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Efficacy of an infant formula manufactured from a specific protein hydrolysate derived from whey protein isolate and concentrate produced by Société des Produits Nestlé S.A. in reducing the risk of developing atopic dermatitis

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Abstract

The European Commission asked EFSA to evaluate the efficacy of an infant formula, containing a specific protein hydrolysate derived from whey protein isolate and concentrate and manufactured by Société des Produits Nestlé S.A., in reducing the risk of developing atopic dermatitis in infants with a family history of allergy. This was following the submission of a dossier by Société des Produits Nestlé S.A. to the European Commission, in the context of Regulation (EU) 2016/127. The protein hydrolysate from which the infant formula is produced is included in Annex I and II of Commission delegated Regulation (EU) 2016/127 as suitable protein source for the manufacture of infant and follow-on formulae. This opinion does not cover the assessment of the nutritional safety and suitability of the infant formula or the safety of the food enzymes used in the manufacture of the protein hydrolysate. The Panel considers that, in relation to the effect that is claimed, the infant formula under evaluation is not sufficiently characterised with respect to the molecular weight distribution of peptides. From the human intervention studies submitted, no conclusions could be drawn on the efficacy of the infant formula in reducing the risk of developing atopic dermatitis. The Panel concludes that a cause-and-effect relationship has not been established between the consumption of the infant formula under evaluation and the reduction in the risk of developing atopic dermatitis in infants with a family history of allergy.

Keywords: protein hydrolysate, characterisation, formula, allergy, atopic dermatitis, clinical trial, infants

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1. Introduction

1.1. Background and Terms of Reference as provided by the requestor

1.1.1. Background

Commission Directive 2006/141/EC[1] lays down harmonised rules applicable in the entire EU to infant formulae and follow-on formulae. The Directive allows the use of protein hydrolysates as source of protein in infant formulae and follow-on formulae under certain conditions (Articles 5–7; Annex I, point 2.2; Annex II, point 2.2 and Annex VI).

Commission delegated Regulation (EU) 2016/127[2] transfers the existing rules of Directive 2006/141/EC under the new framework of Regulation (EU) No 609/2013 of the European Parliament and of the Council[3] and revises them, based on the opinion of the European Food Safety Authority (EFSA) of 2014.[4] In that opinion, EFSA noted that '*the safety and suitability of each specific formula containing protein hydrolysates has to be established by clinical studies. Information on protein sources and the technological processes applied should also be provided. In this context, the Panel notes that one particular formula containing partially hydrolysed whey protein has been evaluated for its safety and suitability by the Panel (…) and has been authorised for use by Directive 2006/141/EC*'. EFSA also noted that '*the criteria given in Directive 2006/141/EC alone are not sufficient to predict the potential of a formula to reduce the risk of developing allergy to milk proteins. Clinical studies are necessary to demonstrate if and to what extent a particular formula reduces the risk of developing short- and long-term clinical manifestations of allergy in at-risk infants who are not exclusively breast fed*'.

Taking into account EFSA's opinion, the delegated Regulation establishes that infant formula and follow-on formula manufactured from protein hydrolysates should only be allowed to be placed on the market if their composition corresponds to the one positively assessed by EFSA so far and prohibits the use of health claims describing the role of infant formula in reducing the risk of developing allergy to milk proteins. The requirements of Commission delegated Regulation (EU) 2016/127 shall apply to infant formula and follow-on formula manufactured from protein hydrolysates from 22 February 2021.

Pursuant to Recital 21 of the Regulation, these requirements may be amended in the future in order to allow the placing on the market of formulae manufactured from protein hydrolysates with a composition different from the one already positively assessed, following a case-by-case evaluation of their safety and suitability by EFSA. In addition, if, after the assessment by EFSA, it is demonstrated that a specific formula manufactured from protein hydrolysates reduces the risk of developing allergy to milk proteins, further consideration will be given to how to adequately inform parents and caregivers about that property of the product.

The requirements of Commission delegated Regulation (EU) 2016/127 shall apply to infant formula and follow-on formula manufactured from protein hydrolysates from 22 February 2021. It can be expected that, before that date, dossiers on formulae manufactured from protein hydrolysates will be presented by food business operators for assessment by EFSA with a view to request possible modifications to the conditions applicable to these products in the delegated Regulation.

In this context, it is considered necessary to ask EFSA to provide scientific advice to the Commission on dossiers on formulae manufactured from protein hydrolysates submitted by food business operators for assessment by EFSA in the future.

EFSA will be informed by the Commission by letter when the applicant has been asked by the Commission to transmit the dossier to EFSA for scientific assessment.

[1] Commission Directive 2006/141/EC of 22 December 2006 on infant formulae and follow-on formulae and amending Directive 1999/21/EC, OJ L 401, 30.12.2006, p. 1.
[2] OJ L 25, 2.2.2016, p. 1.
[3] Regulation (EU) No 609/2013 of the European Parliament and of the Council of 12 June 2013 on food intended for infants and young children, food for special medical purposes, and total diet replacement for weight control and repealing Council Directive 92/52/EEC, Commission Directives 96/8/EC, 1999/21/EC, 2006/125/EC and 2006/141/EC, Directive 2009/39/EC of the European Parliament and of the Council and Commission Regulations (EC) No 41/2009 and (EC) No 953/2009, OJ L 181, 29.6.2013, p. 35.
[4] EFSA NDA Panel (EFSA Panel on Dietetic Products, Nutrition and Allergies), 2014. Scientific Opinion on the essential composition of infant and follow-on formulae. EFSA Journal 2014;12(7):3760.

