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Research Article

A Hypoallergenic Infant Formula Comprising Extensively Hydrolyzed Protein for the Nutritional Treatment of Infants with Cow's Milk Allergy: Safety, Tolerance and Efficacy

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Abstract

Background: There is strong evidence about the role of extensively hydrolyzed formula (eHF) in the treatment of cow's milk protein allergy (CMPA). The main objective of this work was to ensure an adequate nutritional support for cow's milk protein allergic infants.

Methods: An eHF was designed, and this eHF was validated by means of *in vitro* and *in vivo* studies and subsequently in a clinical trial. *In vitro* studies and Active Systemic Anaphylaxis Test (ASA test) established antigenicity. A prospective clinical trial was performed to evaluate the safety and tolerance of this formula for use in infants with proven CMPA. The safety and clinical tolerance was evaluated by means of an oral food challenge provocation test (OFC). Also, LCPUFAs in red blood cells and cytokines in plasma were measured in a subgroup of infants with CMPA.

Results: Of the 47 infants included with proven CMPA, only one showed a positive result in the OFC, meaning that the eHF was tolerated by 98% of the infants. Next, 30 infants were fed with the eHF for 3 months: One infant developed a cutaneous eruption during the 1st month of the follow-up and dropped out. During the 2nd and 3rd month of the intervention, no infants exhibited symptoms. Furthermore, the eHF allowed the infants to experience normal weight gain within the 3 months study period.

Conclusion: In accordance with current guidelines, this eHF was tolerated by more than 90% of infants with proven CMPA.

ABBREVIATIONS

CMPA: Cow's Milk Protein Allergy; eHF: Extensively Hydrolyzed Formula; LCPUFAs: Long Chain Polyunsaturated Fatty Acids; OFC: Oral Food Challenge Provocation Test; DHA: Docosahexaenoic Acid; AA: Arachidonic Acid; WHO: World Health Organization; IgE: Immunoglobulin E

INTRODUCTION

Recent decades have seen an increase in the number of people suffering allergic diseases in developed countries [1] and there is growing evidence of a relation between the development of allergies in early life and its later consequences [2].

The immune system of infants at birth may be fully

developed, but is still immature and has to be programmed. Th2 predominance has to be inverted until a normal adult profile is reached with Th1 predominance. Allergens are proteins that are recognized as "foreign" by the immune system, which triggers an immune/allergic response against these antigens. Cow's milk protein (CMP) may be considered as the first food allergens to come into contact with the newborn [1,3]. Indeed, CMP is the leading cause of food allergy in infants and children younger than three. CMPA seems to peak in the first year of life, with a prevalence of 2% - 3% in the infant population [1]. Most infants with CMPA develop symptoms in the first year of life and approximately 85% become clinically tolerant by the third year of life. CMPA is defined by the occurrence of clinical symptoms related to the abnormal immune response of the host after ingestion of these CM proteins. CMPA can be divided into IgE-mediated and non-IgE-mediated CMPA. In the first case, symptoms usually appear immediately or within two hours after the intake, while in non-IgE-mediated CMPA, symptoms may take a long time to appear - perhaps delayed reactions become manifest only after 48 hours or even 1 week following ingestion [4]. Patients with CMPA develop gastrointestinal symptoms (vomiting, diarrhea, colic, constipation…), skin symptoms (erythema, atopic dermatitis, angioedema…), respiratory symptoms (sibilance, bronchospasm), anaphylaxis, and failure-to thrive [4,5]. In most of the populations studied, there are overlapping symptoms.

The treatment for CMPA in infants involves the complete elimination of CMP from the diet and adequate replacement with a hypoallergenic formula [4-7]. The gold standard and first choice for infants from birth up to six months is human milk and breast-feeding; only in cases where breast-feeding is not possible, an infant formula specially designed for the nutritional treatment of infants can be used [4-7].

Extensively hydrolyzed formulas (eHFs) are recommended for formula-fed infants diagnosed with CMPA. Such formulas are the first option in the nutritional treatment of the infants with CMPA according to current international guidelines [4,5]. Only when infants are allergic to extensively hydrolyzed formulas, a next step could be to use amino acid-based formulas [5].

