

A Double-Blind, Randomized, Crossover Allergy Study of an Extensively Hydrolyzed Casein Formula

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Abstract

Background: Extensively hydrolyzed formulas are used for the dietary management of cow's milk allergy. There is a limited repertoire of commercially available formulas for infants with cow's milk allergy.

Methods: This was a multi-center, double-blind, randomized, crossover clinical study of 75 children younger than 12 years, 11 months with documented cow's milk allergy. The primary outcome was the incidence of reactivity of a new extensively hydrolyzed casein formula and Nutramigen[®] during a Double-Blind Oral Food Challenge. A subset of subjects under the age of three years, 11 months at the time of enrollment received either the new formula or Nutramigen[®] for 16 weeks to investigate tolerance, taste preferences, growth and nutritional variables.

Results: Sixty-one subjects completed both challenges in the per-protocol groups. The new formula was non-inferior to Nutramigen regarding allergic symptoms of reactivity in both per protocol and intent to treat analyses, the latter addressing risk of bias from attrition during DBOFC. Both formulas met the American Academy of Pediatrics criteria for hypoallergenicity and had similar adverse event profiles. The new formula was comparable to Nutramigen[®] in supporting growth and tolerance.

Conclusion: The new hypoallergenic formula broadens access to formulations for dietary management of children with cow's milk allergy. Intent to treat analyses should be included in DBOFC studies of hypoallergenicity to reduce risk of bias from early discontinuation.

Keywords: Infant formula; Infant nutrition; Hypoallergenic; Cow milk allergies; Hydrolyzed casein; Hydrolyzed formulas; Casein ELISA

Introduction

Formulas based on extensively hydrolyzed proteins are recommended for the 2% to 3% of infants who are allergic to cow's milk-based formulas [1,2]. We developed a new, casein-based, extensively hydrolyzed, nutritionally complete infant formula (G19) for the dietary management of infants and children with confirmed cow's milk allergy (CMA).

Alternate diagnostic methods have been systematically reviewed [3], but the double-blind food oral challenges (DBOFC) was reaffirmed as the definitive manner of diagnosing milk allergic subjects by European [4] and American [5,6] expert panels, and for demonstrating hypoallergenicity of extensively hydrolyzed protein formulas [2]. Standardized procedures for such tests have been published [7] but few studies have followed the recommendations for the statistical power.

One important feature of DBOFC not previously addressed is discontinuation of subjects after first oral challenge. There are many reasons unrelated to the formula for early discontinuation, however, if discontinuation is related to formula it introduces potential bias. Intent to treat analyses are needed to address this potential bias.

Methods

Clinical study

We performed a randomized crossover DBOFC trial to show that 95% of proven cow's milk allergic subjects do not react, using a 90% probability of detecting a reaction, per American Academy of Pediatrics (AAP) Guidelines [2]. The first phase of the study was the DBOFC. The two challenges were conducted each on a single day one week apart, and diet was not controlled between challenges. Subjects were

recruited from 22 US pediatric allergy centers in 14 different States; 15 sites enrolled subjects. Subjects from 0 months to 12 years of age with confirmed CMA were eligible for the study if they were in general good health and free from any disease, condition or illness that might interfere with the study evaluations. A subset of young subjects entered a second phase with continued feeding of one blinded formula to assess growth over a 16-week period; this phase was abandoned because of poor recruitment. Informed consent was obtained from caregivers.

The composition of the two formulas was essentially identical in content of macronutrients, vitamins, minerals, and probiotic. The casein hydrolysate for G19 was from FrieslandCampina (Wageningen, The Netherlands). Mass spectrophotometry and reverse phase HPLC analyses showed peptide identity to the peptides in Frisolac[®] AllergyCare (Friesland Nutrition, Leeuwarden, The Netherlands) and molecular weight fingerprint very similar to that in Nutramigen[®]. The hydrolysate was not recognized by antibodies to bovine casein, nor did it provoke anaphylaxis in guinea pigs (unpublished data).