1.1.2. Terms of Reference

In accordance with Article 29 of Regulation (EC) No 178/2002[5], the European Commission requests the European Food Safety Authority to issue scientific opinions on infant and follow-on formula manufactured from protein hydrolysates in particular, depending on the nature of the application, on:

1) the safety and suitability for use by infants of a specific formula manufactured from protein hydrolysates;
   If the formula under evaluation is considered to be safe and suitable for use by infants, the European Food Safety Authority is also asked to advise on the minimum specific criteria on protein source, protein processing and protein quality of the formula that need to be satisfied for the safety and suitability of such formulae to be demonstrated.
2) the product's efficacy in reducing the risk of developing allergy to milk proteins;
3) the product's efficacy in reducing the risk of developing allergy/allergic manifestations to allergens in general.

1.2. Interpretation of the Terms of Reference

The interpretation by the Panel on Nutrition, Novel Foods and Food Allergens (NDA) is that assessment of the safety of food enzymes (or their combination) that are used in the manufacture of the protein hydrolysate, is not to be assessed in this opinion. The assessment of the safety of the individual food enzymes is performed by the EFSA Panel on Food Contact Materials, Enzymes and Processing Aids (CEP) according to the guidance and statements of the CEF/CEP Panel (EFSA CEF Panel, 2009, 2016, 2019).

The interpretation by the NDA Panel is that the Terms of Reference cover, for this particular dossier, the assessment of the product's efficacy in reducing the risk of developing atopic dermatitis in infants with a family history of allergy, as requested by the food business operator.

Therefore, the conclusions of the Panel are related to the infant formula that was evaluated in the clinical studies submitted rather than on the specific protein hydrolysate used to manufacture the infant formula, as constituents of the infant formula other than the protein hydrolysate may also play a role in modifying the risk of allergic manifestations. The conclusions of the Panel are not related to the nutritional safety and suitability of the specific protein hydrolysate, the safety of the protein hydrolysate in general, including the safety of the individual enzymes or their combination, or the safety of the final formula.

2. Data and methodologies

2.1. Data

The assessment of the efficacy of the infant formula[6] in reducing the risk of developing atopic dermatitis in infants with a family history of allergy is based on the data supplied in the dossier submitted to EFSA (EFSA-Q-2019-00529) and the additional information provided by the food business operator upon requests.

A common and structured format for the presentation of dossiers related to infant and follow-on formula manufactured from protein hydrolysates is described in the EFSA scientific and technical guidance for the preparation and presentation of an application for authorisation of an infant and/or follow-on formula manufactured from protein hydrolysates (EFSA NDA Panel, 2017). For the present assessment, Section 3 in the EFSA guidance related to the nutritional safety and suitability of the hydrolysed formula is not applicable. As outlined in the guidance, it is the duty of the food business operator who submitted the dossier to provide all available scientific data which are pertinent to the dossier. The procedure followed by EFSA for handling dossiers on formulae manufactured from protein hydrolysates, the various steps in the procedure and estimated timelines are described online.[7]

[5] Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety, OJ L 31, 1.2.2002, p. 1.

[6] Infant formula means food intended for use by infants during the first months of life and satisfying by itself the nutritional requirements of such infants until the introduction of appropriate complementary feeding.

[7] http://www.efsa.europa.eu/sites/default/files/applications/apdeskapplworkflownutriinfant.pdf

2.2. Methodologies

The assessment follows the methodology set out in the EFSA guidance for the preparation and presentation of an application for authorisation of an infant and/or follow-on formula manufactured from protein hydrolysates (EFSA NDA Panel, 2017). Previous EFSA work (EFSA NDA Panel, 2014) and the regulatory framework[8] were also taken into account.

As outlined in the guidance, 'in order to substantiate the efficacy of a product in reducing the risk of developing allergy to milk proteins, at least one human intervention study on the incidence of allergy to milk proteins in the target population for which the formula is intended [...] should be provided'. Similar to health claims, data from studies in animals or other model systems alone cannot substitute for human data (EFSA NDA Panel, 2016), but 'should be provided if they may help to establish the potential of the hydrolysed formula to reduce the risk of developing allergy to milk proteins' (EFSA NDA Panel, 2017).

3. Assessment

3.1. Characterisation of the infant formula

The infant formula which is the subject of the dossier is an infant formula marketed in liquid and in powder form. The protein hydrolysate from which the infant formula is produced is included in Annex I and II of Commission delegated Regulation (EU) 2016/127 as a suitable protein source for the manufacture of infant and follow-on formulae.

Protein source of the hydrolysate:

The protein hydrolysate from which the infant formula is manufactured is produced from demineralised sweet whey protein derived from cow's milk that is composed of:

- 63% caseino-glycomacropeptide (CMP)-free whey protein isolate with a minimum protein content[9] of 95% of dry matter, and
- 37% sweet whey protein concentrate with a minimum protein content[9] of 87% of dry matter.