According to international guidelines [6-8], to claim that a hypoallergenic formula is suitable for the nutritional treatment of infants with CMPA, it is necessary to test the hypoallergenicity by means of clinical trials and to demonstrate that this formula is tolerated by at least 90% (with 95% confidence) of infants with CMPA. Only some eHFs and amino acid-based formulas (AAFs), which are considered non-allergenic, meet these criteria. However, from an organoleptic point of view, these formulas are characterized by an unpleasant taste and bitterness [5-7,9], Which could negatively affect their acceptance by infants and even parents. It is important therefore to try to improve the palatability of these products in order to avoid problems related with the (optimal) growth of infants with CMPA due to the low intake of formula.

Related to the prevention of allergies in the early stages of life and taking into account the functional properties of some nutrients, a lot has been published on the role of docosahexaenoic acid (DHA), probiotics, prebiotics and others. There have even been studies in which pregnant women, lactating women and their infants have been treated with DHA to study the prevention of allergies [10,11]. However, there is still a gap about the role of DHA in the tertiary prevention of allergy. Only *in vivo* animal studies seem to have evaluated the action mechanisms [12] and some clinical trials have mentioned the possible role of DHA in the tertiary prevention or treatment of allergy [13,14].

The **main objective** of the present study was to assess the safety, tolerance and efficacy of a new eHF (20% hydrolyzed casein and 80% hydrolyzed whey proteins) in a group of infants diagnosed with CMPA according to current scientific guidelines [6,7]. **Secondary outcomes** included the analysis of selected plasma cytokines and long chain polyunsaturated fatty acids (LC-PUFAs) in red blood cells in a subgroup of infants diagnosed with CMPA, before and after nutritional treatment with the eHF.

MATERIALS AND METHODS

Study population

This was a prospective, open, multicenter study conducted between January 2010 and December 2012 in different hospitals in Spain (Hospital Virgen de las Nieves (Granada), Hospital Vall D'Hebrón (Barcelona); Hospital Infant a Sofía and Hospital Gregorio Marañón (Madrid)). The study was approved by Ethics Committee of the La Paz Hospital acting as the leading center, and by similar bodies of each participating center. A written informed consent was obtained from each infant's parents before the challenge testing with the new extensively hydrolyzed formula. This study was performed according to Good Clinical Practice guidelines and conform the Helsinki Declaration. The inclusion criteria were: Infants diagnosed with CMPA based on clinical history of IgE-mediated CMPA, acute cutaneous symptoms (erythema, urticaria, angioedema), which seemed to be related with the intake of cow's milk, a positive test in IgE antibodies specific to cow's milk and or milk protein (skin prick test (SPT) and/or measure-specific IgE antibodies) and OFC test, except when the challenge was contraindicated in accordance with recent guidelines [4]. Infants were excluded if they had any serious gastrointestinal tract diseases other than CMPA or lactose intolerance, congenital cardiovascular, kidney, liver diseases, and other diseases which could mask the study results.

Study design

Forty seven infants (26 boys, 21 girls; age 4.6 ± 1.8 months (mean ± SD) who had been previously diagnosed with CMPA, were selected for the study. A diagnosis of immediate hypersensitivity to CMPA was made according to the following criteria: suggestive clinical history based on the occurrence of acute symptoms within 2 hours after the ingestion of a cow's milk-based formula, milk-specific immunoglobulin IgE as indicated by positive SPT or high serum milk specific IgE levels and/or positive challenge procedure.

Skin prick tests were performed according to standard procedures with 5% wt/vol whole cow's milk (Alergia e Immunología Abelló SA, Madrid), and α-lactalbumin, β-lactoglobulin, seroalbumin and casein (Sigma® Chemi cal). Each protein powder (10 mg) was dissolved in 1 mL of phosphate-buffered saline (PBS). Mean wheal diameters were determined after 15 minutes. Histamine (1 mg/100 ml) and PBS served as

positive and negative controls, respectively. Wheal areas > 3 mm in diameter were accepted as positive reactions.

Serum-specific IgE antibodies to whole milk and milk proteins were measured by a commercially available enzyme linked immunoabsorbent assay (CAP System, Pharmacia Diagnostic, Uppsala, Sweden). The test result was considered positive when more than 0.35 kU/l were detected.