CMA was confirmed using criteria previously reported [8,9]. Any of four manners of confirming CMA was sufficient for inclusion:

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Received October 06, 2018; **Accepted** October 29, 2018; **Published** November 09, 2018

Citation: Barber C, Prieto PA, Wallingford JC (2018) A Double-Blind, Randomized, Crossover Allergy Study of an Extensively Hydrolyzed Casein Formula. J Nutr Food Sci 8: 736. doi: [10.4172/2155-9600.1000736](https://doi.org/10.4172/2155-9600.1000736)

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1. A positive challenge to cow's milk within six months of study screening, or
2. Within 90 days of study entry, serum IgE to cow's milk of >3 kUA/L and a convincing history of allergic symptoms to cow's milk, or
3. Within 90 days of study entry, a positive skin prick test (SPT) of >8 mm, or >5 mm with a convincing history of allergic symptoms to cow's milk, or
4. Within 90 days of study entry, a positive ImmunoCAP[®] (Quest Diagnostics[®], Secaucus, New Jersey, US) test defined as >5 kUA/L for cow's milk protein for subjects age <1 years or 15 kUA/L for subjects age >1 years.

These criteria were not all stated at study initiation and were modified twice by protocol amendments. One month after study launch, the SPT (criterion # 3) was added. Six months into the study positive ImmunoCAP[®] test was added (criterion # 4), and the criterion of a positive SPT (criterion # 3) was adjusted according to subject age [10]. Also this protocol amendment modified the initial age at enrollment (0-36 months) to 0-12 years 11 months to increase recruitment.

Subjects were excluded for clinically significant abnormal findings in their medical history, laboratory, or physical exam at screening; use of medications that could interfere with the DBOFC, intolerance to EHC formula, or tolerance to foods containing cow's protein. A third party labeled formulas and randomized subjects with a block size of four to receive "kits" for DBOFC and Triangle Taste Test. In the Triangle Taste Test [11], a separate randomization scheme was prepared to determine if a difference could be perceived among three blinded containers of formula, two of which were the same. Study site personnel were blinded to the subject assignment.

Each DBOFC was performed using increasing amounts of formula (1.5 ml, 4.5 ml, 15 ml, 45 ml, 150 ml) fed in approximately 15-minute intervals. Subjects were monitored continuously during the DBOFC, and symptoms were recorded after each dose. A standardized symptom scoring system was used to assess changes from baseline in a four-category Likert-type response scale: 0 none, 1 light, 2 moderate, 3 severe [7]. Using defined thresholds by organ system, each test was scored by the investigator as pass (small or no change in symptoms) or fail (exceeding the threshold for reactivity). Subjects completing both challenges comprised the Per Protocol population (PP). The Intent-to-Treat (ITT) safety population was defined by protocol as all subjects who were randomized. A modified Intent-to-Treat population (mITT) was established to analyze subjects who had documented reactivity status to at least one challenge (excluding subjects who were randomized but did not have at least one documented challenge).

Statistics

The non-inferiority of reactivity of G19 to Nutramigen[®] was tested as follows: The hypothesis being tested was: $H_0: p_{G19A} - p_{Nutramigen} \geq 10\%$. The incidence of reactivity was anticipated to be 5%. The primary effectiveness outcome was met if the 1-sided 95% upper bound for the difference in reactivity incidence was less than 10% [12]. To assess whether the formulas met the AAP criteria for hypoallergenicity, the 95% 1-sided confidence bound for reactivity incidence rate was estimated *via* the Wald standard error with continuity correction (SAS v.9.1.3). Setting the Type I error to 5%, a sample size of 60 evaluable subjects provided a 90% power for a one-sided non-inferiority test of the difference in reactivity with a 10% non-inferiority margin [13].

Secondary endpoints

Growth was determined by changes from baseline in weight-for-age Z-scores and in length-for-age Z-scores, using WHO reference data [14]. Serum IgE antibodies to total milk protein, casein, beta-lactoglobulin, alpha-lactalbumin and bovine serum albumin were performed at Phadia (Thermo Fisher, Waltham, MA, USA). In the Triangle Taste Test, caregivers tasted each of three blinded formulas and stated if one was preferred [11]. Caregivers were provided with a diary to record any symptoms of allergy between visits.

Approvals and monitoring

The protocol and informed consent form were approved by each study center's Independent Ethics Committee or Institutional Review Board in conformance with the International Conference on Harmonization guidelines on Good Clinical Practice. The trial was registered at clinicaltrials.gov (NCT00938483). Medical monitoring for serious AEs was performed by Perrigo Medical Affairs, with strict recording and reporting requirements for the investigators. All subjects with AEs were followed by the investigator until the AE was resolved, became clinically insignificant, became stable, or the subject was lost to follow-up.

