LETTER TO THE EDITOR

Peptide size profile and residual immunogenic milk protein or peptide content in extensively hydrolyzed infant formulas

To the Editor,

Current clinical food allergy guidelines recommend an extensively hydrolyzed formula (EHF) as the first-line treatment in nonbreast-fed infants with cow's milk protein allergy (CMPA).[1,2] This recommendation is based on the assumption that EHF is tolerated by at least 90% of infants with CMPA.[3] Several studies have reported allergic reactions to EHF.[4,5] This includes the Dutch EuroPrevall cohort study which achieved adequate symptom control in <50% of EHF-treated infants with CMPA.[4] We hypothesized that the observed residual allergenicity was due to insufficient milk protein hydrolysis and/or contamination with milk allergens.[3,6] In order to better understand the observed variability in clinical efficacy, we aimed to characterize a representative sample of marketed EHF with regard to their peptide molecular weight (MW) profile, content of residual immunogenic cow's milk proteins or peptides, and in vitro allergenicity.

Between 2014 and 2018, we collected samples (cans) of 76 commercially available whey- and casein-based EHF (EHF-W and EHF-C) products positioned for the management of CMPA, from 9 manufacturers. To determine possible between- and within-batch variation, samples from different production batches, as well as multiple cans of the same batch, were analyzed when available; Table S1. Product samples were coded and blinded for analysis. Peptide size distribution analysis was performed by size-exclusion, high-pressure liquid chromatography. As a surrogate marker for potential allergenicity, an arbitrary cutoff of >1200 Da was chosen (equivalent to the MW of 10-12 amino acids). Immunogenic peptides or proteins (IPP) derived from bovine beta-lactoglobulin (BLG) and casein were quantified by high-sensitivity ELISA (Euroclone Spa, Pero, Italy). In addition, a subset of 9 EHF products with a range of percentages of peptides with a MW > 1200 Da was assessed for residual BLG-induced in vitro allergenicity, using a humanized rat basophilic leukemia cell degranulation assay. This assay was developed with IgE directed against "allergenic" immunodominant regions/epitopes on bovine BLG, also recognized by serum IgE from CMPA infants.[7] A detailed description of the laboratory methods is provided in Table S2.

Characterization of the MW profiles found that 89%-100% of EHF peptides were <2400 Da; Table 1. Three clusters were observed for the content of IPP with a MW > 1200 Da: <5% (Group 1; n = 14), 5%-15% (Group 2; n = 12), and > 15% (Group 3; n = 7); Figure 1. All EHF-C analyzed were in Group 1. There was variability in the content of peptides <240 Da (6 to 38%) and <600 Da (37 to 88%); Table 1. For some products (W1, W21, and C12), significant MW profile differences were noticed between or within batches; Table S3.

Residual BLG-derived IPP were detected in 4 of 12 (33%) EHF-C and 18 of 21 (86%) EHF-W products. Four EHF-W products (W1, W2, W3, and W4) showed residual BLG-IPP exceeding the limit of quantification (0.01mg/kg) by 20-fold, including one product with an IPP content of >2000 times the quantification limit; Table 1. One of 12 (8%) and 2 of 21 (10%) EHF-C and EHF-W tested positive for casein-derived IPP, respectively. Two samples showed significant between- and within-batch variation for both casein- and BLG-IPP contents (C12 and W1). Three further EHF-W products (W2, W3, and W4) displayed noticeable between- and within-batch variation for residual BLG-IPP content; Figure S1.

A positive relationship between residual BLG-IPP content and the percentage of peptides >1200 Da was found (R^2 = 0.65); Figure S2A. An inverse association was demonstrated for peptides with a MW < 240 Da (R^2 = 0.89); data not shown. For the subset of 9 EHF (2 EHF-C and 7 EHF-W), BLG-induced RBL cell degranulation levels varied depending on the content of peptides >1200 Da; Table 1. No BLG-induced in vitro allergenicity was detected for the 3 samples in Group 1 (C1, C4, and W7). The remaining 6 EHF-W in Groups 2 and 3 induced a dose-dependent degranulation with a calculated residual allergenicity ranging from 272 to 1881 µg BLG/g protein. These exploratory data suggest a close relationship between residual BLG-induced in vitro allergenicity and the content of peptides with a MW > 1200 Da (R^2 = 0.79); Figure S2B.

We characterized the physicochemical profiles of a representative sample of marketed EHF-W and EHF-C products. There was significant variability in the MW profile of peptides, residual BLG- and casein-IPP contents, and in vitro allergenicity, with significant batch-to-batch or within-batch variation observed for some products. These findings are in keeping with earlier studies

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TABLE 1 Number of analyzed samples (*n*) per product, peptide MW distribution (median, maximum deviation from the median), residual BLG- and casein-derived IPP contents (median, maximum deviation from median) and, where available, BLG-induced in vitro allergenicity (one sample analyzed per product). The table separates EHF-W and EHF-C categories. Within each category, samples were ordered by decreasing residual BLG content

