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BURDOCK GROUP
Consultants

July 06, 2017

Paulette M. Gaynor, Ph.D.
Deputy Division Director
Office of Food Additive Safety (HFS-200)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, MD 20740-3835

RE: Lacprodan® OPN-10 GRAS Notification

Dear Dr. Gaynor:

In accordance with 21 CFR §170, Subpart E – Generally Recognized As Safe (GRAS) Notice, I am submitting, as the agent of the notifier, Arla Foods Ingredients Group P/S (AFI), Soenderhoej 10-12, DK-8260 Viby J, Denmark, a notification of the conclusion of GRAS status for Lacprodan® OPN-10 for use in non-exempt cow's milk-based infant formula and cow's milk-based powdered beverages targeted for children 1 – 3 years of age, at an estimated upper consumption level of 39.5 mg/kg body weight/day (90th percentile). This is typically used in the U.S. to represent a long-term or "lifetime averaged" daily intake estimate.

Best regards,

(b) (6)

Ray A. Matulka, Ph.D.
Director of Toxicology
Burdock Group

1. Signed Statements and Certification

In accordance with 21 CFR §170, Subpart E – Generally Recognized As Safe (GRAS) Notice, I, Ray A. Matulka, Ph.D., am submitting a GRAS notification for the use of Lacprodan® OPN-10 as specified in this notification, as the agent of the notifier (Arla Foods Ingredients Group, P/S).

Notifier:

Arla Foods Ingredients Group P/S
Soenderhoej 10-12
DK-8260 Viby J, Denmark

Agent of the Notifier:

Ray A. Matulka, Ph.D.
Director of Toxicology
Burdock Group
859 Outer Road
Orlando, FL 32814
Telephone: 407-802-1400
Facsimile: 407-802-1405
Email: rmatulka@burdockgroup.com

A. Name of the Notified Substance

For the purposes of this GRAS Notification, the name used to describe the ingredient is:

Lacprodan® OPN-10

Lacprodan® OPN-10 (OPN-10) is to be used as an ingredient to provide a supplementary source of osteopontin (OPN) in the diets of infants and children ages 1 – 3 consuming cow's milk-based infant formula and powdered beverages, respectively.

B. Conditions of Use

Lacprodan® OPN-10 may be used to provide a supplementary source of osteopontin in the diets of infants and children ages 1 – 3 consuming cow's milk-based infant formula and powdered beverages, respectively, resulting in an estimated 90th percentile consumption level of 39.5 mg/kg body weight/day.

C. Basis of GRAS Determination

Pursuant to 21 CFR §170.3, a conclusion of GRAS status through scientific procedures, in accordance with §170.30(a) and (b), was found for the use of Lacprodan® OPN-10 as an ingredient in food under its intended conditions of use.

D. Premarket Approval Exemption

Arla Foods Ingredients Group P/S (Arla), has concluded, based on the views of an independent Expert Panel, that Lacprodan® OPN-10, produced from cow's milk, is generally recognized as safe (GRAS) as a food ingredient for non-exempt cow's milk-based infant formula for infants and powdered beverages for children ages 1 – 3 and therefore, is exempt from the requirement of premarket approval of the Federal Food, Drug and Cosmetic Act, under the conditions of its intended use.

E. Availability of Information

The data and information that serve as a basis for this conclusion of GRAS status are available for FDA review and copying at reasonable times at:

Burdock Group
859 Outer Road
Orlando, FL 32814
Telephone: 407-802-1400
Facsimile: 407-802-1405
Email: rmatulka@burdockgroup.com

Alternatively, a copy of the data and information that serve as a basis for this conclusion of GRAS status may be sent *via* electronic format or on paper to FDA upon request.

F. Freedom of Information Act Exemption

No information in this notice is exempt from disclosure under the Freedom of information Act, 5 U.S.C. 522.

G. Certification

The undersigned author of this document hereby certifies that, to the best of their knowledge, this document is a complete, representative and balanced representation of all available information, favorable as well as unfavorable, known by the author to be pertinent to the evaluation of the safety and GRAS status of the use of the substance.

Signed,

(b) (6) _____ Date July 6, 2017

Ray A. Matulka, Ph.D.
Director of Toxicology
Burdock Group
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H. United States Department of Agriculture, Food Safety Inspection Service Review

Where applicable, as required by §170.270, FDA is authorized to send any trade secrets to the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture (USDA) or ask FDA to exclude any trade secrets from the copy of the GRAS notice that will be sent to FSIS.

2. Detailed Information about the Identity of the Notified Substance

A. Identity of Lacprodan® OPN-10

Lacprodan® OPN-10 (OPN-10) is an osteopontin (OPN)-based product isolated from bovine whey and produced by commonly used ultrafiltration and ion exchange chromatography (IEC) processes in the whey industry to fractionate whey. OPN-10 has a minimum of 78% protein based on conventional calculations (N*6.38) and minimum 86% protein based on appropriate correction factors (N*7.17) included in calculations for this ingredient (further discussed below) (de Boer, 2014). OPN comprises at least 95% of the total protein in the OPN-10 fraction.[1] OPN-10 is produced as a source of OPN that can be used as an ingredient to provide infants and young children (ages 1 – 3) with a supplementary source of OPN in their diets.

A.1. Osteopontin in human milk

The goal of any infant formula manufacturer is to provide a nutritious, growth-promoting formula that strives to mimic, as closely as possible, the composition and function of human milk, to infants 0 – 12 months of age. Human milk contains low levels of many different proteins that are believed to contribute to the overall benefits of breast feeding (Lönnerdal, 2014). Compared with formula-fed infants, breast fed infants have a reduced frequency of infections (*e.g.*, otitis media, diarrhea) (Dewey *et al.*, 1995) and greater gastrointestinal comfort[2] (Vivatvakin *et al.*, 2010). Breast feeding is preferred to formula feeding for the first six months (American Academy of Pediatrics, 2012), "...followed by continued breast feeding as complementary foods are introduced, with continuation of breast feeding for one year or longer as mutually desired by mother and infant" (American Academy of Pediatrics, 2012). However, when breast feeding is not sufficient or a viable option, the composition of infant and follow-on formula that most approximates the composition and function of human breast milk in nutrient value are advantageous. Infant formula is defined by the European Commission (EC, 2013) as "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". Infant follow-on formula is also defined by the EC (EC, 2013) as "food intended for use by infants when appropriate complementary feeding is introduced and which constitutes the principal liquid element in a progressively diversified diet of such infants."[3] Basic research into infant nutritional needs have significantly increased in the past few decades, contributing to infant formulas providing nutritional value and biological components more in line with human breast milk (Anderson *et al.*, 1982; Raikos and Dassios, 2014). The evaluation of human milk components has greatly increased over the past two decades and novel components that may be important for infant growth and development have been characterized. Some of these components include docosahexaenoic acid (DHA), arachidonic acid and lactoferrin (Lönnerdal, 2014; Richard *et al.*, 2016). This work on milk characterization has paved the way for identification of similar proteins in bovine and human milk fractions.

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[1] Average OPN content in OPN-10 is 99%.

[2] Gastrointestinal comfort was defined in terms of stool consistency, gastric emptying time and gastrointestinal symptoms, which may include gas, bloating and borborygmi.

[3] As stated by EFSA (2014), the World Health Organisation (2002) has defined "complementary feeding" as "the process starting when breast milk alone is no longer sufficient to meet the nutritional requirements of infants" so that "other foods and liquids are needed, along with breast milk".

Osteopontin (OPN) is an acidic phosphorylated glycoprotein with a wide variety of reported functions, including the maintenance of gut integrity through effects on tight junctions which help to maintain the intestinal mucosal barrier, maintaining gut architechure, modulating intestinal inflammation, cell adhesion, bone remodeling, blood vessel growth, regulation of calcium oxalate formation and cell signaling (Sodek *et al.*, 2000; Kahles *et al.*, 2014; Toyonaga *et al.*, 2015; Kanwar *et al.*, 2016). OPN has been associated with human immune responses (Nau *et al.*, 2000; Konno *et al.*, 2011) that include opsonin activity in a mouse model (Schack *et al.*, 2009b) and aids in the migration of inflammatory cells to wound sites and cytokine expression (Brown *et al.*, 1992; Lund *et al.*, 2009). OPN was identified in human milk by Senger *et al.* (1989) and has subsequently been identified in bovine milk, albeit at lower levels than in the former (Sørensen and Petersen, 1993). In human milk, OPN comprises approximately 1 – 3% (wt/wt)[4] of the total amount of milk proteins, which is approximately equivalent to 100 – 300 mg/L, averaged at 138 mg/L (Schack *et al.*, 2009). Bovine milk contains approximately 15 – 20 mg/L OPN (Schack *et al.*, 2009; Lönnerdal, 2011), which is approximately five-to-ten-fold less then OPN found in human milk (Lönnerdal, 2011). Commercial infant formula (obtained from South Korea and Denmark) naturally contains 5 – 13 mg/L OPN, substantially less than human milk (Schack *et al.*, 2009).

The GRAS dossier that is the subject of this notification summarizes the scientific evidence that supports the general recognition that OPN-10, an ingredient composed mainly of bovine OPN, is safe for human consumption as a food ingredient in term nonexempt milk-based infant formula (which includes formula for infants 6 – 12 months of age) and in milk-based powdered beverages targeted for children 1 – 3 years of age (termed toddlers), commonly referred to as follow-on formula.

A.2. Description of OPN-10

OPN-10 is a proprietary food ingredient produced by Arla Food Ingredients (AFI) containing OPN isolated from bovine whey processed under current Good Manufacturing Practices (cGMP). OPN-10 contains at least 78% protein (N * 6.38), greater than 95% of which is bovine whey-isolated OPN. OPN-10 contains less than 11% ash, and maximum 1.0% of lactose and maximum 1.0% of fat.

A.3. Mechanisms of action

The scientific community has evaluated human milk components, including the identification and isolation of novel glycoproteins, such as OPN. OPN is a highly phosphorylated, multifunctional glycoprotein with an open, flexible structure. Experimentally, OPN has been found to have a variety of actions, including involvement in mammary gland development and differentiation in animal models and may help inhibit precipitation of calcium in milk, as well as possibly reduce the risk of calcium precipitation in the kidneys that form kidney stones (OPN is secreted by tubule cells and papillary epithelium) (Sodek *et al.*, 2000). In newborn mice nursing dams lacking milk OPN, impaired cognitive development, reduced brain OPN and reduced myelin-related proteins were reported (Jiang and Lönnerdal, 2016). In infant rhesus monkey fed formula with added bovine milk OPN, intestinal analysis found OPN positively regulated intestinal proliferation and cell migration from jejunal tissue samples, as well as cellular chemotaxis (Donovan *et al.*, 2014; Jiang and Lönnerdal, 2016). In a mouse model for colitis, oral

[4] wt/wt = weight/weight

administration of bovine-derived OPN positively modulated lower intestinal markers of immune responses (diarrhea and fecal blood) to dextran sulfate (Kanwar *et al.*, 2016).

OPN may have a positive impact on nervous tissue repair functions, as suggested by studies in mice with experimentally induced spinal cord injuries (Hashimoto *et al.*, 2007), and may play a role in the inhibition of scar formation in connective and other tissues (Sodek *et al.*, 2000). In addition, *in vitro* studies with isolated mouse osteoclasts show that OPN may help regulate bone mineralization processes and bone remodeling by osteoclasts (Rodriguez *et al.*, 2014). OPN appears to aid in the regulation of biomineralization *via* osteoblasts while having little direct effect on osteoblast cell development, as seen in an *in vitro* study using mouse primary osteoblast cultures (Holm *et al.*, 2014). OPN may also be secreted by macrophages to serve as adhesion proteins for the facilitation of phagocytosis during tissue remodeling (Mckee and Nanci, 1996).

OPN has been closely associated with some functions involving the immune response, including binding integrin receptors expressed on inflammatory cells (*e.g.*, neutrophils, macrophages and mast cells), promoting chemotaxis or cell activation (especially as an important early regulator of Th1-mediated immunity) (Ashkar *et al.*, 2000), and acting as an opsonin by enhancing phagocytosis *in vitro* through a novel (aXb2 integrin) OPN receptor (Schack *et al.*, 2009b). Nau *et al.* (2000) found that OPN expression after infection by mycobacteria is inversely proportional to patient outcome; that is, "patients who do well after an infection by mycobacteria express high levels of OPN." Patients with localized infection tended to have a higher OPN expression. The authors of the study concluded that "osteopontin expression correlates with an effective immune and inflammatory response when humans are challenged by a mycobacterial infection and that osteopontin contributes to human resistance against mycobacteria" (Nau *et al.*, 2000). Overall, there is no single known specific function of OPN. Experimental evidence indicates that OPN may have multiple, beneficial actions on different biological systems.

B. Method of Manufacture

OPN is extracted from bovine whey, a cheese or casein production byproduct, utilizing commonly known processes of the whey industry. The whey used as a starting material for the production of OPN-10 conforms to the European Union Food Hygienic Guidelines and EU Regulation 853/2004,[5] which allows for the use of only U.S.-approved pesticides and veterinary drugs.[6] Furthermore, except for the demineralized water, all the remaining processing aids (*i.e.*, sodium chloride,[7] sodium hydroxide,[8] hydrochloric acid[9] and calcium chloride[10]) used in the production of OPN-10 are food grade and approved for infant formula. The raw material - milk and whey - comes from plants that are all authorized by authorities that regulate dairy product facilities. All plants have implemented Hazard Analysis and Critical Control Points (HACCP) and comply with current Good Manufacturing Practices (cGMP) which is regulated in the European Union under Regulation (EC) No 852/2004 on the hygiene of foodstuffs and consistent with U.S. GMPs for infant formula (see 21CFR §110).

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[5] http://faolex.fao.org/docs/pdf/eur63427.pdf; site last visited September 29, 2015.
[6] (Global MRL Database, 2015)
[7] 21CFR§182.1: Substances that are generally recognized as safe.
[8] 21CFR§184.1763 Sodium hydroxide.
[9] 21CFR§182.1057 Hydrochloric acid.
[10] 21CFR§184.1193 Calcium chloride.

At the point of entry to the factory, an analytical control assessment is made of the raw materials: temperature, pH, sensory tests and antibiotics detection. The plant producing OPN-10 is certified under DS / EN ISO 50001: 2011, ISO 22000: 2005 / TS 22002-1: 2009 and FSSC 22000, which all control: (1) Implemented quality control systems, which include cGMP and HACCP; (2) Raw material analytical control (*e.g.*, temperature, pH, sensory tests and detection of antibiotics); and (3) The physicochemical and microbiological characteristics of the final product. AFI has been certified for the development, production, and sales of products from whey and lactose by the Danish Authorities and by FDA.[11] The production process is summarized in Figure 1.

Raw skim milk is received and tested at the cheese or casein production facility at the start of every production lot, where it undergoes a pasteurization step that is controlled with a rejection valve. This is a critical control point at the dairy facility, eliminating the risk of pathogens. The milk is then processed to produce cheese or casein, which also yields whey (containing OPN). The whey is kept in storage tanks kept at 5°C. The whey then undergoes pasteurization (minimum 72.5°C/15 seconds) or microfiltration (ceramic membrane < 1.4 μm), and ultrafiltration where the temperature is kept below 12°C and processing through an anion exchange column containing Q sepharose big beads, the feed is adjusted to the correct pH and concentration by inline adjustment with demineralized water, NaOH or HCl depending on the need. There is a heat treatment step prior to the drying process. The column first eluted with a weak (< 0.2 M) NaCl solution to remove impurities from the column then is rinsed with demineralized water before the product is eluted with an approximately 1 M NaCl solution. Importantly, the ion exchange columns are designed to prevent carry-over of the resins into the OPN eluate. Firstly, there is a 10μm column frit retaining the resin, which has a diameter of 100 – 300μm. Secondly, there is a 50μm sieve tube filter whereby the sieve tube filter is visually examined before and after production to ensure that any resin is retained if the 10μm column frit fails. If the filter is found damaged the product is submitted to rigorous testing to ensure product quality. The ultrafiltration step removes the residual low molecular weight contaminants in the OPN. The eluent then goes through additional ultrafiltration steps to concentrate the OPN; $CaCl_2$ is added to normalize the counterions surrounding the protein and heat treated at greater than 72°C for 15 seconds, conditions that are also requirements for pasteurized milk described in the Grade "A" Pasteurized Milk Ordinance (FDA, 2016).

The OPN-containing filtrate is then spray dried to produce the OPN-10 product in powder form. The powder is then passed through a 1 mm sieve and passed through a rotating magnet before bagging, and the filled bags are passed through a separate metal detector before palleting. AFI performs finished product analysis for *Salmonella* according to federal regulations. OPN-10 is greater than 95% OPN of total protein. The final OPN-10 product is packed in polyethylene bags and stored at room temperature (approximately 20°C). OPN-10 is manufactured in a manner consistent with the production of other bovine milk-related products.

[11] Inspection number FEI 3004285668; inspection on 22/08/2012.

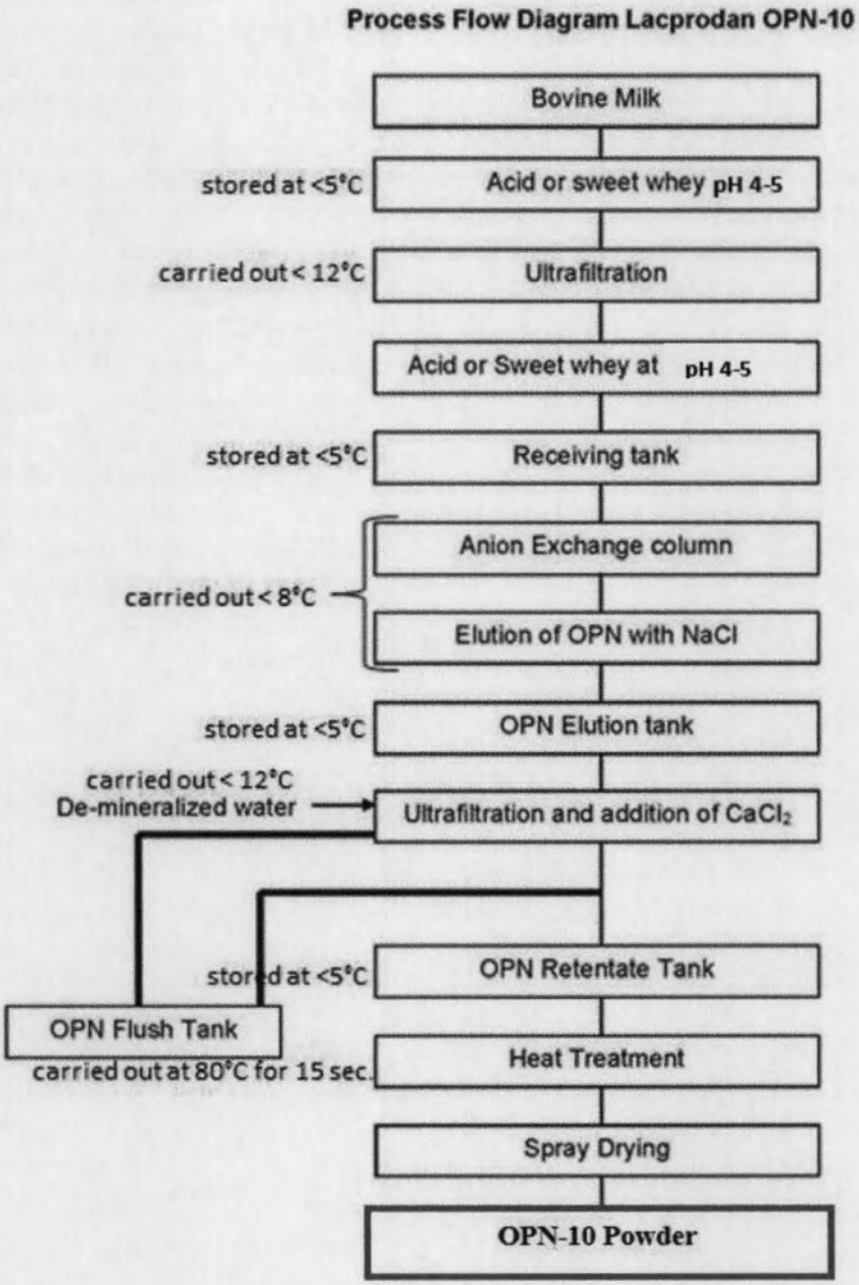

Figure 1. OPN-10 production scheme

C. Specifications

OPN-10 is a bovine-whey-derived ingredient that contains OPN, a highly-phosphorylated glycoprotein with an open and flexible structure. OPN is composed of 262 amino acids and contains sialic acids in conjugated glycosylations. The protein contains 23 moles of phosphorus *per* mole protein and has a high calcium-binding ability. The physical and chemical properties and specifications for OPN-10 are provided in Table 1. Analysis of five nonconsecutive lots confirms that OPN-10 meets specifications and is manufactured according to current Good Manufacturing Practices (cGMP) *per* 21 CFR §106.96 (Appendix I, individual lot analysis table). The protein content is calculated from nitrogen analysis using the Kjeldahl nitrogen method and is calculated by the fixed nitrogen factor of 6.38 for milk products.[12]

The conversion factor of 6.38 is commonly utilized for milk protein analysis. However, the full length bovine OPN was determined to have 262 amino acids, with a molecular weight of approximately 33.9 kDa, of which 29.3 kDa are amino acids, and approximately 1.7 kDa are phosphorylations and 2.9 kDa are O-linked glycosylations. According to AMLDI MS, the most abundant N-terminal fragment has a molecular weight of approximately 19.8 kDa, 16 kDa of which are amino acids, 0.9 kDa are phosphorylations and 2.9 kDa are O-linked glycosylations, with 194 nitrogen atoms (2,716 g N/mol protein). In the full length protein, 362 nitrogen atoms are found, equivalent to 5,068 g N/mol protein. In OPN-10, the predominant molecule (80%) as measured by gel permeation chromatography is a large N-terminal fragment, the full length protein comprising only 20%. The average molecular weight for OPN in OPN-10 is 22,900 g/mol. The full length OPN molecule nitrogen content is 14.9%, leading to a nitrogen conversion factor of 6.69 for the Kjeldahl method. The N-terminal fragment is composed of 13.72% nitrogen, giving a nitrogen conversion factor of 7.29. Utilizing the *percentages* of full length and fragments in OPN-10 and the amount of nitrogen in each, the conversion factor for the Kjeldahl method for OPN-10 is 7.17. OPN has a unique amino acid composition and more post-translational modifications than average milk proteins, and therefore the amount of OPN contained in OPN-10 is calculated by multiplying the nitrogen content by the nitrogen factor of 7.17 to correct for a unique OPN composition and taking into account the 95% purity of OPN in OPN-10 (*i.e.*, X*7.17*0.95) (de Boer, 2014).

AFI continusly monitors the level of pesticide residues, contaminants and heavy metals in our products. Levels have never been detected above the limit set by FDA in the regulations on these substances. All analysis are done by an external laboratory using validated analytical methods.

AFI continusly monitors the whey used to produce OPN-10, and OPN-10 has been tested for the content of heavy metals on an annual basis. The levels found in the raw milk correspond to the levels found in the analysis of OPN-10. OPN-10 was analyzed for *C. sakazakii*, with none reported. As pasteurization of the milk and adherence to strict manufacturing processes makes *C. sakazakii* contamination highly unlikely, analysis for this bacteria will only be conducted on an intermittent basis on the OPN-10. No contamination has been found in any material produced. The testing of the raw milk has been done for more then ten years and increased heavy metal levels above regulatory limits for commercial milk have never been seen (ARLA, 2016).

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[12] http://www.fao.org/docrep/006/y5022e/y5022e03.htm; site last visited December 23, 2014.

Table 1. Specifications of Lacprodan® OPN-10.

Analysis	Method	Specification	Batch Analysis Results (n = 5 lots) Range	Average
Protein (N*6.38) as is (%)	Kjeldahl	≥ 78%	78.3 – 79.46	78.80
OPN as a % of total protein (%)	HPLC	≥ 95%	97.2 – 99.9	97.75
Ash (%)	NMKL 173	≤ 11%	9.2 – 9.7	9.44
Moisture (%)	ISO 6731	≤ 5.5%	4.1 – 5.0	4.5
Lactose (%)	IDF 79B	≤ 1.0%	0.1	0.1
Fat (as is) (%)	ISO 3889	≤ 1.0%	0.2	0.2
Solubility index[13] (ml)	ADPI 916	≤ 1.0 ml	< 1.0	N/A
Scorched particles**	ADPI Bulletin 916	A	A	A
Minerals				
Sodium (%)	ICP	0.5 – 1.5%	1.07 – 1.27	1.19
Chloride (%)	ISO 5943	≤ 0.2%	0.04 – 0.1	0.06
Phosphorus (%)	ICP	1.4 – 2.2%	1.84 – 1.95	1.87
Calcium (%)	ICP	1.6 – 2.7%	2.2 – 2.37	2.29
Heavy Metals#				
Arsenic (mg/kg)	ICP-MS (EPA 200.8)	< 0.5	< 0.010 – 0.10	0.04
Cadmium (mg/kg)	ICP-MS (EPA 200.8)	< 0.05	< 0.01*	< 0.01
Lead (µg/kg)	ICP-MS (EPA 200.8)	< 50.0	13 – 16	14.3
Mercury (mg/kg)	ICP-MS (EPA 200.8)	< 0.05	< 0.005	< 0.005
Selenium (µg/kg)	ICP-MS (EPA 200.8)	5.0	15 – 29.5	
Microbiological load				
Total plate count (cfu/g)	ISO 4833	≤ 5,000	< 1000	< 1000
Mold/yeast (cfu/g)	ISO 6611	≤ 100	< 10	N/A
Bacillus cereus (cfu/g)	ISO 7932	< 50	< 10 – 40	N/A
Staphylococcus aureus (coag +) (cfu/g)	ISO 6888	Not detected	Not detected	Not detected
Enterobactericeae (cfu/g)	ISO 21528	≤ 10	<10 – 10	<10
Salmonella (cfu/g)	BAX®	Not detected/125 g	Not detected/125 g	Not detected/125 g
C. sakazakii (cfu/10 g)##	ISO/TC 34/SC 9	Not detected/10 g	Not detected/10 g	Not detected/10 g

*Limit of detection; **Must meet the American Dairy Products Institute Scorched Particle Standards for Dry Milks (GEA, 2006); ADPI=American Dairy Products Institute; BAX® = Baxter International; cfu = colony forming unit; EPA=Environmental Protection Agency; N/A = not applicable; HPLC=High-Pressure Liquid Chromatography; ICP=Inductively coupled plasma; ICP/MS=Inductively coupled plasma/mass spectrometry; IDF=International Dairy Federation; ISO=International Organization for Standardization; NMKL=NordVal International Denmark; #Heavy metal analysis conducted annually; ##*C. sakazakii* analysis conducted on an intermittent basis.