Results

Subject disposition

Enrollment was from May 2014, through December 2016. A total of 134 subjects were screened. There were 56 screen failures as follows: for 34 subjects, CMA could not be confirmed; 11 subjects had confirmed CMA, but the subjects had clinically significant abnormal medical or laboratory findings or were unable to provide an adequate blood sample for testing. Of 78 subjects enrolled, the ITT population, consent was withdrawn by two prior to randomization; one subject was inadvertently unblinded. 75 subjects were randomized and experienced at least one challenge. Of the randomized subjects, there were 15 who discontinued before completing both challenges (the modified ITT population); seven in the G19-first group and seven in the Nutramigen[®]-first group. In the G19-first group, two discontinued because of adverse reactions to the first OFC and 5 passed the first OFC but discontinued. Among these five, two completed all doses of the first challenge with no symptoms. Seven subjects in the Nutramigen[®]-first group did not complete both challenges; two discontinued because of adverse reactions during the first OFC and five passed the OFC but discontinued. Of these five, three reached the highest dose of the first OFC with no symptoms, two discontinued at lower doses with no symptoms. Sixty subjects completed the DBOFC per protocol; 27 were fed G19 first (46%) and 33 Nutramigen[®]-first (54%).

Baseline demographic data are summarized in Table 1. There was a slight excess of boys relative to girls in each group. Most subjects were Caucasian, and the mean age in each group was about 3 years old. Baseline ImmunoCAP[®] test results are shown in Table 2, separated for inspection into age groups under and over 1 year, because milk allergy presents by 1 year of age [5]. Average and median values for total milk IgE and for IgE specific for each of four milk proteins were "high positive" (3.5 to 17.5 kUA/l) or positive very high" (above 17.5 kUA/l) according to criteria used by Ibero et al. [9] and were comparable between groups (overall scores). Among individual milk proteins, IgE to casein gave the highest mean and median values. Table 2 also illustrates that randomization created comparable ImmunoCAP scores in the total sample and below one year as well.

Table 3 shows the results of the DBOFC testing for the PP and mITT populations. In the PP population one subject first fed G19 and one subject first fed Nutramigen[®] reacted to the test product. From the primary analysis in PP population, the one-sided 95% upper bound of the difference of paired proportions or reactivity between G19 and Nutramigen[®] was 5.5%. Because the 1-sided 95% upper bound of the difference of paired proportions was less than 10%, G19 was non-inferior to Nutramigen[®]. Non-inferiority was also proven for the mITT population; the 1-sided 95% upper bound of the difference or paired proportions of reactivity between G19 and Nutramigen[®] was 7.3%.

Among the 3 subjects who reacted to Nutramigen[®], (2 mITT, 1 PP, Table 3), one reacted to 4.5 ml, one to 45 ml and one to 150 ml of the formula; this was similar for subjects who reacted to G19, two at 15

ml, and one to 150 ml of the new formula. Baseline ImmunoCap[®] data were available for three subjects; one who reacted to G19 had very high KU/L for total milk protein and each of the individual milk proteins. However, the other G19 reactor had scores above the median only for alpha-lactalbumin. The subject with ImmunoCap[®] data who reacted to Nutramigen[®] had scores above the median for four of the five measures. Most subjects who had high or very high ImmunoCAP[®] scores did not react to either formula.

Table 4 shows that both G19 and Nutramigen[®] satisfied the AAP criteria for labeling a formula as hypoallergenic. In the PP population, 59 of 60 subjects (98.3%) did not respond represent of the subjects; the lower end of the 95% confidence interval (CI) was calculated to be 0.943, so at least 90% of allergic subjects did not respond, with a 95%