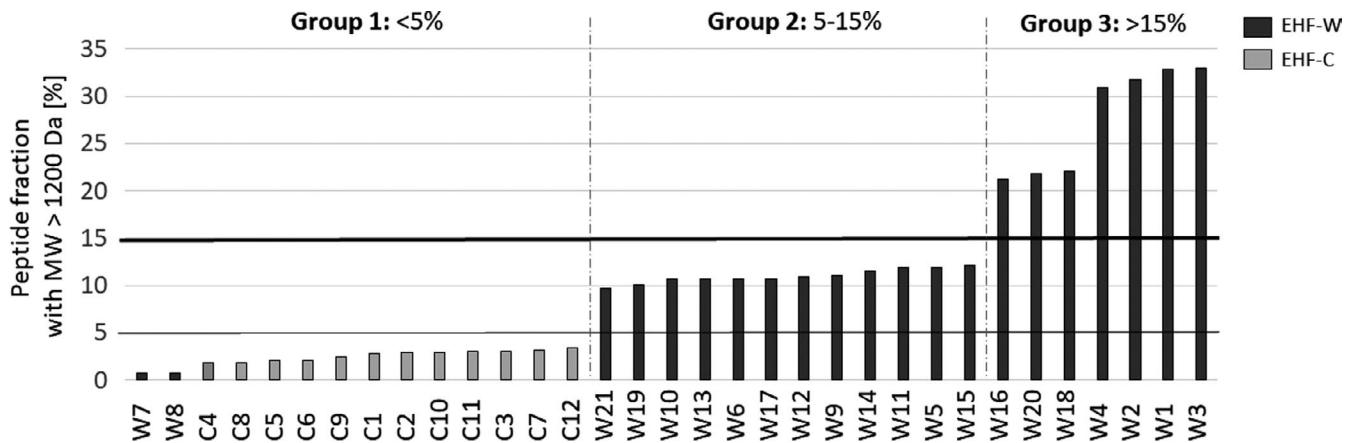| Product	Coding	n	Peptide MW distribution [%]						BLG and Casein [mg/kg]				BLG allergenicity [μg/g]
			<240 Da	<600 Da	<1200 Da	<2400 Da	MW Max Dev.	>1200 Da	BLG Median	BLG Max Dev.	Casein Median	Casein Max Dev.	
Friso PEP	W1	3	6	38	67	91	38	33	20.30	20.29	0.5	0.6	747
Picot® Pepti Junior®/ Croissance 3	W4	3	13	37	69	91	4	31	0.27	7.57	<0.2	0.1	1465
Picot® Pepti Junior 2	W3	2	13	39	67	89	3	33	0.21	0.10	<0.2	0.0	
Picot® Pepti Junior 1	W2	4	12	37	68	91	4	32	0.20	0.09	0.3	0.1	1881
Pepti Junior®	W20	1	17	49	78	95		22	0.06		<0.2		
Aptamil® Pepti Junior	W16	1	16	49	79	95		21	0.06		<0.2		
Nutrilon® 1 Allergy Digestive Care	W18	1	15	48	78	95		22	0.05		<0.2		
Aptamil™ Pepti 1	W5	4	22	60	88	98	2	12	0.05	0.03	<0.2	0.0	626
Nutrilon™ Pepti 2	W19	1	22	63	90	98		10	0.05		<0.2		
Nutrilon® Pepti 1 ProExpert	W14	4	22	60	89	98	1	11	0.04	0.02	<0.2	0.0	
Aptamil™ Pepti 2	W6	1	22	62	89	98		11	0.04		<0.2		
Nutrilon™ 1 Allergy Care	W11	1	22	60	88	98		12	0.04		<0.2		
Nutrilon™ 2 Allergy Care	W12	1	22	61	89	98		11	0.04		<0.2		
Nutrilon® Pepti 2 ProExpert	W15	4	23	60	88	98	1	12	0.04	0.00	<0.2	0.0	425
Nutrilon Pepti 1	W13	4	23	61	89	98	2	11	0.03	0.03	<0.2	0.0	
Aptamil® Allerpro™ 1 Gold+	W9	4	22	55	89	98	2	11	0.03	0.02	<0.2	0.0	
Pepticate®	W21	4	23	60	90	98	26	10	0.03	0.01	<0.2	0.0	272
Aptamil® Allerpro™ 2 Gold+	W10	3	24	57	89	98	2	11	0.02	0.02	<0.2	0.0	
Galliagene®	W17	1	22	62	89	98		11	<0.01		<0.2		
Althéra®	W7	6	34	86	99	100	4	1	<0.01	0.01	<0.2	0.0	<10.8
Alfaré®	W8	5	34	86	99	100	3	1	<0.01	0.01	<0.2	0.0	
Damira® 2000	C12	3	20	77	97	99	7	3	0.06	0.05	0.6	0.4	
Nutriben® Hidrolizada 1	C2	1	18	78	97	100		3	0.05		<0.2		
Allernova AR	C11	2	19	77	97	99	1	3	0.02	0.01	<0.2	0.0	
Friso Pep AC	C3	1	22	78	97	99		3	<0.01		<0.2		
Nutramigen® Lipil 1	C7	1	31	85	97	98		3	0.01		<0.2		
Similac® Alimentum	C1	3	38	88	97	99	2	3	<0.01	0.00	<0.2	0.0	<10.8
Nutramigen	C4	2	34	83	98	99	0	2	<0.01	0.00	<0.2	0.0	<10.8
Nutramigen LGG®	C5	1	36	87	98	99		2	<0.01		<0.2		
Nutramigen LGG® 1	C6	1	32	87	98	99		2	<0.01		<0.2		
Pregestimil Lipil	C8	1	33	87	98	99		2	<0.01		<0.2		
Nutramigen LGG® 2	C9	1	33	86	98	99		2	<0.01		<0.2		
Nutramigen® Lipil 2	C10	1	34	87	97	98		3	<0.01		<0.2		