A food challenge test for CMP was performed if not contraindicated. Unblinded controlled challenge tests were performed. The infants were given a standard infant formula starting with 2 ml. The quantities administered were increased up to 5 and 10 ml on the same day and then to 25, 50 and 100 ml on subsequent days. If no reaction occurred, the test was considered negative. The results were considered positive for immediate hypersensitivity to cow's milk when objective clinical manifestations (urticaria, erythema, angioedema, vomiting, diarrhea, anaphylaxis and others) occurred within 2 hours of ingestion of the standard infant formula. Challenge tests were not performed in infants with histories of anaphylactic reactions and evidence of specific IgE antibodies since the probability of a positive provocation in such cases is 90% [4,15].

Extensively hydrolyzed formula

The tested formula based on extensively Hydrolyzed casein (20%) and extensively hydrolyzed whey proteins (80%) derived from cow's milk, contained a molecular weight distribution in which 26% of the peptides had between 1000 to 3000 Da and 64% had a molecular weight of less than 1000 Da. The mean molecular weight was 989 Da and the highest was less than 2150 Da. It contained extensively hydrolysates as protein source, lactose and maltodextrin as carbohydrate sources, vegetable fats and added vitamins, minerals, DHA and AA and nucleotides. The composition in normal dilution is shown in Table (1). Its composition complied with the European Directive 2006/141 for infant and follow-on formulas and European Directive 1999/21 for foods for special medical purposes. *In vitro* tests (ELISA assays, molecular weight distribution) indicated the hypoallergenicity of the eHF Table (2), as did an *in vivo* animal study carried out at the Institute of Nutrition of the University of Granada. An Active Systemic Anaphylaxis test (ASA test) was carried out and the quality protein efficiency ratio (AOAC 960.48) [16] of the hydrolysates used as source of protein in the composition of this eHF was calculated (Table 3).

Safety and tolerance measures

The safety and tolerance of the study formula were assessed after recruitment by means of an OFC test. Infants tolerant to the study formula were followed up for approximately 3 months. The percentage of infants who developed allergic symptoms and the percentage of allergic symptoms related to the study formula during the first three months of feeding were also evaluated.

An evaluation of growth parameters was made by means of weight and height gain measurements from the first visit up to 3 months later. Weight for age, length for age and weight for length were also calculated as z-scores according to the WHO Child Growth Standards) [17].

Table 1: Average nutritional composition of the study formula.

			/100 ml
Energy		Kcal	69
Protein		g	1.8
Fat		g	3.6
	Linoleic acid	mg	585.1
	Linolenic acid	mg	53.6
	Arachidonic acid	mg	16.8
	Docosahexaenoic acid	mg	16.8
Carbohydrates	g	7.4	
	Lactose	g	6.3
	Maltodextrins	g	1.1
Nucleotides		mg	3.4

Table 2: Antigenic Characterization of the extensively hydrolyzed infant formula.

Parameters	Data
Highest Molecular Weight (MW) (Da)	2,150
% MW > 3,000 Da	0
% 3,000 > MW > 1,000 Da	36
% MW < 1,000 Da	64
MW Med. Da	989
% Free amino acids MW < 200 Da	3

Abbreviations: Molecular Weight (MW) Daltons (Da)

Table 3: Characterization of the Hydrolysates.

Indexes	Extensively	Extensively Hydrolyzed
	Hydrolyzed	Casein
	Whey	
% MW Distribution > 3,000 Da	0	0
Active Anaphylaxis test	Pass	Pass
% Biological value BV	72.99 ± 5.54	75.27 ± 6.12
Protein efficacy ration PER	3.62 ± 0.23	3.69 ± 0.26

Abbreviations: PER: Protein Efficacy Ratio; BV: Biological Value

Follow up visits were carried out 1 month, 2 months and 3 months after enrolment. At each visit, the researcher drew up a general clinical history and evaluated the tolerance to the study formula and any adverse events, examining symptoms in a daily notebook kept by the parents. Investigators also assessed all adverse events (AEs) for severity (mild, moderate, serious) and relation (unlikely, possible or likely) to the study formula. AEs were considered serious if they were fatal, life threatening, caused permanent harm or required extended in-patient treatment. A relation was considered probable if there was a temporary relationship with the ingestion of the formula or might be due to another cause, however improbable.