Demographic data	Group	G19-first (N=35)	Nutramigen [®] - first (N =40)	Overall (N =75)
Gender	Male	21 (60.0%)	24 (60.0%)	45 (60.0%)
	Female	14 (40.0%)	16 (40.0%)	30 (40.0%)
Ethnicity	Hispanic or Latino	7 (20.0%)	5 (12.5%)	12 (16.0%)
	Not Hispanic or Latino	28 (80.0%)	35 (87.5%)	63 (84.0%)
Race	Caucasian	21 (60.0%)	22 (55.0%)	43 (57.3%)
	Black or African American	7 (20.0%)	10 (25.0%)	17 (22.7%)
	Asian	1 (2.9%)	4 (10.0%)	5 (6.7%)
	Native Pacific Islander	1 (2.9%)	1 (2.5%)	2 (2.7%)
	Other	5 (14.3%)	3 (7.5%)	8 (10.7%)
Age (months)	N	35	40	75
	Mean (STD)	57.2 (47.02)	47.2 (41.08)	51.9 (43.94)
Weight (kg)	N	35	40	75
	Mean (STD)	19.6194 (12.64320)	17.7099 (12.13964)	18.6010 (12.33017)
Length/Height/Visit 3 (cm)	N	8	17	25
	Mean (STD)	76.863 (14.9713)	77.024 (12.5624)	76.972 (13.0610)

Table 1: Baseline demographic data for the Intent-to-Treat (ITT) analysis population at screening of the subjects unless otherwise noted.

Variable	Statistic	Received G19A first (N=35)			Received nutramigen [®] first (N=40)		
		≤ 1 Year	>1 Year	Overall	≤ 1 Year	>1 Year	Overall
Milk IgE (KU/L)	N	4	28	32	2	30	32
	Mean	19.218	8.884	10.176	16.120	15.624	15.655
	STD	12.9652	16.7931	16.5512	16.5180	20.4262	19.9781
	Median (Min, Max)	22.850 (1.47, 29.70)	3.625 (0.34, 88.50)	4.785 (0.34, 88.50)	16.120 (4.44, 27.80)	4.685 (0.23, 74.60)	4.880 (0.23, 74.60)
Casein (KU/L)	N	4	21	25	2	28	30
	Mean	5.123	8.548	8.000	2.975	12.095	11.487
	STD	4.5199	19.3659	17.7969	3.9386	20.2001	19.6416
	Median (Min, Max)	4.930 (0.13, 10.50)	0.900 (0.10, 88.80)	2.280 (0.10, 88.80)	2.975 (0.19, 5.76)	2.405 (0.13, 86.80)	2.405 (0.13, 86.80)
β-Lacto globulin (KU/L)	N	4	25	29	2	28	30
	Mean	0.630	5.039	4.431	4.625	5.433	5.379
	STD	0.3196	11.9844	11.2033	4.7164	8.9454	8.6782
	Median (Min, Max)	0.620 (0.31, 0.97)	1.040 (0.14, 53.40)	0.950 (0.14, 53.40)	1.040 (0.19, 7.96)	0.920 (0.11, 34.00)	1.150 (0.11, 34.00)
α-Lact-albumin	N	4	24	28	1	29	30
	Mean	17.728	3.463	5.501	4.170	4.923	4.898
	STD	31.9711	5.2098	12.7487	-	10.9042	10.7154
	Median (Min, Max)	2.600 (0.11, 65.60)	1.525 (0.11, 22.70)	1.525 (0.11, 65.60)	4.170 (4.17, 4.17)	1.140 (0.19, 52.30)	1.225 (0.19, 52.30)
Bovine Serum Albumin (KU/L)	>100	0	1	1	0	0	0
	N	4	17	21	2	16	18
	Mean	19.325	4.215	7.093	25.015	5.080	7.295
	STD	24.2415	8.8587	13.7074	35.1927	8.1485	13.1529
Median (Min, Max)	12.335 (0.73, 51.90)	0.520 (0.12, 36.10)	0.730 (0.12, 51.90)	25.015 (0.13, 49.90)	1.885 (0.19, 31.00)	1.885 (0.13, 49.90)	

Table 2: Total milk IgE and immunoCAP[®] values at screening by age for the phase I intent-to-treat analysis population.

