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Safety of bovine milk osteopontin as a Novel food pursuant to Regulation (EU) 2015/2283

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Abstract

Following a request from the European Commission, the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) was asked to deliver an opinion on bovine milk osteopontin (bmOPN) as a novel food (NF) pursuant to Regulation (EU) 2015/2283. The NF concerns OPN derived from bovine whey. The NF is intended to be used at a maximum use level of 151 mg/L, in infant formula (IF), follow-on formula (FoF) and ready-to-eat dairy-based meals for children up to 35 months of age. As compared to the concentrations naturally present in cow's milk and concentrations found in IF on the market reported in the literature, the proposed use level of the NF represents an about 10-fold higher concentration of bmOPN. The intended use levels of the NF would provide bmOPN at a concentration within the range of human milk (hm) OPN. In a 6-month study, 14, 72 and 140 mg bmOPN/L in reconstituted (as consumed) IF were given to 279 infants in order to study possible effects on frequency and severity of adverse events, and growth, formula intake and stool consistency. Despite that a number of inconsistencies and limitations were noted in the study report, the Panel considers that the results obtained from this study do not raise safety concerns. Considering the source of the NF, that neither the toxicological studies nor the provided infant study do raise safety concerns, and the low bmOPN plasma levels in infants resulting from the consumption of the NF, the Panel considers that the margin of exposure (i.e. 36) between the NOAEL of the subchronic toxicity study (1,200 mg/kg bw per day) and the highest P95 estimate for infants (33.4 mg/kg bw per day) is sufficient. The Panel concludes that the NF is safe under the proposed conditions of use.

Keywords: Novel Foods, bovine milk osteopontin, whey protein, infants and young children, infant formula, follow-on formula

Requestor: European Commission

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1. Introduction

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

On 27 March 2020, the company Arla Foods Ingredients Group P/S submitted a request to the Commission in accordance with Article 10 of Regulation (EU) No 2015/2283 to place on the EU market bovine milk osteopontin (bmOPN).

Bovine milk osteopontin is produced as a source of osteopontin that can be used as an ingredient to provide infants and young children with a supplementary source of OPN in their diets. Bovine milk osteopontin is proposed for use in infant formulas, follow-on formula, and milk-based drinks for young children in the European Union (EU), including ready-to-drink and reconstituted formula products.

In accordance with Article 10(3) of Regulation (EU) 2015/2283, the European Commission asks the European Food Safety Authority to provide a scientific opinion on bmOPN.

2. Data and methodologies

2.1. Data

The safety assessment of this Novel Food (NF) is based on data supplied in the application and information submitted by the applicant following EFSA requests for supplementary information.

During the assessment, the Panel identified additional data which were not included in the application (Sodek at al., 2000; Lund et al., 2009; Bissonette et al., 2012; Reza et al., 2013; Lamort et al., 2019).

Administrative and scientific requirements for NF applications referred to in Article 10 of Regulation (EU) 2015/2283 are listed in the Commission Implementing Regulation (EU) 2017/2469[1].

A common and structured format on the presentation of NF applications is described in the EFSA guidance on the preparation and presentation of an NF application (EFSA NDA Panel, 2016). As indicated in this guidance, it is the duty of the applicant to provide all of the available (proprietary, confidential and published) scientific data (including both data in favour and not in favour) that are pertinent to the safety of the NF.

This NF application includes a request for protection of proprietary data in accordance with Article 26 of Regulation (EU) 2015/2283. The data requested by the applicant to be protected comprise: certificates of analyses and batch testing (annex II of the dossier), stability reports (annex IV), information on the concentration of osteopontin in commercial infant formula (annex V) and the unpublished study reports on the following studies (annex VIII): a bacterial reverse mutation assay (Kvistgaard et al., 2012), an *in vitro* mammalian chromosome aberration test (Kvistgaard et al., 2013a), an *in vivo* micronucleus test (Kvistgaard et al., 2013b), a subchronic oral toxicity study in rats (Lina, 2007) and a study in infants (Peng and Lönnerdal, 2013).

2.2. Methodologies

The assessment follows the methodology set out in the EFSA guidance on NF applications (EFSA NDA Panel, 2016) and the principles described in the relevant existing guidance documents from the EFSA Scientific Committee. The legal provisions for the assessment are laid down in Article 11 of Regulation (EU) 2015/2283 and in Article 7 of the Commission Implementing Regulation (EU) 2017/2469.

The legal provisions for the assessment of food for specific groups are laid down in Regulation (EU) 609/2013[2] and, respectively, in Commission Delegated Regulation 2017/1798[3] in the case of total diet replacement for weight control, in Commission Delegated Regulation (EU) 2016/128[4] food for special medical purposes and in Commission Delegated Regulation (EU) 2016/127[5] as regards the specific compositional and information requirements for infant formula and follow-on formula and as regards requirements on information relating to infant and young child feeding.

This assessment concerns only the risks that might be associated with the consumption of the NF under the proposed conditions of use, and is not an assessment of the efficacy of the NF with regard

[1] Commission Implementing Regulation (EU) 2017/2469 of 20 December 2017 laying down administrative and scientific requirements for applications referred to in Article 10 of Regulation (EU) 2015/2283 of the European Parliament and of the Council on novel foods. OJ L 351, 30.12.2017, pp. 64–71

[2] https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32013R0609&from=en

[3] https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32017R1798&from=en

[4] https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32016R0128&from=en

[5] https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32016R0127&from=en

to any claimed benefit. This assessment also is not an assessment on whether the NF is suitable as stipulated by Regulation (EU) No 609/2013.

3. Assessment

3.1. Introduction

In accordance to Article 3 of the Novel Food Regulation (EU) 2015/2283, the NF falls under category (v) food consisting of, isolated from or produced from animals or their parts, except for animals obtained by traditional breeding practices which have been used for food production within the Union before 15 May 1997 and the food from those animals has a history of safe food use within the Union.

The NF which is the subject of the application is bovine milk osteopontin (bmOPN) isolated from bovine whey or milk by ultrafiltration and ion exchange chromatography. The NF consists of at least 76.5% protein (N × 6.38), of which at least 84.5% is bmOPN. The NF is intended to be used as an ingredient for infant- and follow-on formula (IF and FoF), and formula for young children from 1 to 3 years of age.

Osteopontin was first identified in 1986 in osteoblasts and plays an important role in bone metabolism and homoeostasis (Si et al., 2020). It is expressed by various cells and is present in most tissues and body fluids (Christensen et al., 2010). It interacts with a number of integrins via specific motifs within the molecule. Osteopontin also contains calcium-binding and CD44-binding motifs. Its biological function depends on post-translational modifications, in particular phosphorylation and glycosylation and also on proteolytic cleavage, thus on the presence of proteases and peptidases. Through its interaction with integrin, OPN also mediates cell migration and adhesion and has been described to play a role in tumour growth, inflammatory and other immunological processes (Lund et al., 2009; Castello et al., 2017; Lamort et al., 2019).

Proteases known to cleave OPN include thrombin, cathepsins, kallikrein and plasmin. Although the latter has been reported to be the principal protease in milk, the major cleavage site of bmOPN appears to be specific for thrombin (Christensen and Sorensen, 2014).

3.2. Identity of the NF

The NF is produced from bovine whey and contains at least 76.5% protein (N × 6.38), of which at least 84.5% is bmOPN. The NF contains also other other milk proteins, up to 11% ash and a moisture content below 9.5%. Bovine milk OPN is an acidic highly phosphorylated glycoprotein with an open and flexible structure. According to the applicant, bmOPN in the NF comprises two fractions: the full length OPN (MW 33.9 kDa), composed of 262 amino acids, and the shorter N-terminal fragment (MW 19.8 kDa), composed of 150 amino acids. These MWs have been calculated on the basis of the comprised amino acids and the 3 O-linked glycosylation and 28 phosphorylation sites of the full length, and the 3 O-linked glycosylation and 16 phosphorylation sites of the N-terminal fragment, respectively. These MWs are consistent with the MWs reported by Christensen and Sørensen (2014) when using matrix-assisted laser desorption ionisation mass spectrometry (MALDI-MS).[6]

As measured by high-performance liquid chromatography (HPLC) gel filtration, the proportions of full-length bmOPN to the N-terminal fragment are approximately 25:75.