The demineralised sweet whey protein is used in the further manufacture of the protein hydrolysate [redacted]

Certificates of analysis for 11 batches of each of the raw materials were provided, i.e. for the whey protein isolate and the whey protein concentrate mentioned above. Individual intact proteins in whey have been identified in the dossier. On request from EFSA, the food business operator confirmed that the information provided on these individual intact proteins is based on publicly available data on protein composition of whey as presented in Farrell et al. (2004).

Protein processing to obtain the protein hydrolysate:

The protein hydrolysate and the infant formula in which the protein hydrolysate is used are manufactured under ISO 9001:2015 (quality management systems) and ISO 22000:2005 (food safety management systems). This is indicated in the certificates provided by the food business operator.

In order to produce the hydrolysate used in this infant formula, a two-stage hydrolysis process is applied whereby at each stage a trypsin preparation is added to the whey protein solution. Food enzyme preparations from [redacted] that was used to produce the infant formula investigated in the human intervention studies was presented by the food business operator as directly pertinent to the assessment of the efficacy of the formula. [redacted]

The trypsin preparation from porcine pancreas contains [redacted] trypsin (EC 3.4.21.4.) [redacted]. On request from EFSA, the food business operator explained that the trypsin preparation, [redacted] is added in amounts of at least [redacted] United

[8] https://ec.europa.eu/food/safety/labelling_nutrition/special_groups_food/children_en
[9] The content of the crude protein is calculated as Kjeldahl-N × 6.38.

States Pharmacopoeia (USP) units/g protein. [redacted]

Information on the absolute amount of food enzyme, expressed in mg/g substrate protein, has also been provided by the food business operator and is dependent on the amount of the food enzyme needed to reach a minimum reference enzyme activity per unit protein substrate weight. [redacted]

The hydrolytic process is conducted at [redacted] at a [redacted] The process is performed in two hydrolytic steps (i.e. the food enzyme preparation is added twice) with a heat treatment step ([redacted]) in between to denature the protein that would not yet be hydrolysed, after which the solution is cooled down again to [redacted]. The first hydrolytic step has a duration of [redacted] and the second a duration of [redacted]. The hydrolysis is concluded with a heat treatment of [redacted].

This last heat treatment inactivates [redacted] of trypsin, as documented in a report of one of the internal laboratories of the food business operator that is certified by ISO 17025:2005 (general requirements for the competence of testing and calibration laboratories). This report describes the calibration and provides results for [redacted] batches of powdered and liquid formula (batches of unspecified date). It is explained that the internal target is residual active trypsin below [redacted] in the finished product, [redacted]. The method for the residual trypsin measurement was described by the food business operator. The limits of quantification obtained from the lowest and highest trypsin concentrations of the calibration curve correspond to [redacted] of inactivation of trypsin. The residual activity of [redacted] was not measured.

[redacted]

Degree of hydrolysis and molecular weight distribution of peptides, content of free amino acids and residual proteins in the protein hydrolysate:

Data on the degree of hydrolysis (DH), the molecular weight distribution of peptides and the residual protein content have been provided only for the final formula (to which free amino acids are added) and not for the protein hydrolysate. These data are, therefore, described below in the context of the description of the characteristics of the final infant formula.

With respect to free amino acids in the protein hydrolysate, the food business operator indicated that no free amino acids are released during the hydrolysis process, due to the endoprotease specificity of the food enzyme preparation.

Manufacturing process of the infant formula:

In order to produce the powder formula under evaluation, the liquid hydrolysate is pre-heated, mixed with free amino acids, minerals, vitamins, carbohydrates, oils and optionally nucleotides. It is then heated again, evaporated, homogenised and evaporated again. Thereafter, additional ingredients are added before spray drying, as well as afterwards (dry-mixed with the powder) before sieving and final packing under protective atmosphere.

For liquid formula, which is also the subject of the dossier, after the addition of the last ingredients, the steps of the second heat treatment, evaporation and spray drying (applied in the case of the production of the powder) are replaced by sterilisation (ultra-high temperature treatment) before aseptic homogenisation, and aseptic filling.

Degree of hydrolysis and molecular weight distribution of peptides, content of free amino acids and residual proteins in the infant formula:

The food business operator based the calculation of the DH on the equation DH (%) = $h/h_{tot} \times 100$, where h is the number of cleaved peptide bonds and h_{tot} is the total number of peptide bonds present in the proteins before cleavage. The value of h is derived by measuring the increase in free amino groups in the source material ($AN_{final}–AN_{initial}$). Free amino nitrogen (AN) is determined by the reaction with trinitrobenzenesulfonic acid (TNBS),[10] and total nitrogen (TN) is determined by the Kjeldhal method. Free AN and TN before hydrolysis were measured in five batches of each of the

[10] TNBS method is an assay of the chromophore specifically formed by the reaction of TNBS with free amino groups.

source materials (i.e. the sweet whey protein concentrate and the CMP-free whey protein isolate mentioned earlier) and a weighted average was used. Free AN and TN after hydrolysis were determined in five batches of the final formula. In order to derive h, the AN/TN values were converted into mmol/g protein and AN before hydrolysis ($AN_{initial}$) was subtracted from AN after hydrolysis (AN_{final}). The total number of peptide bonds in the source material, h_{tot}, was derived from the amino acid analysis of four batches of each of the source materials, as sum of the mmol amino acids/g protein. The Panel notes that AN_{final} was measured in the formula which contains additional free amino acids instead of in the protein hydrolysate. Therefore, the Panel notes that the data presented by the food business operator do not represent DH and are only considered to approximate DH. Data have been provided for five different formula brands (one sample each) containing the protein hydrolysate, showing values in the range of [redacted].