Population	G19	Nutramigen®	G19 – Nutramigen Difference	95% 1-Sided Upper Confidence Bound	p-Value
PP	1/60 (1.7%)	1/60 (1.7%)	0.0%	5.5% ¹	<0.001 ²
mITT	3/68 (4.4%)	3/68 (4.4%)	0.0%	7.3% ³	0.008 ³

Note: ¹95% 1-sided upper confidence bound calculated via Fleiss: $(\hat{p}_{NPS-G19A} - \hat{p}_{Nutramigen}) + 1.645 \cdot s\hat{e}(\hat{p}_{NPS-G19A} - \hat{p}_{Nutramigen}) + (1/n)$; ²p-value calculated based on rearranging the confidence interval formula given in Fleiss (2003), using the non-inferiority margin of 0.10: $1 - \Phi^{-1}\{0.10 - (\hat{p}_{NPS-G19A} - \hat{p}_{Nutramigen}) - (1/n)\} / s\hat{e}(\hat{p}_{NPS-G19A} - \hat{p}_{Nutramigen})$; If there were no subjects in either discordant cell (Present/Absent or Absent/Present), then the standard error, 95% 1-sided upper confidence bound, and p-value are undefined. ³p-value and 95% 1-sided upper confidence bound were calculated based on the difference of proportions among the 2 study formulas without considering paired response and using the non-inferiority margin of 0.10.

Table 3: Primary efficacy endpoint analysis: incidence of reactivity to G19 and Nutramigen® for the per-protocol and modified intent-to-treat analysis populations.

Population	Formula provided	Non-reactivity incidence (%)	1-sided lower 95% confidence bound ¹
PP	G19	59/60 (98.3%)	0.943
	Nutramigen®	59/60 (98.3%)	0.943
mITT	G19	65/68 (95.6%)	0.908
	Nutramigen®	66/69 (95.6%)	0.908

¹AAP criteria for labeling a formula as hypoallergenic are satisfied if the 95% 1-sided lower confidence bound for reactivity incidence is greater than 0.90.

Table 4: AAP criteria for hypoallergenicity for the phase I per-protocol (PP) and modified intent-to-treat (mITT) analysis populations.

TEAE	Categories	NPS-G19A(N=68) n (%)	Nutramigen (N=68) n (%)	G19 vs Nutramigen® p value ¹ n (%)
Summary	Subjects who consumed any formula with ≥ 1 TEAE	29 (42.6)	33 (48.5)	0.491
	Subjects with ≥ 1 Serious TEAE	2 (2.9)	1 (1.5)	0.559
	Subjects with ≥ 1 Related (Possibly, Probably, Definitely) TEAE	20 (29.4)	20 (29.4)	1.000
	Subjects with ≥ 1 TEAE associated with the DBOFC	20 (29.4)	18 (26.5)	0.702
	Subjects discontinuing/interrupting formula due to TEAE	6 (8.8)	3 (4.4)	0.301
	Deaths	0	0	NC ²
Severity	Subjects with ≥ 1 Mild TEAE	17 (25.0)	17 (25.0)	0
	Subjects with ≥ 1 Moderate TEAE	7 (10.3)	11 (16.2)	3
	Subjects with ≥ 1 Severe TEAE	5 (7.4)	5 (7.4)	0
	Subjects with ≥ 1 Not Related TEAE	3 (4.4)	8 (11.8)	5
	Subjects with ≥ 1 Unlikely Related TEAE	6 (8.8)	5 (7.4)	1
Relationship	Subjects with ≥ 1 Possibly Related TEAE	8 (11.8)	9 (13.2)	1
	Subjects with ≥ 1 Probably Related TEAE	6 (8.8)	5 (7.4)	1
	Subjects with ≥ 1 Definitely Related TEAE	6 (8.8)	6 (8.8)	0

Note: ¹p-value from Chi-square test; significance defined as p ≤ 0.05

²NC=Non-calculable

Subjects reporting more than one TEAE was only counted once under the strongest relationship and/or severity.

Table 5: Summary of treatment-emergent adverse events (TEAE) for the safety population.

CI. The same analyses for the mITT populations showed that 95.6% of the subjects did not respond, where the lower end of the 95th centile was 0.908.

Safety

Approximately 43% and 50% of all Phase 1 subjects experienced at least one treatment-emergent adverse event (TEAE) on G19 or Nutramigen®, respectively (Table 5). However, most of these subjects did not exhibit changes in symptom severity or number of systems to be scored a positive reaction to the test food [7]. There were no significant differences between the treatment groups in number of subjects with at least one adverse event (p=0.491), the severity of TEAEs (p=0.729), the relationship of TEAEs to the study formula (p=0.682), and the number of TEAEs associated with DBOFCs (p=0.702). There were 6 subjects in G19 and 3 subjects in Nutramigen® that discontinued or interrupted the formula due to a TEAE (p=0.301).