3.3. Production process

Pasteurised or microfiltered bovine whey protein concentrate is collected in a storage tank prior to the isolation of the bmOPN protein. The whey fraction is diluted with demineralised water to standardise the protein concentration. Additionally, the whey can also be pH-adjusted with low

[6] According to the SDS gel electrophoresis performed after HPLC gel filtration, conducted by the applicant, the apparent MWs of the full-length OPN and the N-terminal fragment were approximately 60 and 40 kDa, respectively, in line with the results reported by Bissonnette et al. (2012). Phosphorylation has a major effect on the migration of both proteins, and dephosphorylation can induce significant shifts on SDS-PAGE. In addition, the discrepancy in the size of OPN proteins may depend on the type of acrylamide gradient used for SDS-PAGE so that a difference between the two major forms secreted in milk was found to be of 20 kDa. The anomalous mobility of highly acidic proteins on SDS-Page has also been observed for other proteins (Tiwari et al., 2019).

concentrations of NaOH or HCl to achieve a defined pH before it is loaded onto an anion exchange column.

The prepared whey fraction is loaded onto the column that binds the phosphorylated bmOPN while the other whey components such as lactose and whey proteins, predominantly alpha-lactalbumin and beta-lactoglobulin, pass through. After a certain time, a premixed solution of NaCl in demineralised water is used as eluent to desorb the bmOPN from the column.

The bmOPN-containing eluent is collected in a storage tank before it undergoes ultrafiltration to remove low molecular weight components including NaCl. As the bmOPN is concentrated during the ultrafiltration, $CaCl_2$ is added to the retentate to neutralise the counterions surrounding the protein. The final bmOPN-containing retentate is spray dried to produce a powder that is subsequently sieved and passed through a rotary magnet prior to final packaging in polyethylene bags.

Certificates of analyses were provided for the four raw materials used (NaOH, HCl, NaCl and $CaCl_2$). According to the applicant, the NF is manufactured in compliance with the principles of Hazard Analysis Critical Control Points (HACCP) and Good Manufacturing Practice (GMP) and the producing plant is certified under DS/EN International Organization for Standardization (ISO) 50001:2011, 22000:2005/TS 22002-1:2009 and Food Safety System Certification (FSSC) 22000.

The Panel considers that the production process is sufficiently described and does not raise safety concerns.

3.4. Compositional data

The NF consists of approximately 80% protein (analysed by the Kjeldahl method and when using the default nitrogen factor of 6.38 for milk products), about 9% ash, 4% moisture and residual amounts of lactose (about 0.1%) according to the testing of five batches provided by the applicant (Table 1). Certificates of analyses and information on the methods were provided.

Table 1: Batch results from five batches of the NF

Parameters	Batch number					Range	Method
	1	2	3	4	5		
Protein as is							
(N × 6.38), %*	80.5	79.6	80.1	80.0	79.6	79.6–80.5	ISO 8968-3/IDF 20-3
bmOPN as % of protein							
(N × 6.38)*	> 88.5	> 88.5	> 88.5	> 88.5	> 88.5	> 88.5	Ion exchange HPLC
bmOPN area							
(% of total area)	87.9	89.0	89.1	88.0	91.3	87.9–89.1	HPLC Gel filtration
Full-length bmOPN							
(% of bmOPN area)	26.5	25.6	25.9	25.4	26.4	25.4–26.5	HPLC Gel filtration
N-terminal fragment							
(% of bmOPN area)	73.5	74.4	74.1	74.6	73.6	73.5–74.6	HPLC Gel filtration
Other milk proteins (% of total area)	12.1	11.0	10.9	11.4	8.7	8.7–12.1	HPLC Gel filtration
Lactose, %	0.1	0.1	0.1	0.1	0.1	0.1	ISO 5765-2/IDF 79-2 enzymatic
Fat, %	0.1	0.1	0.1	0.2	0.2	0.1–0.2	ISO 1736 gravimetric
Ash, %	9.0	9.1	9.2	8.8	9.2	8.8–9.2	NMKL 173 gravimetric
Moisture, %	3.9	4.0	3.9	4.0	4.2	3.9–4.2	ISO 6731
Insolubility index (mL)	0.10	0.10	0.10	0.10	0.10	0.10	ISO 8156 /IDF 129 gravimetry

HPLC: High-Performance Liquid Chromatography; IDF: International Dairy Federation; ISO: International Organization for Standardization; NMKL: NordVal International Denmark, OPN: osteopontin.

*: On the basis of the amino acids contained in OPN, the applicant has proposed to calculate the protein with a factor of 7.17 which would result to a higher proportion of protein in the NF (about 12% higher, i.e. protein content about 90%).

The same batches were also analysed for their mineral contents (Table 2).

Table 2: Mineral contents of five batches

Parameters	Batch number					Range	Method
	1	2	3	4	5		
Sodium, %	0.99	1.19	1.17	1.14	1.20	0.99–1.20	ICP-OES
Phosphorus, %	1.81	1.79	1.89	1.74	1.83	1.74–1.89	ICP-OES
Chloride, %	0.05	0.05	0.05	0.05	0.05	0.05	ISO 5943/IDF 88
Calcium, %	2.42	2.19	2.34	2.22	2.18	2.18–2.42	ICP-OES
Magnesium, %	0.01	0.01	0.01	0.01	0.01	0.01	ICP-OES
Potassium, %	0.05	0.05	0.05	0.05	0.05	0.05	ICP-OES

ICP-OES: Inductively coupled plasma-optical emission spectrophotometer; IDF: International Dairy Federation; ISO: International Organization for Standardization.

Furthermore, the applicant provided analytical results on heavy metals (Tables 3 and 4), microbiological contamination (Table 5) and aflatoxin M1 (Table 6).

Table 3: Heavy metal analyses for five batches of the NF

Parameters	Batch number					Method
	6[(a)]	7[(a)]	8[(a)]	9[(b)]	10[(b)]	
Arsenic, mg/kg	< 0.1	< 0.01	< 0.01	< 0.01	< 0.01	MS
Cadmium, mg/kg	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	MS
Lead, mg/kg	0.016	0.014	0.013	0.0033	0.120	MS
Mercury, mg/kg	< 0.005	< 0.005	< 0.005	< 0.005	< 0.005	MS

MS: mass spectroscopy.
(a): Analysed by ICP-HRMS = inductively coupled plasma-high resolution mass spectrometer (ISO 17294m:2005).
(b): Analysed by ICP-MS = Inductively coupled plasma-mass spectrometry (ISO 17294m:2016).

With respect to batch No 10, the applicant indicated the processing aid $CaCl_2$ as the source for the lead contamination. As a consequence, the applicant added a specific control point related to $CaCl_2$ in the HACCP plan as to avoid this problem. Five subsequent production batches showed lead concentrations below the specification limit (Table 4).

Table 4: Lead analyses for five batches of the NF

Parameters	Batch number					Method
	1	2	3	4	5	
Lead, mg/kg	0.004	0.037	0.004	0.020	0.016	ICP-MS (ISO 17294m:2016).

ICP-MS: Inductively coupled plasma-mass spectrometry; ISO: International Organization for Standardization.

Table 5: Microbiological analyses for five batches of the NF

Parameters	Batch number					Method
	1	2	3	4	5	
Total Plate Count, 30°C, CFU/g	< 100	< 100	< 100	< 100	400	ISO 4833-1
Aerobic thermophilic count, CFU/g	< 100	< 100	< 100	< 100	< 100	ISO 4833-1, 55°C for 48 hrs
Yeast/Mould, CFU/g	< 10	< 10	< 10	< 10	< 10	ISO 6611
Bacillus cereus, CFU/g	< 10	< 10	< 10	< 10	< 10	ISO 7932
Sulfur-reducing clostridia, CFU/g	< 10	< 10	< 10	< 10	< 10	ISO 15213
Staphylococcus aureus, per g	Absent	Absent	Absent	Absent	Absent	ISO 6888-1
Enterobacteriaceae, CFU/g	< 10	< 10	< 10	< 10	< 10	ISO 21528-2

Parameters	Batch number					Method
	1	2	3	4	5	
Salmonella, per 250	Absent	Absent	Absent	Absent	Absent	BAX®

BAX®: Baxter International; CFU: colony forming unit; ISO: International Organization for Standardization.

Information was provided on the accreditation of the laboratories that conducted the analyses presented in the application.

Table 6: Aflatoxin M1 Analyses for five batches of the NF

Parameters	Batch number				
	9	10	11	12	13
Aflatoxin M1 (μg/kg)	0.01*	< 0.01[(a)]	< 0.01[(b)]	< 0.01[(b)]	< 0.01[(b)]

(a): Method: LC-FLD (liquid chromatography fluorescence detector).
(b): Method: LC-MS/MS (liquid chromatography with tandem mass spectrometry).

The Panel considers that the information provided on the composition is sufficient for characterising the NF.

3.4.1. Stability

The applicant performed stability tests with the bulk material on eight batches and on two samples of infant formula to which the NF was added.