FIGURE 1 Percentage of peptides with MW > 1200 Da. EHF-W and EHF-C products are depicted in dark gray and light gray bars, respectively. The black lines represent the 5% and 15% threshold. Three EHF groups were identified according to the fraction of peptides with a MW > 1200 Da

demonstrating significant heterogeneity among marketed EHF regarding their peptide composition and clinical safety.[5,8,9]

The enzymatic hydrolysis and heat treatment used during manufacturing of EHF are designed to disrupt the vast majority of allergenic epitopes. The final product safety of an EHF relies on multiple processes, including effective protein hydrolysis, the removal of residual allergenic peptides or proteins by ultrafiltration (for some products) and ongoing quality management. The significant residual BLG- or casein-derived IPP found in some EHF products suggests incomplete hydrolysis and/or contamination during manufacturing. Furthermore, the significant batch-to-batch or within-batch variation observed may be due to inadequate quality management for some EHF products. The content of peptides with a MW > 1200 Da appeared to closely correlate with both the residual BLG-IPP content and BLG-induced in vitro allergenicity. The percentage of peptides with a MW > 1200 Da may therefore provide a useful reference for comparison of the residual allergenicity between EHF products. This is particularly relevant for EHF-W because the globular, three-dimensional structure of whey proteins renders the final peptide profile highly dependent on hydrolysis conditions. By contrast, caseins are more easily hydrolyzed.

Our report has several limitations. Firstly, the products analyzed represent a selection of commercially available EHF. Secondly, our findings only apply to the product characteristics at the time of sampling, and recipes or quality management standards may have changed since. Importantly, the clinical implications of our findings are at this stage uncertain, and further studies are needed. Despite these limitations, our survey highlights the need for a more meaningful definition of EHF products. Efforts should be made to standardize analytical methods for residual allergen detection, improve quality control measures during EHF manufacturing, and define minimum clinical evidence requirements for product safety.

CONFLICT OF INTEREST

The authors declare the following potential conflicts of interest: SN, AJ, MK, and RH are salaried employees of Nestlé Health Science, Switzerland, which sponsored the study. The other authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Improving timely access to food allergy care: A pragmatic controlled trial

To the Editor,

As rates of food allergy rise, specialist allergy services struggle to manage demand[1] and waiting times to access such services increase.[2] In many regions, allergy care is primarily delivered by allergists, due to limited allergy training opportunities for general pediatricians and primary care physicians. At the Royal Children's Hospital (RCH), Melbourne, Australia, children referred to the Department of Allergy and Immunology for suspected food allergy wait around 12 months (with the exception of patients with anaphylaxis or aged under one who are seen sooner).[3]

The diagnosis of food allergy relies on clinical history together with demonstration of allergen-specific IgE (sIgE). sIgE may be detected by skin prick test or serum levels of sIgE (ssIgE) and both are applied in the clinical setting, either alone or together.[4] Recent data have confirmed the reliability of ssIgE testing for the diagnosis of food allergies, paving the way for a decentralized model of care.[5] In 2011/12, our team developed and piloted a Clinical Decision Support Program to upskill community general pediatricians in the diagnosis and management of simple food allergy using ssIgE testing. Pediatricians who completed our training course were able to manage 80% of children independently without referral to an allergist.[6]

We have now conducted a large, pragmatic, controlled trial to determine whether the community-based model of care can reduce time to assessment compared with standard specialist hospital-based care and deliver care that is of comparable safety and quality.

In this pragmatic controlled trial which commenced in 2015, we compared a control cohort (CC) with an intervention cohort (IC). CC families received standard hospital-based care at the RCH Allergy Clinic, and IC families were offered the opportunity to see a community-based general pediatrician who had completed the online Clinical Decision Support Training Program. IC families who elected not to take up an appointment remained in the IC for analysis and received standard hospital-based care and are still considered as part of the IC. Children aged 0-12 years newly referred to the RCH Allergy Clinic with suspected food allergy were eligible. Training included three 1-hour webinars, with specific guidance on when to test for simple food allergy using ssIgE testing, management of IgE- and non–IgE-mediated food allergy and eczema in the context of suspected/known food allergy, and provision of allergy resources and management plans if appropriate. The accompanying online Clinical Decision Support Program consisted of two flowcharts (one each for IgE- and non–IgE-mediated food allergy) which covered