C.1. Allergenicity

OPN-10 is to be added only to cow's milk-based infant formulas and cow's milk-based liquid and powdered drinks for children 1 – 3 years of age. The OPN-10-containing products will be labeled as containing or produced from milk; this labeling should mitigate any reasonable

[13] A measurement of the amount of product sediment after the application of low centrifugal forces under specified conditions-a high value indicates that the product is less soluble (US Dairy Export Council, 2016).

question concerning allergenicity beyond that declared for milk-based products. Bovine-derived OPN is already found in cow's milk-based infant formulas (Schack *et al.*, 2009) and the addition of OPN-10 is only to increase the level of OPN intake to OPN concentrations found in breast milk, with a high homology between human and bovine OPN, the major protein in OPN-10. The amount of OPN-10 in an infant formula or toddler product is approximately 1% of total protein and between 0.1 to 0.2% of total milk solids. Therefore, it is not likely that OPN-10 would elicit an allergic response apart from that of a response to cow's milk.

C.2. Stability

OPN-10 has been analyzed for stability and was found to be stable for approximately three years if kept under cool (5 – 20°C), dry conditions away from strong odors (Table 2). The OPN-10 was packaged in polyethylene bags, standard for storage of OPN-10. OPN-10 is composed primarily (> 95%) of OPN; therefore, the protein content of OPN (as evaluated with a nitrogen conversion factor of 7.17) and moisture were monitored periodically on more than five batches of OPN-10, measured at day 0 and after approximately 151 weeks. OPN stability was quantified by HPLC, relative to both total protein and total dry matter. At day 0 the content expressed as %OPN in protein in the OPN-10 product vary from 96.99 – 100.16 % showing that there is low variability in the content of OPN between different batches of OPN-10 as well as low uncertainty of the analytical method. OPN-10 did increase in moisture content over the three-year period, but no degradation of the OPN protein was found in the OPN-10 product. Heat stability is an issue when a protein has defined tertiary structure. Lack of tertiary structure for OPN is an advantage for the intended application of addition to infant formula consumed by infants 0 – 12 months of age and milk-based beverages targeted for children ages 1 – 3 (commonly referred to as toddlers). Almost all infant formulas are either spray dried (powder) or use intense heat for a short period treatment (liquid) to control the quality and minimize microbial contamination. The fluid structure of OPN is expected to preserve the OPN molecule during the manufacture of infant formula containing OPN-10. The lack of a decrease in OPN concentration during the heating and drying treatment for the production of OPN-10 indicates that OPN contained in OPN-10 resists heat-induced degradation (Table 2).

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Table 2. Stability of OPN-10 (ARLA, 2015)

Analysis	Batch Number	Analysis (Date)	OPN	PROT(7.17)	Water	Protein	Purity	OPN	Loss of OPN	Loss of OPN	Duration
			% of powder	% of powder	% of powder	% of dry matter	OPN % of protein	% of dry matter	% of original dry matter	% loss by protein	Weeks
Initial	(b) (4)	11-30-2011	89.60	89.62	4.78	94.12	99.98	94.10			
Final	(b) (4)	10-28-2014	86.30	84.79	8.77	92.94	101.78	94.60	-0.53	-1.80	151
Initial	(b) (4)	09-30-2011	89.00	90.11	4.71	94.55	98.77	93.39			
Final	B400239000	10-28-2014	86.10	85.54	8.53	93.52	100.65	94.13	-0.79	-1.90	160
Initial	(b) (4)	11-30-2011	88.97	89.22	4.68	93.59	99.72	93.33			
Final	(b) (4)	10-28-2014	86.90	85.24	8.66	93.32	101.95	95.14	-1.94	-2.23	151
Initial	(b) (4)	11-30-2011	88.60	88.10	4.83	92.57	100.57	93.10			
Final	(b) (4)	10-28-2014	86.10	85.22	9.01	93.66	101.03	94.63	-1.64	-0.46	151
Initial	(b) (4)	11-04-2011	89.30	89.16	4.44	93.29	100.16	93.44			
Final	(b) (4)	10-28-2014	85.30	84.36	9.44	93.15	101.11	94.19	-0.80	-0.95	155
Initial	(b) (4)	11-18-2011	88.25	89.05	4.89	93.62	99.11	92.79			
Final	(b) (4)	10-28-2014	85.40	85.63	8.97	94.07	99.73	93.82	-1.11	-0.63	153
Initial	(b) (4)	11-30-2011	88.45	88.66	4.52	92.85	99.77	92.64			
Final	(b) (4)	10-28-2014	86.00	85.78	8.70	93.95	100.26	94.19	-1.68	-0.49	151
Initial	(b) (4)	11-30-2011	86.95	89.65	4.64	94.01	96.99	91.18			
Final	(b) (4)	10-28-2014	83.60	85.15	9.03	93.60	98.18	91.90	-0.79	-1.23	151

*HPLC/LCMS for OPN; OPN = osteopontin; PROT = protein

Analysis of the levels of OPN-10 (as determined by OPN levels) when added to infant formula was also conducted utilizing an LC-MS-MS quantitation method at typical and elevated temperature/relative humidity (Table 8). Results show that OPN from OPN-10 is stable in infant formula for at least 90 days at both typical and accelerated time/temperature. Analysis at 180 days (six months) shows that the OPN from OPN-10 is stable at room temperature and typical humidity, but accelerated stability conditions suggest a slight degradation of the OPN protein, which is also seen of other proteins in infant formulas in general. Additional time point analyses will continue to be conducted. The *percent* of bovine OPN contained in the infant formula did not substantially change over time when stored at room temperature for at least six months, and at accelerated conditions at up to six months.

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Table 3. Stability of OPN-10 when added to infant formula.

Analysis	Room Temperature (25 C/60% RH)			Accelerated Stability (40 C/75% RH)		
	Control	Sample 1	Sample 2	Control	Sample 1	Sample 2
Time 0						
OPN (Time 0)* (mg OPN/100 g)	15.5	108	102	15.5	108	102
% OPN at Time 0	100	100	100	100	100	100
30 Days after Time 0						
OPN (mg OPN/100 g)	15	98.4	109	15	91.7	101
% OPN compared to Time 0	96.7	91.1	106.8	96.7	85	99
60 Days after Time 0						
OPN (mg OPN/100 g)	15	110	110	15	103	99.3
% OPN compared to Time 0	96.7	102	108	96.7	95.3	97.3
90 Days after Time 0						
OPN (mg OPN/100 g)	15	110	106	15	107	96
% OPN compared to Time 0	96.7	102	104	96.7	99	94.1
180 Days after Time 0						
OPN (mg OPN/100 g)	16.4	105.6	103.4	13.7	91.9	81.2
% OPN compared to Time 0	105	97.7	101	88.3	85	80

*Analysis values are expressed in mg OPN/100 g infant formula powder (as typically processed)

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3. Dietary Exposure

OPN-10 is being added to term cow's milk-based infant formula to provide OPN at a concentration to mimick the amount of OPN contained in breast milk, as recent research found that breast milk contains on average approximately 138 mg OPN/L (*i.e.*, 0.138 mg/ml milk) and that commercial infant formula (powdered infant formula reconstituted in water as *per* manufacturer's instructions[14]) currently contains approximately 5 – 13 mg OPN/L formula (Schack *et al.*, 2009).

To provide a total of 138 mg OPN/L formula, the amount of OPN already in formula (a conservative 13 mg OPN/L) must be taken into account. The higher level of 13 mg OPN/L formula from the measured range of 5 – 13 mg OPN/L is being utilized so that a minimum amount of OPN-10 is being added to infant formula. OPN-10 is to be added to infant formula to provide up to 125 mg OPN/L formula (*i.e.*, 138 mg OPN/L breast milk minus 13 mg OPN already contained in a liter of infant formula). OPN-10 contains at a minimum 78% protein (based on Nitrogen factor of 6.38) of which at least 95% is the OPN protein. So in order to fortify 125 mg/L of OPN protein, approximately 160 mg/L of OPN-10 (125/0.78) is to be added to correct for purity. Together with innate levels of approximately 13 mg OPN/L, the target in final formula would be 138 mg OPN/L formula. (Schack *et al.*, 2009),

Estimates for the daily intake (EDI) of OPN-10 when added to term cow's milk-based infant formula were calculated based on infant formula consumption data included in the U.S. Department of Agriculture (USDA) and U.S. Department of Health and Human Services (DHHS) National Health and Nutrition Examination Survey (NHANES) 2011-2012 national food survey. The NHANES survey obtains nationally representative nutrition and health data, along with U.S. prevalence estimates for nutrition and health status measures (CDC, 2015b). The survey collects two days of 24-hour dietary recall data, during which participants provide information on types and amounts of foods consumed. The NHANES data set is often utilized in GRAS dossiers to estimate the amount of a certain food ingredient consumed on a daily basis.[15] These data are then analyzed to code individual foods and portion sizes. From the data tables prepared under the NHANES program, a consumption analysis of OPN-10 when added to milk-based infant formulas was conducted, utilizing the amount of milk-based formula consumed and the amount of OPN-10 to be contained in the formula. The resulting information provided a mean and 90th percentile estimated intake of OPN-10 when added to milk-based infant formulas. The NHANES data set does not provide specific data on the age of the infant, other than "less than 1 year of age". From the use of OPN-10 in infant formulas (minus those indicated as made from soy or other plant-based materials) as taken from the NHANES 2011-2012 food survey (Appendix II), the "eaters-only"[16] estimated mean and 90th percentile intakes of OPN-10 were 119.0 and 189.7 mg/person/day, respectively (Table 4).

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[14] The OPN concentration was measured in read-to-feed formulas based on 125 g/L of powder (Schack *et al.*, 2009).
[15] As an example, please see the "no questions" letter provided by FDA for GRAS notification 669; https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm549873.pdf; site last visited April 19, 2017.
[16] "eater's only" data utilizes only those respondents that consumed the specific requested food at least once during the survey period (typically utilized by FDA in determining ingredient consumption);

It is generally accepted that a breast-fed infant has a daily milk intake corresponding to approximately one-sixth of their body weight (Greer, 2001), with an average of 165 mL/kg body weight for their first three months of life (American Academy of Pediatrics, 2015). The greatest exposure to formula ingredients (on a body weight basis [BW]) is during the first few months of life, when the mean body weight of infants from birth to less than one month of age is 4.8 kg, increasing to 5.9 kg from one month of age to less than three months of age and 7.4 kg from three to less than six months of age (EPA. 2011). On a body weight basis, mean and 90th percentile consumption of OPN-10 at less than one month of age would be 24.8 and 39.5 mg/kg BW/day, respectively. While total enery requirements (kJ/day basis) increase with age (higher in boys due to gender weight differences), on a bodyweight basis the energy requirements decrease from a maximum at one month of age, tending to plateau starting at six months of age, and further decreasing as BW increases (Butte, 2005). The energy requirement data indicate that the first few months of infant formula will result in the greatest consumption of OPN-10, on a BW basis.

Table 4. OPN-10: Current intake and predicted intake of OPN-10 following supplementation of milk-derived infant formula at the indicated levels with OPN-10 utilizing NHANES (2011-2012) dataset (CDC, 2015b).

	Per User				
OPN-10 intake from:	**OPN-10 (mg/L infant formula)***	**50th Percentile OPN-10 consumption (mg/infant/day)**	**50th Percentile OPN-10 (mg/kg BW/day)**	**90th Percentile OPN-10 (mg/infant/day)**	**90th Percentile OPN-10 (mg/kg BW/day)**
Current amount of OPN-10 in commercial infant formula	0	0	0	0	0
Possible consumption of OPN-10 as an added ingredient to food at < 1 month of age of the infant	160	119	24.8	189.7	39.5

*Addition of 160 mg OPN-10/L milk-derived infant formula will provide 125 mg OPN/L formula

The World Health Organization (WHO, 2001) recommends exclusive breast feeding for the first six months of life, then partial breast feeding up to two years of age or beyond. Davis *et al.* (2012) conducted a series of surveys concerning breast feeding and the health of infants and toddlers and found that among the Hispanic families surveyed in the US, 36% of the children were breast fed beyond twelve months of age. Toddlers from one to up to three years of age may still consume breast milk or infant formula/complimentary milk-based supplemental beverages as part of the weaning process from infant formula to solid foods, although not as a sole source of nutrients but in decreasing amounts as the amount of solid food consumption increases, and therefore consume OPN during this period, although at levels lower than during the first year of life. The U.S. dietary guidelines for Americans (2010) recommend that intake of 2 cups of milk and milk products (approximately 475 ml or 16 ounces) by children 2 – 3 years of age (regardless of body weight) is sufficient for adequate nutrient intake, stating that "moderate evidence shows that intake of milk and milk products is linked to improved bone health, especially in children and adolescents." The American Heart Association (AHA) also states in their dietary recommendations scientific position that 2 cups milk/day is appropriate for children ages 1 – 3 (AHA, 2014). In

https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm074725.htm; site last visited April 19, 2017.

addition, Maguire *et al.* (2013) found that "two cups (500 ml) cow's milk *per* day maintained 25-hydroxyvitamin D > 75 nmol/L with minimal negative effect on serum ferritin for most children." Higher levels of milk consumption could decrease serum ferritin, possibly through replacement of the consumption of iron-containing foods (Maguire *et al.*, 2013).

Average body weights for children 1 – 3 years of age slowly increase over time, as expected. One-year-old boys weigh an average of 10.31 kg while one-year-old girls weigh an average of 9.52 kg (CDC, 2001). Average body weights for 2 – 3 year-olds range from approximately 12.7 – 14.3 kg for boys and 12.1 – 13.9 kg for girls (CDC, 2001). Utilizing the smallest and greatest body weights of the various age groups, one can determine the age groups that would be consuming the greatest and least amount of OPN from formula. On a bodyweight basis, one-year-old female toddlers who continue to consume formula (considered in the industry as follow-on formula at this age) may consume an average of 49.9 ml formula/kg bodyweight,[17] to 29.8 ml formula/kg bodyweight for three-year-old boys (the age group with the greatest bodyweight). This would result in consumption of OPN from formula in toddlers at up to 6.89 mg/kg bodyweight for one-year-old girls[18] and 4 mg/kg bodyweight for three-year-old boys. These levels are over three-fold lower than levels of OPN consumed by an infant (24.8 mg OPN/kg bodyweight), confirming that the infant has the greatest exposure to OPN from OPN-10, on a bodyweight basis.

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[17] 475 ml formula/9.52 kg body weight = 49.89 ml/kg

[18] 0.138 mg OPN/ml formula * 49.89 ml formula consumed/kg bodyweight = 6.89 mg OPN/kg bodyweight

4. Current, Proposed, and Self-limiting Levels of Use

Neither OPN-10 nor OPN as an isolated substance is currently added to food, including infant formula, in the U.S.

OPN-10 would be added to cow's milk-based infant formula and milk-based liquid and powdered drinks for children 1 – 3 years of age (at 160 mg OPN-10/L) to better reflect the level of OPN contained in human mature breast milk (reported at a mean of 138 mg OPN/L (Schack *et al.*, 2009)). The infant formula would be marketed for full- or near-full-term infants, and OPN-10 would not be added to pre-term-focused or exempt infant formula. OPN-10 would only to be used in the wet blending-spray drying process of the production of infant formula, where ingredients are blended in water, homogenized, pumped to a heat exchanger for pasteurization, and then spray dried into a powdered product. The use of OPN-10 is not self-limiting.

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5. Experience Based on Common Use in Food Before 1958

Lacprodan OPN-10 is not based on common use in food. Lacprodan OPN-10 was not consumed as a food ingredient in food by a significant number of consumers prior to January 1, 1958.

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6. Basis for the Conclusion of GRAS Status

The conclusion that Lacprodan OPN-10 (OPN-10) is GRAS is on the basis of scientific procedures, as described in the Dossier in Support of the Generally Recognized as Safe (GRAS) status of Lacprodan OPN-10 as a Food Ingredien in Infant formula and Toddler beverages, dated June 1, 2017 (ARLA, 2017).

This part of the notification (*i.e.*, Section 6: Basis for the Conclusion of GRAS Status) contains information concerning the characterization, manufacturing process, intake and safety studies conducted, all of which were evaluated by a GRAS Expert Panel to conclude that OPN-10 is GRAS under the intended conditions of use.

6.1 Introduction

Osteopontin (OPN) is an acidic phosphorylated glycoprotein with a wide variety of reported functions, including the maintenance of gut integrity through effects on tight junctions which help to maintain the intestinal mucosal barrier, maintaining gut architechure, modulating intestinal inflammation, cell adhesion, bone remodeling, blood vessel growth; regulation of calcium oxalate formation and cell signaling (Sodek *et al.*, 2000; Kahles *et al.*, 2014; Toyonaga *et al.*, 2015; Kanwar *et al.*, 2016). OPN has been associated with human immune responses (Nau *et al.*, 2000; Konno *et al.*, 2011) that include opsonin activity in a mouse model (Schack *et al.*, 2009b) and aids in the migration of inflammatory cells to wound sites and cytokine expression (Brown *et al.*, 1992; Lund *et al.*, 2009). OPN was identified in human milk by Senger *et al.* (1989) and has subsequently been identified in bovine milk, albeit at lower levels than in the former (Sørensen and Petersen, 1993). In human milk, OPN comprises approximately 1 – 3% (wt/wt)[19] of the total amount of milk proteins, which is approximately equivalent to 100 – 300 mg/L, averaged at 138 mg/L (Schack *et al.*, 2009). Bovine milk contains approximately 15 – 20 mg/L OPN (Schack *et al.*, 2009; Lönnerdal, 2011), which is approximately five-to-ten-fold less then OPN found in human milk (Lönnerdal, 2011). Commercial infant formula (obtained from South Korea and Denmark) naturally contains 5 – 13 mg/L OPN, substantially less than human milk (Schack *et al.*, 2009).

Lacprodan® OPN-10 (OPN-10) is an OPN-based product isolated from bovine whey and produced by commonly used ultrafiltration and ion exchange chromatography (IEC) processes in the whey industry to fractionate whey. OPN-10 has minimum 78% protein based on conventional calculations (N*6.38) and minimum 86% protein based on appropriate correction factors included in calculations (N*7.17) (further discussed below) (de Boer, 2014). OPN comprises at least 95% of the total protein in the OPN-10 fraction.[20] OPN-10 is produced as a source of OPN that can be used as an ingredient to provide infants with a supplementary source of OPN in their diets.

The goal of any infant formula manufacturer is to provide a nutritious, growth-promoting formula that strives to mimic, as closely as possible, the composition and function of human milk as much as possible, to infants 0 – 12 months of age. Human milk contains low levels of many different proteins that are believed to contribute to the overall benefits of breast feeding (Lönnerdal, 2014). Compared with formula-fed infants, breast fed infants have a reduced frequency of infections (*e.g.*, otitis media, diarrhea) (Dewey *et al.*, 1995) and greater

[19] wt/wt = weight/weight
[20] Average OPN content in OPN-10 is 99%.

gastrointestinal comfort[21] (Vivatvakin *et al.*, 2010). Breast feeding is preferred to formula feeding for the first six months (American Academy of Pediatrics, 2012), "followed by continued breast feeding as complementary foods are introduced, with continuation of breast feeding for one year or longer as mutually desired by mother and infant" (American Academy of Pediatrics, 2012). However, when breast feeding is not sufficient or a viable option, the composition of infant and follow-on formula that most approximates the composition and function of human breast milk in nutrient value are advantageous.

Infant formula is defined by the European Commission (EC, 2013) as "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". Infant follow-on formula is also defined by the EC (EC, 2013) as "food intended for use by infants when appropriate complementary feeding is introduced and which constitutes the principal liquid element in a progressively diversified diet of such infants."[22] Basic research into infant nutritional needs have significantly increased in the past few decades, contributing to infant formulas providing nutritional value and biological components more in line with human breast milk (Anderson *et al.*, 1982; Raikos and Dassios, 2014). The evaluation of human milk components has greatly increased over the past two decades and novel components that may be important for infant growth and development have been characterized. Some of these components include docosahexaenoic acid (DHA), arachidonic acid and lactoferrin (Lönnerdal, 2014; Richard *et al.*, 2016). This has paved the way for identification of similar proteins in bovine and human milk fractions. This dossier is a summary of the scientific evidence that supports the general recognition that OPN-10 is safe for human consumption as a food ingredient in term nonexempt infant formula (which includes formula for infants 6 – 12 months of age, commonly referred to as follow-on formula) and in milk-based powdered beverages targeted for children 1 – 3 years of age (termed toddlers).

6.1.1. Description of the Ingredient

6.1.1.1. Osteopontin

Generic OPN (CAS No. 106441-73-0) is a phosphoprotein known by several synonyms including, but not limited to: 2ar Peptide; 44 kDa phosphoprotein; Bone sialoprotein 1; Bone sialoprotein I; Early T-lymphocyte activation-1 protein; Eta-1 protein; Eta-1-Op gene product; Eta-1-Op protein; Phosphoprotein I, 2aR; Secreted phosphoprotein 1; Sialoprotein 1 and Urinary stone protein.

OPN obtained from multiple species has been found to be a product of a single gene, but alternative splice variants have been described in various tissues: OPN-a, OPN-b and OPN-c (Figure 2) (He *et al.*, 2006; Shen and Weber, 2014). The OPN-a form, which represents full-length native OPN, is present in milk (human and bovine) and is constitutively present in a variety of non-malignant tissues (Patani *et al.*, 2008; Bissonnette *et al.*, 2012; Shen and Weber, 2014). Sorensen *et al.* (2003) purified and characterized both intact and fragmented OPN from human milk from

[21] Gastrointestinal comfort was defined in terms of stool consistency, gastric emptying time and gastrointestinal symptoms, which may include gas, bloating and borborygmi.

[22] As stated by EFSA (2014), the World Health Organisation (2002) has defined "complementary feeding" as "the process starting when breast milk alone is no longer sufficient to meet the nutritional requirements of infants" so that "other foods and liquids are needed, along with breast milk".

four to eight mothers at different stages of lactation and found that intact OPN migrated as approximately 60 kDa in SDS-PAGE gels, similar to the migration of bovine OPN.

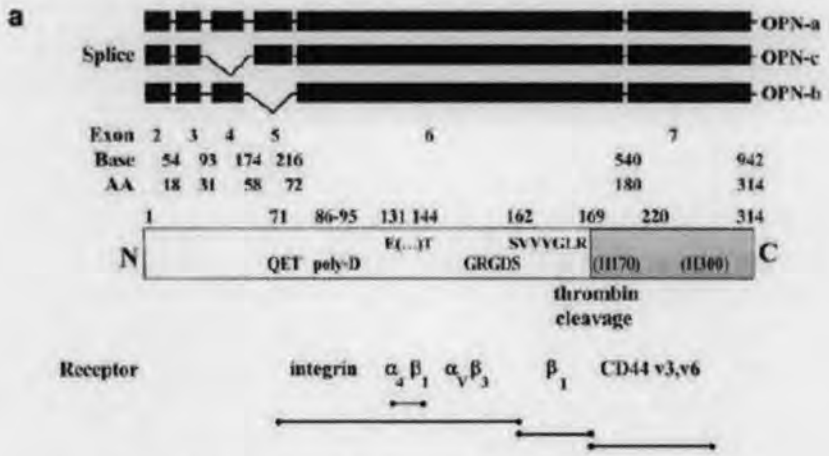

Figure 2. Osteopontin splice variants (He *et al.*, 2006).

The OPN-c form (which lacks exon 4) has been associated with the occurrence of breast cancer and increased capabilities of metastasis in cancer cell studies (Sivakumar and Devaraj, 2014). There are also some indications that the OPN-b form (which lacks exon 5) is upregulated in certain cancers (Hartung and Weber, 2013). The OPN-a form has never been associated with such malignant properties. OPN expression is affected by a number of substances including cytokines, hormones (*e.g.*, vitamin D3 and estrogen), and growth factors. Several inflammatory mediators and growth factors such as interleukin-1, tumor necrosis factor *alpha* and platelet-derived growth factor are known to stimulate OPN transcription in embryonic chick osteoblasts *in vitro via* activation of protein kinase C (Toma *et al.*, 1997; Sodek *et al.*, 2000). OPN without any missing exons (OPN-a) is a substrate for tissue type-2 transglutaminase and the transglutaminase-catalyzed cross-linking is suggested to be important in anchoring of OPN to the extracellular matrix (Prince *et al.*, 1991).

The OPN mRNA is spliced in various ways in human tissues, depending on the expression conditions, while OPN isoforms secreted into bovine milk do not result from alternative splicing, but are the result of post-translational modifications (PTMs). For this present evaluation, the most important form of human OPN is that found in breast milk. While other forms may be produced by a variety of tissues under various conditions, it's important to note that oral administration to the infant, either breast- or formula-fed, is the focus of this assessment. Although useful to mention, the other forms of OPN are of only minor interest to the conclusion of GRAS status for OPN-10. A wide variety of OPN PTMs have been observed in humans including alternative splicing, serine/threonine phosphorylation, glycosylation and tyrosine sulfation, resulting in distinct monomeric isoforms with migration in SDS-PAGE ranging from 41 – 75 kDa with distinct tissue-specific glycosylation (Christensen *et al.*, 2005; Gimba and Tilli, 2013). PTMs have significant effects on the structure and biological properties of proteins, with the different PTMs

on OPN from different tissues and cells reflecting the diverse functions of OPN throughout the body.

OPN in bovine milk has been found to consist of two forms; 1) full-length OPN (262 amino acid residues) with a molecular weight of 33.9 kDa including modifications (migrates as approx. 60 kDa in SDS-PAGE) and, 2) an N-terminal fragment (amino acid residues 1-150) with a molecular weight of 19.8 kDa including modifications (migrates as 35 - 40 kDa in SDS-PAGE). The N-terminal fragment is generated by indigenous proteolytic activity in the mammary gland and milk (Bissonnette *et al.*, 2012; Christensen *et al.*, 2014). Similar cleavage of OPN molecules have been described for OPN in human milk, with similar cleavage sites between bovine and human OPN (Christensen *et al.*, 2010; Bissonnette *et al.*, 2012).