3.4.1.1. Stability of the bulk material

The NF bulk material of eight batches was packaged in polyethylene bags and stored under dry conditions at a temperature ranging from 5°C to 20°C. Their protein content was monitored with the Kjeldahl method (N × 6.38) for 151 weeks (2011–2014). In addition, bmOPN stability was verified by quantifying bmOPN by HPLC relative to both total protein and total dry matter. The protein content was reduced from approximately 80% at start to 76% (range: 80.2–75.07) at the study end. There was also a small decrease of the bmOPN content (% of the powder) from study start (ranging between 87.0 and 89.6) to study end (ranging 83.6–86.9). The moisture content among the eight batches ranged from 4.4% to 4.9% at the start and increased to up to 9.4% at study end. There was no loss of the bmOPN protein per dry substance.

In addition, the applicant provided stability data for bulk material of two batches stored in a Danish warehouse for 30 months. The results obtained from this stability data are consistent with the stability data of the 151-week study, albeit in the latter study, the moisture content increased slightly less (up to 7%). This study included microbiological parameters (total plate count, aerobic thermophilic count, yeasts/mould, *Bacillus cereus, Staphylococcus aureus, Enterobacteriaceae* and *Salmonella*), which were stable over the study duration.

3.4.1.2. Stability when added to infant formula

In another stability study, 0.1% alpha-lactalbumin whey in an IF was substituted by the NF at a concentration of 0.114%. Cans of the NF-containing IF and a control IF without the NF were stored at room temperature (25°C) and 60% relative humidity, and under accelerated conditions (40°C and 75% relative humidity) for up to 720 days. One sample per can of each formula and for both storage conditions was analysed for their bmOPN content by a liquid chromatography with tandem mass spectrometry (LC-MS/MS) quantitative method over 30-day intervals.

When stored under room temperature conditions, at day 180, there was no or little loss (2.2%) of bmOPN in the two samples of IF with the added NF. At day 360, the losses were 2.6% and 5.6%, and at day 720, the losses were 2.3% and 8.4%, respectively.

Under accelerated conditions, the loss of bmOPN was higher in the two samples: 14.9% and 20.4% at day 180, 24.7% and 27% at day 360, and 24.7 and 51.6% at day 720.

The Panel notes a continuous but slow loss of bmOPN over time when added to infant formula stored at room temperature. Under accelerated conditions (40°C/75% RH), the NF is not stable during storage over 6 months or longer.

The Panel notes that according to the stability data of the NF when stored as bulk material and at temperatures ranging from 5°C to 20°C, none of the eight batches met the proposed minimum

specification limit for protein, i.e. at least 76.5% of the NF (protein content by Kjeldahl with N × 6.38) after 151 weeks of storage. The shortfall was small however (0.2–1.5%).

The Panel considers that the data provided sufficient information with respect to the stability of the NF and that the provided stability data do not raise safety concerns.

3.5. Specifications

The specifications of the NF are indicated in Table 7.

Table 7: Specifications of the NF

Parameter (unit)	Limits
Protein % as is (N × 6.38)	76.5–80.5
bmOPN	≥ 84.5
Full-length OPN (% of bmOPN)	≥ 15
N-terminal fragment (% of bmOPN)	≥ 70
Other milk proteins % of protein	≤ 14.5
Lactose (%)	≤ 1.0
Fat (%)	≤ 1.0
Ash (%)	≤ 11
Moisture %	< 9.5
Insolubility index (mL)	≤ 1.0
Heavy metals	
Arsenic mg/kg	< 0.5
Cadmium mg/kg	< 0.05
Lead mg/kg	< 0.05
Mercury mg/kg	< 0.05
Aflatoxin M1 μg/kg	< 0.1
Microbiological contaminants	
Total plate count, 30°C CFU/g	≤ 5,000
Mould/yeast CFU/g	≤ 100
Bacillus cereus CFU/g	< 50
Sulfur-reducing Clostridia CFU/g	< 10
Staphylococcus aureus	absent/g
Enterobacteriaceae CFU/g	< 10
Salmonella spp.	absent/25 g

CFU: colony forming units; bmOPN: bovine osteopontin.

*: On the basis of the amino acids contained in OPN, the applicant has proposed to calculate the protein with a factor of 7.17 which would result to 12% higher limits for protein in the NF (~ 86–90.5).

Noting that food business operators who place an IF or FoF on the market with the NF must comply with the maximum limit set for Aflatoxin M1 which is 0.025 μg per kg of reconstituted formula, and considering that the maximum use level of the NF (i.e. 151 mg per litre reconstituted formula) would represent about 1/6622 of the reconstituted IF and FoF, the Panel finds the proposed maximum limit for Aflatoxin M1 of < 0.1 is not of concern.

The Panel considers that the information provided on the specifications of the NF is sufficient and does not raise safety concerns.

3.6. History of use of the NF and/or of its source

3.6.1. History of use of the source

3.6.1.1. OPN content in bovine milk and commercial infant formula

Schack et al. (2009) analysed the concentration of bmOPN in cow's milk and cow's milk-based commercial IF using an in-house developed enzyme-linked immunosorbent assay (ELISA). According to the authors, the polyclonal antibodies employed have been produced by immunisation with highly

purified full-length bmOPN, but the values measured would also comprise cleaved OPN and the antibodies did not cross-react with other milk proteins. The concentration of bmOPN in a pooled milk sample collected over a period of 4 months from a local Danish dairy was 18 mg/L, whereas the concentration in five infant formulae (two from South Korea, three from Denmark) ranged between 5 mg/L and 13 mg/L, when reconstituted according to the manufacturers' instructions.

According to the applicant, in 18 infant formulae available in South Korea, Vietnam and China between 2010 and 2014, the bmOPN concentration ranged from 2 to 110 mg bmOPN/L of formula (Mintel, 2014 [unpublished]). The applicant also measured bmOPN concentrations ranging between 40 and 79 mg/L in four infant formulae available in the EU and Asian markets, analysed by LC-MS/MS.

3.6.1.2. OPN content in human milk

Schack et al. (2009) studied also the OPN concentration in human breast milk. Samples were obtained from 29 mothers (aged 22–37 years) at the maternity ward of a hospital in Denmark. The milk was collected by breast pumping and 2 mL from each milking during one day was pooled to obtain 'whole-day milk'. Only one sample per woman was taken at different days postpartum (pp), ranging between day 6 and day 58. Antibodies for the in-house human milk (hm) OPN ELISA and the ELISA itself were developed as analogue to the bmOPN ELISA described in Section 3.6.1.1 but were produced and designed to detect hmOPN. The hmOPN concentrations among these samples ranged between 18 and 322 mg/L with a mean value of 138 mg/L (SD ± 79). There was no apparent trend or relationship observed between the hmOPN concentration in the breast milk and the day pp for that period. The Panel notes the limitations of this study, in particularly that only one sample representing one day was analysed per individual and the limited timeframe.

In a more recent study by Jiang and Lönnerdal (2019), breast milk samples of 12 mothers were sampled daily from day 1 to 14, and then on month 1, 2, 4, 6, and 12 pp. One breast was completely emptied at each sampling, making the sample representative of one meal. Human milk OPN levels were analysed with a commercially available ELISA which used goat polyclonal antibodies induced with hmOPN. Human milk OPN levels were declining from day 1. For the first 8 days, concentrations of about 160–220 mg/L were found, which decreased to about 60 mg/L at the end of month 1. There was little, if any, further decline noted until month 12.

Also Zhang et al. (2021) measured longitudinal changes of hmOPN concentrations. Milk samples were obtained from 105 Chinese women at four time points: during the first days (1–5), day 8–14 and month 1 and 6 pp. Samples were always taken between 9 and 11 am, preferably by hand. Human milk OPN was measured by ultraperformance liquid chromatography. According to the reported results, the mean hmOPN concentrations were 718, 586, 450 and 236 mg/L, respectively. Trend and changes from one time point to the subsequent were statistically significant.

The study by Nagatomo et al. (2004), who analysed with another ELISA kit human whey from breast milk samples collected at day 3–7 (n = 20), about 1 month pp (n = 20), 4–7 months pp (n = 21) and approximately 14 months pp (n = 16), showed also an inverse relationship between the hmOPN concentration and the stage of lactation. The article provided only the limited information on the sampling (i.e. that 'breast pumps' have been used). The samples were obtained at various time points pp derived from different mothers which limits the weight and significance of the results.

Decreasing hmOPN concentrations in breast milk by the lactation stage has also been reported by Bruun et al. (2018). The hmOPN concentrations were higher in the breast milk of mothers in China (median: 266.2 mg/L; n = 76) and South Korea (median: 216 mg/L; n = 117) collected at week 4 pp than from Japanese mothers (median: 185.0 mg/L, n = 118) collected 9 weeks pp. The lowest hmOPN concentration was found in breast milk of Danish mothers (median: 99.7 mg/L; n = 318) sampled after 17 weeks pp. This article reported also a second study with repeated measurements over a limited period of time with women in China (n = 75) and Japan (n = 33), which indicated slowly decreasing hmOPN levels in breast milk from week 4 to 13 and week 7 to 25 pp, respectively.