Blood analysis

As mentioned above, total and/or cow's milk specific immunoglobulin E (IgE), were determined by fluorescence enzyme immunoassay in the diagnosis, and a range of cytokines

in plasma (IL-4, IL-8, IL-13, IL-17A, IL-10, INF-ɣ, TGF-β), were measured by means of immunoassay with a MILLIplexTM kit using the Luminex 200 system based in the xMap technology, as described by Kellar et al., [18]. These measures were carried out in a subgroup at baseline and after 3 months of nutritional intervention with the study formula at the Institute of Nutrition of the University of Granada.

Several biochemical parameters were also measured in a subgroup of infants diagnosed with CMPA. Docosahexaenoic acid (C22:6 w-3, DHA) and arachidonic acid (AA) (C20:5 w-6, AA) were measured in red blood cells by gas chromatography coupled to a flame ionization detector, as described by Olza et al. [19], at the Institute of Nutrition of the University of Granada

Infants were included after the diagnosis was confirmed in accordance with recent guidelines [8]. Included infants were fed the eHF for 3 months. Weaning foods were introduced following pediatricians' advice with specific recommendations to avoid products containing cow's milk.

Statistical analysis

This study was performed to investigate the hypoallergenicity of an eHF according to the criteria established in scientific guidelines [6,7], according to which at least 90% of CMPA infants should tolerate the formula with a confidence of 95%. Following the statistical procedure suggested by the AAP guidelines, in a study with a binomial outcome (reaction *versus* no reaction), the sample size can be determined by calculating a binomial CI for p (the probability of having a reaction). In the case of 0 observed reactions, the upper 95% CI for p is <0.10 when the sample size is 29 participants. In this study, considering possible dropouts of 20%, the target was to recruit 36 diagnosed infants [20].

Statistical analysis was carried out using the SPSS program, version 18.0 (SPSS Inc, Chicago, IL, USA). A Student t test was used for the comparative study of quantitative variable. Wilcoxon's non parametric test was used for any quantitative variables that did not follow a normal distribution. Differences were considered statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

Outcomes

The baseline characteristics, symptoms and diagnosis of the diagnosed CMPA infants are shown in Table (4). All of them had an immediate type of reaction: approximately 81% of the infants showed cutaneous eruption, 30% tract symptoms such as vomiting, 21% pruritus, 17% urticaria and 11% angioedema, whereas anaphylaxis, abdominal pain, colic or thrive failure were less common (2%). CMPA was confirmed in all the patients according to the diagnosis criteria. 79% of patients had a positive skin prick test result to some of the milk proteins. An OFC test with CMP was carried out in 37 of infants (79%), while ten (21%) were not challenged with cow's milk because provocation was contraindicated.

The safety and tolerance of this formula were evaluated by means of an OFC test. Only one of the subjects experienced an immediate anaphylaxis after provocation with the eHF, while the formula was tolerated by 46 of the 47 infants, a tolerance level of

Table 4: Baseline characteristics, symptoms and diagnosis.

Characteristics at enrolment	
Sex: male/female	26/21
Age (mean±SD) months	4.6±1.8
Prevalence % of symptoms	
Cutaneous eruption	81
Vomiting	30
Pruritus	21
Urticaria	17
Angioedema	11
Diarrhoea	9
Sibilance, bronchospasm	4
Colic, abdominal pain, rhinorrhoea, conjunctival hyperaemia, anaphylaxis, thrive failure, colic, abdominal pain, others	2
Positive SPT to β-lactoglobulin, n (%)	77
Skin Prick Test: Positive to casein, n (%)	66
Skin Prick Test: Positive to α- lactalbumin, n (%)	66
Skin Prick Test: Positive to seroalbumin, n (%)	11
Skin Prick Test: Positive to cow's milk, n (%)	81
Skin Prick Test: Positive to some CM proteins, n (%)	79
Positive serum IgE to β-lactoglobulin, n (%)	67
Positive serum IgE to casein, n (%)	48
Positive serum IgE to α-lactoallbumin, n (%)	59
Positive serum IgE to seroalbumin, n (%)	19
Positive serum IgE to cow's milk, n (%)	74