OPN was purified from samples of colostrum and milk from cattle, analyzed for size using two polyclonal antibodies for human OPN (hOPN2 and hOPN4) and for the ability of the bovine OPN to be cleaved by chymosin (Kumura *et al.*, 2004). The antibody hOPN2 recognized two distinct bands of 60 and 40 kDa in SDS-PAGE whereas hOPN4 recognized only a 60 kDa protein band, *via* Western blot analysis. Through additional analysis, the authors concluded that the signal observed at 40 kDa was "the result of a deletion of the C-terminal region of OPN" (Kumura *et al.*, 2004). This may be analogous to OPN in human milk, as human milk contains both a 75 kDa protein fragment and a 35 kDa component of OPN (Senger *et al.*, 1989). The discrepancy in observed migration can be explained by differences in the SDS-PAGE systems used.

Bovine milk OPN contains reactive glutamine residues that were identified as Gln34 and Gln36 (Sorensen *et al.*, 1994). These residues are not present in the OPN-c splice variant that is implicated in metastasis processes (He *et al.*, 2006). This has been suggested to be an essential reason for the pathological role of OPN-c, as it may not be cross-linked with the extracellular matrix and thus it can instead play a role in cell migration (Sivakumar and Devaraj, 2014). The OPN sequence is highly conserved among species as shown in Figure 3. The overall acidic nature of the protein with many aspartate and glutamate residues is highly conserved among analyzed sequences. The transglutaminase-reactive glutamines, Gln34 and Gln36 are conserved among all species. A conserved region of consecutive acidic residues, mainly aspartates (residues 70 – 78 in bovine OPN), which has been suggested to regulate hydroxyapatite (HA) formation (Oldberg *et al.*, 1986) is present in all known OPN sequences. The integrin-binding sequence RGD (res. 136 – 138 in bovine OPN) is also conserved in all sequences analyzed in different species. The alignment of OPN from different species in Figure 3 signifies that the amino acids modified in human milk OPN are highly conserved among species, including cattle, sheep, goats and even water buffalo, which is a significant source of milk in Southeast Asia (Christensen *et al.*, 2012; Dubey *et al.*, 2015)()()(). For example, a serine or threonine is found in other mammalian OPN sequences at 23 of the 36 positions that are phosphorylated in human milk OPN, indicating that phosphorylation of this conserved region is essential for OPN function (Christensen *et al.*, 2012).

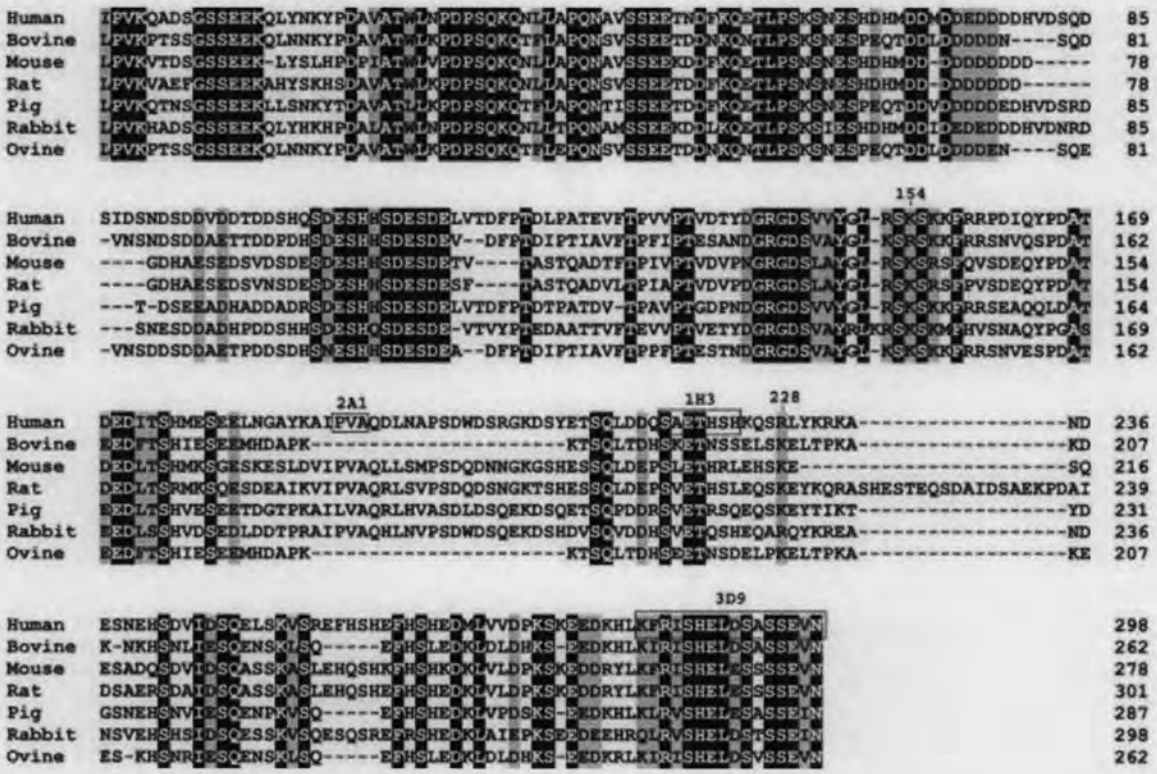

Figure 3. Comparison of mammalian OPN sequences. The alignment pattern was created to maximize alignment of identical and/or similar amino acid residues. Those amino acids that are identical in all OPNs are highlighted in black, and structurally similar amino acids are highlighted in gray. The localization of the epitopes of the monoclonal anti-OPN antibodies 2A1, 1H3, and 3D9 (27) are boxed. The positions of Lys154 and Arg228 which are the C-terminal residues of nmOPN and rOPN-228C, respectively, are indicated (Christensen *et al.*, 2012).

OPN is an intrinsically disordered protein (IDP) produced in a variety of tissues in the body (Platzer *et al.*, 2011; Kurzbach *et al.*, 2013). IDPs make up a class of biologically active proteins that lack defined tertiary and often secondary structure unless bound to a ligand or receptor (Dunker *et al.*, 2001). IDPs (including OPN) exert biological functions by means of motifs and posttranslational modifications in the primary amino acid sequence (*e.g.*, integrin binding sequences, calcium binding motifs), and are not dependent on any tertiary structure to be functional, but gains higher order structure upon binding to receptors or ligands, such as p27-cyclin-CDK complex formation during the cell cycle (Oldberg *et al.*, 1986; Mohan *et al.*, 2006; Collins *et al.*, 2008; Babu, 2016). Therefore, it is not possible to conduct x-ray crystallography procedures on OPN as it does not have a stable tertiary structure. However, a computer-generated model of the hypothetical structure and SAXS[23] measurements have been completed (Figure 4), based on assumptions from the amino acid consensus sequence of mammalian (human, rat, mouse, cow, rabbit and pig) OPN proteins and secondary structure predictions of OPN (Sodek *et al.*, 2000). Heat stability is an issue when a protein has defined tertiary structure that aids its

[23] SAXS = small-angle X-ray scattering (Kikhney and Svergun, 2015).

functionality. Predictions of disorder in thermostable proteins found that greater than two-thirds of these thermostable proteins had substantial areas of disorder, demonstrating that "disordered proteins, as a structural class, are more heat stable and soluble than their folded counterparts, consistent with their sequence features and the principles of amino acid solubility" (Van Der Lee *et al.*, 2014). All infant formulas are either spray dried (powder), use elevated heat (typically 135 – 150 C) treatment (liquid) for a very short time (5 – 15 seconds), ohmic heating (not increasing the product temperature) to control the quality and minimize microbial contamination. Therefore the fluid structure and potential function of OPN may hypothetically preserve its structure during the manufacture of infant formula containing OPN-10.

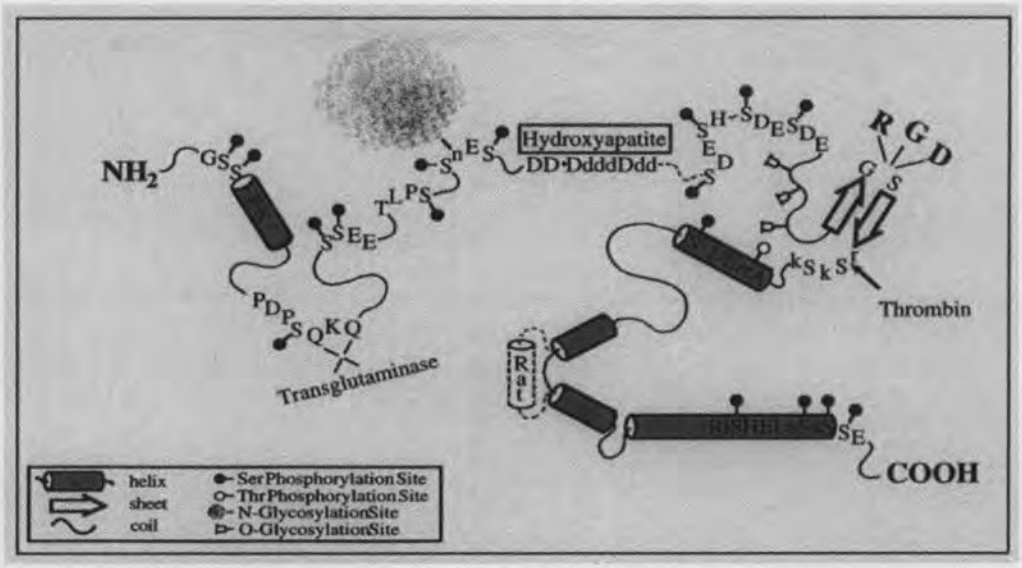

Figure 4. Hypothetical structure of osteopontin, based on the amino acid sequence and secondary structure prediction (Sodek *et al.*, 2000).

The diverse functions of OPN may be largely dependent on post-translational modification (PTM) of the OPN molecule. The different types of PTM include phosphorylation, glycosylation and proteolytic processing, resulting in PTM of OPN being highly tissue- and cell-specific (Sodek *et al.*, 2000). Human OPN contains up to 34 phosphoserine, two phosphothreonine and five O-glycosylated threonine residues, with the degree of phosphorylation of human milk OPN significantly higher than human urine OPN, rat bone OPN, chicken osteoblast OPN, , OPN from transformed murine fibroblasts and murine osteoblast OPN (Christensen *et al.*, 2005; 2007; 2008). However, bovine milk OPN phosphorylation containing 28 phosphate groups is similar to human milk OPN phosphorylation, suggesting similar biological functions may be manifest (Figure 5) (Sorensen *et al.*, 1995; Christensen *et al.*, 2005). The phosphorylations in bovine milk are clustered in groups of three (with highly conserved serine-based motifs), with unphosphorylated regions of 11 – 32 amino acids in between the phosphorylation sites, and the phosphorylation sites are highly conserved among species. Sorensen *et al.* (1995) concluded that the conservation of serines "indicates that the phosphorylation of osteopontin at specific sites is essential for the function of the protein". In addition, a screening of cDNA libraries from human mammary gland and milk

cells found that only full-length OPN mRNA was detected, which suggests that the variation in phosphorylation and heterogeneity is not the result of alternative splicing of the OPN transcript.

There are structural and amino acid differences among the human and the bovine form of OPN, as there are with all milk proteins from different species. But all important structural sequences and motifs are conserved (and functional when we substitute one with the other in cellular assays) (Sorensen *et al.*, 1995). In addition, human and bovine OPN are cleaved at the integrin binding regions in response to enzymatic release. This cleavage probably enhances the integrin binding properties of OPN of both species (Christensen *et al.*, 2010; Christensen *et al.*, 2014).

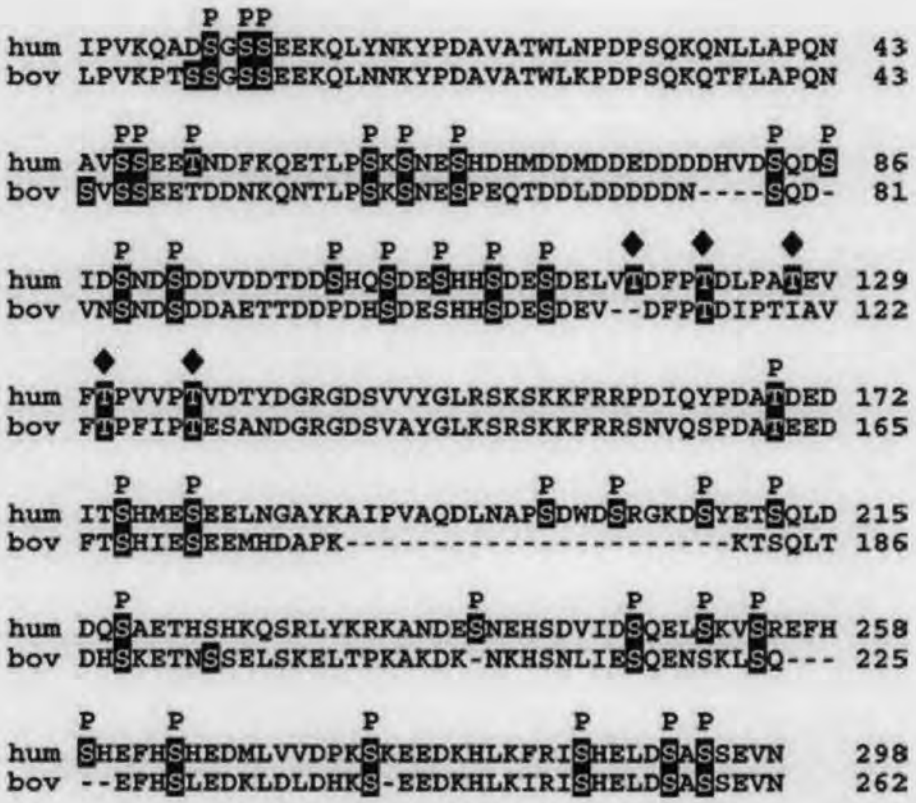

Figure 5. Alignment of human and bovine OPN sequences. Modified residues are highlighted and introduced gaps are indicated by broken lines. P denotes identified phosphorylation and filled diamonds symbolize glycosylations (Christensen *et al.*, 2005).

OPN may be synthesized internally by a variety of cell types including fibroblasts, osteoblasts, osteocytes, chondrocytes, dendritic cells, macrophages, smooth and skeletal muscle, endothelial cells and may be transformed (*e.g.*, neoplastic) cells at nanogram/ml levels, while OPN is secreted into human milk at a mean of 138 mg/L (discussed in detail below). OPN is deposited as a layer at the luminal surfaces of specific populations of epithelial cells in a variety of organs (including, but not limited to breast, gastrointestinal (GI) tract, brain, liver, lung, bone, cardiac tissue, joints, placenta, gall bladder, pancreas, urinary and reproductive tracts and kidney) and may

have multiple roles in the developing body (Brown *et al.*, 1992). OPN is found in the extracellular matrices of teeth and bones (Sodek *et al.*, 2000) and is a component of the normal elastic fibers of skin and aorta (Baccarani-Contri *et al.*, 1995). OPN is also present in body fluids including blood, urine (as uropontin), bile, bovine seminal fluid, and milk (Brown *et al.*, 1992; Min *et al.*, 1998; Anborgh *et al.*, 2011; Kolbach *et al.*, 2012). Among the bodily fluids, the highest level of OPN measured is found in breast milk (Schack *et al.*, 2009; Anborgh *et al.*, 2011; Kolbach *et al.*, 2012).

6.1.1.1.Quantification of OPN in milk

Three commercial enzyme-linked immunosorbant assays (ELISA) kits for measuring human osteopontin dominate the commercial market and the scientific literature (IBL[24], R&D systems[25], Assay Designs[26]). In addition, some laboratories have developed and validated in-house ELISA methods for measuring osteopontin. There exist no commercial available ELISAs for measurement of bovine osteopontin. There are many monoclonal antibodies against human, mouse and rat OPN available on the market, but only 1 – 2 of these cross-react with the bovine milk OPN.[27] The heterogeneity of bovine milk osteopontin with regard to modifications and proteolytical processing, makes it very difficult to generate antibodies that capture all OPN forms. For quantification purposes, mass spectroscopy is used and is the best approach to quality control for OPN-10; antibody-based methods are not used for quality control or specifications.

It must be noted that the reliability of the IBL OPN ELISA has been questioned (Plumer *et al.*, 2008; Schack *et al.*, 2009). Nagatomo *et al.* (2004) analyzed the content of osteopontin in human milk using the IBL ELISA. By this method they found surprisingly high concentrations of OPN in human breast milk. Osteopontin concentrations in early milk (3 – 7 d) and in mature milk (one month postpartum) were found to be 1,493.4 mg/L and 896.3 mg/L, respectively (Nagatomo *et al.*, 2004). Though it has previously been reported that the OPN concentration in milk is quite high (Senger *et al.*, 1989; Sørensen *et al.*, 2003), these values, which suggest that OPN should constitute more than 10% of the total protein in breast milk, seemed to be highly overestimated.

Plumer *at al.* (2008) developed an in house OPN ELISA based on mouse monoclonal antibodies. These antibodies were generated in house utilizing recombinant human full-length OPN, and OPN trilevel controls purchased from R&D Systems. The ELISA was thoroughly validated for accuracy and sensitivity with characterized OPN standards (recombinant OPN from R&D Systems), after which it was tested against the three available commercial ELISAs. The Plumer in-house ELISA was the most accurate, while the Assay Designs ELISA over-estimated the standard values from 1.2-fold to 3.6-fold (Figure 6). The R&D ELISA gave results very similar to the in-house ELISA. The IBL ELISA kit gave extremely high measurements for these same standards (13 – 18-fold higher).

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[24] Immuno-Biological Laboratories; Gunma, Japan (IBL, 2015)
[25] R & D Systems, Minneapolis, MN (R & D Systems, 2017)
[26] Assay Designs is now a part of Enzo Life Sciences (Enzo Life Sciences, 2017)
[27] Personal communication (Sorensen, 2016)

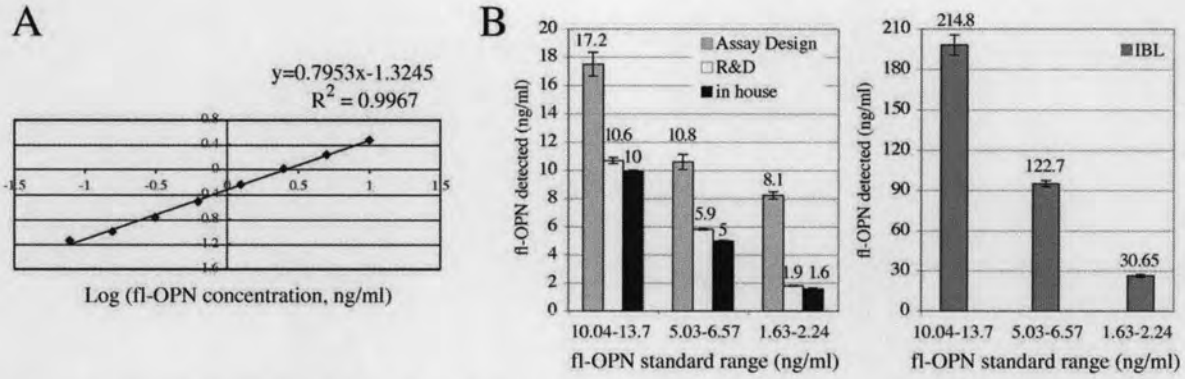

Figure 6. Comparison of OPN ELISA tests. A) Assay evaluating the quantification of recombinant fl-OPN using monoclonal antibodies 2F10 and 1F11-biotin. 2F10 reacts to an epitope on the N-terminal of human OPN and 1F11-biotin reacts with an epitope on the C-terminal side of human OPN. The optical density of the color product is proportional to the quantity of human OPN. The measuring range is from 78 pg/ml to 10 ng/ml. B) Commercially prepared human OPN standards of high (10.1 ng/ml-13.1 ng/ml), medium (5 ng/ml-6.6 ng/ml) or low (1.7 ng/ml-2.3 ng/ml) concentrations were used in four ELISA tests: the in-house test, and those commercially available from Assay Designs, R&D Systems (Quantikine) and IBL (Plumer *et al.*, 2008).

Independently from the Plumer in-house OPN ELISA, an ELISA was established using polyclonal antibodies against human milk OPN (Schack *et al.*, 2009). Like in the Plumer ELISA, the accuracy of this ELISA was validated against characterized OPN standards (in this case human milk OPN obtained, purified and verified in-house). Importantly, this ELISA was compared to the commercial ELISAs from IBL and R&D systems by analyses of 14 individual human milk samples with each of the ELISAs (Table 5). Although there was variation in the OPN concentrations, the mean was consistent with the Plumer study. The Schack in-house ELISA showed that the IBL ELISA strongly overestimated the response for OPN in the samples by up to 10-fold, whereas the results obtained with the Schack ELISA was comparable to those obtained with the R&D ELISA (Schack *et al.*, 2009).

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Table 5. Osteopontin (OPN) concentrations in breast milk samples analyzed with an in-house-developed ELISA and ELISA from R & D Systems and IBL[1] (Schack *et al.*, 2009).

	OPN concentration (mg/L)		
Sample	**In-house**	**R & D[2]**	**IBL[3]**
1	66	86	582
2	257	309	4,075
3	201	182	1,962
4	65	71	394
5	46	66	250
6	22	25	172
7	102	152	601
8	114	227	1,342
9	67	63	203
10	212	267	2,810
11	105	98	255
12	157	144	1,356
13	185	182	1,447
14	103	141	996
Mean ± SD	122±70[a]	144±83[a]	1,175±1,137[b]

[a,b]Mean values within a row with unlike superscript letters are significantly different ($P < 0.01$).
[1]Regression equations: In-house versus R &D: 1.07x + 13.88 (r2 = 0.81); in-house versus IBL: 14.9x - 638.30 (r2 = 0.85); R & D versus IBL: 12.65x - 640.11 (r2 = 0.85).
[2]R & D Systems, Abingdon, UK.
[3]IBL (Immuno-Biological Laboratories), Gunma, Japan.

In general, the Plumer *et al.* (2008) and Schack *et al.* (2009) in-house ELISAs, even though they use very different antibodies against OPN, both showed results that were in line with the results from the R&D ELISA (which uses a third set of antibodies). Both studies show that the IBL assay strongly overestimates (by 10-fold) the concentration of osteopontin.

There are several explanations to why the IBL kit overestimates the OPN. These have been discussed (Plumer *et al.*, 2008; Schack *et al.*, 2009). First it should be clearly stated the IBL kit is not validated for use in milk samples, which could explain why it overestimates OPN in this media as seen in the Nagatomo study (Nagatomo *et al.*, 2004). However, this does not explain why the ELISA also overestimates the OPN in plasma (Plumer *et al.*, 2008) or simply when an OPN standard in any media is measured (Plumer *et al.*, 2008; Schack *et al.*, 2009). The IBL ELISA assay uses a polyclonal antibody as capture antibody and the R&D ELISA has a polyclonal detecting antibody, which makes it possible that there is more wide recognition of epitopes including potential post-translational modifications. The conclusion is that the IBL ELISA is less specific for the OPN molecule and is neither validated nor suitable for measuring OPN in milk samples.

Schack *et al.* (2009) reported OPN levels in human milk from 29 women (mean age 29.6 ± 3.5 years and 20.4 ± 11.3 days post-partum). The authors utilized purified OPN from human milk (isolated using reversed-phase HPLC, dialyzation, and gel-filtration chromatography to obtain the full-length OPN protein). The OPN fraction was then lyophilized to obtain a > 98% pure OPN protein. The purity verified by SDS-PAGE, reversed phase-chromatography, and N-terminal

sequencing. Antibodies (IgG fraction) to this purified OPN were obtained and specificities of the antibodies were checked and verified by Western blotting and purified OPN (no cross-reactivity with other milk proteins was noted).

The determination of OPN content in the human milk was conducted using two different ELISA methods: an in-house developed ELISA utilizing antibodies generated against human milk OPN, and a commercially available human OPN ELISA from R&D Systems. The in-house developed ELISA gave an OPN mean (± standard deviation) value of 138 ± 79 mg OPN/L breast milk (obtained from mothers 6 – 58 days post partum) with a total protein concentration of 8,062 ± 3,680 mg/L breast milk and a mean value of OPN/total protein of 2.1 ± 1.4% (*w/w*). This is considerably higher than in bovine milk, and which the authors analyzed and reported at 18 mg OPN/L bovine milk using an ELISA specifically developed for bovine OPN using antibodies raised against bovine milk OPN (Schack *et al.*, 2009). The R&D Systems ELISA showed a comparable level of OPN in human breast milk of 144 ± 83 mg/L (mean ± standard deviation). There was a large variation in the OPN content of the milk samples analyzed (there also was substantial variation in the protein content of the samples). The authors did not give any clear explanation as to why the variation occurred, other than human individual variation. There did not seem to exist any correlation between days after delivery and the content of OPN in the milk, and it is not known whether this was to be expected. The protein content in some of the milk samples was very low; OPN content was not correlated with protein concentration. The authors could not rule out that there may have been problems in measuring these samples, but if the samples with less than 4 g/l protein are left out of the calculations, an OPN average of 147 mg/l was still achieved.[28]

To confirm the OPN concentration determined *via* ELISA, purified full-length human milk OPN was quantified by amino acid analysis and a sample containing 10 ng OPN was measured by an in-house ELISA (please see above) and two commercial ELISAs purchased from R&D Systems and IBL and used according to manufacturers methods. The protein concentrations in the milk samples were measured using Bradford analyses with BSA as the external standard using a Bio-Rad[29] protein assay kit. The results of the in-house ELISA confirmed OPN content at 10 ng/L breast milk, while the R&D ELISA measured 13 ng OPN/L breast milk. Conversely, the IBL ELISA showed a content of 114 ng/L breast milk, which confirmed the Schack *et al*'s hypothesis that the IBL ELISA overestimates the content of OPN in milk. Plotting of the data from Schack *et al.* (2009) demonstrates that there is little relationship between OPN concentration and days postpartum or duration of lactation (Figure 7). The concentration of OPN in human breast milk is as much as nine times that of bovine milk and 28 times that of commercial infant formula (Schack *et al.*, 2009).

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[28] Personal communication (Sorensen, 2016)
[29] Richmond, CA.