A study which investigated the relationship between hmOPN levels and maternal and infant characteristics reported a mean hmOPN concentration of 137.1 (SD ± 56.8) mg/L in 85 Turkish women measured during the third month of lactation. This study employed a commercial ELISA with polyclonal antibodies to detect hmOPN (Aksan et al., 2021).

The Panel notes that the studies investigating hmOPN levels in breast milk, differed regarding their study design, population groups (ethnicity), the covered period, the sampling, the sample preparation and also the employed analytical method. Four studies indicate that hmOPN concentrations in human breast milk decline over time, with highest concentrations found in early lactation and slowly decreasing concentrations later during lactation. Noting the population group, the study design and

the covered period, the Panel considers that the study by Jiang and Lönnerdal (2019) provided reliable figures regarding time course.

3.6.2. History of use of the NF

The applicant did not provide any data about the use of the NF in other countries.

According to the applicant, a self-affirmed Generally Recognised as Safe (GRAS) dossier (2017) submitted to the U.S. FDA has been withdrawn by the applicant as a consequence of concerns expressed by the US FDA (2017) about the absence of generally recognised clinical endpoints to determine the safety of supposedly bioactive ingredients such as the NF.

3.7. Proposed uses and use levels and anticipated intake

3.7.1. Target population

The target population proposed by the applicant are infants and young children up to 35 months of age.

3.7.2. Proposed uses and use levels

The applicant intends to market the NF for use in infant formulae (up to 6 months included), follow-on formulae (age 6–12 months) and formula for young children (aged 1–3 years) at a use level of up to 151 mg/L (as consumed) in ready-to-drink and reconstituted products. The rationale for the proposed maximum use levels is based on considerations of the applicant about achieving similar levels of OPN as those occurring on average in mature human breast milk (see Section 3.6.1.2). The applicant gives most weight to the study provided by Schack et al. (2009) and also considered that there is little relationship between OPN concentration and days pp or duration of lactation.

When considering the maximum specification protein (N × 6.38) concentration in the NF (i.e. 80.5%) and considering that about 90% of the protein fraction is composed of bmOPN, the proposed maximum use level of 151 mg of the NF per litre of a reconstituted, ready to be consumed formula, corresponds to about 109 mg bmOPN per litre formula (Table 8).

Table 8: Food categories and maximum use levels intended by the applicant

FoodEx2 level	FoodEx2 code	Food category	Max use level (mg NF/100 g)
3	A0EQM	Infant formula	151 mg/L in the final product ready for use, marketed as such or reconstituted as instructed by the manufacturer
3	A0EQL	Follow-on formula	
3	A03RH	Ready-to-eat dairy-based meal for children	

The Panel considers that the maximum proposed use level of 151 mg bmOPN/L for the proposed food groups represents a concentration which is within the range of concentrations reported for hmOPN in human breast milk for the first two weeks of lactation. However, the proposed maximum use level would be about 3-fold higher than the hmOPN levels at month 1–12 of lactation when considering the results of the study by Jiang and Lönnerdal (2019).

3.7.3. Anticipated intake of the NF

EFSA performed an intake assessment of the anticipated daily intake of the NF based on the applicant's proposed uses and maximum proposed use levels (Tables 9 and 10), using individual data from the EFSA Comprehensive European Food Consumption Database (EFSA, 2011). The lowest and highest mean and 95th percentile anticipated daily intake of the NF (on an mg/kg body weight (bw) basis), among the EU dietary surveys, are presented in Table 9.

Table 9: Intake estimate (mg per kg bw per day) resulting from the use of the NF as an ingredient in the intended food categories at the maximum proposed use levels (151 mg/L as consumed)

Population group	Age (years)	Mean intake (mg/kg bw per day)		P95th intake (mg/kg bw per day)	
		Lowest[(a)]	Highest[(a)]	Lowest[(b)]	Highest[(b)]
Infants	< 1	2.8	14.4	9.8	33.4
Young children[(c)]	1–< 3	0.2	4.3	2.1	10.1
Other children	3–< 10	0.00	0.2	0.0	1.7

(a): Intakes are assessed for all EU dietary surveys available in the food comprehensive database on 10/6/2021. The lowest and the highest averages observed among all EU surveys are reported in these columns.
(b): Intakes are assessed for all EU dietary surveys available in the food comprehensive database on 10/6/2021. The lowest and the highest P95th observed among all EU surveys are reported in these columns (P95th based on less than 60 individuals are not considered).
(c): Referred as 'toddlers' in the EFSA food consumption comprehensive database (EFSA, 2011).

Table 10: Intake estimate (mg per day) resulting from the use of the NF as an ingredient in the intended food categories at the maximum proposed use levels (151 mg/L as consumed)

Population group	Age (years)	Mean intake (mg per day)		P95th intake (mg per day)	
		Lowest[(a)]	Highest[(a)]	Lowest[(b)]	Highest[(b)]
Infants	< 1	21.5	83.8	82.0	167.6
Young children[(c)]	1–< 3	2.3	50	21.4	116.0
Other children	3–< 10	0	3.9	0	30.2

(a): Intakes are assessed for all EU dietary surveys available in the food comprehensive database on 10/6/2021. The lowest and the highest averages observed among all EU surveys are reported in these columns.
(b): Intakes are assessed for all EU dietary surveys available in the food comprehensive database on 10/6/2021. The lowest and the highest P95th observed among all EU surveys are reported in these columns (P95th based on less than 60 individuals are not considered).
(c): Referred as 'toddlers' in the EFSA food consumption comprehensive database (EFSA, 2011).

The estimated daily intake of the NF for each population group from each EU dietary survey is available in the excel file annexed to this scientific opinion (under supporting information).

3.8. Absorption, distribution, metabolism and excretion (ADME)

3.8.1. *In vitro* digestion studies

With the objective to investigate the effects of gastric proteases on the digestion of human and bovine milk proteins, Chatterton et al. (2004) incubated pooled human breast milk samples and bovine whey protein concentrates with human neonatal gastric juice at pH values ranging from pH 2.0 to 6.5. Based on the results obtained with a Western immunoblot, which employed polyclonal antibodies, the authors noted that even at pH 2, intact glycosylated and phosphorylated hmOPN, represented by a major protein band of about 78 kDa, resisted gastric digestion. In addition, hmOPN peptides with MWs of about 34 kDa and 23 kDa were seen between pH-values of 3.0–6.5, and 3.5–6.5, respectively. Bovine mOPN resisted gastric digestion in a similar manner to hmOPN with additional smaller cleavage fragments resisting digestion. Full-length bmOPN was represented by a 60-kDa band and fragments were observed at about 30 (N-terminal fragment) and about 20 kDa (C-terminal fragment).

In another *in vitro* digestion modelling, Christensen et al. (2020) incubated hmOPN and bmOPN under human gastrointestinal conditions using digestive enzymes (pepsin, trypsin and chymotrypsin), acidity, volumes of media and bile salt concentrations for up to 60 min. Both hmOPN as well as bmOPN resisted gastric digestion, but were cleaved into smaller protein fragments under intestinal conditions. Glycosylated fragments were able to retain integrin-binding properties investigated by an *in vitro* cell adhesion assay, similarly to undigested OPN. The authors noted that the resistance to gastric digestion was dependent on the glycosylation integrin-binding sites (the glycosylations protected OPN against proteolytic cleavage in the integrin-binding region).

In another *in vitro* study, Dall'Asta et al. (2015) simulated gastrointestinal infant digestion of milk. Breast milk samples from six women were incubated with several digestive juices at 37°C at different phases. The digestive fluids employed human α-amylase, porcine mucin and pepsin, β-galactosidase from *Aspergillus oryzae*, porcine pancreatin, bovine bile and other constituents. Fourier-Transform-Mass Spectrometry (FTMS) was employed to analyse the profile of the peptide fraction obtained from the *in vitro* digestion. The authors reported that after digestion, 28 different peptides with an average of seven amino acid residues and an average MW of 984.4 Da could be identified to originate from hmOPN.

3.8.2. Animals

A study with 3- and 10-week-old OPN-deficient (knock-out) mice showed similar bmOPN plasma levels 1 and 4 hours after administration of a single oral dose of 50 mg per mouse (Rittling et al., 2014). Bovine milk OPN plasma levels were measured with an ELISA using polyclonal anti-bmOPN antibodies 1, 4, 8 and 24 hrs after dosing. In the 10-week-old mice, bmOPN could be detected also 24 h after dosing at a plasma concentration of approximately 40% and 25% of the concentration found after 1 and 4 h, respectively. The highest concentration was measured in the 10-week-old mice 4 h after dosing (~ 4.8 μg/mL).