Detailed descriptions of the internal methods of analyses of free AN and TN used, the corresponding validation files and the analysis table for the batches of the source materials and final formula were submitted by the food business operator. Amino acids were analysed using in-house methods in a laboratory of the food business operator that is accredited for this analysis (certificate of accreditation ISO 17025:2005 'general requirements for the competence of testing and calibration laboratories' provided).

In the original dossier, with respect to residual individual intact proteins in the protein hydrolysate, an analysis using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of one batch of the final infant formula, compared with protein standards, showed that no [redacted] and peptides/proteins above the molecular weight of [redacted] were present in the formula (the SDS–PAGE method was described). However, according to a new more sensitive method, i.e. lithium dodecyl sulfate–polyacrylamide gel electrophoresis (LDS–PAGE) provided by the food business operator, [redacted]. The validated method is not quantitative and the limit of detection was not formally assessed, but given at 1–2.5 mg/kg equivalent protein by the food business operator.

The food business operator also provided several experiments aimed at demonstrating a reduced content of [redacted] in the formula. The levels of [redacted] measured by ELISA were below 0.8 mg equivalent/g equivalent protein which was a pre-specified threshold set by the food business operator. This threshold was chosen to comply with the criteria laid down in Annex IV of Commission Directive 2006/141/EC, i.e. less than 1% of nitrogen containing substances in the formulae should be immunoreactive protein. In addition, an experiment measuring the residual [redacted] in response to the formula compared with intact [redacted]. [redacted] Although [redacted], there was considerable variability within the groups receiving the formula. This prevents any definitive conclusion being drawn from the [redacted] on the allergenicity of the formula. Although [redacted], the Panel notes that [redacted] though detectable by LDS-PAGE, has not been quantified and other allergenic proteins present in whey have not been investigated (e.g. [redacted]).

The molecular weight distribution of peptides in the final formula (to which free amino acids are added, depending on regulatory requirements) was analysed by size-exclusion chromatography with UV detection, as described in the publication by Johns et al. (2011). The chromatographic column used was a [redacted] on an Agilent 1260 HPLC system and with a UV wavelength of [redacted]. However, the analytical method applied by the food business operator differed from the published method in the use of different protein and peptide standards to calibrate the elution time axis. The food business operator explained that a calibration was obtained considering the elution time of a number of standard proteins, peptides and free amino acids. Data on the molecular weight distribution in [redacted] batches of the commercial and prototype protein formulae used ([redacted]) from different production sites were provided. Based on these data, the food business operator derived the following target ranges for the molecular weight distribution of peptides in the final infant formula:

< 1,000 Da: [redacted]
1,000–2,500 Da: [redacted]

2,500–5,000 Da: [redacted]
> 5,000 Da: [redacted]

The Panel notes that the ranges presented by the food business operator within each of the molecular weight categories are very broad. The variability in size distribution of the peptides within the individual categories that may be encountered may have an impact on the presence of the epitopes of allergens which, as a consequence, may vary between different batches of the formula. Therefore, the Panel considers that the data provided are insufficient to characterise the protein hydrolysate in relation to the effect that is claimed, i.e. the reduction of the risk of developing atopic dermatitis in infants with a family history of allergy.

The batch-to-batch variability of the molecular weight distribution of peptides as well as the average peptide size distribution over time (based on historical data) of the protein hydrolysate for each of the [redacted] food enzyme preparations could not be provided by the food business operator [redacted] However, data were provided for 5 or 6 independently produced batches of formula per food enzyme preparation (no certificate of analysis was provided). The protein content of the formulae (both powder and liquid) is 1.9 g/100 kcal (1.27 g/100 mL). The amino acid profile of the infant formula complies with the profile described in Annex IIIB of Regulation (EU) No 2016/127, which was established for this formula when it was included in the Annex to the before-mentioned Regulation.

With respect to Maillard reaction products, the internal release norms for reactive[11] and blocked[12] lysine in the final liquid and powdered formula, measured by ion-exchange chromatography according to an internal method (described by the applicant and presented as validated in-house), have been provided in the dossier.

The contents of alpha-linolenic acid (ALA), docosahexaenoic acid (DHA), iodine and vitamin D correspond to the required content according to Directive 2006/141/EC. The nutrient content of all other nutrients of the formula is in line with Regulation (EU) No 2016/127.

With respect to stability, the food business operator explains that a number of samples from each manufacturing batch are always analysed at the end of shelf-life, according to internal standards. Products are stored in their original pack, in rooms at 20°C with the humidity considered representative of the yearly average climate conditions of the geographical area in which the formula is sold.