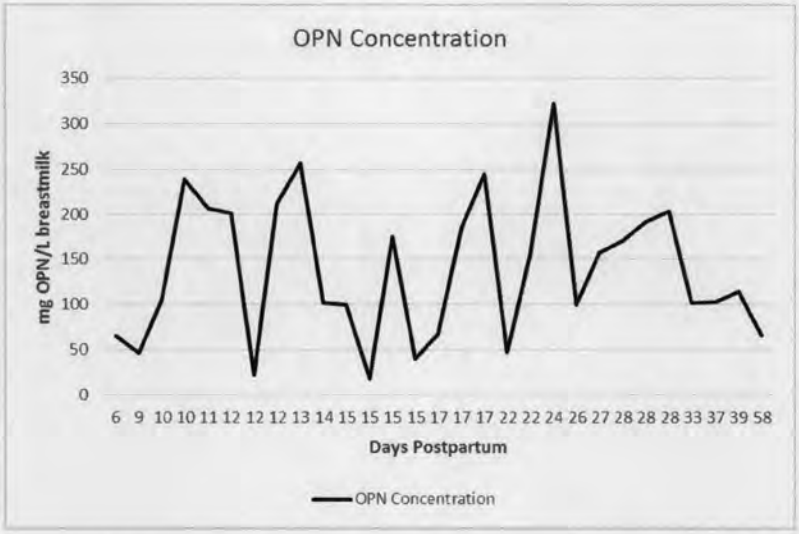

Figure 7. OPN concentration in breast milk *vs.* days postpartum (29 samples, from mothers aged 22 – 37 years; mean = 29.6 years; ethnicity not stated) (Schack *et al.*, 2009).

Additional research found that OPN levels varied according to period of lactation, with the production level of OPN in early milk (median of 1493 mg/L), and mature milk (median ranging from 413 – 896 mg/L). These levels were substantially higher than that quantified in colostrum (median of 2.7 mg/L). Interestingly, all levels were greater than maternal plasma (median of 0.339 mg/L) (Nagatomo *et al.*, 2004). However, further research has shown that the reagents used in the Nagatomo *et al.* study (IBL ELISA) were not validated for milk OPN and overestimated the OPN concentration by approximately a factor of ten, as discussed above (Schack *et al.*, 2009). Earlier work by Senger *et al.* (1989) estimated OPN levels ranging from 3 – 10 mg/L in maternal milk. The estimation was based on the yields from purification by ion-exchange, barium citrate adsorption and a final step of reverse-phase HPLC. These authors assumed that the recoveries from their isolation and centrifugation processes for purification were 100% and their back calculation to OPN levels in human milk is 100% accurate, which is nearly impossible for these techniques and the authors did not include an internal standard to assess recovery. Sun *et al.* (2010) estimated that OPN levels in bovine milk average 8.7 mg/L, which is consistent with the understanding that levels of bovine milk OPN are less than human milk OPN, and comparable to the content of OPN in bovine milk reported by Schack *et al.* (18 mg/L).

Sun *et al.* (2010) utilized a novel three-step extraction procedure that included anion exchange chromatography, one step hydroxyapatite chromatography and a two-step hydroxyapatite procedure, along with fractionation by first adsorbing the protein on to DEAE-Sephacel resin and subsequent fractionation by step-wise elution with NaCl, to remove contaminating proteins and further concentration of the purified protein. In addition, Sun *et al.* (2010) used a goat-anti-bovine ELSA kit produced by "ADL, American" to detect the purified OPN from bonvine milk. The company "ADL, American" could not be located to verify the antibodies use, but could be referring to Assay Design, which would indicate that antibodies for human OPN could have been used, also explaining why they found lower reactivity with the bovine OPN. Similar concentrations of OPN have been found in human milk from mothers delivering at

near full-term as from mothers delivering as early as 26 weeks of gestation (Dhanireddy *et al.*, 1993). However, Dhanireddy *et al.* (1993) utilized a quantitative gel technique (*i.e.*, semi-quantitative in nature). The milk was from mothers of preterm infants and has not been confirmed that breast milk from mothers of preterm babies would have an OPN level similar to mothers of term infants.

Overall, the studies conducted to determine OPN levels in human and bovine milk have shown that several experimental and commercially-available assays can be utilized to determine reliable OPN levels in milk, while at least one other assay (by IBL) consistently provides unrealistically high OPN results. Utilizing validated ELISA methods, the concentration of OPN in bovine milk has been found to be approximately 18 mg OPN/L bovine milk. One study has also been conducted to determine OPN levels in human milk utilizing a validated ELISA assay that provided OPN concentrations comparable with a commercially-available ELISA assay. The study found that for the breast milk samples analyzed, human milk OPN concentrations averaged at 138 mg/L.

6.1.1.2.Description of OPN-10

OPN-10 is a proprietary food ingredient produced by Arla Food Ingredients (AFI) containing OPN isolated from bovine whey processed under current Good Manufacturing Practices (GMP). OPN-10 contains at least 78% protein (N * 6.38), greater than 95% of which is bovine whey-isolated OPN. OPN-10 contains less than 11% ash, and maximum 1.0% of lactose and maximum 1.0% of fat.

6.2. History of use

Research on human milk and bovine milk composition led to a process of isolating the OPN fraction from bovine milk in the past decade. The addition of OPN from bovine milk to infant formula is to attain similar OPN levels found in breast milk (found at a mean of 138 mg/L) in infant formulas (for those infants ages 0 – 12 months), which includes follow-on formulas[30] specifically marketed to infants 6 – 12 months of age, and for children 1 – 3 years of age.

Country specific regulations on milk fractions and their use in infant formulations vary widely across the globe. OPN blended with whey protein concentrates (WPC) may be permitted by some countries for use in infant and follow-on formulas. Through publically available databases, it has been found that bovine osteopontin is added and claimed in infant formulas in several Asian markets including North and South East Asia and China since 2010 (Mintel, 2014). The addition levels vary anywhere from 21 to 110 mg/L of formula. The variation in levels also depends on the target population (0 – 6 months, 6 – 12 months, 1 – 3 years, 3 years and above). OPN-10 is a product that has not been previously utilized as an ingredient added to infant or follow-on formula, foods marketed specifically to children ages 1 – 3, or other foods in the United States. Therefore, OPN-10 is a new food ingredient for the United States market.

Bovine-derived OPN is present in milk-based commercial infant formula at a level of approximately 5 – 13 mg/L (approximately 0.15% of total protein content) (Schack *et al.*, 2009).

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[30] Follow-on formula (aka follow-up formula) has been defined by the Codex Alimentarius Commission as "a food intended for use as a liquid part of the weaning diet for the infant from the 6th month on and for young children" (CODEX, 2011).

OPN is present in bovine milk at a concentration of approximately 15 – 20 mg/L (approximately 0.05% of the total protein content) (Schack *et al.*, 2009).

6.3. Current uses

Neither OPN-10 nor OPN as an isolated substance is currently added to food, including infant formula, in the U.S.

6.4. Mechanisms of action

The scientific community has evaluated human milk components, including the identification and isolation of novel glycoproteins, such as OPN. OPN is a highly phosphorylated, multifunctional glycoprotein with an open, flexible structure. Experimentally, OPN has been found to have a variety of actions, including involvement in mammary gland development and differentiation in animal models and may help inhibit precipitation of calcium in milk, as well as possibly reduce the risk of calcium precipitation in the kidneys that form kidney stones (OPN is secreted by tubule cells and papillary epithelium) (Sodek *et al.*, 2000). In newborn mice nursing dams lacking milk OPN, impaired cognitive development, reduced brain OPN and reduced myelin-related proteins were reported (Jiang and Lönnerdal, 2016). In infant rhesus monkey fed formula with added bovine milk OPN, intestinal analysis found OPN positively regulated intestinal proliferation and cell migration from jejunal tissue samples, as well as cellular chemotaxis (Donovan *et al.*, 2014; Jiang and Lönnerdal, 2016). In a mouse model for colitis, oral administration of bovine-derived OPN positively modulated lower intestinal markers of immune responses (diarrhea and fecal blood) to dextran sulfate (Kanwar *et al.*, 2016).

OPN may have a positive impact on nervous tissue repair functions, as suggested by studies in mice with experimentally induced spinal cord injuries (Hashimoto *et al.*, 2007), and may play a role in the inhibition of scar formation in connective and other tissues (Sodek *et al.*, 2000). In addition, *in vitro* studies with isolated mouse osteoclasts show that OPN may help regulate bone mineralization processes and bone remodeling by osteoclasts (Rodriguez *et al.*, 2014). OPN appears to aid in the regulation of biomineralization *via* osteoblasts while having little direct effect on osteoblast cell development, as seen in an *in vitro* study using mouse primary osteoblast cultures (Holm *et al.*, 2014). OPN may also be secreted by macrophages to serve as adhesion proteins for the facilitation of phagocytosis during tissue remodeling (Mckee and Nanci, 1996).

OPN has been closely associated with some functions involving the immune response, including binding integrin receptors expressed on inflammatory cells (*e.g.*, neutrophils, macrophages and mast cells), promoting chemotaxis or cell activation (especially as an important early regulator of Th1-mediated immunity) (Ashkar *et al.*, 2000), and acting as an opsonin by enhancing phagocytosis *in vitro* through a novel (aXb2 integrin) OPN receptor (Schack *et al.*, 2009b). Nau *et al.* (2000) found that OPN expression after infection by mycobacteria is inversely proportional to patient outcome; that is, "patients who do well after an infection by mycobacteria express high levels of OPN." Patients with localized infection tended to have a higher OPN expression. The authors of the study concluded that "osteopontin expression correlates with an effective immune and inflammatory response when humans are challenged by a mycobacterial infection and that osteopontin contributes to human resistance against mycobacteria" (Nau *et al.*, 2000). Overall, there is no single known specific function of OPN. Experimental evidence indicates that OPN may have multiple, beneficial actions on different biological systems.

6.5. Regulatory Status

A search of the website for the Food and Drug Administration did not reveal any similar substances having been approved for use in infant formula. Under section 912 of the FDA Amendments Act of 2007 amended Section 301(II) of the Federal Food, Drug, and Cosmetic Act, there is a prohibition of the introduction of any food into interstate commerce to which has been added an approved drug, licensed biological, or a drug or biological product for which substantial clinical investigations have been instituted and for which the existence of such investigations has been made public. Our search of the FDA website did not reveal any reference to OPN as a drug and no published clinical studies were located that would lead us to believe a substantial clinical investigation had been conducted and made public that would indicate a contemplated drug use of OPN.

The addition of substances to infant formula to mimic those levels found in human breast milk is well-documented. Several GRAS notifications indicate the agency has reviewed the GRAS status of substances added to infant formula that minic substances found in breast milk, including (but not all-inclusive) the addition of 2-FL (GRN 571 and GRN 546) cow's milk-derived lactoferrin (GRN 546) and galacto-oligosaccharides (GOS) (GRN 495).

6.6. Proposed use or uses

OPN-10 would be added to cow's milk-based infant formula and milk-based liquid and powdered drinks for children 1 – 3 years of age (at 160 mg OPN-10/L, refer to calculations in section 6.8) to better reflect the level of OPN contained in human mature breast milk (reported at a mean of 138 mg OPN/L (Schack *et al.*, 2009)). The infant formula would be marketed for full- or near-full-term infants, and OPN-10 would not be added to pre-term-focused or exempt infant formula. OPN-10 would only to be used in the wet blending-spray drying process of the production of infant formula, where ingredients are blended in water, homogenized, pumped to a heat exchanger for pasteurization, and then spray dried into a powdered product. The pasteurization step destroys harmful bacteria such as *Cronobacter sakazakii*[31] that is ubiquitous in nature. Therefore there is a very low potential for *C. sakazakii* to be a contaminant in OPN-10 and in the subsequent final infant formula product.[32] Importantly, the FDA established specific protocols to assure each batch of infant formula is not contaminated with this organism.[33]

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[31] *Cronobacter* multi-species complex (formerly *Enterobacter sakazakii*) is a group of gram-negative bacteria that exists in the environment and which can survive in very dry conditions. The natural habitat for *Cronobacter* is not known. It has been found in a variety of dry foods, including powdered infant formula, skimmed milk powder, herbal teas, and starches. It has also been found in wastewater. *Cronobacter* illnesses are rare, but they are frequently lethal for infants and can be serious among people with immunocompromising conditions and the elderly (CDC, 2015a). http://www.cdc.gov/cronobacter/technical.html; site last visited March 25, 2016.

[32] http://www.fda.gov/ohrms/dockets/ac/03/briefing/3939b1_tab4b.htm; site last visited September 28, 2015.

[33] https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm289378.htm; site last visited May 12, 2017.

6.7. DESCRIPTION, SPECIFICATIONS AND MANUFACTURING PROCESS

6.7.1. Description, Characterization and Specifications

OPN-10 is a bovine-whey-derived ingredient that contains OPN, a highly-phosphorylated glycoprotein with an open and flexible structure. OPN is composed of 262 amino acids and contains sialic acids in conjugated glycosylations. The protein contains 23 moles of phosphorus *per* mole protein and has a high calcium-binding ability. The physical and chemical properties and specifications for OPN-10 are provided in Table 1. Analysis of five nonconsecutive lots confirms that OPN-10 meets specifications and is manufactured according to current Good Manufacturing Practices (cGMP) per 21 CFR §106.96 (Appendix I, individual lot analysis table). The protein content is calculated from nitrogen analysis using the Kjeldahl nitrogen method and is calculated by the fixed nitrogen factor of 6.38 for milk products.[34]

The conversion factor of 6.38 is commonly utilized for milk protein analysis. However, the full length bovine OPN was determined to have 262 amino acids, with a molecular weight of approximately 33.9 kDa, of which 29.3 kDa are amino acids, and approximately 1.7 kDa are phosphorylations and 2.9 kDa are O-linked glycosylations. According to AMLDI MS, the most abundant N-terminal fragment has a molecular weight of approximately 19.8 kDa, 16 kDa of which are amino acids, 0.9 kDa are phosphorylations and 2.9 kDa are O-linked glycosylations, with 194 nitrogen atoms (2,716 g N/mol protein). In the full length protein, 362 nitrogen atoms are found, equivalent to 5,068 g N/mol protein. In OPN-10, the predominant molecule (80%) as measured by gel permeation chromatography is a large N-terminal fragment, the full length protein comprising only 20%. The average molecular weight for OPN in OPN-10 is 22,900 g/mol. The full length OPN molecule nitrogen content is 14.9%, leading to a nitrogen conversion factor of 6.69 for the Kjeldahl method. The N terminal fragment is composed of 13.72% nitrogen, giving a nitrogen conversion factor of 7.29. Utilizing the percentages of full length and fragments in OPN-10 and the amount of nitrogen in each, the conversion factor for the Kjeldahl method for OPN-10 is 7.17. OPN has a unique amino acid composition and more post-translational modifications than average milk proteins, and therefore the amount of OPN contained in OPN-10 is calculated by multiplying the nitrogen content by the nitrogen factor of 7.17 to correct for a unique OPN composition and taking into account the 95% purity of OPN in OPN-10 (*i.e.*, X*7.17*0.95) (de Boer, 2014).

AFI continusly monitors the level of pesticide residues, contaminants and heavy metals in our products. Levels have never been detected above the limit set by FDA in the regulations on these substances. All analysis are done by an external laboratory using validated analytical methods.

AFI continusly monitors the whey used to produce OPN-10, and OPN-10 has been tested for the content of heavy metals on an annual basis. The levels found in the raw milk correspond to the levels found in the analysis of OPN-10. OPN-10 was analyzed for *C. sakazakii*, with none reported. As pasteurization of the milk and adherence to strict manufacturing processes makes *C. sakazakii* contamination highly unlikely, analysis for this bacteria will only be conducted on an intermittent basis on the OPN-10. No contamination has been found in any material produced. The

[34] http://www.fao.org/docrep/006/y5022e/y5022e03.htm; site last visited December 23, 2014.

testing of the raw milk has been done for more then 10 years and has never seen increased heavy metal levels above regulatory limits for commercial milk (ARLA, 2016).

Table 6. Specifications of Lacprodan® OPN-10.

Analysis	Method	Specification	Batch Analysis Results (n = 5 lots) Range	Average
Protein (N*6.38) as is (%)	Kjeldahl	≥ 78%	78.3 - 79.46	78.80
OPN as a % of total protein (%)	HPLC	≥ 95%	97.2 - 99.9	97.75
Ash (%)	NMKL 173	≤ 11%	9.2 - 9.7	9.44
Moisture (%)	ISO 6731	≤ 5.5%	4.1 - 5.0	4.5
Lactose (%)	IDF 79B	≤ 1.0%	0.1	0.1
Fat (as is) (%)	ISO 3889	≤ 1.0%	0.2	0.2
Solubility index[35] (ml)	ADPI 916	≤ 1.0 ml	< 1.0	N/A
Scorched particles**	ADPI Bulletin 916	A	A	A
Minerals				
Sodium (%)	ICP	0.5 - 1.5%	1.07 - 1.27	1.19
Chloride (%)	ISO 5943	≤ 0.2%	0.04 - 0.1	0.06
Phosphorus (%)	ICP	1.4 - 2.2%	1.84 - 1.95	1.87
Calcium (%)	ICP	1.6 - 2.7%	2.2 - 2.37	2.29
Heavy Metals#				
Arsenic (mg/kg)	ICP-MS (EPA 200.8)	< 0.5	< 0.010 - 0.10	0.04
Cadmium (mg/kg)	ICP-MS (EPA 200.8)	< 0.05	< 0.01*	< 0.01
Lead (µg/kg)	ICP-MS (EPA 200.8)	< 50.0	13 - 16	14.3
Mercury (mg/kg)	ICP-MS (EPA 200.8)	< 0.05	< 0.005	< 0.005
Selenium (mg/kg)	ICP-MS EN ISO 17294m:2016	< 0.15		0.06***
Microbiological load				
Total plate count (cfu/g)	ISO 4833	≤ 5,000	< 1000	< 1000
Mold/yeast (cfu/g)	ISO 6611	≤ 100	< 10	N/A
Bacillus cereus (cfu/g)	ISO 7932	< 50	< 10 - 40	N/A
Staphylococcus aureus (coag +) (cfu/g)	ISO 6888	Not detected	Not detected	Not detected
Enterobactericeae (cfu/g)	ISO 21528	≤ 10	<10 - 10	<10
Salmonella (cfu/g)	BAX®	Not detected/125 g	Not detected/125 g	Not detected/125 g
C. sakazakii (cfu/10 g)##	ISO/TC 34/SC 9	Not detected/10 g	Not detected/10 g	Not detected/10 g

*Limit of detection; **Must meet the American Dairy Products Institute Scorched Particle Standards for Dry Milks (GEA, 2006); ADPI=American Dairy Products Institute; BAX® = Baxter International; cfu = colony forming unit; EPA=Environmental Protection Agency; N/A = not applicable; HPLC=High-Pressure Liquid Chromatography; ICP=Inductively coupled plasma; ICP/MS=Inductively coupled plasma/mass spectrometry; IDF=International Dairy Federation; ISO=International Organization for Standardization; NMKL=NordVal International Denmark; #Heavy metal analysis conducted annually; ***selenium analysis conducted on a semi-annual basis, current data based on single analysis ##*C. sakazakii* analysis conducted on an intermittent basis.

[35] A measurement of the amount of product sediment after the application of low centrifugal forces under specified conditions-a high value indicates that the product is less soluble (US Dairy Export Council, 2016).

6.7.2. Manufacturing process

OPN is extracted from bovine whey, a cheese or casein production byproduct, utilizing commonly known processes of the whey industry. The whey used as a starting material for the production of OPN-10 conforms to the European Union Food Hygienic Guidelines and EU Regulation 853/2004,[36] which allows for the use of only U.S.-approved pesticides and veterinary drugs.[37] Furthermore, except for the demineralized water, all the remaining processing aids (*i.e.*, sodium chloride,[38] sodium hydroxide,[39] hydrochloric acid[40] and calcium chloride[41]) used in the production of OPN-10 are food grade and approved for infant formula. The raw material - milk and whey - comes from plants that are all authorized by authorities that regulate dairy product facilities. All plants have implemented Hazard Analysis and Critical Control Points (HACCP) and comply with current Good Manufacturing Practices (cGMP) which is regulated in the European Union under Regulation (EC) No 852/2004 on the hygiene of foodstuffs and consistent with U.S. GMPs for infant formula (see 21CFR §110).

At the point of entry to the factory the raw materials, an analytical control assessment is made: temperature, pH, sensory tests and antibiotics detection. The plant producing OPN-10 is certified under DS / EN ISO 50001: 2011, ISO 22000: 2005 / TS 22002-1: 2009 and FSSC 22000, which all control: (1) Implemented quality control systems, which include cGMP and HACCP; (2) Raw material analytical control (*e.g.*, temperature, pH, sensory tests and detection of antibiotics); and (3) The physicochemical and microbiological characteristics of the final product. AFI has been certified for the development, production, and sales of products from whey and lactose by the Danish Authorities and by FDA.[42] The production process is summarized in Figure 1.

Raw skim milk is received at the cheese or casein production facility, where it passes a pasteurization step that is controlled with a rejection valve, which is tested at the start of every production lot. This is a critical control point at the dairy facility, eliminating the risk of pathogens. The milk is then processed to produce cheese or casein, which also gives whey (containing OPN). When the whey is kept in storage tanks the temperature is set to 5°C. The whey then undergoes pasteurization (minimum 72.5°C/15 seconds) or microfiltration (ceramic membrane < 1.4 μm), and ultrafiltration where the temperature is kept below 12°C and processing through an anion exchange column containing Q sepharose big beads, the feed is adjusted to the correct pH and concentration by inline adjustment with demineralized water, NaOH or HCl depending on the need. There is a heat treatment step prior to the drying process. The column first eluted with a weak (< 0.2 M) NaCl solution to remove impurities from the column then is rinsed with demineralized water before the product is eluted with an approximately 1 M NaCl solution. Importantly, the ion exchange columns are designed to prevent carry-over of the resins into the OPN eluate. Firstly, there is a 10μm column frit retaining the resin, which has a diameter of 100 – 300μm. Secondly, there is a 50μm sieve tube filter whereby the sieve tube filter is visually examined before and after production to ensure that any resin is retained if the 10μm column frit

[36] http://faolex.fao.org/docs/pdf/eur63427.pdf; site last visited September 29, 2015.
[37] (Global MRL Database, 2015)
[38] 21CFR§182.1: Substances that are generally recognized as safe.
[39] 21CFR§184.1763 Sodium hydroxide.
[40] 21CFR§182.1057 Hydrochloric acid.
[41] 21CFR§184.1193 Calcium chloride.
[42] Inspection number FEI 3004285668; inspection on 22/08/2012.

fails. If the filter is found damaged the product is submitted to rigorous testing to ensure product quality. The ultrafiltration step removes the residual low molecular weight contaminants in the OPN. The eluent then goes through additional ultrafiltration steps to concentrate the OPN; $CaCl_2$ is added to normalize the counterions surrounding the protein and heat treated at greater than 72°C for 15 seconds, conditions that are also requirements for pasteurized milk described in the Grade "A" Pasteurized Milk Ordinance (FDA, 2016).

The OPN-containing filtrate is then spray dried to produce the OPN-10 product in powder form. The powder is then passed through a 1 mm sieve and passed through a rotating magnet before bagging, and the filled bags are passed through a separate metal detector before palleting. AFI performs finished product analysis for *Salmonella* according to federal regulations. OPN-10 is greater than 95% OPN of total protein. The final OPN-10 product is packed in polyethylene bags and stored at room temperature (approximately 20°C). OPN-10 is manufactured in a manner consistent with the production of other bovine milk-related products.

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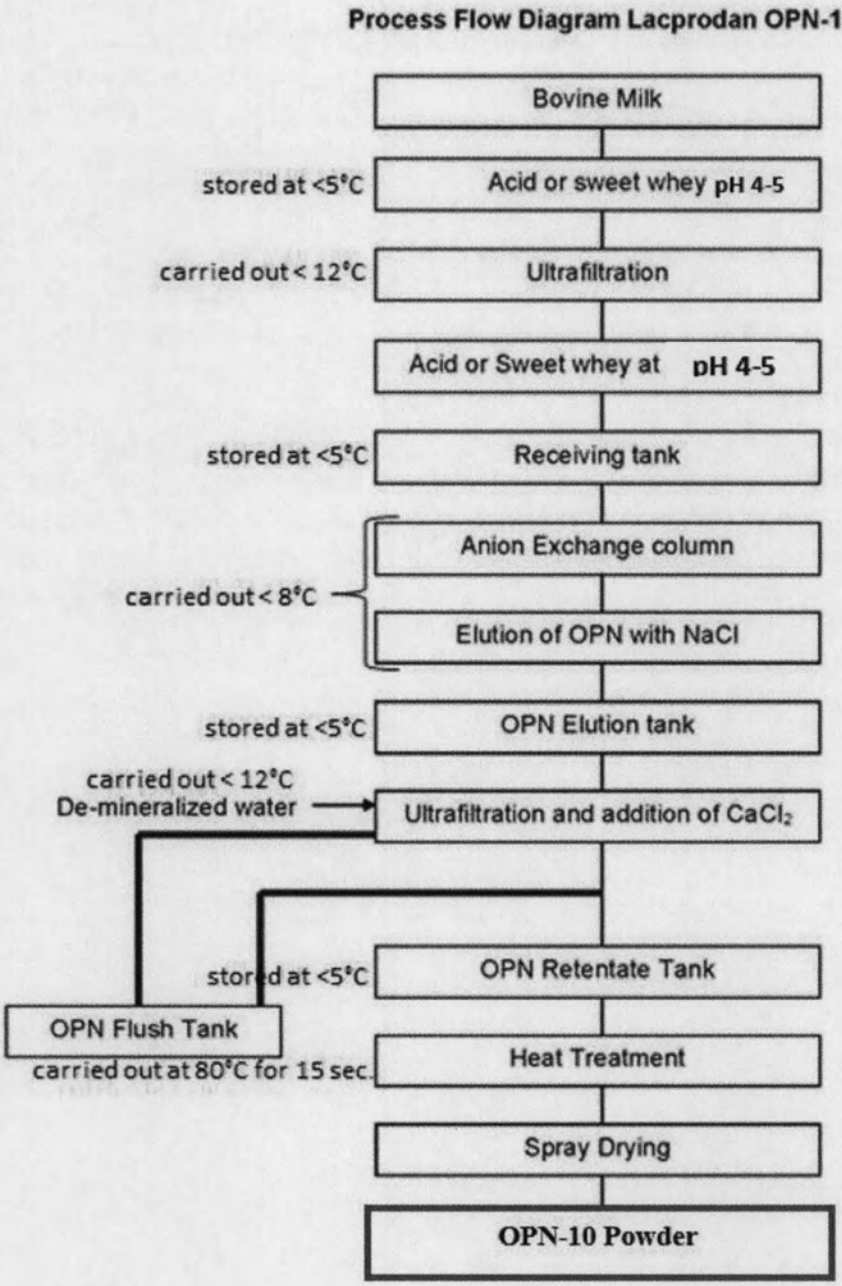

Figure 8. OPN-10 production scheme

6.7.3. Allergenicity

OPN-10 is to be added only to cow's milk-based infant formulas and cow's milk-based liquid and powdered drinks for children 1 – 3 years of age. The OPN-10-containing products will be labeled as containing or produced from milk; this should mitigate any reasonable question

concerning allergenicity beyond that declared for milk-based products. Bovine-derived OPN is already found in cow's milk-based infant formulas (Schack *et al.*, 2009) and the addition of OPN-10 is only to increase the level of OPN intake to OPN concentrations found in breast milk, with a high homology between human and bovine OPN, the major protein in OPN-10. The amount of OPN-10 in an infant formula or toddler product is approximately 1% of total protein and between 0.1 to 0.2% of total milk solids. Therefore, it is not likely that OPN-10 would elicit an allergic response apart from that of a response to cow's milk.