Jiang et al. (2019) showed that murine milk (mm)OPN can be found in the brain of OPN-deficient (knock-out) mouse pups nursed by wild-type dams, which indicates that mmOPN is absorbed from murine milk. The authors also showed that wild-type mouse pups nursed by wild-type dams had higher OPN levels in the brain as compared to wild-type mouse pups nursed by OPN-deficient dams, which also suggests absorption of mmOPN by the suckling pups. Brain mRNA levels for OPN were not different between the wild-type pups nursed by either wild-type or OPN-deficient dams, suggesting that the OPN absorbed from the milk did not interfere with OPN expression in the brain. Furthermore, in another experiment described in this article, radioactivity was found in several organs, including brain and liver in wild-type pups fed via oral gavage with purified hmOPN radiolabelled with ^{125}iodine. The authors noted that the detection of radioactivity in the brain would not necessarily represent full-length, intact OPN.

A recent study of Jiang et al. (2021) investigated digestion and absorption of bmOPN, recombinant bOPN (rbOPN) and recombinant hOPN (rhOPN) produced by *Chlamydomonas reinhardtii* administered by oral gavage at a dose of 12 mg/kg bw to 12 days old OPN-deficient (knock-out) mouse pups. Full-length OPN was found in the stomach and small intestine 30 min after dosing. OPN peptides were formed in both digestive organs with similar profiles for the three OPNs, except the two recombinant OPNs had an additional 20 kDa band observed in a Western blot. Another set of 12-day-old mice also received by oral gavage the three OPNs but biotinylated, at the same dose (12 mg/kg bw) in order to investigate absorption and to distinguish between exogenous OPNs and OPN produced endogenously in plasma. Three hours after the gavage blood samples were taken and investigated for biotinylated OPN and OPN fragments by an ELISA. All three exogenous OPNs were detected in the plasma at similar concentrations (ranging between 16.6 and 18.2 ng/mL). In addition, uptake of the three biotinylated OPNs by human intestinal epithelial cells (HIECs) *in vitro* without significant differences was shown in this study.

3.8.3. Human

Jiang and Lönnerdal (2019) investigated the effect of bmOPN added to a commercially available IF fed to three groups of infants on their hOPN and bmOPN plasma levels. Zero, 65 or 130 mg bmOPN were added per litre of IF resulting in measured concentrations of 14, 72 and 140 mg/L IF, respectively. In addition, a group of breastfed (BF) infants was studied. Each group comprised 25 infants to study the effect on hOPN plasma levels. For each dose group, eight infants per group were enrolled to study bmOPN plasma concentration. These investigations were a follow-up of the infant study described below in Section 3.10.6.1 (Study in infants with the NF) (Peng and Lönnerdal, 2013; Lönnerdal et al., 2016). Infant plasma samples were obtained at month 1, 4 and 6. Bovine milk OPN and hOPN were measured with two commercially available ELISA tests without cross-reactivity of the two kits (i.e. the hOPN ELISA did not detect bmOPN and vice versa).

At the first measurement around initiation of the study at 1 month of age, no statistically significant differences were found among the four infant groups regarding demographics, medical history, physical examination, anthropometric measures and morbidity. According to the figures provided by Jiang and Lönnerdal (2019), the low-dose group (without added bmOPN) had the lowest hOPN plasma

concentration (~ 175 μg/L) already at this first measurement at month 1, about 27% lower than the BF group and the mid- and high-dose groups. The study authors noted that some infants of the mid- and high-dose groups had started receiving the study formulas already a few days prior to 1 month of age, meaning that no real baseline data were available prior dosing.

In the samples obtained from 4-month-old infants, plasma hOPN concentrations were statistically significantly higher in the BF, the mid- and the high-dose groups as compared to the low-dose group, and no statistically significant differences were seen between the BF, mid- and high-dose groups. At 6 months of age, BF infants had statistically significantly the highest plasma hOPN concentration, and the low-dose infants still had the lowest hOPN concentration, statistically significantly lower than the other three groups. According to the study author and to the applicant, this could indicate that dietary bmOPN increased endogenous hOPN production in infants. The Panel notes, however, that no baseline data were available and baseline-adjusted statistical analyses could not be performed which would account for differences already present before the test formula feeding of bmOPN started. In fact, as noted above, the hOPN plasma concentration of the low-dose group was already the lowest at the first measurement at month 1. Given the absence of baseline-adjusted statistical analyses, no conclusion can be drawn on whether dietary bmOPN had an impact on endogenous OPN production in this study as suggested by the study authors.

No bmOPN was detected in the plasma of BF and low-dose groups. Infants of the mid-dose group had mean plasma levels of about 3 and 2 μg bmOPN/L at 4 and 6 months of age, respectively. Infants of the high group had mean plasma levels of about 5 and 4 μg bmOPN/L at 4 and 6 months of age, respectively. These measured bmOPN plasma concentrations are about 5% or less of the plasma levels measured for hOPN at the respective time points.

The Panel considers that the study by Jiang and Lönnerdal (2019) does not show that bmOPN added at 65 or 130 mg/L to IF has a significant, if any, impact on infants' hOPN plasma levels, and that resulting bmOPN plasma levels are small, i.e. 5% or less of the infants' hOPN plasma levels.

Overall, the Panel notes that *in vitro*, animal and human studies indicate that orally consumed human and bovine OPN is at least partially resistant to digestion and can be absorbed and enter circulation. The Panel also considers that the available animal and human studies do not provide evidence that orally consumed OPN would have an impact on endogenously produced OPN.

3.9. Nutritional information

According to the proposed use level and the resulting highest P95 estimate (approximately 168 mg bmOPN per day), the resulting protein intake from the NF would be negligible. The Panel considers that the NF is not nutritionally disadvantageous.

3.10. Toxicological information

The applicant provided three genotoxicity studies (all performed with the same batch No. B470239) and a subchronic toxicity study on the NF, which were conducted in compliance with OECD principles of GLP (OECD, 1998a) and in accordance with the test guidelines No. 471, 473, 474 and 408 from the Organisation for Economic Co-operation and Development (OECD) (OECD, 1997a,b,c, 1998a,b). In addition, the applicant provided a study report on a teratogenicity study, which was not performed and reported in compliance with the respective OECD Guidance document and GLP. All toxicological studies provided by the applicant are listed in Table 11.

Table 11: List of toxicological studies (all unpublished) with the NF

Reference	Type of study	Test system	Dose
Kvistgaard et al. (2012)	Bacterial reverse mutation test	*S. Typhimurium* TA98, TA100, TA1535, TA1537 and *E. coli* WP2 uvrA	Up to 5,000 μg/plate (absence and presence of S9 mix), (batch No. B470239)
Kvistgaard et al. (2013a)	In vitro chromosomal aberration test, human lymphocytes	Human lymphocytes	1,000, 3,000, 5,000 μg/mL (absence and presence of S9 mix), (batch No. B470239)
Kvistgaard et al. (2013b)	In vivo micronucleus test	NMRI mice	2,000 mg/kg bw per day (oral gavage) (batch No. B470239)

Reference	Type of study	Test system	Dose
Lina (2007)	90-day repeated dose oral toxicity study	Wistar rats	1,200 mg/kg bw per day (batch No. 91538-batch 1)
Chinese CDC (2009)	Teratogenicity study	Wistar rats	630, 1,250 or 2,500 mg/kg bw per day at gestation days 7–16, gavage (batch 200811009)

3.10.1. Genotoxicity

In the *in vitro* bacterial reverse mutation assay, *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* strain WP2uvrA were treated with the NF (Kvistgaard et al., 2012). In this study, the test item was non-mutagenic at concentrations up to 5,000 μg/plate, in the absence or presence of metabolic activation.

In an *in vitro* mammalian chromosome aberration test with human peripheral blood lymphocytes, no increase in the number or type of aberrant metaphases was detected up to 5,000 μg/mL, either in the presence or absence of a metabolic activation system (Kvistgaard et al., 2013a). No cytotoxicity was observed at any concentration. The results of the study indicated that the NF is not clastogenic at doses up to 5,000 μg/mL.

In an *in vivo* mammalian micronucleus test with Naval Medical Research Institute (NMRI) mice the test item did not induce structural and/or numerical chromosomal damage in the immature erythrocytes at a dose of 2,000 mg/kg bw given per oral gavage (Kvistgaard et al., 2013b).

Even though an *in vitro* micronucleus test, as recommended in the EFSA Scientific Opinion on genotoxicity testing strategies (EFSA Scientific Committee, 2011), was not conducted, the Panel considers that given the source, the production process and the nature of the NF, and the results of the provided studies, there are no concerns with regard to genotoxicity.