The Panel considers that, in relation to the effect that is claimed, the infant formula under evaluation is not sufficiently characterised with respect to the molecular weight distribution of peptides.

3.2. Efficacy of the formula in reducing the risk of developing atopic dermatitis in infants with a family history of allergy

The food business operator provided results of three human intervention studies reported in nine publications as directly pertinent to the assessment of the efficacy of the formula (Vandenplas et al., 1992, 1995; Marini et al., 1996; von Berg et al., 2003, 2007, 2008, 2010, 2013, 2016). None of them were claimed proprietary by the food business operator hence none were accompanied by their study reports. The food business operator also provided, as supportive evidence, 11 human studies and 12 animal studies.

As described in the scientific and technical guidance (EFSA NDA Panel (2017)), 'the Panel cannot set specific requirements with respect to the duration of the intervention and/or the duration of the follow-up. However, the Panel considers that reducing the risk of developing allergy during at least the first year of life would be clinically significant for the target population. Claims on the reduction of the risk of allergic disease for longer periods of time would require longer follow-ups'.

Study by Vandenplas et al.

The study reported in Vandenplas et al. (1992, 1995) was described as a randomised study with a follow-up of five years without providing information on the methodology used for randomisation. Following a request from EFSA to provide additional information with respect to the randomisation procedure, the food business operator provided some clarifications they received from the authors of the study. These indicated that subjects were allocated to groups in a non-random manner (exact

[11] Lysine present in proteins/peptides with a free amino group.
[12] Lysine present in proteins/peptides for which the amino group has reacted with reducing sugars.

methodology claimed confidential). The Panel considers that no conclusion can be drawn from this non-randomised study for the scientific substantiation of the effect.

Study by Marini et al.

The study by Marini et al. (1996) was described as a randomised, single-blinded (caregivers were aware of the group allocation) controlled trial. The trial was carried out in infants with two atopic parents and who were either fed the formula under evaluation or a control formula manufactured from intact cow's milk protein. Details on the randomisation procedure were neither provided in the publication nor provided by the food business operator upon request from EFSA. Randomisation took place once formula-feeding was introduced. The publication reports that 279 infants were enrolled in the study. According to the food business operator, 155 were randomised to formula-feeding (either intervention or control), while 124 were included in an exclusively breast-fed reference group.

Atopic dermatitis was diagnosed according to Moore et al. (1985), when scaly, erythematous and itchy eczematous rash was localised in at least two of the following areas: face and scalp, behind the ears and at the flexural folds.

Results are presented in the publication in terms of cases of atopic dermatitis in subgroups of infants who were exclusively formula-fed, those who received breast milk in addition to the two study formulae (around 63% of all formula-fed infants; separated by study formula) and those who were exclusively breast-fed. No statistical analyses were presented on these subgroups.

A multivariable logistic regression analysis was presented in the paper, in which feeding intact cow's milk formula showed higher odds for developing atopic dermatitis at 1 year of age (odds ratio (OR) 3.2, 95% confidence interval (CI) 1.1; 9.2). However, the Panel notes that it is unclear to which feeding this was compared and whether exclusively breast-fed infants were part of this analysis or not.

A factor called 'breast/hydrolysed feeding' (with an unclear comparator) was not statistically significantly related to the health outcome atopic dermatitis and no separate analysis was presented for the intervention formula. No results were presented for the outcome at 2 and 3 years of age, when it was also assessed. The Panel notes that it is unclear for which covariates the analysis was adjusted. The Panel also notes that no correction for multiple statistical comparisons was made in the analysis of this study, in which the primary endpoint had not been specified and multiple study parameters were investigated.

The Panel considers that, owing to the methodological limitations of this study and the associated analysis, i.e. single-blind study design, unclear randomisation procedure and unclear statistical analysis, no conclusion can be drawn from this study for the scientific substantiation of the effect.

GINI study

The **German Infant Nutritional Intervention (GINI) study** (von Berg et al., 2003, 2007, 2008, 2010, 2013, 2016) was a randomised, controlled, double-blind, two-centre intervention study. It was undertaken on healthy term infants less than 14 days of age, with a birthweight $\geq$ 2,500 g, with at least one family member (mother, father or sibling) with an atopic disease, and with no prior consumption of cow's milk-based formula. In this study, three different formulae manufactured from hydrolysed protein, among them the formula under evaluation, were compared with a formula manufactured from intact cow's milk protein with respect to their effect on reducing the risk of developing atopic manifestations.

The Panel notes that von Berg et al. (2003) reports for the formula under evaluation the percentage of peptides in the fractions $<$ 1,500 Da as 54% and in the fraction of $>$ 6,000 Da as 18%. This contrasts with the target ranges for the molecular weight distribution of peptides of the formula provided by the food business operator. According to these target ranges, [redacted] of all peptides are in the fraction of $>$ 5,000 Da. Upon request from EFSA, the food business operator explained that the article by von Berg et al. (2003) contains no information on the analytical methods used to assess the molecular weight distribution of peptides and therefore, it is not possible to compare the results reported by von Berg et al. (2003) with the target ranges used by the food business operator. The food business operator states that the production process of the infant formula used in the study by von Berg et al. (2003) is identical to the one described in the dossier. The Panel considers that, based on the information available, it cannot be ascertained that the formula used in the study by von Berg et al. (2003) was the same as the one under evaluation.