6.7.4. Stability

OPN-10 has been analyzed for stability and was found to be stable for approximately three years if kept under cool (5 – 20°C) dry conditions away from strong odors (Table 7). The OPN-10 was packaged in polyethylene bags, standard for storage of OPN-10. OPN-10 is composed primarily (> 95%) of OPN; therefore, the protein content of OPN (as evaluated with a nitrogen conversion factor of 7.17) and moisture were monitored periodically on more than five batches of OPN-10, measured at day 0 and after approximately 151 weeks. OPN stability was quantified by HPLC, relative to both total protein and total dry matter. At day 0 the content expressed as %OPN in protein in the OPN-10 product vary from 96.99 – 100.16 % showing that there is low variability in the content of OPN between different batches of OPN-10 as well as low uncertainty of the analytical method. OPN-10 did increase in moisture content over the three-year period, but no degradation of the OPN protein was found in the OPN-10 product.

Heat stability is an issue when a protein has defined tertiary structure. Lack of tertiary structure for OPN is an advantage for the intended application of addition to infant formula consumed by infants 0 – 12 months of age and milk-based beverages targeted for children ages 1 – 3 (commonly referred to as toddlers). Almost all infant formulas are either spray dried (powder) or use intense heat for a short period treatment (liquid) to control the quality and minimize microbial contamination. The fluid structure of OPN is expected to preserve the OPN molecule during the manufacture of infant formula containing OPN-10. The lack of a decrease in OPN concentration during the heating and drying treatment for the production of OPN-10 indicates that OPN contained in OPN-10 resists heat-induced degradation (Table 7).

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Table 7. Stability of OPN-10 (ARLA, 2015)

Analysis	Batch Number	Analysis (Date)	OPN	PROT(7.17)	Water	Protein	Purity	OPN	Loss of OPN	Loss of OPN	Duration
			% of powder	% of powder	% of powder	% of dry matter	OPN % of protein	% of dry matter	% of original dry matter	% loss by protein	Weeks
Initial	(b) (4)	11-30-2011	89.60	89.62	4.78	94.12	99.98	94.10			
Final	(b) (4)	10-28-2014	86.30	84.79	8.77	92.94	101.78	94.60	-0.53	-1.80	151
Initial	(b) (4)	09-30-2011	89.00	90.11	4.71	94.55	98.77	93.39			
Final	(b) (4)	10-28-2014	86.10	85.54	8.53	93.52	100.65	94.13	-0.79	-1.90	160
Initial	(b) (4)	11-30-2011	88.97	89.22	4.68	93.59	99.72	93.33			
Final	(b) (4)	10-28-2014	86.90	85.24	8.66	93.32	101.95	95.14	-1.94	-2.23	151
Initial	(b) (4)	11-30-2011	88.60	88.10	4.83	92.57	100.57	93.10			
Final	(b) (4)	10-28-2014	86.10	85.22	9.01	93.66	101.03	94.63	-1.64	-0.46	151
Initial	(b) (4)	11-04-2011	89.30	89.16	4.44	93.29	100.16	93.44			
Final	(b) (4)	10-28-2014	85.30	84.36	9.44	93.15	101.11	94.19	-0.80	-0.95	155
Initial	(b) (4)	11-18-2011	88.25	89.05	4.89	93.62	99.11	92.79			
Final	(b) (4)	10-28-2014	85.40	85.63	8.97	94.07	99.73	93.82	-1.11	-0.63	153
Initial	(b) (4)	11-30-2011	88.45	88.66	4.52	92.85	99.77	92.64			
Final	(b) (4)	10-28-2014	86.00	85.78	8.70	93.95	100.26	94.19	-1.68	-0.49	151
Initial	(b) (4)	11-30-2011	86.95	89.65	4.64	94.01	96.99	91.18			
Final	(b) (4)	10-28-2014	83.60	85.15	9.03	93.60	98.18	91.90	-0.79	-1.23	151

*HPLC/LCMS for OPN; OPN = osteopontin; PROT = protein

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Analysis of the levels of OPN-10 (as determined by OPN levels) when added to infant formula was also conducted utilizing an LC-MS-MS quantitation method at typical and elevated temperature/relative humidity (Table 8). Results show that OPN from OPN-10 is stabile in infant formula for at least 90 days at both typical and accelerated time/temperature. Analysis at 180 days (six months) shows that the OPN from OPN-10 is stable at room temperature and typical humidity, but accelerated stability conditions suggest a slight degradation of the OPN protein, which is also seen of other proteins in infant formulas in general. Additional time point analyses will continue to be conducted. The *percent* of bovine OPN contained in the infant formula did not substantially change over time when stored at room temperature for at least six months, and at accelerated conditions at up to six months.

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Table 8. Stability of OPN-10 when added to infant formula.

Analysis	Room Temperature (25 C/60% RH)			Accelerated Stability (40 C/75% RH)		
	Control	Sample 1	Sample 2	Control	Sample 1	Sample 2
Time 0						
OPN (Time 0)* (mg OPN/100 g)	15.5	108	102	15.5	108	102
% OPN at Time 0	100	100	100	100	100	100
30 Days after Time 0						
OPN (mg OPN/100 g)	15	98.4	109	15	91.7	101
% OPN compared to Time 0	96.7	91.1	106.8	96.7	85	99
60 Days after Time 0						
OPN (mg OPN/100 g)	15	110	110	15	103	99.3
% OPN compared to Time 0	96.7	102	108	96.7	95.3	97.3
90 Days after Time 0						
OPN (mg OPN/100 g)	15	110	106	15	107	96
% OPN compared to Time 0	96.7	102	104	96.7	99	94.1
180 Days after Time 0						
OPN (mg OPN/100 g)	16.4	105.6	103.4	13.7	91.9	81.2
% OPN compared to Time 0	105	97.7	101	88.3	85	80

*Analysis values are expressed in mg OPN/100 g infant formula powder (as typically processed)

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6.8. Estimated Daily Intake of OPN-10 at the proposed Use Levels in Term Cow's Milk-Based Infant Formulas

OPN-10 is being added to term cow's milk-based infant formula to provide OPN at a concentration to mimick the amount of OPN contained in breast milk, as recent research found that breast milk contains on average approximately 138 mg OPN/L (*i.e.*, 0.138 mg/ml milk) and that commercial infant formula (powdered infant formula reconstituted in water as *per* manufacturer's instructions[43]) currently contains approximately 5 – 13 mg OPN/L formula (Schack *et al.*, 2009).

To provide a total of 138 mg OPN/L formula, the amount of OPN already in formula (a conservative 13 mg OPN/L) must be taken into account. The higher level of 13 mg OPN/L formula from the measured range of 5 – 13 mg OPN/L is being utilized so that a minimum amount of OPN-10 is being added to infant formula. OPN-10 is to be added to infant formula to provide up to 125 mg OPN/L formula (*i.e.*, 138 mg OPN/L breast milk - 13 mg OPN already contained in a liter of infant formula). OPN-10 contains at a minimum 78% protein (based on Nitrogen factor of 6.38) of which at least 95% is the OPN protein. So in order to fortify 125 mg/L of OPN protein, approximately 160 mg/L of OPN-10 (125/0.78) is to be added to correct for purity. Together with innate levels of approximately 13 mg OPN/L, the target in final formula would be 138 mg OPN/L formula. (Schack *et al.*, 2009),

Estimates for the daily intake (EDI) of OPN-10 when added to term cow's milk-based infant formula were calculated based on infant formula consumption data included in the U.S. Department of Agriculture (USDA) and U.S. Department of Health and Human Services (DHHS) National Health and Nutrition Examination Survey (NHANES) 2011-2012 national food survey. The NHANES survey obtains nationally representative nutrition and health data, along with U.S. prevalence estimates for nutrition and health status measures (CDC, 2015b). The survey collects two days of 24-hour dietary recall data, during which participants provide information on types and amounts of foods consumed. The NHANES data set is often utilized in GRAS dossiers to estimate the amount of a certain food ingredient consumed on a daily basis.[44] These data are then analyzed to code individual foods and portion sizes. From the data tables prepared under the NHANES program, a consumption analysis of OPN-10 when added to milk-based infant formulas was conducted, utilizing the amount of milk-based formula consumed and the amount of OPN-10 to be contained in the formula. The resulting information provided a mean and 90th percentile estimated intake of OPN-10 when added to milk-based infant formulas. The NHANES data set does not provide specific data on the age of the infant, other than "less than 1 year of age". From the use of OPN-10 in infant formulas (minus those indicated as made from soy or other plant-based materials) as taken from the NHANES 2011-2012 food survey (Appendix II), the "eaters-only"[45] estimated mean and 90th percentile intakes of OPN-10 were 119.0 and 189.7 mg/person/day, respectively (Table 4).

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[43] The OPN concentration was measured in read-to-feed formulas based on 125 g/L of powder (Schack *et al.*, 2009).
[44] As an example, please see the "no questions" letter provided by FDA for GRAS notification 669; https://www.fda.gov/downloads/Food/IngredientsPackagingLabeling/GRAS/NoticeInventory/ucm549873.pdf; site last visited April 19, 2017.
[45] "eater's only" data utilizes only those respondents that consumed the specific requested food at least once during the survey period (typically utilized by FDA in determining ingredient consumption);

It is generally accepted that a breast-fed infant has a daily milk intake corresponding to approximately one-sixth of their body weight (Greer, 2001), with an average of 165 mL/kg body weight for their first three months of life (American Academy of Pediatrics, 2015). The greatest exposure to formula ingredients (on a body weight basis [BW]) is during the first few months of life, when the mean body weight of infants from birth to less than one month of age is 4.8 kg, increasing to 5.9 kg from one month of age to less than three months of age and 7.4 kg from three to less than six months of age (EPA. 2011). On a body weight basis, mean and 90th percentile consumption of OPN-10 at less than one month of age would be 24.8 and 39.5 mg/kg BW/day, respectively. While total enery requirements (kJ/day basis) increase with age (higher in boys due to gender weight differences), on a bodyweight basis the energy requirements decrease from a maximum at one month of age, tending to plateau starting at six months of age, and further decreasing as BW increases (Butte, 2005). The energy requirement data indicate that the first few months of infant formula will result in the greatest consumption of OPN-10, on a BW basis.

Table 9. OPN-10: Current intake and predicted intake of OPN-10 following supplementation of milk-derived infant formula at the indicated levels with OPN-10 utilizing NHANES (2011-2012) dataset (CDC, 2015b).

	Per User				
OPN-10 intake from:	**OPN-10 (mg/L infant formula)***	**50th Percentile OPN-10 consumption (mg/infant/day)**	**50th Percentile OPN-10 (mg/kg BW/day)**	**90th Percentile OPN-10 (mg/infant/day)**	**90th Percentile OPN-10 (mg/kg BW/day)**
Current amount of OPN-10 in commercial infant formula	0	0	0	0	0
Possible consumption of OPN-10 as an added ingredient to food at < 1 month of age of the infant	160	119	24.8	189.7	39.5

*Addition of 160 mg OPN-10/L milk-derived infant formula will provide 125 mg OPN/L formula

The World Health Organization (WHO, 2001) recommends exclusive breast feeding for the first six months of life, then partial breast feeding up to two years of age or beyond. Davis *et al.* (2012) conducted a series of surveys concerning breast feeding and the health of infants and toddlers and found that among the Hispanic families surveyed in the US, 36% of the children were breast fed beyond twelve months of age. Toddlers from one to up to three years of age may still consume breast milk or infant formula/complimentary milk-based supplemental beverages as part of the weaning process from infant formula to solid foods, although not as a sole source of nutrients but in decreasing amounts as the amount of solid food consumption increases, and therefore consume OPN during this period, although at levels lower than during the first year of life.

The U.S. dietary guidelines for Americans (2010) recommend that intake of 2 cups of milk and milk products (approximately 475 ml or 16 ounces) by children 2 – 3 years of age (regardless of body weight) is sufficient for adequate nutrient intake, stating that "moderate evidence shows that intake of milk and milk products is linked to improved bone health, especially in children and

https://www.fda.gov/Food/GuidanceRegulation/GuidanceDocumentsRegulatoryInformation/IngredientsAdditivesGRASPackaging/ucm074725.htm; site last visited April 19, 2017.

adolescents." The American Heart Association (AHA) also states in their dietary recommendations scientific position that 2 cups milk/day is appropriate for children ages 1 – 3 (AHA, 2014). In addition, Maguire *et al.* (2013) found that "two cups (500 ml) cow's milk *per* day maintained 25-hydroxyvitamin D > 75 nmol/L with minimal negative effect on serum ferritin for most children." Higher levels of milk consumption could decrease serum ferritin, possibly through replacement of the consumption of iron-containing foods (Maguire *et al.*, 2013).

Average body weights for children 1 - 3 years of age slowly increase over time, as expected. One-year-old boys weigh an average of 10.31 kg while one-year-old girls weigh an average of 9.52 kg (CDC, 2001). Average body weights for 2 - 3 year-olds range from approximately 12.7 – 14.3 kg for boys and 12.1 – 13.9 kg for girls (CDC, 2001). Utilizing the smallest and greatest body weights of the various age groups, one can determine the age groups that would be consuming the greatest and least amount of OPN from formula. On a bodyweight basis, one-year-old female toddlers who continue to consume formula (considered in the industry as follow-on formula at this age) may consume an average of 49.9 ml formula/kg bodyweight,[46] to 29.8 ml formula/kg bodyweight for three-year-old boys (the age group with the greatest bodyweight). This would result in consumption of OPN from formula in toddlers at up to 6.89 mg/kg bodyweight for one-year-old girls[47] and 4 mg/kg bodyweight for three-year-old boys. These levels are over three-fold lower than levels of OPN consumed by an infant (24.8 mg OPN/kg bodyweight), confirming that the infant has the greatest exposure to OPN from OPN-10, on a bodyweight basis.

6.9. Absorption, Distribution, Metabolism and Elimination (ADME)

OPN-10 is composed of > 95% OPN, and therefore information concerning the ADME of OPN is relevant to the discussion of the safety of OPN-10.

Rittling *et al.* (2014) conducted a study to show the effects of OPN on bone health in mice, but only the relevance to distribution will be discussed here (this study did not conform to FDA guidelines for performance of acute toxicity studies). In a single dose study, OPN (provided as OPN-10) was administered to OPN deficient mice ($OPN^{-/-}$) that were three weeks and ten weeks of age ($n = 3$ mice/group, gender not provided) and OPN levels were evaluated in plasma and bone at 0, 1, 4, 8 and 24 hours post-dose (Rittling *et al.*, 2014). The mice were administered 50 mg OPN/mouse orally (approximately 2000 mg/kg BW). OPN was detected in the plasma of both age groups utilizing competitive ELISA analysis (antibodies utilized in this assay were those utilized by Schack *et al.*, (2009), although the time curves for elimination were slightly different. Peak plasma levels of OPN occurred at approximately four hours post-dose (Figure 9). Similar results occurred between the different age groups, and although OPN was not detected in the plasma of 10-week old mice eight hours after feeding, there was a significant amount of OPN in the plasma of the ten-week-old mice twenty-four hours post dosing. A rationale for this variation in OPN metabolism was not provided by the authors. The maximal amount of OPN detected in the plasma of the ten-week-old mice was higher than in the three-week-old mice (6888 ± 2132 ng/ml *vs.* 3092 ± 1387 ng/ml, respectively), but the difference was not statistically significant ($P = 0.351$). This confirms that OPN from OPN-10 is absorbed when given orally (Rittling *et al.*, 2014).

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[46] 475 ml formula/9.52 kg body weight = 49.89 ml/kg

[47] 0.138 mg OPN/ml formula * 49.89 ml formula consumed/kg bodyweight = 6.89 mg OPN/kg bodyweight

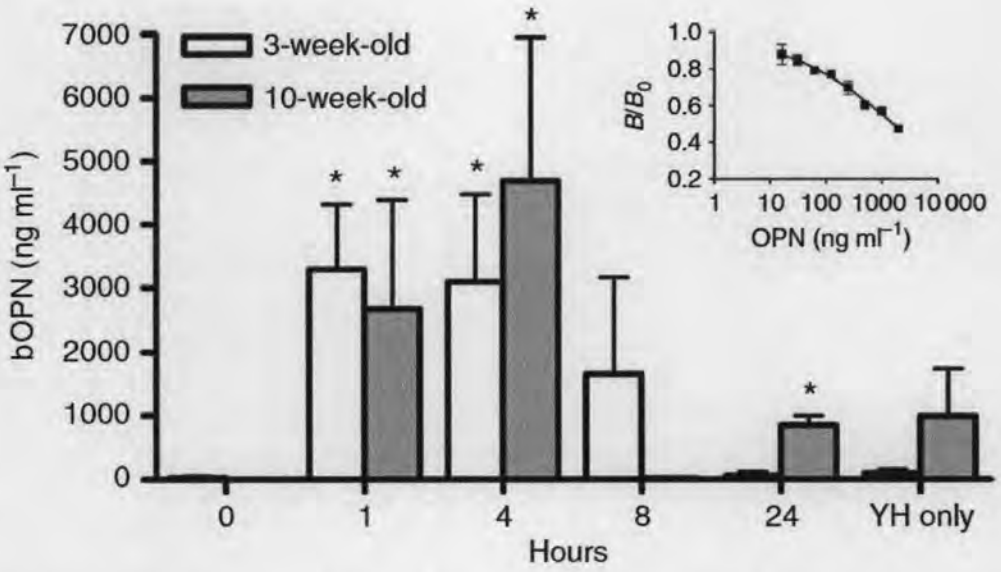

Figure 9. OPN reactivity in plasma of OPN (-/-) mice fed OPN. bOPN, 50mg per mouse, was dissolved in YooHoo chocolate milk (which contains bOPN) and administered orally in a bolus over a period of 15-20 min to OPN deficient mice. Plasma was collected at different times after feeding and assayed for anti-OPN antibody reactivity using a competition ELISA. Data for both 3-week old (open bars) and 10-week old (filled bars) are shown. Inset: representative standard curve using Lacprodan-OPN10 as standard; *n* = 3 *per* group YH=YooHoo. *$P < 0.05$ as compared with time 0, determined by Mann-Whitney test (Rittling *et al.*, 2014).

Chatterton *et al.* (2004) evaluated the effect of *in vitro* digestion on OPN and its stability. For the study, gastric juice from a neonate and aspirates from two infants aged eight days and 28 days at 1 and 3 hours following bolus ingestion of human milk, were collected. An aliquot of the gastric juice (10 µl) was added to 900 µg of mature human milk and incubated, then analyzed by SDS PAGE. OPN resisted digestion in the neonatal gastric juice at pH down to 3.0 (the human OPN antibody recognized the ~78 kDa, 34 kDa and 23 kDa OPN bands). In the same study design, human OPN was resistant to digestion with gastric aspirates from human infants, with intact (~75 kDa size) human milk OPN stable to proteolysis at acid levels greater or equal to pH 4.0, while the smaller fragments of ~34 and ~23 kDa were slightly degraded by the gastric proteases. Bovine whey protein concentrate was also evaluated for digestion of different proteins, with bovine OPN also resisted digestion at pH 4.0, similar to human OPN proteolytic activity. While OPN may be somewhat resistant to stomach acid and proteases (*e.g.*, pepsin), further digestion by intestinal proteases is likely.

Contrary to Chatterton *et al.* (2004), Dall'Asta *et al.* (2015) found that OPN is digested by pepsin and pancreatin into at least 28 different peptides, an average of seven amino acid residues in the permeate with an average molecular weight of 984.4 Da[48] for these peptides, when utilizing an *in vitro* model for infant digestion of milk utilizing a model replicating the digestive tract (*i.e.*,

[48] Dall'Asta *et al* (2015) does not specify the units for the molecular weight of the peptides, but considering that the intact OPN molecule is 33 – 44 kDa (including post-translational modifications), it is assumed that the fragments were measured in the Dalton range.

saliva, gastric juice, duodenal juice and bile juice). Dietary OPN is expected to be degraded by intestinal proteases and the resulting amino acids absorbed, similar to any other dietary protein (Henschel *et al.*, 1987; Tang and Wong, 1987).

OPN-10 is manufactured from whey protein consisting of proteins that are naturally present in cow's milk (> 90% protein matter). Therefore the metabolic fate of the protein OPN-10 found in whey protein following the consumption of products containing the ingredient is expected to be largely the same as that following the consumption from cow's milk or any other food source containing cow's milk-derived protein (*e.g.*, dairy products, powdered milk whey protein products).

6.10. Safety Evaluation

6.10.1. Acute studies

OPN-10 was orally consumed (when added to a Yoo-Hoo milk product vehicle) in a single-dose study to $OPN^{-/-}$ mice (a mixed 129S1/S7 background mouse bred in-house, used at three or ten weeks of age, gender not provided) at three mice/group, with each group killed one, four, eight and 24-hours post dose for plasma analysis of OPN content) at 50 mg/mouse, which is approximately equivalent to 2000 mg/kg BW, assuming a mouse weighs 25 g (Rittling *et al.*, 2014). The plasma levels of OPN significantly increased at one and four hours post-dose, then returned to near pre-dose levels. No adverse effects of OPN consumption under conditions examined were noted by the authors. Although this study was not a safety study but part of an evaluation on absorption and effects on tumor formation in a preclinical model, the study gives some information on safety of OPN-10 when consumed as an acute bolus dose at 2000 mg/kg BW in mice, which is approximately 50-fold greater than the anticipated consumption of 39.5 mg/kg bw/day when added to term infant formula.

6.10.2. Subchronic studies

A 13-week dietary toxicity study with the objective to evaluate the safety of OPN-10 was performed in Wistar rats (six weeks old at study initiation) according to the OECD[49] Guidelines for Testing of Chemicals, Number 408 "Health Effects, Repeated Dose 90-Day Oral Toxicity Study in Rodents (Kvistgaard *et al.*, 2014). Four groups of ten males and females/group received constant levels (adjusted for body weight) of OPN-10 in a standardized diet (Rat & Mouse Breeding Diet, RM3 from SDS Special Diets Services, Witham, England) at concentrations of 0, 0.5, 1, or 2% (providing an approximate constant intake of 0, 0.3, 0.6 and 1.2 g/kg BW, respectively). OPN-10 was homogeneously mixed into the feed and was stable for the duration of the study. All animals were evaluated prior to the start of the study and then again at the study's conclusion for clinical and ophthalmologic changes, as suggested by OECD guidelines for this particular dietary study. Water was provided to the animals *ad libitum*. All animals completed the study and there were no clinical signs or ophthalmologic changes attributed to administration of OPN-10. There were two inconsequential (albeit statistically significant at $P < 0.05$) decreases in food consumption in females (low dose at days 21 – 25 and mid dose at days 46 – 49); no changes were noted in males.

The mean overall (Days 0 – 91) food intake of males provided feed containing 0.5%, 1.0% or 2.0% OPN-10 was 19.9, 19.9, 19.8 and 20.3 g/day, while mean food intake of the corresponding

[49] Organisation for Economic Co-operation and Development

female groups was 12.5, 12.8, 12.3 and 12.8 g/day, respectively. There were no changes in water consumption for either sex (with the exception of decreased water consumption at days 38 - 39 for the mid-dose females). There were no statistical differences in mean BW between any treatment groups compared to control (within the same sex)[50] for the duration of the study (Figure 10 and Figure 11). The mean overall (Days 0 – 91) daily intake of OPN-10 in male rats fed dietary concentrations of 0.5%, 1% and 2% was 301, 596 and 1208 mg/kg BW/day, respectively and, resulted in consumption of 223.0, 441.6 and 895.1 mg OPN/kg BW/day, respectively. For the same dietary concentrations, the mean overall daily intake of OPN-10 in female rats was 322, 637 and 1272 mg/kg BW/day, respectively, which corresponded to 238.6, 472.0 and 942.6 mg OPN/kg BW/day, respectively (Kvistgaard *et al.*, 2014). The highest dose consumed by the rats (1272 mg/kg bw/day OPN-10) is approximately 32-fold greater than the anticipated consumption of 39.5 mg/kg bw/day OPN-10 when added to term infant formula.

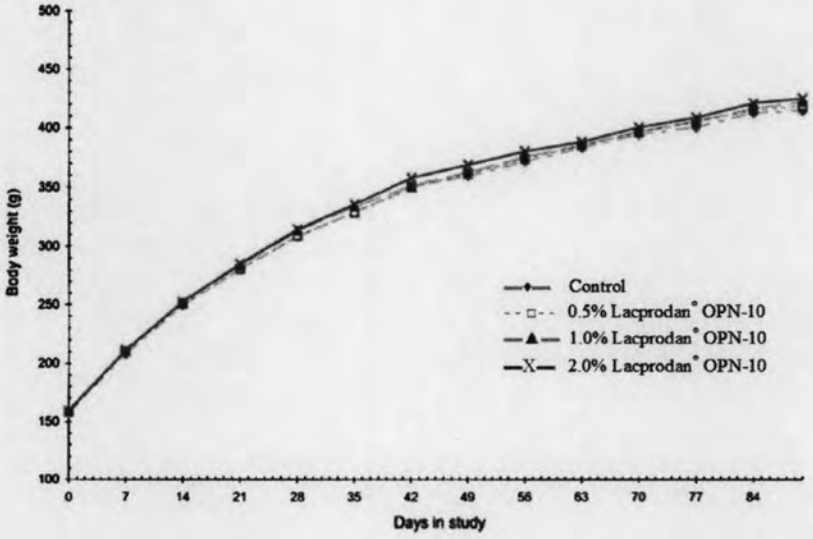

Figure 10. Mean body weights (g) of male rats provided OPN-10 for 13 weeks (Kvistgaard *et al.*, 2014).