3.10.2. Subchronic toxicity

The applicant provided a 90-day repeated dose toxicity study by Lina (2007) with Wistar rats in compliance with the OECD principles of GLP (OECD, 1998a) and according to OECD Guidance No 408 (OECD, 1998b). Groups of 10 male and 10 female 6-weeks old rats received 0 (rodent diet without the NF), 300, 600 or 1,200 mg/kg bw per day of the NF (batch number 91538-batch) incorporated into the rodent diet for thirteen weeks.

There were no deaths during the 90 days, no test item-related clinical signs observed in the weekly assessment and no ophthalmological changes observed at the end of the treatment. In the functional observation battery (FOB) assessments, no changes were observed.

There were no differences in body weight, food and water consumption between test item groups and the controls in both sexes.

The only statistically significant differences in haematology were slightly, by 2% and 1%, increased mean corpuscular haemoglobin concentration in the low- and mid-dose group of male rats, respectively. Given the small difference, the lack of a dose-response and the absence of a statistical difference between the high dose and control group, the noted differences are considered incidental. There were no other statistically significant differences found in the haematological endpoints including total and differential white blood cells.

Clinical chemistry parameters did not show statistically significant differences.

In males of the low- and mid-dose group, a statistically significant increase in urinary density associated with a decreased urinary volume in low-dose was noted, but neither of the two urine parameters were changed in the high-dose males. The Panel considers this finding incidental.

There was a statistically significant increase (by about 10%) of the relative kidney weight in the mid dose group of female rats. The absolute kidney weight of this group was also increased (by about 6.6%) but was not statistically significant from the control group. Considering that also the highest dose group of female rats did not differ from the control group, the Panel considers this finding incidental. There were no differences observed for the absolute or relative kidney weight in any of the male rats.

Macroscopic examination at necropsy and microscopic examination of the organs did not reveal any adverse effects caused by the test substance.

The Panel notes that this 90 day study was performed in 2007 and did not include thyroid-stimulating hormone (TSH), the two thyroid hormones triiodothyronine (T3) and thyroxine (T4) and thyroid gland weight, which became later a required parameter (OECD, 2018). As required, the histology of the thyroid gland was investigated for the highest dose of both sex and did not provide an indication for a toxicological effect.

In response to an EFSA request to investigate whether serum samples of the rats were still available, the applicant responded that serum samples from the 90-day study were no longer available for analysis of TSH, T3, or T4.

The Panel considers that the highest dose tested, i.e. 1,200 mg/kg bw of the NF, is the no observed adverse effect level (NOAEL) of this subchronic study.

3.10.3. Reproductive and developmental toxicity

In a (non-GLP) teratogenicity study, 3-month-old mated female Wistar rats (n = 15 per group) were given the NF (batch number 200811009) at 0, 630, 1,250 or 2,500 mg/kg bw per day dissolved in distilled water and by oral gavage on gestation days 7 to 16 (Chinese CDC, 2009). Day 0 of gestation was determined upon detection of a vaginal plug. The pregnant rats were weighed on day 0, 7, 12, 16 and 20. On day 20, the animals were killed and bled. The number of implantations, the number of live fetuses, the number of dead fetuses and the number of fetuses absorbed, were recorded. All fetuses were examined for abnormalities and measured for length and weight. One half of the fetuses were subject to dissection and examined for abnormalities in the brain, cerebrum, ventricles, diencephalons, tongue, cleft palate, nose, eyes, maxilla, medulla oblongata, spinal cord and all respiratory, digestive and urinary organs. Effects on bones was examined in the other half of fetus, including ossification retardation, lack of skeletal integration, bifurcation, rib count increases or decreases, shape abnormalities or arrangement disorder of the skull, cervical, thoracic, lumbar, or sacral vertebrae, pelvis, limbs, sternum, or ribs.

For each of the 15 mated rats per group, the study report noted 13 pregnant rats in the control and high dose group and each 12 pregnant rats in the low and mid group. There were no statistically significant differences recorded for the body weight of the treated rats compared to controls. The same applied to the body weight and body length of the fetus, the number of live fetuses, absorbed fetuses and dead fetuses and the loss rate of the fetuses of the test vs. control group. There were no abnormalities observed in the visceral examination. Abnormalities of fetal skull (incomplete ossification) and the sternum ('dotted shapes') were reported for fetuses of all groups, but without dose-response, qualitative or statistically significant quantitative differences.

The Panel considers that the presented study did not indicate teratogenic effects of the test item. However, this study was not performed under GLP and did also not meet respective OECD standards. The shortcomings include that the test item was administrated only at the gestation days 7–16 (instead of day 0 until the day of sacrifice) and the low number of pregnant rats (n = 12 or 13) used in this study (instead of 20 female rats with implantation sites at necropsy according to OECD (2018).

3.10.4. Human data

3.10.4.1. Study in infants with the NF

Effects of the NF added to an infant formula was studied over 6 months with 279 infants in a double-blind randomised trial in Shanghai, China (Peng and Lönnerdal, 2013, study report; Lönnerdal et al., 2016; NCT00970398). The safety objectives were the frequency and severity of adverse events (measured by recorded adverse events and concomitant medications). Other endpoints were growth (body length, bw and head circumferences, food intake, stool consistency and wellbeing recorded by 3 days questionnaires. All safety endpoints were assessed at each of the study visits at 1, 2, 3, 4, 5 and 6 months of age. Additional endpoints were plasma cytokines (measured at month 1, 4 and 6), haematology, iron status, plasma amino acids, insulin, blood urea nitrogen (BUN) and antibody titre against tetanus at month 6 to a vaccination with a trivalent vaccine comprising diphtheria, pertussis and tetanus at month 4.

Infants included in the study were less than 1 month of age with a gestational age of 37–42 weeks at birth, weighed between 2.5 kg and 4 kg, without asphyxia and infections at birth, exclusive formula-feeding or, for the breast-fed reference group (BF), exclusive breast-feeding at inclusion with the mother's intention to continue like that until 6 months of age. Recommendations of feeding only

small amounts (taste portions) of complementary foods between 4 and 6 months of age were given to all parents/caregivers.

Formula-fed (FF) infants were stratified for sex. According to the study protocol, 240 infants (120 male, 120 female) should be fed from 1 to 6 months of age with one of the following three formulas: a commercially available IF without added NF ('low dose' bmOPN group because the IF contained 14 mg bmOPN/L), or with the same IF used as for the low dose but with added 65 or 130 mg/L of the NF-containing bmOPN (with 89% purity), respectively. The total content of bmOPN in the formulae fed to the three groups, was 14 (low dose), 72 (mid dose) and 140 (high dose) mg bmOPN/L, respectively (Lönnerdal et al., 2016). A fourth group of 80 (40 male, 40 female) exclusively BF infants should be also enrolled according to the study protocol.

Formula intake was measured by the parents and tracked in a food diary. A diary was also kept for clinical symptoms, stool consistency and frequency, illnesses, medication use or hospitalisation. Blood samples were obtained at 1, 4 and 6 months of age and assessed for red and white blood cell concentrations, serum ferritin, C-reactive protein (CRP), amino acids, blood urea nitrogen, cytokine levels, i.e. interleukin (IL-2, IL-6, IL-8, IL-10, IL-12, IL-15) and tumour necrosis factor, insulin and tetanus-specific antibodies. No baseline plasma samples were analysed.

The study protocol and informed consent form were reviewed and approved by the Institutional Review Boards at University of California, Davis (UCD) and Fudan University, Shanghai prior to the screening or enrolment of any study participants.

Results

According to the study report, there were no true baseline values. The reason is given in the published article by Jiang and Lönnerdal (2019), referred to in Section 3.8.3, who noted that *'some infants in the F65 and F130 groups had started receiving the study formulas a few days prior to 1 month of age'*.

Although according to the study protocol and synopsis of the study 320 infants were enrolled and analysed, the study report provided only information about 279 enrolled infants: 67, 67, 70 and 75 infants were randomly assigned to the low-, mid-, high-dose and BF group. Among them, 28 infants did not complete the study: 1, 6, 7 and 14 in the low-, mid-, high-dose and BF group, respectively. Therefore, 251 infants completed the study at month 6 (66, 61, 63 and 61 in the low-, mid-, high-dose and BF group, respectively).