Randomisation of study participants was performed based on a computer-generated list, stratified by single or double heredity (parents only) of atopy and study site. The Panel notes that insufficient

information was provided in the publications on whether the allocation of subjects to groups was appropriately concealed or not.

Mothers in all groups were encouraged to exclusively breast-feed for at least 4 months, but preferably for 6 months. Study formula could be introduced at the discretion of the mother and was provided up to 6 months of age. Regarding blinding, study formulae were identically labelled and coded with four different letters for each of the four formulae. Caregivers were advised not to introduce complementary foods before 4 months of age. They were asked, when complementary feeding was to be started, not to introduce more than one food per week and to avoid dairy products, hen's egg, soy, fish, nuts, tomatoes and citrus fruits during the first year of life. The type of milk fed in the first 6 months of life and the timing of introduction of complementary foods was assessed through weekly diaries kept by caregivers and cross-checked by structured interviews.

In von Berg et al. (2003), it is reported that clinical examinations were carried out at 1, 4, 8 and 12 months of age together with a structured interview conducted by a study physician to elicit information on the presence or absence of diagnostic criteria for atopic dermatitis modified from Hanifin and Rajka (1980). These criteria were '1) typical morphology and distribution of skin lesions (face, neck and scalp, flexural folds, hands, and extensor sides of the extremities); 2) pruritus (signs of scratching); and 3) tendency toward chronicity (duration $\geq$ 14 days, chronically relapsing, or both)'. The fulfilment of the criteria was cross-checked by a second specifically trained allergologist. The Panel understands that only if both physicians agreed on the fulfilment of all three criteria, atopic dermatitis was diagnosed.

In von Berg et al. (2008), it is described that at, and after the age of one year up to 15 years (i.e. at 1, 2, 3, 4, 6, 10 and 15 years of age), the diagnosis of atopic dermatitis was based on caregivers' reports on physician's diagnosis using a modification of the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire. EFSA requested the food business operator to clarify whether the ascertainment of atopic dermatitis at 1 year of age was based on a physician's diagnosis or caregivers' reports of symptoms and whether the outcome ascertainment was different in von Berg et al. (2003, 2008). In response to this last request, EFSA received a letter from the investigators of the GINI study in which it was clarified that both methods of outcome ascertainment (i.e. physician's diagnosis and caregivers' reports) were used at 1, 2 and 3 years of age. The investigators also provided a cumulative analysis of incident cases of atopic dermatitis based on clinical diagnosis by the study physicians in the population of children with complete follow up until 3 years of age.

The Panel notes the potential bias that is introduced into the outcome ascertainment based on caregivers' reports of symptoms. Therefore, the Panel does not consider it as an adequate method of assessing the development of atopic dermatitis. As a consequence, no conclusions can be drawn from the analyses at time points where the outcome assessment was based only on caregivers' reports of symptoms, i.e. all analyses after three years of age. The results from these time points are therefore not reported in the present opinion.

The primary outcome of the study was the presence of atopic manifestations (i.e. at least one of the following: atopic dermatitis, allergic urticaria or food allergy with manifestations in the gastrointestinal tract) at 1 year of age. Sample size calculations were based on the following assumptions: 1) infants exclusively breast-fed or who drop-out during the study would approximately amount to 50% and 2) the prevalence of atopic manifestations at the end of the study in the intervention and control groups would be 30% and 20%, respectively. To obtain a power of 80% at a two-sided significance level of 5%, 600 infants per group had to be enrolled (without considering drop-outs and exclusively breast-fed infants 313 infants had to be included).

Finally, 557 infants were randomised to the group receiving the formula under evaluation (intervention group) and 556 to the formula manufactured from intact cow's milk protein (control group). According to the publication by von Berg et al. (2003), 315 and 328 infants received the study formulae, 269 and 286 completed the 12 months follow-up, and 241 and 256 were compliant with the study protocol (i.e. per protocol (PP) population). In subsequent publications on the same study population (von Berg et al., 2008, 2010, 2013), the number of infants included in the PP population was not corroborated. Following a request from EFSA to clarify this discrepancy, the food business operator indicated that the PP population in this study also included subjects with missing data at intermediate time points. Therefore, the food business operator explained that the number of subjects in the PP population was variable and could increase from one assessment time point to the next, depending on whether a measurement was available or not.

For the analysis at 1 year of age (von Berg et al., 2003), the reasons for drop-outs were given overall for all four study groups together, with an indication that no significant differences between

groups were observed. A total of 42% of infants were exclusively breast-fed during the first four months of age and not considered in the PP analysis. The exclusively breast-fed infants (that were excluded from the analysis) differed in characteristics from those who had received formula with respect to the following: higher prevalence of atopic dermatitis in the family, higher rate of caesarean section, higher parental education, higher number of siblings, higher number of mothers older than 30 years of age, less smoking mothers, less homes with pets, and less participants from one of the study sites. Following a request from EFSA to comment to what extent the principle of randomisation is still respected with removal of this high number of subjects, the food business operator indicated that exclusively breast-fed infants were equally distributed among groups and that all but 3% of those were followed-up and included in the intention-to-treat (ITT) analysis, which showed results in the same direction as the PP analysis.