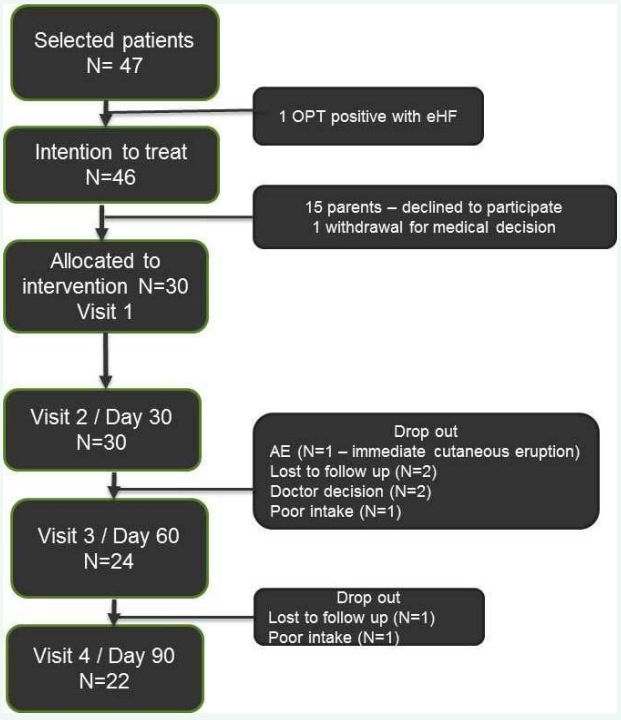

Figure 1 Flow Chart.

98% (95% CI: 94-100%) (Figure 1). This means that more than 90% of the infants tolerated the formula, thus complying with the requirements established by various scientific bodies [6,7].

Furthermore, 30 out of 47 infants with confirmed CMPA were included in the follow-up study after the written informed consent was signed by the parents; 15 infants were not included because the parents declined to participate and 1 infant for a medical decision (Figure 1). Before the end of the study, 2 infants dropped out due to poor intake, with the argument that infants did not like the taste of the formula; 3 for non-attendance at follow-up visits, 2 infants because of their doctor's decision and one infant due to an adverse reaction. As a result, the dropout rate was 27% (8 infants out of 30).

With reference to the evolution of the number of episodes (symptoms) and adverse events, after the first month of treatment, one infant experienced immediate cutaneous eruption (of moderate intensity) and dropped out since their doctor considered this adverse event probably related to consuming the formula. During the following two months no infants dropped out for intolerance or showed allergic symptoms related to the consumption of the formula (Table 5). At the end of the follow-up, no serious adverse event related to the study formula had been recorded. From the beginning to the 3rdmonth of the dietary treatment with the study formula, the number of symptoms gradually became fewer (Table 5). 11 symptoms were recorded during the first months of life in 3 of the 30 patients with CMPA. After the 2ndand 3rd months of treatment, no symptoms were observed.

Regarding the nutritional efficacy of a hypoallergenic formula, this should be demonstrated by a longitudinal study of weight and height development in at least 20 infants [8]. In our case, 30

Table 5: Evolution of the symptoms according to medical criteria.

	At inclusion, Before challenge N=30	1 months	2 months	At 3 months
		N=30	N=24	N=22
Number of infants with Episodes	30	3	0	0
% of infants with episodes	100	10	0	0
Number of total symptoms	52	11	0	0
% of total symptoms	100	21	0	0
Number of infants with Cutaneous eruption	25	2	0	0
% of infants with cutaneous eruption	87	10	0	0
Number of infants with pruritus	10	2	0	0
% of infants with pruritus	33	7	0	0
Number of infants with vomiting	10	3	0	0
% of infants with vomiting	33	10	0	0
Number of infants with diarrhoea	2	1	0	0
% of infants with diarrhoea	7	3	0	0

Table 6: Anthropometric data at inclusion and after 3 months of feeding with extensive hydrolyzed formula.

	At inclusion	At 3 months	
	N=30	N=22	
Age (months)			P (intra-groups)
Mean ± SD	4.4 ± 1.7	7.6 ± 1.7	
Range	1-9	4 - 12	
Weight (kg)			
Mean ± SD	7.0 ± 1.5	8.8 ± 1.3	<0.001*
Min- Max	2.81-9.6	7.0-11.9	
% Weight Gain	125		
Weight for age z score			
Mean ± SD	0.2 ± 1.0	0.6 ± 1.0	< 0.05**
Length (cm)			
Mean ± SD	63.9 ± 4.8	71.7 ± 3.8	<0.001*
Min- Max	50.0 - 74.0	66.0-78.0	
Length for age z score			
Mean ± SD	0.3 ± 1.1	1.2 ± 1.3	<0.01**
Weight for Length z score			
Mean ± SD	0.2 ± 1.5	0.1 ± 1.1	0.733**

*Student's t Test
**Wilcoxon's Test

infants were fed with the study formula for at least 1 month and twenty two for 3 months. The growth parameters were evaluated with the intention of treating them as z-scores according to the WHO Child Growth Standards (Table 6). By the third month of feeding with the study formula, the weight for age and length for age had increased (Table 6). The *z*-score of weight for age was always positive and close to the median, meaning there was no risk of under nutrition or obesity.