The reasons for the discontinuations of (only) 26 infants were given in the information provided in the study report for the exclusions from the per protocol population: withdrawal of blood-draw consent (1, 3, 1 and 7 for the low-, mid-, high-dose and BF group, respectively), inability to come to follow-up visits (0, 2, 3, 1, respectively), mother started to have sufficient milk (2 in the mid-dose group), insufficient breast milk (3 in the BF group) and 'not well adapted to formula' causing gastrointestinal problems (3 in the high-dose group). The study report neither provided information on how many infants were comprised in total in the per-protocol (PP) population, nor the number of infants for each of the four groups were given. The published article on this study however indicates that each group comprised '60–65 per group' (Lönnerdal et al., 2016). Considering these numbers and despite a note in the study report that all 279 infants were included in the safety analysis, the study report provided results and statistical analyses only for the PP population including endpoints related to safety. Following a request by EFSA, the applicant confirmed that for the statistical analyses including the safety endpoint 'adverse events' only the PP population was analysed, i.e. the three drop-outs for gastrointestinal reasons were not included in the statistical analysis of the endpoint 'adverse events'.

With regard to the 'adverse events', the only statistically significant differences for the PP population between the FF infants and the group BF were the incidence (% of months) and the prevalence (number per 100 days) of pyrexia, noting that both were statistically lower in the mid- and high-dose groups as compared to the low-dose group, indicating that bmOPN did not have a detrimental effect on this endpoint. No significant differences were reported between formula fed infants for the prevalence of diarrhoea, gastrointestinal problems, vomiting, infections, respiratory, eye or skin problems. No deaths and no serious adverse events occurred in this study. The study report does not comment on the outcome regarding concomitant medication. There was no statistically significant difference in body weight, body length or head circumference reported between the FF infants. There was also no statistically significant difference in formula intake among the FF groups.

In response to the question raised by EFSA to the applicant on the results for the endpoint of concomitant medication, the applicant responded that this endpoint was actually not studied.

The mid- and high-dose group had statistically significantly higher insulin plasma levels than the low-dose group at month 4. At month 6, however, the high-dose group had the lowest insulin plasma levels, statistically significant compared to the mid-dose group, but not when compared to the low-dose group. At month 4 and 6, BUN was significantly higher in the high-dose group than in the mid-dose group, but not when compared to the low-dose group.

No significant differences were observed for the white blood cell (lymphocytes, eosinophils, basophils, neutrophils and monocytes) differential counts between the FF groups analysed by flow cytometry. The applicant also referred to a published article by West et al. (2017) who studied subsets of lymphocytes in the plasma samples obtained from the infants of the study by Peng and Lönnerdal (2013). The authors of that publication and the applicant noted that the high-dose group had a statistically significant increased T-cell proportion among lymphocytes as compared to the low- and mid-dose group. Additional analyses revealed that this higher proportion of T cells in the high-dose group was not associated with a statistically significantly increased proportion of either of the two studied subtypes, T-helper cells and cytotoxic T cells. Furthermore, the observed proportion of T cell in the F2 group was not statistically significantly higher compared to the breastfed infants.

At month 6, the mid-dose groups (61.8 pg/mL $\pm$ 56.6) and high-dose group (46.2 pg/mL $\pm$ 17.3) had statistically significant higher mean values for interleukin-2 as compared to the low-dose group (29.9 pg/mL $\pm$ 13.1). However, there was no dose-response among the FF groups and the mean values for all formula-fed groups remained within one standard deviation of the BF group (46.4 pg/mL $\pm$27.6). Interleukin-10 mean values at month 6 were statistically significantly lower in the mid group (20.1 pg/mL $\pm$ 45.0) and high-dose group (7.26 pg/mL $\pm$ 6.86) compared to the low-dose group (146 pg/mL $\pm$ 103), but also this parameter remained within one standard deviation of the result for the BF group (28.5 $\pm$ 20.5). C-RP plasma concentration did not differ statistically significantly among the FF groups.

Infants were vaccinated with a trivalent vaccine (diphtheria, pertussis, tetanus) at 4 months of age. The antibody titre (IgG) against tetanus was measured at 6 months of age (Lönnerdal et al., 2016). There was no significant difference between BF and FF infants, but among the FF groups, the mid-dose group had fewer antibodies than the low-dose group. There were no statistically significant differences between the high-dose group and any of the other groups.

Overall, the Panel notes that a few statistically significant results have been reported among the FF groups. The reported differences were inconsistent at different time points measured and/or without dose-response and appear to be incidental among the many parameters studied related to haematology, blood chemistry and immunological endpoints measured at multiple time points. In addition, the reported differences were within the physiological range and were not associated with functional consequences (e.g. no effects on antibody responses). The Panel considers that no cause for concern is evident, even if no definitive conclusion can be drawn on these differences. The lack of baseline data in this study adds to the obstacles for the interpretation of the results.

The Panel considers that the results obtained for the formula intake, growth, weight and head circumferences do not raise safety concerns and the results for the reported adverse events suggest that there was no difference between the tolerability of a commercially available infant formula with a 'background' bmOPN content of 14 mg/L (i.e. without added bmOPN) and the same formula with added bmOPN resulting in contents of about 80 and 140 mg/L, respectively. With regard to the other studied endpoints (including haematological, blood chemistry and immunological endpoints), although a few reported differences were noted, the Panel considers they do not raise safety concerns.

Overall, some limitations and reporting issues were noted, such as no information was given why only 279 infants were finally enrolled in the study although the study protocol and the study synopsis noted 320 infants and even though 28 drop-outs were reported, reasons were provided only for 26. Furthermore, the number of infants analysed in the PPT population was not reported. Another limitation of this study is that it did not statistically analyse the full safety population which included three infants in the high-dose group who dropped out because of gastrointestinal problems.

Given that the subchronic toxicity study provided and described in Section 3.10.2 did not include thyroid hormones, EFSA asked the applicant whether serum samples from the provided 6-month study in infants would still be available to analyse them for TSH, T3 and T4. Since no serum was available anymore from the applicant for such additional analyses, the applicant reviewed the scientific literature regarding the relationship between bmOPN and thyroid function.

In the response provided by the applicant, it is noted that evidence exists in the literature of increased hOPN plasma levels among patients with hyperthyroidism (increased T3 and T4 and decreased TSH) as well as of hOPN downregulation in hypothyroid patients (Alwakeel et al., 2012;

Reza et al., 2013; Spaulding, 2013; El-Zawawy et al., 2020). Reviewing the role and function of OPN in bone metabolism and noting this correlation between OPN and thyroid hormone plasma levels, these authors investigated whether plasma OPN could serve as a marker for thyroid function. Reza et al. (2013) and Sodek at al. (2000) noted the expression of OPN in preosteoblasts, osteoblasts, osteocytes and chondrocytes (among other cells), the function of OPN by serving as a linking protein ('bridge') between the cells and hydroxyapatite through RGD (R = Arginine, G = Glycine, D = Aspartate) and polyaspartic acid motifs, and its prominent presence in the mineralised extracellular matrices of bones. Wei et al. (2016) noted the correlation of OPN plasma levels with bone turnover markers. Tuchendler and Bolanowski (2014) reviewed the literature about the role of thyroid hormones in bone metabolism and noted that overt hyperthyroidism leads to decreased bone mineral density and increased fracture risk. Tuchendler and Bolanowski (2014) also noted the extensive expression of thyroid hormone receptors in bone and the catabolic effects of thyroid hormones on bone metabolism. These authors also noted that hyperthyroidism increased bone turnover and loss of bone mineral density. The applicant also argued that increased OPN plasma levels have been reported also for patients suffering from Graves' disease where increased T3 and T4 production is triggered by thyroid-stimulating immunoglobulins (El-Zawawy et al., 2020). According to the applicant's view, the available evidence would suggest that increased OPN plasma levels in patients suffering from hyperthyroidism is rather a consequence than a cause of the increased T3 and T4 levels. The applicant also noted that there were no indications for increased thyroid hormone levels such as differences in feed intake, body weight, neurobehavioural endpoints, histopathology of the bone or thyroid gland observed in the 90-day rat study compared to the control group. The same would apply for the 6-month study with infants with respect to their food intake, body weight, growth and clinical observations. Finally, the applicant stressed that the intended maximum use level for the NF to IF and FoF would not exceed concentrations of hOPN in human breast milk.

The Panel agrees that there are no indications from the provided animal and human studies which would indicate increased thyroid hormone production and also concurs with the applicant's interpretation of the literature on the association between OPN plasma levels and thyroid hormones.

3.10.4.2. Other human studies

In a cross-sectional study, 42 healthy children and 51 children with asthma were enrolled. The asthma group comprised of 23 children up to an age of 5 years of age and 28 children older than 5 years of age (Akelma et al., 2014). For the > 5-year age asthmatic group, but not for the younger asthmatic children, OPN serum levels (median: 7.2, range: 3.4–14.5 ng/mL) were statistically significantly higher as compared to the healthy control children (median: 6.0, range: 3.2–7.3 ng/mL). In addition, among the 28 asthmatic children who were older than 5 years of age those who also suffered from allergic rhinitis (n = 15) had statistically higher OPN serum levels as compared to those (n = 13) without allergic rhinitis.