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[50] The statistical analysis did not include analysis for differences between the sexes, as the body weights of male and female rats are naturally different, with males being substantially larger than females as the rats age, which would give a false positive effect.

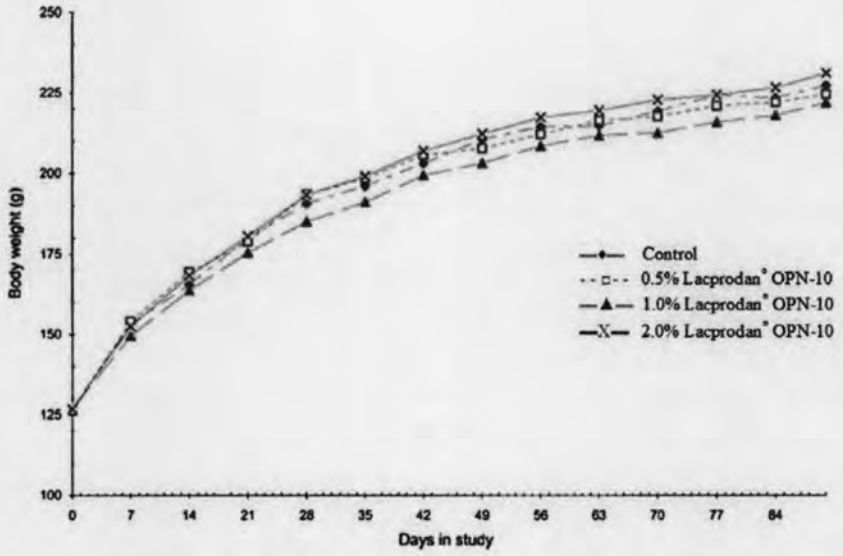

Figure 11. Mean body weights (g) of female rats provided OPN-10 for 13 weeks (Kvistgaard *et al.*, 2014).

At the termination of the study, hematological findings were likewise unremarkable (Table 10), but a statistically significant ($P < 0.05$) increase (compared to control) in mean MCHC was noted for low and mid dose males, but not in the high-dose male group or any of the treated female groups. Because this effect was not dose-dependent and did not correlate with significant changes in MCH or MCV, the effect was not considered toxicologically relevant. No changes were noted in total white count or differential white blood cell counts; nor for percent reticulocytes, thrombocytes or prothrombin time.

Clinical chemistry findings at study termination did not show any statistically significant ($P > 0.05$) changes in either sex at any dose for alkaline phosphatase, alanine aminotransferase, *gamma*-glutamyl transpeptidase, total bilirubin, total protein, albumin, plasma glucose, cholesterol, phospholipids, triglycerides, creatinine, urea, inorganic phosphate, calcium, chloride, potassium or sodium. In females, there was a trend (trend analysis utilizing the Williams test at $P < 0.05$) towards an increase in aspartate aminotransferase (AST) for the mid and high dose groups over control value, but an ANOVA/Dunnett's test did not reveal statistical differences ($P > 0.05$) between the test groups and the control group and the effect was not dose-dependent and was within historical (normal) controls (Table 11). There was no significant change in AST values in the male dose groups. There was also a significant ($P < 0.05$) trend (compared with the control group) towards an increase in the albumin/globulin ratio in all female test groups, this effect was not dose-dependent in nature, was within historical control values, and was not considered toxicologically relevant. There were no changes in males for any of the clinical chemistry parameters.

Analysis of the urine (Table 12) found that there was a statistically significant decrease in urine volume (compared to control value) for the low dose males. Also, for the 0.5% and 1.0% male dose group, there was a statistically significant increase in urine density that corresponded to the decreased urinary volume; no changes were noted in the high-dose (2.0%) male dose group. The urinary density was within known norms for this sex and strain of rat (1.022 - 1.05 kg/L) and

so these minor variations were not considered toxicological in nature. No other difference in urinalysis parameters were observed between treated and control animals, with the exception of a significant ($P < 0.05$) increase in urinary protein in males in the 0.5% OPN-10 dose group. No changes were noted in the urine of treated females, compared to the corresponding control group.

Table 10. Hematological analysis of male and female rats administered OPN-10 for 13 weeks (Kvistgaard *et al.*, 2014).

Nominal dose	Control	0.5% OPN-10	1.0% OPN-10	2.0% OPN-10	Historic Control Ranges (TNO, 2016)#
Dependent variable			**Male**		
Hematology					
RBC (10^{12}/L)	9.265±0.457	9.214±0.337	9.166±0.239	9.3006±0.450	8.85 - 10.0
Hb (mmol/L)	9.68±0.42	9.72±0.34	9.85±0.26	9.79±0.33	9.16 - 10.2
PCV (L/L, x 10^3)	476.6±20.4	473.0±22.4	480.3±15.2	480.5±20.5	447 - 477
MCV (fL)	51.47±1.82	51.34±1.47	52.40±1.00	51.67±1.41	50.3 - 54.8
MCH (fmol)	1.045±0.036	1.055±0.029	1.075±0.030	1.052±0.028	1.01 - 1.14
MCHC (mmol/L)	20.30±0.09	20.56±0.42*	20.53±0.30*	20.37±0.22	19.9 - 20.70
Reticulocytes (%)	2.154±0.251	2.183±0.258	2.333±0.186	2.217±0.207	0.818 - 2.454
Thrombocytes (10^9/L)	1076.2±80.8	1049.4±88.3	1034.4±70.7	1025.2±118.7	525 - 1117
Prothrombin Time (s)	32.92±2.07	35.71±10.92	32.54±2.52	32.35±1.90	32.9 - 40.6
White Blood Cell Counts					
WBC (10^9/L)	11.07±2.13	12.78±2.77	11.41±3.18	11.96±2.80	9.6 - 12.6
Lymphocytes (10^9/L)	9.57±2.05	11.34±2.77	10.03±2.92	10.45±2.64	8.4 - 11.5
Neutrophils (10^9/L)	1.08±0.37	0.94±0.34	0.93±0.21	1.01±0.37	0.7 - 1.2
Eosinophils (10^9/L)	0.130±0.018	0.158±0.033	0.147±0.041	0.167±0.055	0.09 - 0.15
Basophils (10^9/L)	0.095±0.033	0.124±0.056	0.097±0.043	0.107±0.045	0.08 - 0.15
Monocytes (10^9/L)	0.213±0.067	0.230±0.071	0.208±0.061	0.216±0.083	0.03-0.18#
Lymphocytes (%)	86.10±3.93	88.30±4.06	87.61±1.95	87.12±3.34	84.6 - 90.6
Neutrophils (%)	9.92±3.90	7.63±3.82	8.39±1.60	8.82±3.41	5.9 - 10.9
Eosinophils (%)	1.20±0.23	1.25±0.18	1.31±0.28	1.42±0.45	0.9 - 1.5
Basophils (%)	0.84±0.16	0.93±0.20	0.81±0.17	0.86±0.19	0.6 - 1.4
Monocytes (%)	1.92±0.48	1.86±0.70	1.89±0.66	1.80±0.40	0.8-3.8#
			Female		
Hematology					
RBC (10^{12}/L)	8.794±0.338	8.703±0.459	8.544±0.297	8.704±0.404	8.39 - 9.32
Hb (mmol/L)	9.81±0.41	9.62±0.30	9.50±0.30	9.71±0.26	9.09 - 10.06
PCV (L/L, x 10^3)	472.3±23.1	460.6±18.0	455.6±15.4	467.7±12.2	451 - 475
MCV (fL)	53.72±1.83	52.96±1.23	53.35±1.55	53.79±1.70	51.8 - 53.9
MCH (fmol)	1.115±0.039	1.108±0.034	1.113±0.031	1.117±0.039	1.07 - 1.15
MCHC (mmol/L)	20.77±0.26	20.88±0.26	20.86±0.30	20.77±0.29	20.3 - 21.1
Reticulocytes (%)	2.235±0.539	1.971±0.313	1.997±0.317	2.168±0.548	0.866 - 2.525
Thrombocytes (10^9/L)	961.0±77.9	1017.3±94.3	1024±93.1	1030.9±101.9	530 - 1081
Prothrombin Time (s)	34.91±2.22	33.91±1.78	34.43±2.46	34.84±1.01	28.6 - 37.1
White Blood Cell					
WBC (10^9/L)	7.10±1.31	7.96±1.97	7.63±2.27	7.97±2.23	6.2 - 9.0

Table 10. Hematological analysis of male and female rats administered OPN-10 for 13 weeks (Kvistgaard *et al.*, 2014).

Nominal dose	Control	0.5% OPN-10	1.0% OPN-10	2.0% OPN-10	Historic Control Ranges (TNO, 2016)#
Lymphocytes (10^9/L)	6.14±1.01	7.13±1.82	6.69±2.07	7.23±2.11	5.3 - 7.9
Neutrophils (10^9/L)	0.72±0.73	0.55±0.16	0.70±0.34	0.45±0.13	0.5 -0.8
Eosinophils (10^9/L)	0.088±0.038	0.107±0.030	0.079±0.044	0.093±0.032	0.06 - 0.1
Basophils (10^9/L)	0.043±0.013	0.051±0.019	0.060±0.034	0.056±0.029	0.0 - 0.09
Monocytes (10^9/L)	0.114±0.045	0.122±0.036	0.130±0.086	0.131±0.066	0.02-0.16
Lymphocytes (%)	87.08±8.01	89.41±2.42	87.53±4.67	90.48±1.84	84.9 - 90.7
Neutrophils (%)	9.47±7.84	6.95±2.17	9.10±4.72	6.00±1.71	6.3 - 11.6
Eosinophils (%)	1.24±0.58	1.37±0.34	1.01±0.50	1.21±0.44	0.8 - 1.6
Basophils (%)	0.58±0.09	0.63±0.08	0.75±0.24	0.66±0.16	0.6 - 1.4

Number of animals = 10/group. Statistical Analysis: ANOVA/Dunnet's: * = $p < 0.05$; ** = $p < 0.01$; ANOVA = analysis of variance; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCV = Mean corpuscular volume; PCV = Packed cell volume; RBC = Red blood cell count; WBC = Total white blood cell count; #Historic control range obtained from Charles River of the same strain and age of rat (Charles River, 2016).

Table 11. Clinical chemistry analysis of male and female rats administered OPN-10 for 13 weeks (Kvistgaard *et al.*, 2014).

Nominal dose	Control	0.5% OPN-10	1.0% OPN-10	2.0% OPN-10	Historic Control Ranges (TNO, 2016)
Dependent variable					
		Males			
Clinical chemistry					
ALP (U/L)	106.9±30.6	100.5±22.7	108.9±29.8	111.4±20.8	80 - 220
AST (U/L)	70.3±10.0	68.9±9.5	69.0±11.8	67.9±7.4	56 - 79
ALT (U/L)	51.2±7.7	48.5±10.4	48.3±11.7	50.6±9.4	24 - 51
GGT (U/L)	0.81±0.63	0.71±0.61	0.59±0.53	0.82±0.61	0.22 - 1.34
BILI (µmol/L)	2.28±0.19	2.18±0.23	2.28±0.20	2.31±0.17	2.11 - 2.28
Total Protein (g/L)	67.1±1.6	67.4±2.4	67.5±0.7	68.2±2.7	66.7 - 72.4
Albumin (g/L)	35.8±0.8	35.5±0.8	35.6±0.8	35.9±1.4	33.5 - 36.9
Albumin/Globulin	1.146±0.036	1.117±0.070	1.117±0.053	1.113±0.043	0.995 - 1.146
Glucose (mmol/L)	8.411±1.004	9.140±1.310	8.33±0.757	8.615±1.525	7.554 - 10.404
Cholesterol (mmol/L)	1.896±0.227	2.033±0.358	2.024±0.277	2.012±0.323	1.896 - 2.446
Phospholipids (mmol/L)	1.483±0.148	1.549±0.331	1.602±0.165	1.554±0.175	1.476 - 1.768
Triglycerides (mmol/L)	0.980±0.256	1.107±0.736	1.119±0.297	0.990±0.374	0.878 - 1.468
Creatinine (µmol/L)	31.5±1.9	32.5±2.4	33.7±2.5	33.4±2.5	28.2 - 32
Urea (mmol/L)	5.79±0.51	5.98±1.10	6.27±1.12	6.40±0.83	4.53 - 6.38
PO_4 (mmol/L)	2.700±0.153	2.668±0.242	2.538±0.172	2.606±0.157	2.285 - 2.753
Ca (mmol/L)	2.937±0.075	2.938±0.063	2.907±0.057	2.963±0.093	2.863 - 3.119
Cl (mmol/L)	98.6±1.0	98.1±1.3	98.2±1.7	98.6±1.2	97.5 - 99.4
K (mmol/L)	5.53±0.28	5.34±0.48	5.45±0.34	5.49±0.40	5.09 - 5.77
Na (mmol/L)	151.7±0.9	151.5±1.4	151.9±1.4	151.9±1.3	149.8 - 154.3
		Female			
ALP (U/L)	86.0±23.1	77.0±13.5	78.1±16.9	71.1±8.8	56 - 502

Table 11. Clinical chemistry analysis of male and female rats administered OPN-10 for 13 weeks (Kvistgaard *et al.*, 2014).

Nominal dose	Control	0.5% OPN-10	1.0% OPN-10	2.0% OPN-10	Historic Control Ranges (TNO, 2016)
Dependent variable					
AST (U/L)	64.6±15.9	73.1±12.0	79.1±13.1†	74.9±10.9†	60 - 100
ALT (U/L)	45.3±15.8	43.4±12.1	47.3±12.8	47.6±13.2	22 - 65
GGT (U/L)	0.40±0.52	0.75±0.49	0.36±0.47	0.56±0.54	0 - 0.92
BILI (µmol/L)	2.33±0.22	2.33±0.27	2.42±0.36	2.42±0.35	1.96 - 2.72
Total Protein (g/L)	67.6±2.7	67.4±2.1	66.4±0.8	68.0±2.9	65.2 - 72.1
Albumin (g/L)	37.8±1.5	38.4±1.3	38.3±0.9	38.7±1.6	34.8 - 40.1
Albumin/Globulin	1.273±0.074	1.325±0.049†	1.363±0.059†	1.323±0.059†	1.133 - 1.341
Glucose (mmol/L)	7.636±1.394	6.506±0.856	6.633±0.725	7.687±1.727	6.785 - 8.185
Cholesterol (mmol/L)	2.125±0.394	2.249±0.332	1.948±0.301	1.893±0.345	1.505 - 2.199
Phospholipids (mmol/L)	1.867±0.255	1.973±0.218	1.773±0.227	1.818±0.234	1.519 - 2.149
Triglycerides (mmol/L)	0.736±0.217	0.758±0.205	0.690±0.176	0.796±0.207	0.52 - 1.267
Creatinine (µmol/L)	38.7±1.9	37.4±3.6	38.2±1.9	38.1±3.9	31.8 - 42.3
Urea (mmol/L)	6.43±1.15	6.26±0.51	7.00±1.36	6.93±1.13	5.42 - 7.92
PO_4 (mmol/L)	1.878±0.346	1.971±0.348	1.908±0.332	1.964±0.314	1.832 - 2.513
Ca (mmol/L)	2.881±0.048	2.862±0.083	2.850±0.061	2.901±0.083	2.798 - 2.998
Cl (mmol/L)	103.4±2.5	103.2±1.2	102.8±2.3	102.4±2.2	101.6 - 104.5
K (mmol/L)	5.53±0.34	5.64±0.33	5.66±0.28	5.45±0.44	5.04 - 5.9
Na (mmol/L)	151.2±1.9	151.0±1.2	150.8±1.7	150.5±1.5	149.1 - 151.9

Number of animals = 10/group. Statistical Analysis: ANOVA/Dunnet's: * = $p < 0.05$; ** = $p < 0.01$; Trend analysis (William's test): † = $p < 0.05$; ALT = Alanine aminotransferase; ALP = Alkaline phosphatase; ANOVA = analysis of variance; AST = Aspartate aminotransferase; BILI = Bilirubin (total); Ca = Calcium; Cl = Chloride; GGT = *Gamma* glutamyl transferase activity; Hb = Hemoglobin; K = Potassium; Na = Sodium; PO_4 = Inorganic phosphate.

Table 12. Urinalysis of male and female rats administered OPN-10 for 13 weeks (Kvistgaard *et al.*, 2014).

Nominal dose	Control	0.5% OPN-10	1.0% OPN-10	2.0% OPN-10
Dependent variable				
		Male		
Microscopic analysis of the Urine				
RBC (0-5)	0.0	0.0	0.0	0.0
WBC (0-5)	0.0	0.0	0.0	0.0
Epithelial cells (0-5)	1.0	1.0	1.0	1.2
AMOR (0-5)	1.0	1.0	1.0	1.0
Crystals (0-5)	2.0	1.8	1.8	1.7
Casts (0-5)	0.0	0.0	0.0	0.0
Bacteria (0-5)	3.1	3.0	3.1	3.4
Worm eggs (0-1)	0.0	0.0	0.0	0.0
Sperm (0-1)	0.2	0.2	0.5	0.3
Quantitative/Semi-quantitative observations of the urine (16 hr samples) collected on Day 84-85				
Volume (mL)	7.12±1.17	5.20±1.35*	5.62±1.40	6.95±2.11
Density (g/L)	1,0320±6.3	1,047.1±8.7**	1,041.7±7.3*	1,032.2±13.3
pH	6.85	6.80	6.55	6.85

Table 12. Urinalysis of male and female rats administered OPN-10 for 13 weeks (Kvistgaard *et al.*, 2014).

Nominal dose	Control	0.5% OPN-10	1.0% OPN-10	2.0% OPN-10
Dependent variable				
Protein (Range 0-3)	0.5	1.3*	1.0	0.6
Glucose (0-4)	0.0	0.0	0.0	0.0
Ketones (0-3)	0.0	0.0	0.0	0.0
Urobilinogen (0-4)	0.0	0.0	0.0	0.0
Bilirubin (0-3)	0.0	0.0	0.0	0.0
Occult blood (0-4)	0.0	0.0	0.0	0.0
		Female		
Microscopic in the Urine				
RBC (0-5)	0.0	0.0	0.0	0.0
WBC (0-5)	0.0	0.0	0.0	0.0
Epithelial cells (0-5)	1.0	1.0	1.0	1.0
AMOR (0-5)	1.1	1.0	1.0	1.0
Crystals (0-5)	0.3	0.1	0.1	0.3
Casts (0-5)	0.0	0.0	0.0	0.0
Bacteria (0-5)	2.1	2.2	2.0	2.2
Worm eggs (0-1)	0.0	0.0	0.0	0.0
Sperm (0-1)				
Quantitative/Semi-quantitative observations of the urine (16 hr samples) collected on Day 84-85				
Volume (mL)	4.90±1.85	4.30±1.79	4.03±1.35	5.02±0.94
Density (g/L)	1,032.2±011.4	1,036.2±11.9	1,037.2±12.2	1,029.5±6.0
pH	5.90	5.40	5.65	5.90
Protein (Range 0-3)	0.0	0.1	0.0	0.0
Glucose (0-4)	0.0	0.0	0.0	0.0
Ketones (0-3)	0.0	0.0	0.0	0.0
Urobilinogen (0-4)	0.0	0.0	0.0	0.0
Bilirubin (0-3)	0.0	0.0	0.0	0.0
Occult blood (0-4)	0.0	0.0	0.0	0.0

Number of animals = 10/group. Statistical Analysis: ANOVA/Dunnet's: * = $p < 0.05$; ** = $p < 0.01$; Trend analysis (William's test): †= $p < 0.05$; AMOR = amorphous material; ANOVA = analysis of variance; RBC = Red blood cell count; WBC = Total white blood cell count.

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There were no dose dependent effects in absolute or relative organ weights of male rats (Table 13). The absolute and relative kidney weights of the kidneys of females in all treatment groups showed a statistically significant upward trend ($P < 0.05$), but the ANOVA/Dunnett's test was significant ($P < 0.05$) only for the relative kidney weight of the 1.0% dose group and therefore was not a dose-dependent effect (historical control data for kidney weights for this strain of rat ranges from 1.195 – 1.452 g, while the historical control data for the relative kidney weight ranges from 5.82 – 6.36 g/kg BW). The absolute and relative thymus weights showed a statistically significant trend ($P < 0.05$) towards an increase in females provided feed containing 2.0% OPN-10, but an ANOVA/Dunnett's test did not indicate a statistically significant ($P < 0.05$) difference between test groups and the control group (the historical control data for thymus weight for this strain of rat ranges from 0.25 - 0.33 g, while the relative thymus weight ranges from 1.13 – 1.42 g/kg BW).

There were no microscopic findings related to administration of OPN-10 at any dose in males or females. Trends towards decreased urine volume, increased urine density and increased kidney and thymus weights had no histologic correlates and therefore were not considered treatment-related. This 13-week study by Kvistgaard *et al.* (2014) indicates that OPN-10 was tolerated at up to 2% of the diet, corresponding to a NOAEL of 1208 and 1272 mg/kg BW/day in male and female rats, respectively, the highest dose tested, which is approximately 32-fold greater than the anticipated consumption of 39.5 mg/kg bw/day when added to term infant formula. This study showed that no adverse effects were seen when OPN-10 was consumed at levels many times greater than what would be provided to infants, and evaluated numerous different toxicological parameters necessary for the determination of a NOAEL.

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Table 13. Absolute and relative organ weights of rats administered OPN-10 for 13 weeks (Kvistgaard *et al.*, 2014).

	Control	0.5% OPN-10	1.0% OPN-10	2.0% OPN-10
		Males		
Body weight (g)	392.76±29.14	396.77±23.66	399.27±23.05	403.01±33.67
Brain (g)	1.893±0.190	1.917±0.082	1.919±0.080	1.969±0.035
Brain (g/kg BW)	4.858±0.680	4.846±0.343	4.817±0.269	4.913±0.379
Heart (g)	1.233±0.120	1.238±0.054	1.277±0.097	1.324±0.114
Heart (g/kg BW)	3.160±0.401	3.129±0.217	3.200±0.196	3.289±0.194
Liver (g)	10.576±1.059	10.270±1.116	10.638±0.916	11.013±1.447
Liver (g/kg BW)	26.93±1.92	25.84±1.84	26.63±1.34	27.26±2.13
Thymus (g)	0.4133±0.1631	0.4182±0.0551	0.4076±0.0750	0.4499±0.0967
Thymus (g/kg BW)	1.074±0.507	1.057±0.145	1.025±0.193	1.115±0.216
Kidneys (g)	2.214±0.172	2.215±0.156	2.203±0.167	2.303±0.221
Kidneys (g/kg BW)	5.649±0.411	5.587±0.321	5.519±0.279	5.717±0.327
Adrenals (g)	0.0444±0.066	0.0447±0.0094	0.0439±0.0060	0.0447.0098
Adrenals (g/kg BW)	0.1145±0.0234	0.1138±0.0288	0.1099±0.0137	0.1113±0.0240
Spleen (g)	0.7090±0.0593	0.7597±0.0881	0.7328±0.0546	0.7659±0.0808
Spleen (g/kg BW)	1.807±0.110	1.915±0.220	1.837±0.152	1.899±0.096
Testes (g)	3.276±0.461	3.089±0.865	3.335±0.433	3.276±0.391
Testes (g/kg BW)	8.428±1.546	7.846±2.275	8.358±1.052	8.191±1.224
Epididymides (g)	1.433±0.132	1.292±0.210	1.322±0.188	1.407±0.151
Epididymides (g/kg BW)	3.673±0.496	3.275±0.605	3.324±0.507	3.506±0.396
		Females		
Body weight (g)	212.48±13.71	209.72±11.73	207.17±14.72	214.77±13.81
Brain (g)	1.757±0.041	1.744±0.042	1.750±0.073	1.750±0.067
Brain (g/kg BW)	8.302±0.619	8.334±0.375	8.437±0.517	8.173±0.482
Heart (g)	0.751±0.041	0.765±0.049	0.785±0.080	0.790±0.058
Heart (g/kg BW)	3.542±0.236	3.652±0.222	3.790±0.280	3.684±0.278
Liver (g)	4.807±0.418	4.964±0.388	4.884±0.469	5.003±0.373
Liver (g/kg BW)	22.67±2.14	23.67±1.57	23.60±1.76	23.37±2.21
Thymus (g)	0.2584±0.0341	0.2726±0.0378	0.2640±0.0442	0.2995±0.0435†
Thymus (g/kg BW	1.217±0.129	1.300±0.172	1.275±0.191	1.400±0.232†
Kidneys (g)	1.265±0.068	1.327±0.090†	1.348±0.061†	1.342±0.071†
Kidneys (g/kg BW)	5.963±0.276	6.341±0.475†	6.523±0.323†	6.265±0.444†
Adrenals (g)	0.0545±0.0065	0.0561±0.0059	0.0581±0.0065	0.0596±0.0088
Adrenals (g/kg BW)	0.2573±0.0333	0.2689±0.0376	0.2813±0.0331	0.2785±0.0457
Spleen (g)	0.4575±0.1029	0.4502±0.0418	0.4371±0.0557	0.4353±0.0458
Spleen (g/kg BW)	2.143±0.374	2.143±0.126	2.103±0.153	2.033±0.263
Ovaries (g)	0.0696±0.0108	0.0699±0.0115	0.0718±0.0093	0.0762±0.0147
Ovaries (g/kg BW)	0.3291±0.0586	0.3325±0.0439	0.3476±0.0449	0.3585±0.0845
Uterus (g)	0.7699±0.3083	0.5679±0.1868	0.6458±0.1998	0.6492±0.2884
Uterus (g/kg BW)	3.597±1.352	2.707±0.847	3.101±0.841	3.035±1.381

Number of animals = 10/group; data are presented as mean ±SD; †= $P < 0.05$, Trend analysis (William's test)

A three-month feeding study was conducted in newborn rhesus monkeys to investigate the effect of consumption of OPN-10[51] on infant growth, bone mineral density (BMD) and immune function, compared to infant formula and breast fed rhesus monkey infants (Donovan *et al.*, 2014). Although not designed as a safety study, this study provides information on intake of OPN-10 in nonhuman primates, evaluating growth and development parameters consistent with human newborns. The newborn monkeys were obtained at birth from a breeding colony at the California Regional Primate Research Center (University of California, Davis). The infants were divided into three groups: Group 1 (control) consumed commercially available infant formula[52] ($n = 6$); Group 2 consumed commercial infant formula that was supplemented with OPN-10 (providing 125 mg OPN/L milk; $n = 6$); and Group 3 was breast fed (breast fed controls; $n = 4$).[53] The infants were maintained in the study for three months. No solid food was given during the study period. The control formula was a commercially available whey-based formula containing 5.3 g fat, 1.9 g protein and 11 g carbohydrate/100 kcal.