Approximately 75% of the Phase II safety population had at least one TEAE, the most frequent of which were constipation, diarrhea, abdominal pain upper, dermatitis, pruritus, rash, vomiting, asthma, food allergy and hypersensitivity. Overall, the formula groups were quite similar.

Six subjects experienced serious TEAEs, 2 of whom continued in the study and 4 of whom withdrew from the study. No other serious AEs were reported.

Growth

Of the 28 subjects eligible for Phase II, 13 were assigned to G19, 7 to Nutramigen®, and 8 discontinued without taking product. WAZ increased from -0.44 to -0.24 in the G19 group and from -0.45 to -0.41 in control. LAZ increased from 0.29 to 0.41, and from -0.96 to -0.65 in G19 and control groups, respectively.

Sensory evaluation of formulas

Triangle Taste Tests showed caregivers failed to correctly identify the two of three samples that were the same (p=0.116). There was no difference in preference for taste or color. Significantly more participants (49.2%) preferred the odor of Nutramigen® than G19 (25.4%), while 25.4% showed no preference (p<0.05). Mouthfeel was not different between formulas.

Discussion

Stringent enrolment criteria established IgE-mediated CMA in our subjects. Blinding of formulas and investigators, comparable baseline

data of subjects first fed G19 and control formulas, and the crossover design all minimized potential bias. Sample sizes were adequate. The assessment of reactivity in mITT analyses avoided attrition bias. All subjects in the mITT population who reacted were enrolled after the protocol amendments, suggesting neither changes in age of subjects nor criteria demonstrating CMA introduced bias. Thus, design elements and results validate the conclusion that G19 is non-inferior to Nutramigen[®].

Both G19 and Nutramigen[®] met the AAP criteria for hypoallergenicity in both the PP and mITT analyses. Other researchers have shown comparable reactivity of serum from CMA subjects to Nutramigen[®] and Frisolac[®], the latter having the same hydrolysate as G19 [15].

The PP population included one subject in each group who failed to react to the first challenge to hydrolysate but reacted to the second challenge. Thus, despite efforts to make identical formulations using similar hydrolysates, there remain subtle differences in formulation [9]. G19 could be useful for the subset of subjects who do not tolerate Nutramigen[®] and vice versa.

This EHF contains the probiotic *Lactobacillus rhamnosus* (HN001, DR20[®], Fonterra Group Inc.) which is closely related to the probiotic used in Nutramigen[®] [16] reported to promote gastrointestinal health [17] and may promote earlier development of tolerance to cow's milk protein in some, mainly non-IgE CMA subjects [16]. The effect of G19 with *Lactobacillus rhamnosus* on development of tolerance remains to be determined.

Not all studies on hypoallergenic formulas have attained the statistical power of the present study. Burks et al. [8] had only 32 subjects per group in a comparison of an amino acid formulation to a commercially available EHC formula; Martin-Esteban et al. [18] studied 34 subjects. Because a difference in reactivity of only one or two subjects can change the conclusion about hypoallergenicity [18], larger studies mainly from Europe [9,16,19,20] are more reliable. Our study, with others testing the same hydrolysate [19-21] provide a solid foundation for use of G19 in CMA.

Despite the small number of subjects, the improvements in WAZ and LAZ over four months of feeding is consistent with the adequacy of G19, like Nutramigen[®] to support infant growth and long-term health [21-25]. Similar TEAE and Triangle Taste Test results for sensory scores suggest comparable consumer tolerance and acceptability of G19 and Nutramigen[®].

Caregivers of infants with milk allergy are challenged to provide complete nutrition at a time of life when breastmilk or infant formula is a mainstay of the diet. There are few commercial formulations available, and they are costly relative to standard formulas because of the additional processing requirements and stringent quality control measures needed to assure absence of milk allergens. This new formulation provides the same nutrition, taste preferences and hypoallergenicity as the leading brand, but is manufactured by a company producing lower cost formulas and will increase consumer choice.

A limitation of the study was the short exposure of subjects to G19 in Phase I. Phase II allowed for observations over 4 months of feeding, long enough to develop allergy to G19 peptides.

In conclusion, G19 was non-inferior to Nutramigen[®], and both formulas met the AAP criteria for hypoallergenicity. With similar indices of tolerance, G19 offers an acceptable alternative to Nutramigen[®] for dietary management of CMA.

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