Baseline characteristics of the PP population were presented (von Berg et al., 2003). No statistically significant differences were observed between the study groups.

No statistically significant effect of consumption of the formula under evaluation was observed on the primary outcome of the study, i.e. on the risk of developing atopic manifestations at 1 year of age (von Berg et al., 2003) and on the cumulative incidence of developing atopic manifestations at 3 years of age (von Berg et al., 2007).

Atopic dermatitis, one of the secondary outcomes of the study, was diagnosed at 1 year of age, as reported in von Berg et al. (2003), in 22/241 infants in the intervention and 38/256 infants in the control group in the PP population (crude OR [95% CI] calculated by EFSA: 0.58 [0.33; 1.01]; adjusted OR 0.56 [0.32; 0.99], multivariable logistic regression, adjusted in the final model for sex, family history of atopic dermatitis and maternal smoking after pregnancy).

According to the letter from the investigators to EFSA, an additional 15 incident cases diagnosed by the study physician occurred during the second and third year of life in the intervention group and 17 in the control group, in those participants who had a complete three-year follow-up (i.e. n = 229 in the intervention and n = 245 in the control group). The adjusted OR for the cumulative incidence of atopic dermatitis, diagnosed by a study physician, from birth to 3 years was 0.60 (95% CI 0.37–0.97; adjusted for the same covariates as the analysis reported above). Breast-feeding as a covariate was not considered in the analysis at 1 year of age, but was included in the analysis as a possible covariate at 3 years of age, without showing statistical significance.

Results for the ITT population (both including and excluding exclusively breast-fed infants) for the prevalence of atopic dermatitis in the first year of life and for the cumulative incidence from birth to three years of age were not statistically significant (von Berg et al., 2007).

No correction for multiple comparisons was carried out for either the ITT or the PP analysis. EFSA requested the food business operator to clarify to what extent conclusions can be drawn from this analysis for the substantiation of the effect, considering that atopic dermatitis was not the primary outcome of the study and that the analysis was not corrected for multiple comparisons. Following this request, the food business operator replied that atopic dermatitis was one of the composite outcomes of atopic manifestations, the primary outcome of the study, and that correcting for multiple comparisons of these correlated outcomes would have led to an overly conservative approach. The Panel notes that there are statistical methods and testing strategies available that allow accounting for the testing of multiple correlated outcomes. The Panel also notes that, in the absence of appropriate corrections, it cannot be excluded that the statistically significant findings of the study with respect to the risk of developing atopic dermatitis is due to the increase in the type 1 error rate associated with carrying out multiple statistical tests.

The Panel notes the methodological limitations of the study with regard to the evaluation of an effect of the formula on the reduction in risk of developing atopic dermatitis (i.e. exclusion of a substantial percentage, 42%, of randomised subjects from PP analyses, all exclusively breast-fed infants, and lack of correction for multiple statistical testing, outcome ascertainment after the age of three years based on caregivers' reports only). The Panel also notes the uncertainty as to whether or not the formula investigated in the study was the same as the formula under evaluation described in Section 3.1. Thus, the Panel considers that no conclusions can be drawn from this study for the scientific substantiation of the effect.

Additional information provided:

The food business operator also presented random effects meta-analyses on the three studies described above and on five studies, including the previous three and two additional studies that were

presented as supportive evidence. The Panel notes that, in the absence of conclusions that can be drawn from the individual studies, no conclusions can be drawn from a meta-analysis thereof.

Eleven human studies provided by the food business operator as supportive evidence all compared the formula under evaluation to formulae manufactured from intact cow's milk protein, unless otherwise stated in the description below. The Panel notes that none of these 11 human studies, presented as supportive evidence, enabled conclusions to be drawn on the effect of the formula on the reduction of risk of developing atopic dermatitis. The reasons are outlined in the following.