Blood samples were obtained at birth and every month and at months 1, 2, and 3 of the study. Hemoglobin, hematocrit and white blood cell differential counts were quantified and plasma urea nitrogen was determined. Growth was determined *via* body weight measurements at birth, 1, 2, and 3 month time points. Crown-rump length was measured at birth and at two months' of age. Body composition and bone density were measured by a DEXA[54] scan, and fat mass (FM), fat-free mass (FFM), and bone mineral density (BMD; g/cm^2) were also determined, at three months of age. At study termination, the infants were euthanized and jejunal samples collected and immediately frozen in liquid nitrogen for later mRNA extraction for a non-toxicological evaluation of mRNA expression in exfoliated intestinal epithelial cells, as previous work suggested that mRNA expression in these cells are different between three-month-old infants that have been formula-fed compared to breast fed infants (Donovan, 2006). As the mRNA expression analysis was not evaluating the safety of the OPN-10 when consumed, the data will not be further discussed.

There were no significant differences in body weight, crown-rump length or BMD between the monkeys fed formula and those fed formula with added OPN-10 (Table 14). The suckling young of most mammalian species consumes milk energy at approximately 225 kcal $BW_{kg}^{0.83}$ (Oftedal, 1984); therefore, the Rhesus monkeys in this study consumed approximately equivalent amounts of OPN-10 (on a bodyweight basis) that would have been consumed by human infants on the same formula. The breast fed monkeys had lower lean body mass ($P < 0.05$) than the OPN-10 and formula-fed (FF) monkeys, which did not differ from one another (Table 14); the authors stated that the decrease in lean body mass is similar to analysis of human infants (Gale *et al.*, 2012)()()(). Mean red blood cell volume (MCV), red blood cell count (RBC), hemoglobin and hematocrit levels did not differ between groups (Table 15). The BF monkeys had a higher percentage of neutrophils than the other two groups ($P < 0.05$), but also a lower percentage of lymphocytes ($P < 0.05$); the apparent differences of these values were not of clinical significance, as between the FF and OPN-10-fed monkeys, there were no differences in the total number of

[51] Also termed bOPN in this publication.

[52] The authors stated that the composition of rhesus milk is similar to that of human milk, and therefore standard infant formulas can be used for infant monkeys until 4 – 5 months of age without modification (Donovan *et al.*, 2014).

[53] Two infants were excluded from the study: one due to illness and one due to maternal illness.

[54] Dual-energy x-ray absorptiometry.

WBC or differential WBC counts.[55] In addition, these types of percentage differences in WBC are consistent with previous findings that formula feeding may alter immune cell composition, compared with breast feeding (Andersson *et al.*, 2009).

Overall, this study shows that the addition of OPN-10 to commercial formula, when compared to infants consuming control infant formula, did not adversely affect growth and body composition in Rhesus monkey infants when consumed for twelve weeks, with no adverse effects in overall growth, hematological parameters and, lean and fat body mass endpoints. The authors conclude that "taken together, the addition of bovine OPN to formula did not produce any adverse effects on onverall neonatal growth or body composition" and that "the lack of difference between the FF and the bovine OPN infants further supports the safety of OPN in neonates" (Donovan *et al.*, 2014).

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[55] Immune challenges or allergenicity studies were not a part of the study protocol.

Table 14. Body weight, crown-rump length and body composition of 3-month-old Rhesus monkeys fed mother's milk or formula with or without 125 mg/l bOPN[1,2] (Donovan *et al.*, 2014).

	FF	FF+OPN	BF
Body Weight (kg)			
Birth	0.53±0.09	0.51±0.06	0.59±0.22
1 month	0.73±0.11	0.68±0.08	0.70±0.28
2 month	0.92±0.09	0.92±0.11	0.82±0.32
3 month	1.29±0.28	1.05±0.13	1.07±0.25
Crown-Rump Length (cm)			
Birth	184±14.0	190±7.8	198±16.3
1 month	221±7.4	215±11.9	214±12.0
2 month	243±15.8	234±15.7	230±11.5
Body Composition at 3 months			
Body Fat (g)	3.1±2.2	2.4±1.6	1.2±1.2
Lean Body Mass (g)	30.1±5.2[a]	29.8±4.7[a]	22.6±2.3[b]
Bone Mineral Density (g/cm^2)	0.19±0.02	0.20±0.01	0.20±0.03

[1] All values are mean ± S.D.; [2]Different letter superscript within row indicate statistical differences at P≤0.05; BF = breast fed; FF = formula-fed; OPN = formula + 125mg bOPN/L obtained from OPN-10; bOPN=bovine-sourced osteopontin.

Table 15. Hematology and differential white blood cell count in 3-mos-old Rhesus monkeys fed mother's milk or formula with or without 125 mg bOPN/L (Donovan *et al.*, 2014)[1,2]

	FF	FF+OPN	BF
RBC (x10^6/µl)	5.2±0.2	5.5±0.4	4.9±0.7
Hemoglobin (g/dl)	12.2±0.6	13.0±0.8	12.2±1.3
Hematocrit (%)	38.4±1.3	41.4±3.8	36.8±4.1
Mean Cell Volume (MCV) (fl)	73.6±2.6	74.2±2.5	75.5±4.9
Platelets (x10^5/µl)	3.3±1.3	3.4±2.6	5.4±1.8
WBC (x10^3/µl)	9.6±3.3	9.5±3.2	9.0±3.1
Segmented Neutrophils (%)	50.2±17.5[a]	35.2±23.3[a]	60.5±6.1[b]
Lymphocytes (%)	47.4±16.6[b]	60.2±21.6[b]	38±7.0[a]
Monocytes (%)	0.6±1.3	2.7±2.0	1.5±1.5
Eosinophils (%)	1.8±1.9	1.0±0.6	1.5±0

[1]All values are mean ± S.D.; [2]Different letter superscript within a row indicate statistical differences at $P \leq 0.05$; BF = breast fed (n = 4); FF = formula-fed (n = 6); OPN; formula + 125mg bOPN/L (n = 6) obtained from OPN-10; RBC = red blood cells; WBC = white blood cells; bOPN = bovine-sourced osteopontin

6.11. Other Studies

A teratogenicity study was conducted to evaluate the effects of OPN-10 on reproduction of pregnant rats or on embryonic or fetal development (Kvistgaard *et al.*, 2014). Healthy three-month old Wistar rats were obtained from the Beijing Vitalriver Laboratory Animal Technology Co. Ltd, and fed diets specific for the rats' nutrition and breeding was purchased from the breeding farm of the Experimental Animal Research Institute Chinese Academy of Medical Science (Beijing, China); food and water was provided *ad libitum*. Male and female rats were mated and the day a vaginal plug was detected was determined as Day 0 of pregnancy. Upon mating, the female rats were housed singly and randomly assigned to be administered 0, 630, 1250 or 2500 mg/kg BW/day OPN-10 (n = 15/group) *via* gavage from Days 7 – 16 of gestation. Not all female

rats that were mated became pregnant; the control and high-dose group contained 13 pregnant dams, while the low- and mid-dose OPN-treated rats each contained 12 dams/group.[56]

The rats were anesthetized, bled and killed on Day 20 of gestation. Feed and water intake was not quantified. The numbers of implantations, live fetuses, dead fetuses, and absorbed fetuses were recorded. The fetuses were examined for abnormalities (*i.e.*, general condition, head, spine, chest, abdomen, tail, limbs and toe development) and weighed and measured. One-half of the fetuses were dissected to permit visceral examination of the brain, cerebrum, ventricles, diencephalons, tongue, cleft palate, nose, eyeballs, maxilla, medulla oblongata, spinal cord, and all organs of the respiratory, digestive and urogenital systems. The other half of the fetuses were examined for the following effects on bone: ossification retardation, lack of skeletal integration,[57] bifurcation, increase or decrease in number of ribs, arrangement disorder or shape abnormality of the skull, cervical, thoracic, lumbar or sacral vertebrae, pelvis, limbs, sternum, ribs or pelvis.

OPN-10 had no adverse effects on reproduction of the pregnant rats or on embryonic or fetal development. There was no effect of OPN-10 on dam body weight at any time point examined ($P > 0.05$) and the pregnant rats in all groups grew well during the test period. There was no effect of the consumption of OPN-10 on the pregnancy rate, number of live fetuses, number of fetuses *per* litter, numbers of absorptions or dead fetuses, fetal loss rate, or fetal body weight or length ($P > 0.05$; Table 16) for any dose group in this rat study. No gross or visceral abnormalities occurred in any group (Table 17). Incomplete ossification of the skull and malformations of the sternum (*i.e.*, dotted shape of number 1, 3, and 4 sternums and missing or dotted shape of number 6 sternum) were observed in control and treated animals. However there were no significant differences ($P > 0.05$) in the number and types of skeletal abnormalities between groups (Table 17). No adverse effects occurred when OPN-10 was administered to pregnant Wistar rats during Days 7 – 16 of pregnancy at up to 2500 mg/kg BW/day. The authors concluded that "under the conditions of this study, the NOAEL for teratogenicity of Lacprodan® OPN-10 was 2.50 g Lacprodan® OPN-10/kg bw (1.85 g OPN/kg bw), the highest dose evaluated" (Kvistgaard *et al.*, 2014). The NOAEL of 2500 mg OPN-10/kg bw/day found in this study may be utilized in the determination of the Acceptable Daily Intake (ADI).

Table 16. Effect of OPN-10 on maternal weight, embryonic survival and fetal growth (Kvistgaard *et al.*, 2014).

	Control	0.63 g/kg BW OPN-10	1.25 g/kg BW OPN-10	2.50 g/kg BW OPN-10
Mated females at initiation	15	15	15	15
Terminal body weight (g)[1]	388.9±49.6	353.9±54.5	363.9±66.2	394.5±36.8
Pregnant females at termination	13	12	12	13
Pregnant females at termination	86.7	80.0	80.0	86.7
Live fetuses	158	132	135	171
Live fetuses per litter[1]	12.2±5.1	11.0±4.9	11.3±5.4	13.2±4.5
Absorptions	4	6	4	4
Dead fetuses	0	0	0	0
Fetal loss rate (%)[2]	2.5	4.4	2.9	2.3
Live fetal weight (g)[1]	3.78±0.43	3.70±0.59	3.80±0.40	3.89±0.64

[56] Authors personal communication, 2016 (Kvistgaard, 2016).
[57] Meant as a lack of skeletal connectivity.

Live fetal length (cm)[1]	3.67±0.36	3.60±0.15	3.69±0.41	3.69±0.30

[1]Data are presented as mean ± standard deviation, analyzed using a chi-squared test (PEMS softwar) or ANOVA (SPSS software; IBM, Armon, New York, USA);; [2] Numbers of absorptions/numbers of live fetuses plus absorptions.

Table 17. Effect of OPN-10 on external, visceral and skeletal development

Treatment Group	Control	0.63 g/kg BW OPN-10	1.25 g/kg BW OPN-10	2.50 g/kg BW OPN-10
	External alterations/fetuses examined			
Head	0/158	0/132	0/135	0/171
Vertebral column	0/158	0/132	0/135	0/171
Chest and abdomen	0/158	0/132	0/135	0/171
Tail	0/158	0/132	0/135	0/171
Limbs and toes	0/158	0/132	0/135	0/171
	Visceral alterations/fetuses examined			
Head	0/76	0/65	0/65	0/81
Esophagus, trachea, medulla	0/76	0/65	0/65	0/81
Chest cavity	0/76	0/65	0/65	0/81
Abdominal cavity	0/76	0/65	0/65	0/81
	Skeletal alterations/fetuses examined			
Abnormal fetuses (%)	15/82 (18.3%)	16/67 (23.9)	18/70 (25.7)	20/90 (22.2%)
Skull abnormalities (%)	5/82 (6.1%)	5/67 (7.5%)	5/70 (7.1%)	11/90 (12.2%)
Sternum abnormalities (%)	11/82 (13.4%)	13/67(19.4%)	15/70 (21.4%)	10/90 (11.1%)
Abnormalities of other bones (%)	0/82 (0%)	0/67 (0%)	0/70 (0%)	0/90 (0%)

[1]Data are presented as mean ± standard deviation, analyzed using a chi-squared test (PEMS softwar) or ANOVA (SPSS software; IBM, Armon, New York, USA); 05; [2] Numbers of absorptions/numbers of live fetuses plus absorptions.

An assessment of gastrointestinal function and resistance to necrotizing enterocolitis (NEC)[58] was conducted with infant formula enriched with OPN and compared to a control formula or bovine colostrum in caesarean-delivered preterm pigs (Møller *et al.*, 2011). The piglets (Large White X Danish Landrace) were administered total parenteral nutrition (TPN) through an arterial catheter within six hours of delivery and continued for 48 hours.[59] During the TPN period, the control piglets (n = 13) were administered enteral doses of sterile deionized water and the OPN group (n = 13) was administered enteral doses of 5 ml/kg BW/three hours of pure OPN diluted in sterile deionized water (2 g/L). Following the TPN period, the control piglets were manually administered oral boluses of standard milk formula (15 ml/kg BW) every three hours, while the second group of piglets was administered oral boluses of enteral milk infant formula enriched with OPN (2.2 g/L OPN). The enteral milk formulas for the control diets were prepared from

[58] Potential clinical signs of NEC were cited as: feeding intolerance, abdominal distension, diarrhea and respiratory distress. When clinical symptoms of NEC were observed in a litter prior to the 1.5 day *in vivo* portion, the pigs were anesthetized, killed, the gastrointestinal tract removed and the small intestine, colon and stomach were weighed empty and, each small intestine segment, stomach and colon were photographed and given a macroscopic pathologic lesion score on a scale from 1 to 6.

[59] Maternal colostrum protects against NEC in the small intestine in preterm pigs, while infant formula may lead to digestive and immune dysfunction and NEC lesions (Møller *et al.*, 2011).

commercially available infant formulas[60] and adjusted to match the energy and protein concentrations found in porcine milk during lactation (Table 18) (Møller *et al.*, 2011).

Table 18. Composition of enteral control formula and enteral formula with added OPN (*per* liter formula) (Møller *et al.*, 2011).

	Control	OPN
Energy (kJ)	4051.85	4099.89
Protein (g)	63.20	61.77
OPN (g)	0.0	2.22
Polycose (g)	44.25	44.88
Dextrose (g)	8.78	8.40
Lactose (g)	0.0	0.02
Protein-bound carbohydrate (g)	0.0	0.24
Total fat (g)	60.15	62.29
Saturated fat (g)	43.94	46.06
Monunsaturated fat (g)	9.21	9.12
Polyunsaturated fat (g)	3.44	3.43

Supplementation of the adjusted commercial infant formula with OPN did not significantly reduce the incidence of NEC (54% and 77% in the OPN and control groups, respectively; $P > 0.05$) although the incidence of NEC in the OPN-administered group was nonsignificantly reduced compared to the control group, while the mean NEC severity score was significantly reduced in the OPN group ($P < 0.01$). There were no significant differences in intestinal dimensions, mucosal proportions, small intestinal villus height or organ weights between groups. The activity of measured enzymes (lactase, sucrase, maltase, dipeptidase A) did not differ between OPN- and control-treated pigs. The study authors concluded that "OPN supplementation was associated with a modest decrease in NEC without any consistent effects on gut structural and functional indices" (Møller *et al.*, 2011).

6.12. Genotoxicity studies

OPN-10 has been evaluated for the potential to produce mutagenic and genotoxic effects in both *in vitro* and *in vivo* systems.

6.12.1. Reverse bacterial mutation assay

The ability of OPN-10 to cause mutations was evaluated in the reverse bacterial mutation assay conducted according to Organization for Economic Co-operation and Development (OECD) protocol and under good laboratory practice (GLP) conditions (Kvistgaard *et al.*, 2014). The ability of OPN-10 to potentially induce mutagenicity in *Salmonella typhimurium* tester strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* WP2 uvr A strain was assessed in the presence and absence of the S9 metabolic activation system mix (Ames *et al.*, 1973).

Based on pre-experiments to evaluate cytotoxicity, 5000 µg/plate OPN (5750 µg/plate OPN-10) was selected as the highest dose for all test strains and conditions. Five lower concentrations (2500, 1000, 316, 100, 31.6, 10.0 and 3.16 µg/plate)[61] were prepared by serially diluting the stock solution with distilled water. Distilled water was the negative control, while the

[60] Pepdite, maxipro, Liquigen; SHS International, Liverpool, UK.

[61] OECD guideline 471 recommended maximum test concentration is 5000 µg/plate, with at least five different concentrations used that are approximately half log intervals between test points for an initial experiment (OECD, 1997a)

positive controls in the absence of S9 mix were (1) 4-nitro-o-phenylene-diamine (4-NOPD) for *S. typhimurium* TA98 and TA1537; (2) sodium azide (NaN_3) for *S. typhimurium* TA100 and TA1535 and; (3) methylmethanesulfonate (MMS) for *E. coli* WP2 uvr A. The positive control in the presence of S9 mix for all strains of bacteria was 2-aminoanthracene (2-AA). Samples of each tested strain were grown in appropriate agar to the late exponential or early stationary phase, then 100 µl bacterial suspension was incubated with either the S9 mix (for testing with metabolic activation) or S9 mix substitution mix, test article formulation or the positive or negative controls. Sterile top agar containing L-histidine and biotin for *S. typhimurium* and tryptophan for *E. coli* was then added to each tube, mixed and poured onto minimal glucose agar plates and incubated at 37 °C for 48 hours.

The plates were evaluated for test article toxicity, as indicated by a clearing or diminution of the background lawn or a reduction in the number of revertants down to a mutation factor of approximately ≤ 0.5 (relative to the solvent control). The results of the bacterial reverse mutation assays are presented in Table 19. The average numbers of revertant colonies *per* triplicate test were similar to the revertant colony averages for the negative (vehicle) controls, for each of the tester strains of *S. typhimurium* and the *E. coli* tester strains of bacteria. In both the plate incorporation and the preincubation experiments, none of the strains incubated with OPN-10 (either with or without metabolic (S9) activation) had biologically relevant or dose-dependent numbers of revertant colonies that were two-fold (or three-fold, depending on the strain) or greater than that of the vehicle control values. The positive control chemicals resulted in revertant colony formation that was several-fold greater than vehicle control values. It was concluded that OPN-10 was not mutagenic in this study at up to 5000 µg/plate. It was concluded from this study that, under the conditions of this study, no observable adverse effects occurred at up to 5000 µg/plate, the highest concentration evaluated.

Table 19. Results of the bacterial reverse mutation assay with OPN-10 (Kvistgaard *et al.*, 2014).

Test substance	Dose (µg/plate)	Average number of revertant colonies/plate ($n = 3$)									
		TA98		TA100		TA1535		TA1537		WP2uvrA	
OPN-10 (+S9)		Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II
	0	29	34	130	107	8	8	7	5	61	59
	31.6	36	34	110	115	9	11	6	7	49	49
	100	35	26	108	127	8	11	7	7	71	55
	316	40	30	123	103	10	9	7	9	80	63
	1000	33	27	118	104	9	12	7	8	61	60
	2500	36	34	115	107	7	9	10	6	66	51
	5000	30	34	140	110	11	13	6	11	61	64
Positive controls											
2-AA (+S9)	2.5	2558	2138	2248	1318	96	107	339	172	240	127
OPN-10 (-S9)		Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II	Expt. I	Expt. II
	0	20	18	91	85	5	13	5	5	59	47
	31.6	30	18	93	98	8	14	6	7	51	45
	100	22	16	92	88	7	13	9	6	54	42
	316	24	22	93	95	4	12	6	7	51	51
	1000	21	22	102	113	5	18	5	6	56	47
	2500	34	16	85	103	6	12	7	8	59	55
	5000	25	17	91	103	4	11	4	9	64	57

Table 19. Results of the bacterial reverse mutation assay with OPN-10 (Kvistgaard *et al.*, 2014).

Test substance	Dose (µg/plate)	Average number of revertant colonies/plate ($n = 3$)									
		TA98		TA100		TA1535		TA1537		WP2uvrA	
Positive controls											
4-NOPD (-S9)	10	323	364								
NaN_3 (-S9)	10			725	573	540	1248				
4-NOPD (-S9)	40							70	58		
MMS	10									452	560

2-AA = 2-aminoanthracene; NaN_3 = sodium azide; 4-NOPD = 4-nitro-o-phenylene-diamine; MMS = methylmethanesulfonate; OPN-10 = Lacprodan® OPN-10; Expt. I = Experiment 1 (Plate incorporation test); Expt. II = Experiment II (Pre-incubation test).

6.12.2. Chromosome aberration assay

OPN-10 was evaluated for the potential to produce structural chromosomal aberrations, both in the presence and absence of S9 metabolic activation (Kvistgaard *et al.*, 2014). The assay, conducted according to OECD guidelines,[62] used human peripheral blood lymphocytes in which the lymphocytes were treated with OPN-10 and then prepared 24 hours later. The treatment interval was four hours (with and without metabolic activation) for the first experiment (Experiment I) and four hours (with metabolic activation) and 24 hours (without metabolic activation) for the second experiment (Experiment II). Slides of the cultures were then prepared with Giemsa solution and 100 metaphases *per* culture were scored for structural chromosomal aberrations.

The concentrations of OPN-10 used in the first study (based on preliminary cytotoxicity assays) were 500, 1000, 2000, 3000, 4000 and 5000 µg/ml OPN, both with and without S9 metabolic activation. Concentrations used for the second experiment (without metabolic activation) were 500, 1000, 2000, 3000, 3500, 4000, 4500 and 5000 µg/ml OPN and (with metabolic activation) 300, 600, 1250, 2500 and 5000 µg/ml OPN. Microscopic analysis was conducted on 1000, 3000 and 5000 µg/ml concentrations without metabolic activation and 1250, 2500 and 5000 µg/ml OPN concentrations with metabolic activation (*i.e.*, four hour treatment, 24 hour incubation period for Experiment II). Analysis of at least 200 well-spread metaphase cells was performed and structural chromosomal aberrations, breaks, fragments, deletions, exchanges and chromosomal disintegrations were recorded. Gaps[63] were recorded but not included in the aberration rate calculations.

In the first experiment (with and without metabolic activation) the aberration rates of the negative control and all OPN-10 dose groups (1000, 3000 and 5000 µg/ml OPN concentrations) were similar (Table 20) and within historical control range for the facility (data not shown). In the second experiment, the aberration rates of the OPN-10 dose groups (with and without metabolic activation) were again within historical control range for the facility and similar to the negative control. No biologically relevant decrease of the relative mitotic index (defined as a greater than 70% decrease) was noted in any of the dose groups evaluated. The criteria for determining a positive result (a concentration-related or reproducible increase in the number of cells with chromosome aberrations for at least one of the dose groups and is higher than the historical range for the *percent* aberrant cells in the negative control group) was never reached for the OPN-10

[62] OECD No. 473: *In vitro* Mammalian Chromosome Aberration Test, adopted 21st July, 1997 (OECD, 1997b).
[63] Defined as an achromatic region occurring in one or both chromatids independent of its width.

concentrations.[64] The positive controls ethylmethanesulfonate (EMS) and cyclophosphamide (CPA) induced distinct and biologically relevant increases in cells with structural chromosomal aberrations. The authors concluded that treatment with OPN-10 did not induce chromosomal aberrations under the conditions of the study (Kvistgaard *et al.*, 2014).

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[64] Statistical analysis was not conducted, as it is not required under OECD guidelines for this study.

Table 20. Chromosome aberration assay results for OPN-10 (Kvistgaard *et al.*, 2014).

Exposure	Concentration (µg/ml)	Number of structural aberrant cells/200 examined				Mean % Aberrant cells Excluding Gaps	Mitotic Index (relative %)
		Chromatid		Chromosome			
		Breaks	Exchanges	Breaks	Exchanges		
Experiment I (-S9)							
Negative control	0	1	0	0	0	1.0	100
OPN-10	1000	3	0	0	0	1.5	78
OPN-10	3000	2	0	0	0	1.5	70
OPN-10	5000	4	0	0	0	2.0	79
Positive control (EMS)	400/600*	10	6	0	0	8.5	64
Experiment I (+S9)							
Negative control	0	0	0	0	0	0.5	100
OPN-10	1000	0	0	0	0	0.0	79
OPN-10	3000	2	0	0	0	1.0	100
OPN-10	5000	1	0	0	0	0.5	85
Positive control (CPA)	5.0	16	6	1	0	13.0	80
Experiment II (-S9)							
Negative control	0	0	1	0	0	0.5	100
OPN-10	1000	1	1	0	0	1.5	102
OPN-10	3000	2	0	0	0	1.0	130
OPN-10	5000	2	0	0	0	1.0	107
Positive control(EMS)	400/600*	25	11	0	1	14.0	106
Experiment II (+S9)							
Negative control	0	0	0	0	0	0.0	100
OPN-10	1250	3	0	0	0	1.5	100
OPN-10	2500	2	0	0	0	1.5	95
OPN-10	5000	1	0	0	0	0.5	96
Positive control (CPA)	5.0	24	5	1	0	11.0	89

*Corresponds to Lots (b) (4) and (b) (4), respectively; CPA = cyclophosphamide; EMS = ethylmethanesulfonate; OPN-10 = Lacprodan® OPN-10

6.12.3. Mouse Micronucleus study

The *in vivo* micronucleus assay[65] was used to detect chromosomal or mitotic apparatus damage to the erythroblasts obtained from the bone marrow of mice that consumed OPN-10 (Kvistgaard *et al.*, 2014). The assay was conducted using male and female Naval Medical Research Institute (NMRI) mice,[66] with groups of mice (n = 5/sex/group) administered one dose of OPN-10 (2300 mg/kg BW, to provide 2000 mg/kg BW OPN) *via* gavage. Negative control mice (n = 5/sex) were administered saline *via* gavage. The positive control (CPA) was injected into the peritoneal cavity of a separate group of mice (n = 5/sex) at 40 mg/kg BW 24 hours prior to obtaining bone marrow. Sampling of the bone marrow cells was carried out 24 and 48 hours after treatment with OPN-10. The mouse bone marrow was washed and mounted on a slide and stained with May-Grunwald/Giemsa stain prior to microscopic examination. Immature erythrocytes (2000) were scored *per* animal for the incidence of micronucleated immature erythrocytes. Cytotoxicity of the test item was assessed by evaluating the ratio between immature (polychromatic) erythrocytes and mature erythrocytes. At least 200 erythrocytes were counted *per* animal and the result was expressed as the proportion of immature, polychromatic erythrocytes among total erythrocytes, termed the relative polychromatic erythrocyte (PCE) count.