The percentages of DHA and AA in red blood cells before and after the nutritional treatment did not show statistical differences although there was a trend to increase after the three months of the treatment (AA: 5.61 ± 2.11 *vs* 7.57 ± 1.51; DHA: 4.50 ± 1.28 *vs* 5.94±1.01), while neither linoleic nor linolenic acid showed statistically significant change or trend (LA: 9.99 ± 2.44 *vs* 10.31 ± 1.18; LNA: 1.16 ± 0.60 vs 1.05 ± 0.57).

In the case of parameters related to the immune system such as cytokines in plasma, IL-8, which is considered an intestinal inflammatory marker, showed a statistical decrease after the 3 months of nutritional treatment with the eHF (mean ± SEM: 33.78 ± 22.58 vs 5.66 ± 1.40 pg/ml, U Mann Whitney U p=0.03). IL-4 and IL-13, cytokines involved in the allergic response (Th2 response), were not detected, and IL-10, a cytokine related to tolerance, tended to increase (8.91 ± 2.37 vs 11.39 ± 3.21 pg/mL). Similarly, baseline eosinophil values were even higher than maximum limits (0 – 700 cells/mm 3) and after the 3rdmonth of nutritional treatment a statistically significant decrease was observed (1458 ± 2570 vs 417 ± 402 cell/mm^3).

This is the first study on the safety, tolerance and efficacy of this eHF in infants with CMPA. The tolerance of this formula was demonstrated by means of the OFC test, the result of which showed a tolerance of 98% (95% CI: 94-100%) (1 out of 47 infants reacted). Martin et al. [28], found a tolerance of 94% for a casein-whey (40-60) protein hydrolysate formula (95% CI: 84.4-100%) (2 out of 33 reacted) and Giamprieto et al. [20], reported a tolerance of 92% (95% CI: 75-100%) in the challenge test with an extensively hydrolyzed whey formula (2 out of 25 infants reacted) (Profylac). These studies confirm that a very small number of infants react to even extensively hydrolyzed formulas. The safety and tolerance of this kind of formula have been extensively established by means of a challenge test [20, 21].

During the follow-up study, one infant in the first month of nutritional treatment, had an immediate cutaneous reaction and dropped out since doctor considered this adverse event was probably related to consumption of the formula although it was of moderate intensity. Reche et al. [22], also observed an immediate reaction of urticaria in an infant fed with an eHF. Indeed, in the study developed by Seppo et al. [23], two infants in the group fed with eHF were seen to have an adverse reaction to the study formula, and Niggemann et al. [24], observed the same frequency of adverse events in an eHF and in an amino acids formula.

The clinical trials developed with the aim of following up the allergy related symptoms seem to provide support for the usefulness of these formulas in the nutritional treatment of infants with CMPA. In our study, from the beginning of the dietary treatment with the study formula, the number of allergic symptoms gradually fell and after the 2ndand 3rd months of treatment, no symptoms were observed. In a recent published study with an extensively hydrolyzed rice formula, the evolution of symptoms was similar to our results [25].

Observational and interventional studies have shown that infants with allergies usually suffer impaired growth compared to healthy infants, so that hypoallergenic formulas should ensure normal development. However, the data available on the nutritional adequacy of eHFs are insufficient. Our data confirm that after three months of feeding with the study formula, the weight for age and length for age increased and had caught up with WHO standards. Recently, Dupont et al. [26], reported that infants with CMPA, fed with an extensively hydrolyzed casein formula, also experienced an improvement in the weight for age z score (-1.1 ± 1.3 to -0.5 ± 1.1), length for age z score (-1.3 ± 1.6 to -0.8 ± 1.2) and weight for length z score (-0.1 ± 0.9 to -0.3 ± 1.0) following the 6 months of feeding such as happened in our study. Other evidence supporting our results concerning adequate growth was provided by the biological values from *in vivo* animals assays. As far as we know, few studies have studied the effect in growth of the nutritional treatment of extensively hydrolyzed formula and, in some cases; we found that different tables of growth had been used (Finnish references growth data, Center for Disease Control and Prevention, Euro Growth Standard). However, it seems that for the first year of life, the best choice for monitoring growth is the WHO Child Growth Standard [17].

