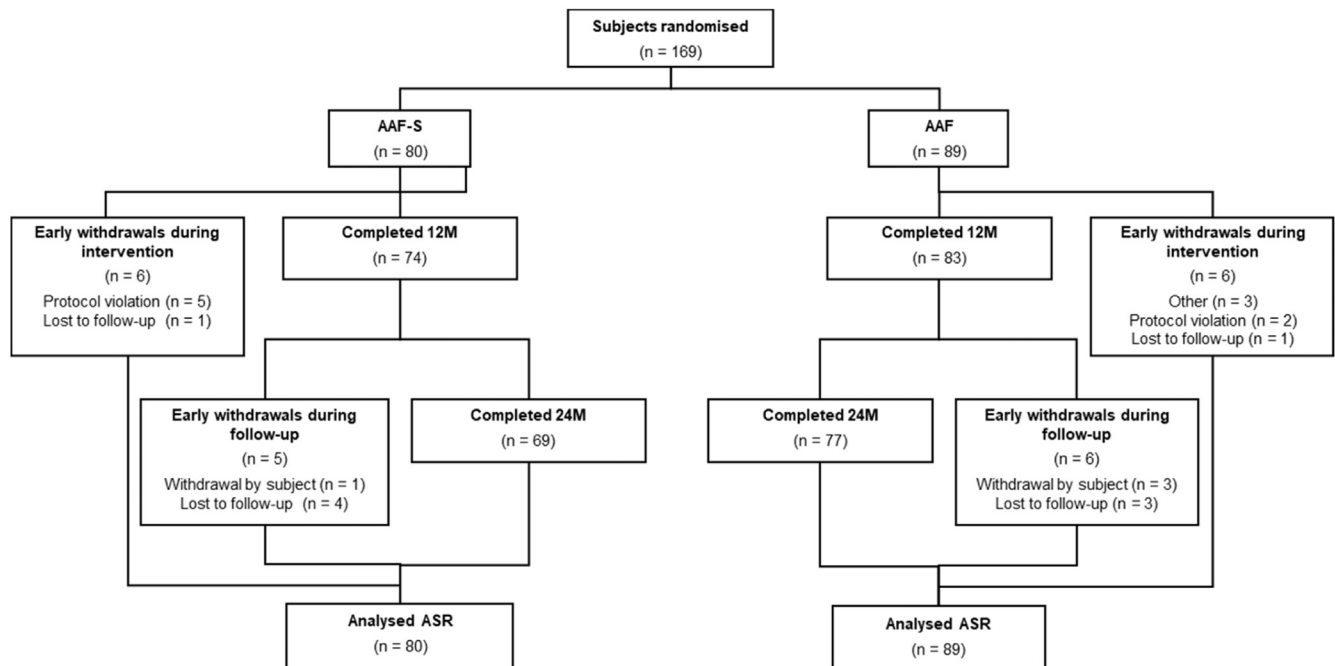


FIG E1. CONSORT diagram showing the flow of subjects in the AAF-S and AAF study arms.

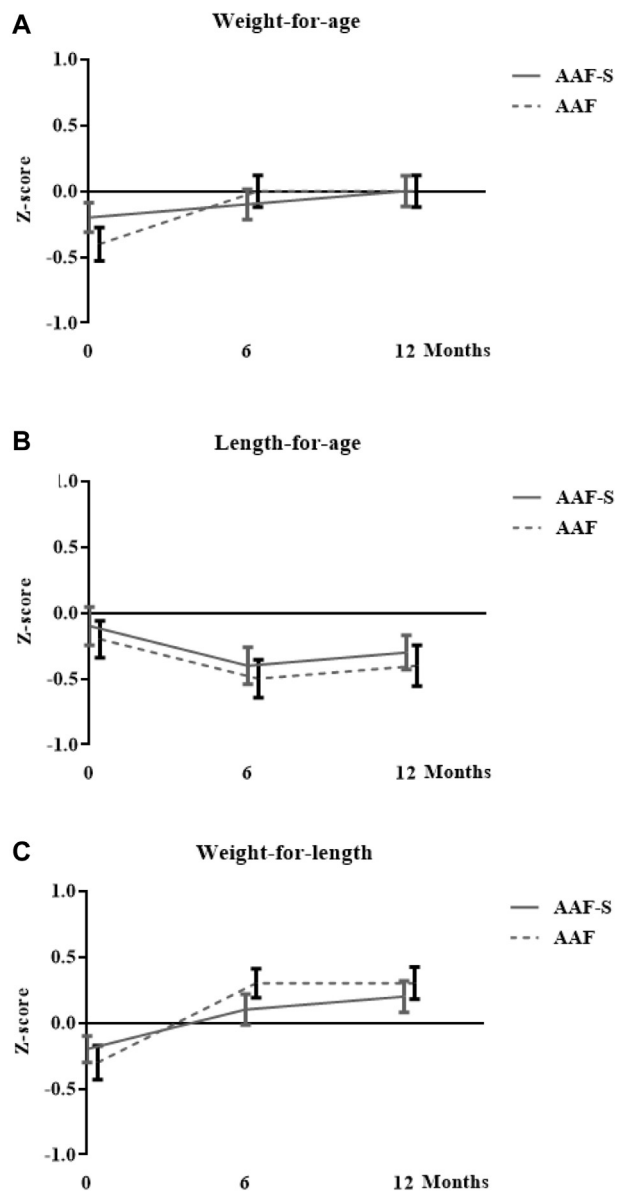


FIG E2. Infant growth. Weight-for-age (A), length-for-age (B), and weight-for-length (C) z scores over time. The z scores were calculated using World Health Organization 2006 growth standards. Values are provided as means \pm SEMs.