- Becker et al. (2004) described a multifaceted allergy intervention (i.e. encasement of mattresses, instructions for washing bedsheets, cleaning of carpets and furniture, keeping pets outside the house, smoking cessation, avoidance of day care, breast-feeding or use of the formula under evaluation), but did not report on the effect of the formula under evaluation and on atopic dermatitis separately from atopy.
- It is unclear whether the study described in the paper by D'Agata et al. (1996) was a non-randomised intervention or an observational study. The investigated outcome was eczema that was not defined, and it is unclear whether this was eczema of all origins or atopic dermatitis.
- de Seta et al. (1994) investigated an outcome that was a combination of atopic dermatitis and cow's milk intolerance, rather than atopic dermatitis alone.
- Exl et al. (2000) presented an analysis in infants fed the formula under evaluation vs intact cow's milk formula, in a non-randomised study. In addition, the intervention consisted of delaying allergen introduction in general and not only of the formula under evaluation. Also, the outcome that was assessed was 'skin symptoms' that included, but was not limited to, atopic dermatitis.
- The study by Fukushima et al. (1997) was not randomised and the diagnosis of atopic dermatitis was based on caregivers' reports in questionnaires, which is not considered by the Panel as an adequate method of outcome ascertainment.
- The study reported in Iikura et al. (1995) and Akimoto et al. (1997) was not randomised and the outcome that was investigated was 'atopic-dermatitis-like symptoms'. It is unclear whether this covered only atopic dermatitis or also other skin diseases.
- Lowe et al. (2011), in an RCT, investigated doctor-diagnosed eczema or any rash that was treated with topical steroid preparation, rather than specifically atopic dermatitis.
- Tsai et al. (1991) carried out an unblinded RCT.
- Vandenplas et al. (1988, 1989) reported on a non-randomised intervention study on dermatological symptoms rather than atopic dermatitis.
- Equally, the study by Sun et al. (2015) was not randomised and atopic dermatitis was diagnosed based on caregivers' reports of a physician's diagnosis, which is not considered by the Panel as an adequate method of outcome ascertainment.
- Finally, the study by Willems et al. (1993) was not appropriately randomised. The outcome that was investigated was not further defined eczema, and it is unclear whether this was eczema of all origin or atopic dermatitis.

The Panel notes that, among the 14 human intervention studies provided, no human intervention studies have been submitted from which conclusions could be drawn for the scientific substantiation of the effect. In the absence of evidence from human clinical studies on the effect of the infant formula in reducing the risk for developing atopic dermatitis, the Panel notes that the animal studies that were also submitted cannot be used for the scientific substantiation of the effect.

The Panel concludes that a cause-and-effect relationship has not been established between the consumption of the infant formula under evaluation and a reduction in the risk of developing atopic dermatitis in infants with a family history of allergy.

4. Conclusions

The Panel concludes that:

- in relation to the effect that is claimed, the infant formula under evaluation is not sufficiently characterised with respect to the molecular weight distribution of peptides;
- a cause-and-effect relationship has not been established between the consumption of the infant formula under evaluation and a reduction in the risk of developing atopic dermatitis in infants with a family history of allergy.

5. Documentation as provided to EFSA

Dossier on the Efficacy of Nestlé's Infant Formulas Manufactured from Partially Hydrolysed 100% Whey Protein in Reduction of the Risk of Developing Atopic Dermatitis. December 2019. Submitted by Société des Produits Nestlé S.A.

Steps taken by EFSA

1) The technical dossier was received by EFSA on 19/07/2019.
2) A letter from the European Commission with the request for a scientific opinion on the safety and suitability for use by infants of an infant and follow-on formula manufactured from protein hydrolysate was received by EFSA on 09/08/2019.
3) The scientific evaluation procedure started on 20/01/2020.
4) On 26/02/2020, the Working Group on Food Allergy of the NDA Panel agreed on a list of questions for the food business operator to provide additional information to accompany the dossier. The scientific evaluation was suspended on 13/03/2020 and was restarted on 16/04/2020.
5) On 24/04/2020, the Working Group on Food Allergy of the NDA Panel agreed on a list of questions for the food business operator to provide additional information to accompany the dossier. The scientific evaluation was suspended on 14/05/2020 and was restarted on 19/06/2020.
6) On 07/05/2020, additional information was spontaneously submitted by the study group of one of the human intervention studies provided by the food business operator.
7) On 28/09/2020, the Working Group on Food Allergy of the NDA Panel agreed on a list of questions for the food business operator to provide additional information to accompany the dossier. The scientific evaluation was suspended on 15/10/2020 and was restarted on 17/12/2020.
8) On 22/01/2021, the Working Group on Food Allergy of the NDA Panel agreed on a list of questions for the food business operator to provide additional information to accompany the dossier. The scientific evaluation was suspended on 01/02/2021 and was restarted on 15/02/2021.
9) During its meeting on 27-29/04/2021, the NDA Panel, having evaluated the data, adopted an opinion on 29/04/2021.

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Abbreviations

ALA	alpha-linolenic acid
AN	amino nitrogen
CEF	EFSA Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids
CEP	EFSA Panel on Food Contact Materials, Enzymes and Processing Aids
CI	confidence interval
CMP	caseino-glycomacropeptide
DH	degree of hydrolysis
DHA	docosahexaenoic acid
EC	number Enzyme Classification Number of Enzyme Commission of the International Union of Biochemistry and Molecular Biology
ELISA	enzyme-linked immunosorbent assay
GINI	German Infant Nutritional Intervention
h	number of cleaved peptide bonds
HPLC	high-performance liquid chromatography
h_{tot}	total number of peptide bonds
IgE	immunoglobulin E
ISAAC	The International Study of Asthma and Allergies in Childhood
ISO	International Organization for Standardization
ITT	intention-to-treat
LDS–PAGE	lithium dodecyl sulfate–polyacrylamide gel electrophoresis
MW	molecular weight
NDA	EFSA Panel on Nutrition, Novel Foods and Food Allergens
OR	odds ratio
PP	per protocol
RCT	randomised controlled trial
SDS–PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TN	total nitrogen
TNBS	trinitrobenzenesulfonic acid
USP	United States Pharmacopeia
UV	ultraviolet