In a case–control study, samples were obtained from 35 mild-to-moderate asthmatics, 19 severe asthmatics and 17 healthy controls in the steady state and in cases of exacerbation (Samitas et al., 2011). Osteopontin serum levels, bronchoalveolar lavage fluid (BALF) and bronchial tissue were investigated by ELISA and immunohistochemistry or immunofluorescence. Reticular basement membrane thickness and goblet cell hyperplasia were also determined. Serum and BALF OPN levels were significantly increased in all asthmatics in the steady state, whereas serum levels decreased during exacerbations. OPN was upregulated in the bronchial tissue of all patients, and expressed by epithelial, airway and vascular smooth muscle cells, myofibroblasts, T-lymphocytes and mast cells. OPN expression also correlated with reticular basement membrane thickness and was more prominent in subepithelial inflammatory cells in severe compared to mild-to-moderate asthma. The authors of this study noted that reasons behind the upregulation of OPN expression in asthma, as well as its role in the development of allergic inflammation and airway remodelling, remain largely unknown and they acknowledged that based on their study, it is difficult to determine a specific mechanism of action.

The Panel considers that based on the presented two studies reporting associations between upregulated OPN and asthma, no conclusions can be drawn on a cause and effect relationship.

Another study referred to by the applicant reported a dose-dependently increased OPN expression in bronchoalveolar cells obtained by lavage from 19 subjects (7 with asthma) who were exposed to different ozone concentrations (Leroy et al., 2015). The OPN expression was higher obtained from the asthma patients.

3.11. Allergenicity

The NF is derived from bovine milk. 'Milk and products thereof' are listed under the Annex II (i.e. substances or products causing allergies or intolerances) of Regulation (EU) No 1169/2011[7].

Panel considers that the intake of the NF can cause allergic reactions similar to those arising from consuming milk and dairy products.

4. Discussion

The NF concerns OPN derived from bovine whey. The NF is intended to be used at a maximum use level of 151 mg/L in IF and FoF and ready-to-eat dairy-based meals for children up to 35 months of age. When considering the maximum specification protein (N × 6.38) concentration of the NF (i.e. 80.5%) and when considering that about 90% of the protein fraction is composed by bmOPN, the proposed maximum use level of 151 mg of the NF per litre of a reconstituted, ready to be consumed formula, corresponds to about 109 mg bmOPN per litre formula.

The highest P95 intake of the NF is 33.4 mg/kg bw per day, estimated for infants. As compared to the concentrations naturally present in cow's milk and concentrations found in IF on the market reported in the literature, the proposed use level of the NF represents an about 10-fold higher concentration of bmOPN. Consultation of the Mintel-Database indicated IFs on the Asian market with a bmOPN concentration of up to 110 mg/L. The intended use levels of the NF would provide bmOPN at concentrations within the range of hmOPN found breast milk.

The NOAEL from the provided subchronic toxicity study in rats was the highest dose tested, i.e. 1,200 mg/kg bw, which provides a margin of exposure of about 36, which is below the default safety factor of 200 as suggested by the EFSA Scientific Committee (2012).

A non-GLP and not OECD-compliant teratogenicity study with rats with the highest dose tested of 2,500 mg/kg bw did not show adverse effects. Some limitations in the study design were noted.

In a 6-month study, three different bmOPN concentrations, i.e. 14, 72 and 140 mg/L, in infant formula were given to 279 infants in order to study possible effects on frequency and severity of adverse events, and growth (body length, body weight and head circumferences), formula intake and stool consistency. Other endpoints were haematology, iron status, plasma amino acids, insulin, blood urea nitrogen (BUN), plasma cytokines and antibody titre against tetanus at month 6 to a vaccination with a trivalent vaccine comprising diphtheria, pertussis and tetanus at month 4. Even though a number of inconsistencies and limitations were noted in the study report, the Panel considers that the results obtained from this study do not raise safety concerns.

The Panel also considers that analyses of the plasma of infants who participated in this 6-month study did not show that the addition of bmOPN to IF has an impact on the hOPN plasma levels of the infants. Moreover, the bmOPN plasma levels resulting from the absorption of the bmOPN from the IF were low, i.e. about 2–5% of the endogenously produced plasma OPN levels.

Considering the source of the NF, that neither the toxicological studies nor the provided infant study raise safety concerns, and the low bmOPN plasma levels in infants resulting from the consumption of the NF, the Panel considers that the margin of exposure (i.e. 36) between the NOAEL of the subchronic toxicity study (1,200 mg/kg bw) and the highest P95 estimate for infants (33.4 mg/kg bw per day) is sufficient.

5. Conclusions

The Panel concludes that the NF, bmOPN, is safe under the proposed conditions of use.

5.1. Protection of Proprietary data in accordance with Article 26 of Regulation (EU) 2015/2283

The Panel could not have reached the conclusion on the safety of the NF under the proposed conditions of use without the data claimed as proprietary by the applicant (certificates of analyses and batch testing (annex II of the dossier), stability reports (annex IV) and the unpublished study reports on the following studies (annex VIII): a bacterial reverse mutation assay (Kvistgaard et al., 2012), an *in vitro* mammalian chromosome aberration test (Kvistgaard et al., 2013a), an *in vivo* micronucleus

[7] https://eur-lex.europa.eu/legal-content/EN/TXT/HTML/?uri=CELEX:32011R1169&from=EN

test (Kvistgaard et al., 2013b), a subchronic oral toxicity study in rats (Lina, 2007) and a study in infants (Peng and Lönnerdal, 2013).

6. Steps taken by EFSA

1) On 09/10/2020 EFSA received a letter from the European Commission with the request for a scientific opinion on the safety of Bovine milk osteopontin. Ref. Ares(2020)5367160.
2) On 09/10/2020, a valid application on Bovine milk osteopontin, which was submitted by Arla Foods Ingredients, was made available to EFSA by the European Commission through the Commission e-submission portal (NF 2020/1698) and the scientific evaluation procedure was initiated.
3) On 23/06/2021, EFSA requested the applicant to provide additional information to accompany the application and the scientific evaluation was suspended.
4) On 24/01/2022, additional information was provided by the applicant through the Commission e-submission portal and the scientific evaluation was restarted.
5) During its meeting on 26/01/2022, the NDA Panel, having evaluated the data, adopted a scientific opinion on the safety of Bovine milk osteopontin as a NF pursuant to Regulation (EU) 2015/2283.

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Abbreviations

ADME	Absorption, distribution, metabolism and excretion
ADPI	American Dairy Products Institute
BmOPN	bovine milk osteopontin
BALF	bronchoalveolar lavage fluid
BF	Breast-fed
BUN	Blood urea nitrogen
Bw	bodyweight
CFU	Colony forming units
$CaCl_2$	Calcium chloride
CoA	Certificates of analysis
CRP	C-reactive protein
Da	Dalton
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FF	Formula fed
FOB	functional operational battery
FoF	Follow-on formula
FSSC	Food Safety System Certification
FTMS	Fourier-Transform-Mass-Spectrometry
GLP	Good Laboratory Practice
GMP	Good Manufacturing Practice
GRAS	Generally recognised as safe
HACCP	Hazard Analysis Critical Control Points
HCl	hydrogen chloride

mOPN	Human milk osteopontin
HIEC	Human intestinal epithelial cells
HPLC	High performance liquid chromatography
ICP-HRMS	Inductively coupled plasma-high resolution mass spectrometer.
ICP-MS	Inductively coupled plasma - mass spectrometry
ICP-OES	Inductively coupled plasma - optical emission spectrophotometer
IDF	International Dairy Federation
IF	Infant formula
IL	interleukin
ISO	International Organization for Standardization
kDa	Kilo Dalton
LC-FLD	Liquid chromatography fluorescence detector
LC-MS/MS	Liquid Chromatography with tandem mass spectrometry
MALDI-MS	Matrix assisted laser desorption ionisation mass spectrometry
Mopn	milk osteopontin
mmOPN	murine milk osteopontin
mRNA	Messenger Ribonucleic Acid
MS	Mass spectroscopy
MW	Molecular weight
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NDA	Panel on Nutrition, Novel Foods and Food Allergens
NF	Novel Food
NMKL	NordVal International Denmark
NMRI	Naval Medical research Institute
NOAEL	no observed adverse effect level
NF	Novel food
OECD	Organisation for Economic Co-operation and Development
OPN	Osteopontin
Pp	Post-partum
PP	Per-protocol
rbOPN	Recombinant bovine osteopontin
rhOPN	Recombinant human osteopontin
T3	triiodothyronine
T4	tetraiodothyronine
TSH	thyroid stimulating hormone
UCD	University of California, Davis
US	United States