AAFs and eHFs are characterized by their bitter taste and Pedrosa et al. [9], found a significant correlation between peptide weight, reflecting the degree of hydrolysis of each formula, and the scores obtained for taste, texture, and overall palatability. Before the end of our study, two infants dropped out on account of poor intake one infant after the second month of the treatment and other one was before the third month of the treatment (Figure 1) with the argument that the infants did not like the taste of the formula. This situation seems to be very common with this kind of formula [6,9,22,25]. One of the main complaints of parents was that infants reject hydrolyzed formulas due to their unpleasant taste. This was further shown in two studies performed with hydrolyzed rice protein formulas [22,25], which are considered an alternative in the case of CMPA [5]. Pedrosa et al. [9], showed that the acceptability of the formulas designed for the nutritional treatment of CMPA depends on the bitter, bad taste, which is usually rejected by infants and seems to be the cause of frequent complaints by parents. For this reason, in this kind of clinical trial in which tolerance is the main outcome, rejection of a formula due to the bitter taste should not be confused with rejection of a formula due to allergic symptoms; in the above studies, the dropout rate was high and one of the main reasons was the taste acceptance of the formula. In the study carried out by Del Gliudice et al. [27], whey eHFs were judged more palatable than casein eHFs and AAFs. This situation was also evident in the study of Pedrosa et al. [9], and even formula designed with whey and casein was the most palatable formula. The formula tested in the present study is the only formula in the market with a whey-casein ratio of 80:20. One reason for this design was to decrease the amount of peptides from casein protein, which are known for their strong bitter taste [9]. The

biological value of these hydrolysates, extensively hydrolyzed whey and extensively hydrolyzed casein, were analyzed, and the growth rate, tolerance and safety in infants with CMPA was tested. Although in recent years, extensively hydrolyzed rice formulas have been increasingly recommended for the treatment of CMPA, the quality of the protein from cow's milk protein is higher than that of hydrolyzed rice protein.

Another outcome in the study formula was the presence of DHA and AA (0.45% of total fatty acids) in similar levels to those of DHA and AA in human milk [28]. It is known that these fatty acids are involved in visual acuity, cognitive development, and that they are precursors of eicosanoid and docosanoid mediators involved in the immune response [29]. In our study, the levels of DHA and AA found in red blood cells in infants fed with the eHF, were similar to the levels found in the red blood cells of infants fed breast milk (DHA: 5.94 ± 1.01% of total FAs vs 5.6 ± 0.7% of total FAs) in the study by Makrides et al., [30]. This is the first work to study the role of DHA and AA in the nutritional treatment of infants with CMPA. While a great number of studies have been published about the role of DHA in the prevention of allergy and supplementation during the gestational and lactating period [10,11], there is still a gap in our knowledge of the role of these LCPUFAs in the tertiary prevention of allergy. To date, only some *in vitro* and *in vivo* animal studies [12] have tested and demonstrated certain roles for DHA and AA in the regulation of the immune response. Currently, no nutritional strategies have been found to be effective as secondary or tertiary preventive measures and more longitudinal studies are needed to clarify these issues.

CONCLUSION

In this study the eHF was well tolerated by infants with CMPA. According to the results obtained in the safety, tolerance and nutritional validation, this eHF formula complied with the requirements established by the scientific bodies (AAP, ESPGHAN). This formula would be useful for the nutritional treatment of infants with proven CMPA.

This formula contains DHA and AA in levels similar to those in human milk and it seems that the presence of these LCPUFA contribute to the development of the correct immune response and the development of tolerance. From a scientific point of view, more research will be necessary to elucidate the role of these fatty acids in the In the tertiary prevention prevention of cow's milk allergy during the first years of life, while it seems that an analysis of cytokines could be an innovative and feasible tool for the more accurate diagnosis of CMPA.

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