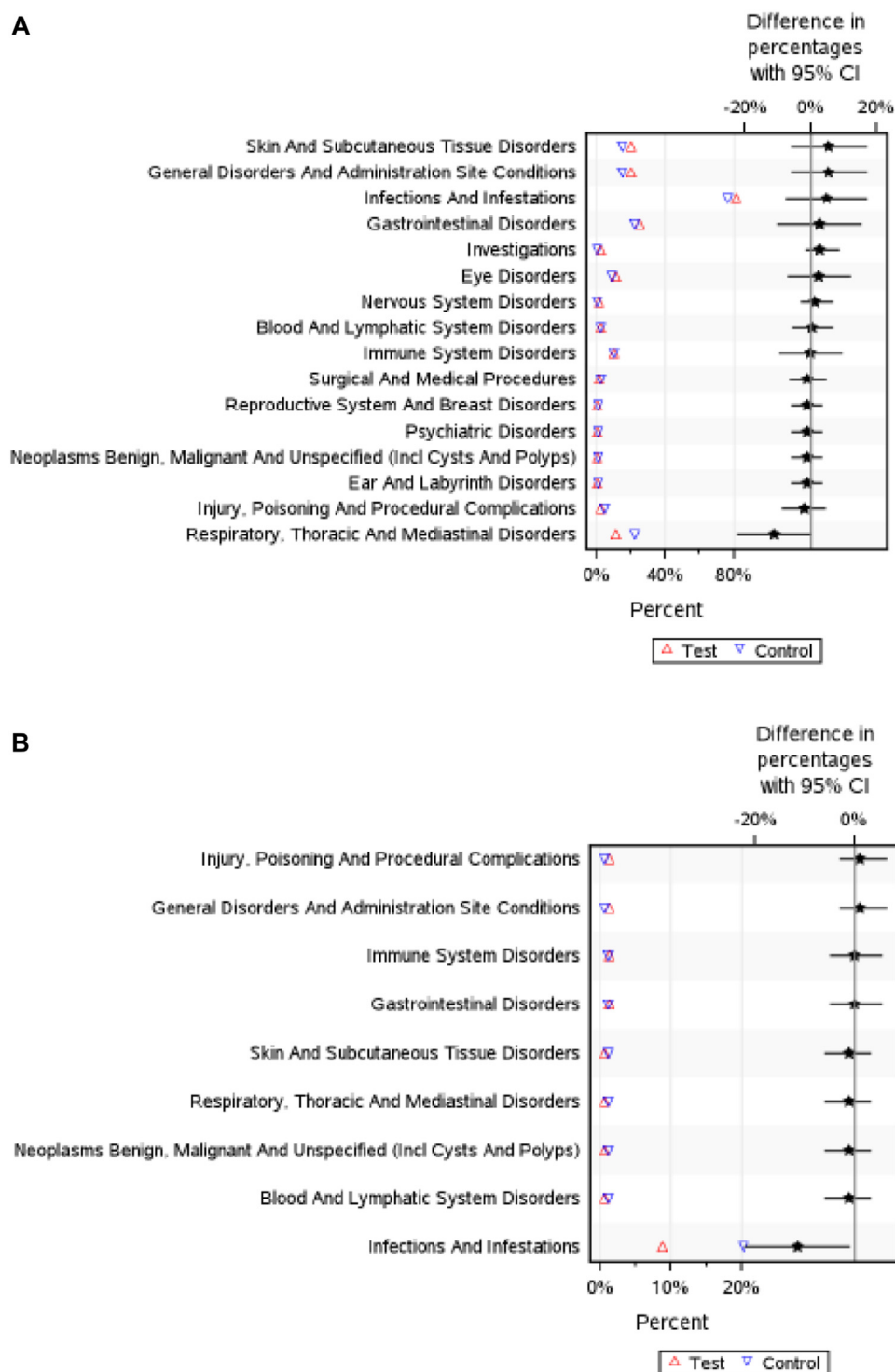


FIG E3. Forest plot showing AEs (all subjects treated) sorted by body system. Forest plots showing confidence limit for the risk difference calculated using the Miettinen-Nurminen method of (A) AEs and (B) SAEs.

TABLE E1. Demographics

| Characteristic | AAF-S (n = 80) | AAF (n = 89) | Total (N = 169) |
|---|-----------------|-----------------|-----------------|
| Age at baseline (months), mean \pm SD | 9.39 \pm 2.29 | 9.33 \pm 2.74 | 9.36 \pm 2.53 |
| Sex | | | |
| Male | 57 (71) | 65 (73) | 122 (72) |
| Female | 23 (29) | 24 (27) | 47 (28) |
| Race | | | |
| Asian | 39 (49) | 44 (49) | 83 (49) |
| Black | 0 | 2 (2) | 2 (1) |
| White | 36 (45) | 38 (43) | 74 (44) |
| Combination of above/other | 5 (6) | 5 (6) | 10 (6) |
| Country of residence | | | |
| Germany | 17 (21) | 15 (17) | 32 (19) |
| Italy | 0 | 5 (6) | 5 (3) |
| Singapore | 8 (10) | 7 (8) | 15 (9) |
| Thailand | 27 (34) | 31 (35) | 58 (34) |
| United States | 6 (8) | 12 (14) | 18 (11) |
| United Kingdom | 22 (28) | 19 (21) | 41 (24) |

Denominator for percentage is number of subjects in treatment group with nonmissing data. Data are presented as no. (%) unless otherwise indicated.