The relative PCE (*i.e.*, the proportion of PCE among total erythrocytes) was determined for each animal in all treatment groups (OPN-10, positive and negative controls). The relative PCE values for the negative controls (both 24 and 48-hour experiments; Figure 12 and Figure 13) were within the historical control range of the laboratory for negative control values (historical control range of relative PCE for male NMRI mice are 0.49 – 0.79 and for female mice are 0.46 – 0.77). The mice administered OPN-10 for 48 hours showed mean relative PCE values of 0.54 (males) and 0.70 (females) which were also within the range of historical negative control values and was not significantly different ($P > 0.05$) from the control group. OPN-10 did not induce micronuclei formation in the bone marrow PCE and was not cytotoxic to the developing cells.

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[65] Conducted according to OECD guidelines for the testing of chemicals, No. 474: Mammalian Erythrocyte Micronucleus Test, adopted July 21, 1997 (OECD, 1997c).

[66] The NMRI mouse strain is typically utilized by the preclinical contract laboratory (BSL, Germany) that conducted the assay, and have as per OECD guidelines, historical control data to validate the use of this mouse model in the micronucleus assay.

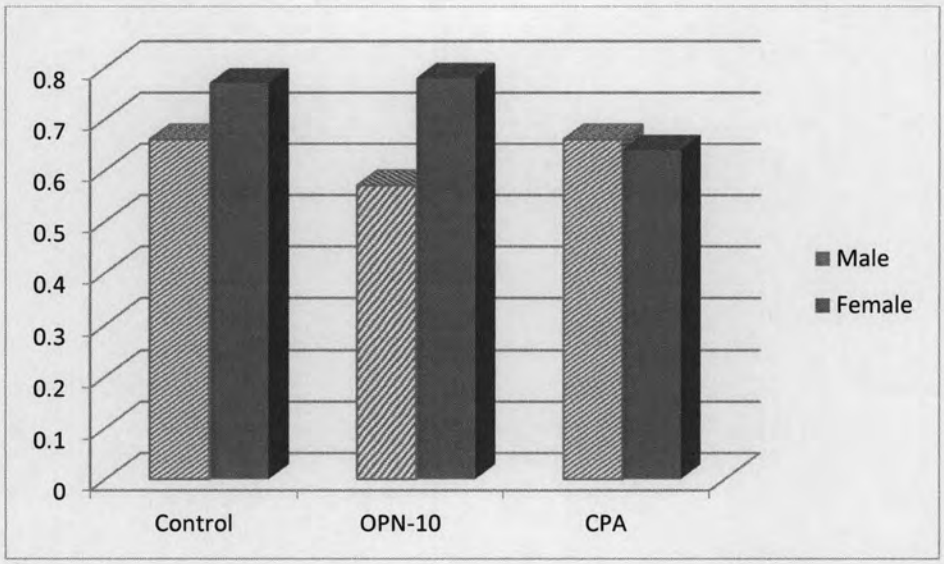

Figure 12. No apparent change in mean relative PCE after 24 hours treatment with Lacprodan® OPN-10 (OPN-10) or cyclophosphamide (CPA); $P > 0.05$ (Kvistgaard *et al.*, 2014).

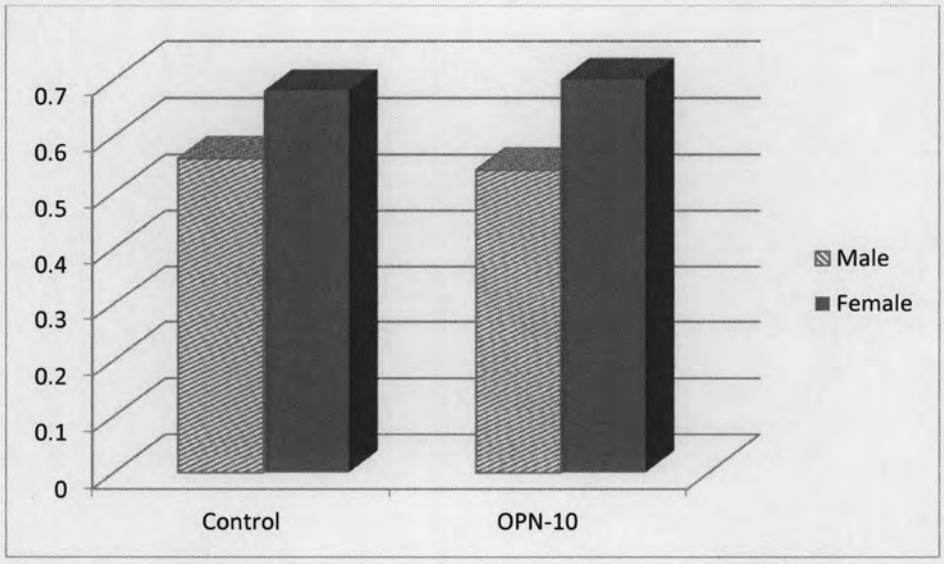

Figure 13. No apparent change in mean relative PCE after 48 hours treatment with OPN-10; $P > 0.05$ (Kvistgaard *et al.*, 2014).

The incidence of micronucleated immature erythrocytes (MNE) was evaluated for all dose groups by scoring 2000 PCE *per* animal. MNE for negative controls were within the range of historical negative controls (males: 0.04 – 0.27; females: 0.04 – 0.29) except for the male mice after 48 hours treatment with vehicle. The increase (0.02%) was not considered biologically or statistically significant by the authors. OPN-10 administration for 24 and 48 hours resulted in mean

MNE values in both male and female mice that were not significantly different ($P > 0.05$) from negative control values (Figure 14 and Figure 15). The CPA treatment induced a statistically significant ($P < 0.05$) increase in micronucleus frequency (3.12% for males and 2.93% for females). OPN-10 at a dose that is approximately 70-fold higher than to be consumed by infants did not induce structural and/or numerical chromosomal damage in the immature erythrocytes in the mouse, and therefore was stated by the authors to be "nonmutagenic with respect to clastogenicity and/or aneugenicity in this assay" (Kvistgaard *et al.*, 2014).

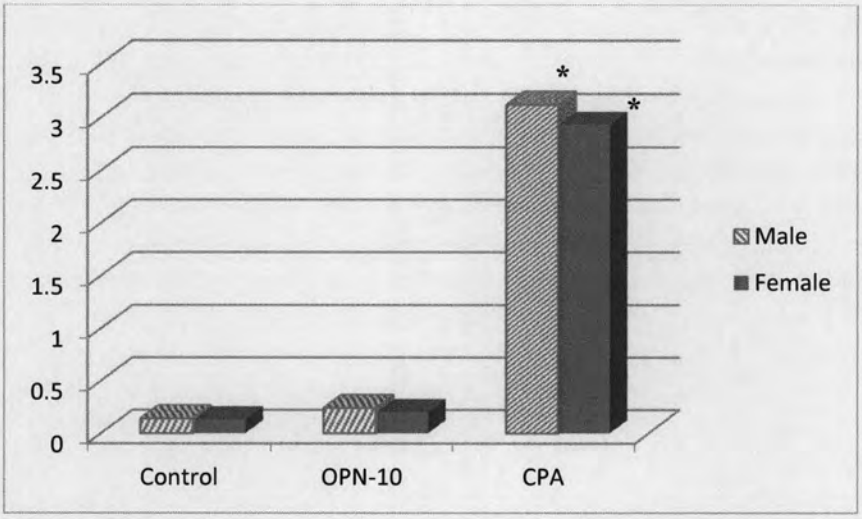

Figure 14. No apparent change in micronucleated RBCs (%) after 24-hour treatment with OPN-10 compared to control, significant increase with cyclophosphamide (CPA); *$P < 0.05$ (Kvistgaard *et al.*, 2014).

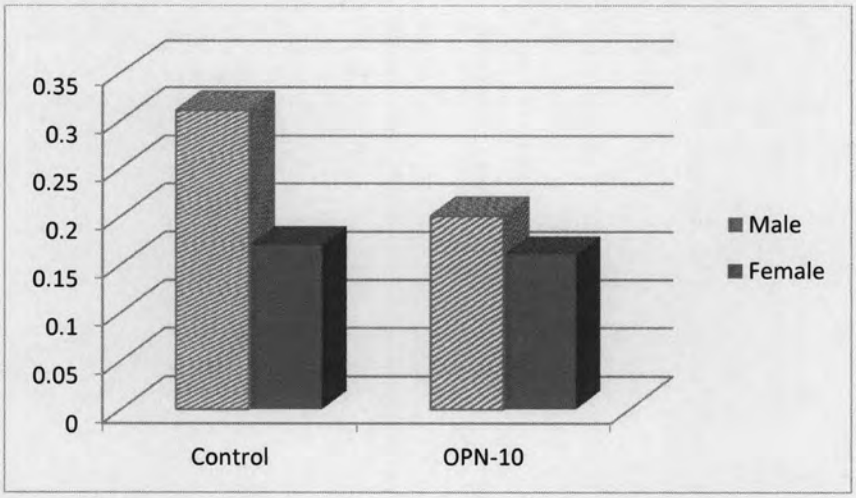

Figure 15. No apparent change in micronucleated RBCs (%) after 48-hour treatment with OPN-10 in mice; $P > 0.05$ between groups (Kvistgaard *et al.*, 2014).

6.13. Carcinogenesis and Inflammation

OPN has been investigated in association with malignant and inflammatory diseases (Rittling and Novick, 1997; Li *et al.*, 2012; Boyerinas *et al.*, 2013; Hsieh *et al.*, 2013; Kumar *et al.*, 2013). The role of OPN is complex and depends on the experimental conditions. In cellular experiments, OPN may facilitate the formation of metastasis in pre-existing tumors, with pre- and post-translational modification and alternative splicing heavily influencing OPN function (Anborgh *et al.*, 2011). Being a cytokine-like protein, produced by a scenario of immune-regulation cells, the role of OPN is most likely that of a regulator of the inflammatory conditions in the tumor microenvironment. Animal studies indicate that tumor growth is reduced in OPN-deficient mice compared with wild type animals (Hayashi *et al.*, 1994), while other recent research indicates that certain variants of OPN positively influence the pathophysiology of cancer (Courter *et al.*, 2010; Shevde and Samant, 2014).

Rittling *et al.* (2014) administered bovine milk-derived OPN (from OPN-10) *via* drinking water to tumor-bearing, immune-competent B6129SF1 mice and evaluated the development of subcutaneous tumor formation and growth. The tumors were induced using ras-transformed mouse embryo fibroblasts injected subcutaneously into the right flank, one site *per* mouse. Five days after fibroblast injection, the mice were administered water containing OPN-10 at 0.3 mg/ml (300 mg/L)[67] for 21 days (approximately equivalent to 75 mg/kg bw/day in the mice). OPN-10 administration (containing approximately 24% full-length OPN) significantly ($P < 0.05$)[68] inhibited tumor growth, with an average decrease in tumor size of about 50% on Day 15 of the study (Figure 16). This study showed that orally administered bovine-derived OPN-10 did not induce tumor formation or increase the growth rate of tumors formed from ras-transformed mouse embryo fibroblasts (Rittling *et al.*, 2014).

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[67] The maximum concentration identified in human milk (Schack *et al.*, 2009).

[68] Significance was determined with T-tests or one-way ANOVA to compare treated and control samples, except for plasma OPN concentrations (Mann-Whitney test was used)

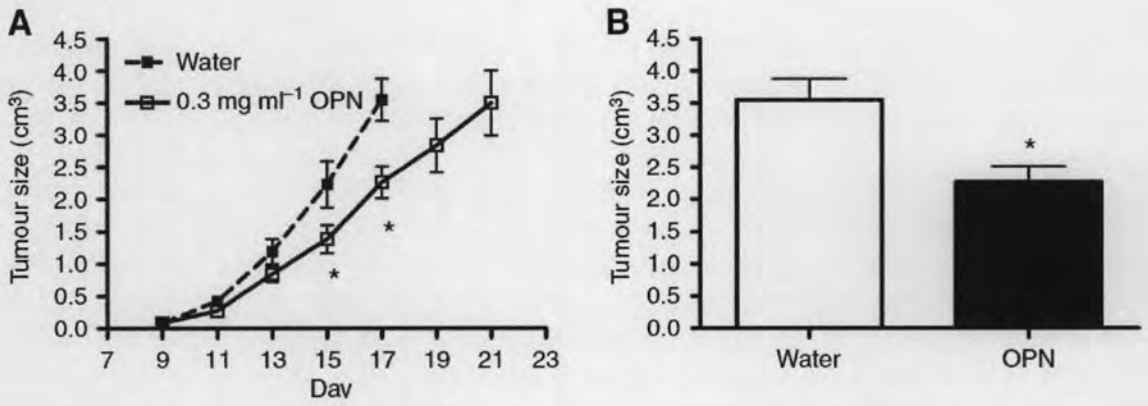

Figure 16. Effect of orally administered bovine OPN on growth of tumor cells (Rittling *et al.*, 2014)**. A. Tumor size in control and OPN-fed mice. B. Mean (± SEM[69]) tumor size on control and OPN-fed tumors on Day 15. *Significantly different at $P < 0.05$.**

An analysis of the concentrations of OPN found in plasma in animal and human carcinogenicity studies, compared with levels of OPN found in human milk (138 µg/ml) and bovine milk (18 µg/ml) show that OPN levels are several magnitudes greater than any levels found in several different cancer studies (ranging from 0.030 – 0.606 µg/ml) (Table 21). The concentration of OPN in human breast milk is 100 – 1000 times higher than the highest concentrations measured in cancer studies. This underscores the fact that mammals would not have conserved a high OPN content in their milk through generations if milk-derived OPN initiated or promoted cancer formation.

Table 21. Concentrations of OPN measured in different experimental studies.

Location	OPN Concentration	Reference
Human milk	138 µg/ml	Schack *et al.* (2009)
Bovine milk	18 µg/ml	Schack *et al.* (2009)
Adult's plasma	0.035 µg/ml	Schack *et al.* (2009)
3-month-old-infant plasma	0.342 µg/ml	Schack *et al.* (2009)
Umbilical cords	0.263 µg/ml	Schack *et al.* (2009)
Breast metastasis	0.142 µg/ml (Control 0.047 µg/ml)	Singhal *et al.* (1997)
Breast metastasis	0.177 µg/ml	Bramwell *et al.* (2006)
Prostate cancer	0.198 µg/ml (No control)	Hotte *et al.* (2002)
Head and neck cancer	0.490 µg/ml (No control)	Petrik (2006)
Renal cancer	0.100-0.126 µg/ml (Control28 µg/ml)	Ramankulov *et al.* (2007)
Squamous carcinoma (skin)	0.606 µg/ml	Shimada *et al.*, (2005)
Glioblastoma	0.030 µg/ml (Control 0.018 µg/ml)	Sreekanthreddy (2010)

As discussed in Section 2 above, OPN obtained from milk goes through post-translational modifications that differ from OPN secreted by inflammatory or other cells throughout the body. Many studies have evaluated the potential role of OPN formed in tissues other than mammary

[69] SEM = Standard error of the mean.

glands on the modulation of a variety of different cellular processes, including bone mineralization and remodeling, tumor progression and inflammatory responses (Sodek *et al.*, 2000). In general, OPN released in the body is a multifunctional extracellular protein that is expressed during the tissue remodeling process in a variety of different cell types and conditions (Subramani *et al.*, 2015). The OPN gene has been found to be upregulated in bronchoalveolar lavage (BAL)-obtained cells from subjects dose-dependently exposed to ozone, with a dose-dependent increase in OPN protein production, indicating a role of OPN during pulmonary inflammation by ozone (Leroy *et al.*, 2015). A study analyzing the characteristics of the OPN secreted in human airways found that airway-secreted OPN undergoes extensive post-translational modification by polymerization and proteolytic fragmentation and, is more fragmented and less polymerized in people with mild or moderate asthma (Arjomandi *et al.*, 2011).

Akelma *et al.* (2014) found that serum OPN levels increase in school-age children with asthma (median age of 61 – 85 months), compared to the control group of the same age profile ($P < 0.004$), which also correlated with higher eosinophil counts, although the neutrophil count, interleukin levels (IL-6, IL-10, IL-13 and IL-17), TGF-B and hsCRP levels were comparable ($P > 0.05$). This study is in agreement with the consensus that OPN is expressed during inflammatory processes related to Th-2-mediated diseases, which includes asthma, although the exact role in this process is still under debate (Akelma *et al.*, 2014). The authors concluded that OPN from inflammatory cells may be regarded as a marker, which increases in Th2-mediated inflammatory diseases (Akelma *et al.*, 2014). This type of inflammatory process is not seen in children that consume bovine-derived OPN. Conversely, OPN expression was not increased during peak-pollen season in grass pollen sensitized allergic rhinitis (AR) subjects during peak season, compared to both nonsensitized control subjects or to AR subjects treated with the intranasal glucocorticocoid fluticasone propionate (O'Neil *et al.*, 2010). OPN consumption by infants *via* breast milk does not increase markers of inflammatory reactions.

Other research has found that OPN decreased cartilage cellular inflammatory reactions induced by lipopolysaccharide, potentially associated with the ERK1/2 signal pathway (Li *et al.*, 2015). Further studies have provided additional evidence that the degree of phosphorylation of OPN that occurs in different cellular conditions greatly influences the OPN function (Christensen *et al.*, 2007; 2008). In general, OPN may be considered a good marker for processes that require tissue remodeling and immunomodulary cellular responses (Denhardt *et al.*, 2001; Subramani *et al.*, 2015).

Altogether, the available data provide no evidence that OPN derived from milk has a possible role in induction of cancer and inflammation in humans (Rittling and Chambers, 2004), but splice variants of OPN (*i.e.*, OPN-c which is not present in milk) may be associated with progression of cancer or an increase in inflammation only through the proliferative effect of different variants of the OPN protein, and not an initiation/promotion effect, as studies stated above show that OPN is not a mutagenic or clastogenic substance in validated, regulatory-compliant studies. Splice variants of OPN occur during the generation of the protein from mRNA, and post-translational changes to the bovine-derived OPN in OPN-10 is extremely unlikely to produce effects similar to the splice variants secreted by other cells in the body.

6.14. Observations in Humans Orally Administered OPN-10

A double-blinded, randomized infant trial was conducted in China to evaluate the effects of the addition of OPN-10, when added to commercial infant formula, on infant growth, nutritional status, health, immune function and cytokine expression over a six-month period (Lönnerdal *et al.*, 2016). Breast fed infants (BF) were evaluated as a reference group, and formula-fed infants (FF) were fed either control formula (BiosTime Premium[70]) or the same formula containing OPN-10 at 65 mg/L (F65) or 130 mg/L (F130). The composition of the control formula is provided in Table 22. The three different formulas were manufactured by the manufacturer (Laiterie de Montaigu, France[71]) and analyzed to confirm the target concentrations for all regulated ingredients and OPN.

The inclusion criteria for the subjects were that the infants were less than one month of age at the start of the study, gestational age at birth of 37 – 42 weeks, birth weight of greater than 2500 g, and absence of chronic illness. The infants must have been exclusively under formula-feeding or breast feeding at inclusion for the respective groups and, the caregivers also had to agree to exclusively breast feed or formula feed (as for the appropriate group) until the infant was six months of age. The study was approved by the Institutional Review Boards at the University of California, Davis (UCD) and Fudan University, Shanghai, as the in-life portion of the trial was conducted solely in China and the samples were analyzed in the U.S. The study was registered as NCT00970398 under ClinicalTrials.gov at the US National Institutes of Health (NIH). The formula-fed infants were stratified for sex and randomized to receive one of the three study formulas from 1 – 6 months of age. The intervention was blinded for both staff and parents/caregivers until the infants completed the study.

Table 22. Nutrient composition of the standard infant formula (F0) (Lönnerdal *et al.*, 2016)

	Target Value		FDA Nutrient Requirements	
Composition	***Per* 100 g powder**	***Per* 100 kcal**	***Minimum***	***Maximum***
Energy (Kcal/kJ)	500/2093		#	#
Protein (g)	11.00	2.20	1.8 (per 100 kcal)	4.5 (*per* 100 kcal)
Whey proteins (g)	7.7	1.5	#	#
Casein(g)	3.3	0.7	#	#
β-lactalbumin (g)	1.9	0.4	#	#
Fat (g)	25.0	5.0	3.3	6.0
Linoleic acid (mg)	3700	740.3	300	#
α-linolenic acid (mg)	450	90.0	#	#
Linoleic acid/α-linolenic acid ratio	8.22	8.22	#	#
DHA (mg)	50	10.0	#	#
ARA (mg)	80	16.0	#	#
% DHA / Total fatty acids	0.21	0.21	#	#
% ARA / Total fatty acids	0.33	0.33	#	#
1,3-Dioleoyl 2-Palmitoyl Triglyceride (g)	4.75		#	#
Carbohydrate (g)	56.7	11.3	#	#
GOS (g)	2.0	0.4	#	#
Nucleotides (mg)	22.0	4.4	#	#
Vitamin A (μgRE)	550	110	250 (IU) (75 μg)	750 (IU) (225 μg)
Vitamin. D (μg)	10.6	2.1	40 IU (1 μg)	100 IU (2.5 μg)

[70] (Mentions légales, 2017)
[71] (Biostime, 2015)

Table 22. Nutrient composition of the standard infant formula (F0) (Lönnerdal *et al.*, 2016)

Composition	Target Value		FDA Nutrient Requirements	
	Per 100 g powder	*Per* 100 kcal	*Minimum*	*Maximum*
Vitamin E (alfa-TE) (mg)	7.0	1.4	0.7 IU (0.47 mg)	#
Vitamin K (μg)	45.0	9.0	4.0	#
Vitamin B1 (μg)	900.0	180.1	40	#
Vitamin B2 (μg)	1002.0	200.5	60	#
Vitamin B6 (μg)	600.0	120.0	35	#
Vitamin B12 (μg)	1.65	0.3	0.15	#
Vitamin C (mg)	100	20.0	8	#
L-carnitine(mg)	9.0	1.8	#	#
Niacin (μg)	4100.0	820.3	250	#
Pantothenic acid (μg)	3500	700	300	#
Folic acid (μg)	120	24.0	4	#
Biotin (μg)	20	4.0	1.5	#
Taurine (mg)	40	8.0	#	#
Choline (mg)	100	20.0	7	#
Inositol (mg)	40	8.0	4	#
Calcium (mg)	351	70.2	60	#
Potassium (mg)	686	137.3	80	200
Phosphorus (mg)	200	40.0	30	#
Calcium/Phosphorus	1.76	1.76	#	#
Iron (mg)	5.0	1.0	0.15	3.0
Zinc (mg)	3.8	0.8	0.5	#
Copper (μg)	400.0	80.0	60	#
Iodine (μg)	120.0	24.0	5	75
Sodium (mg)	150.0	30.0	20	60
Chloride (mg)	331.0	66.2	55	150
Manganese (μg)	176.0	35.2	5	#
Selenium (μg)	15	3.0	2.0	7.0

ARA = arachidonic acid; DHA = Docosahexaenoic acid; GOS = galacto-oligosaccharide; #FDA has no specification for this nutrient/ratio in infant formula (21CFR§107.100)

To assess formula intake and intake of any complementary foods, the parents completed a three-day food diary every month starting at inclusion and ending when the infants were six-months of age. The volume of each formula meal and the volume or weight of any complementary food was also measured. Body weight, length and head circumference were measured at birth and monthly, and the parents completed a daily symptom diary throughout the study, providing information on stool consistency and frequency, any disease symptoms, medication administered or hospitalization. The diaries were collected at each visit. All adverse events were monitored by the study clinician, and dedicated nurses were appointed by the study team, who was responsible for co-ordination of visits, monitoring and counseling on adverse events, collecting care-giver recording of study-related events, and other duties as necessary. The in-life portion of the study was conducted in China; therefore, the Chinese immunization schedule was followed. The published Chinese immunization schedule (Hu *et al.*, 2013) is provided in Table 23.

Table 23. Recommended childhood primary immunization schedule in China (Hu *et al.*, 2013).

	Age							
	Birth	1 m	2 m	3 m	4 m	5 m	6 m	8 m
BCG	Dose 1							
HepB	Dose 1	Dose 2					Dose 3	
OPV			Dose 1	Dose 2	Dose 3			
DPT				Dose 1	Dose 2	Dose 3		
MRV								Dose 1
JEV								Dose 1

BCG = Bacillus Calmette-Guérin; DPT = diphtheria, pertussis and tetanus; HepB = Hepatitis B; JEV = Japanese encephalitis vaccine; MRV = mixed respiratory vaccine; OPV = Oral polio vaccine

Blood samples were obtained (*via* venipuncture) at 1, 4 and 6 months of age. Fresh blood was analyzed for red blood cell (RBC) concentration, serum ferritin and for C-reactive protein (CRP). Plasma was obtained and analyzed for: amino acids,[72] blood urea nitrogen (BUN), cytokine levels,[73] insulin, ferritin and tetanus-specific antibodies. The plasma was stored at -80°C prior to analysis.

Statistical analysis was conducted utilizing SAS for Windows (Cary, NC), with descriptive analysis calculated for all variables. Continuous variables were compared among groups at each time point (*via* ANCOVA[74]), while binary variables were compared with logistic regression and the same covariates. Pairwise comparisons utilized the Tukey-Kramer adjustment. For all procedures, tests were conducted to compare all four groups to each other, or to compare the three formula fed (FF) groups, or the BF group to the combined formula-fed groups. Statistical significance was $P < 0.05$.

Infants were recruited for the study from 2009 through 2013, with 240 FF infants (120 boys and 120 girls) and 80 BF infants (40 boys and 40 girls) recruited from the area surrounding Fudan University, Shanghai, China. There were no significant differences at the start of the study among the groups for birth weight, length or head circumference (Table 24). From the initiation of treatment (one month of age), dropout rates were 2, 10, 11 and 23% in the F0, F65, F130 and BF groups, respectively, with no significant differences among FF groups (Table 25). Insufficient milk was the most common reason for dropouts in the BF group. There were no significant differences in formula intake among the control and F65 and F130 FF groups. When comparing formula intake between formula-consuming groups, intake in the F130 FF group was significantly greater than the F65 FF group (Table 24). Hours sleeping did not differ between the FF groups, although it was significantly less in the BF group (repeated measures; $P < 0.05$).

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[72] Threonine, serine, valine, leucine, glycine, isoleucine, phenylalanine, tryptophan, arginine, histidine, proline, cystine, tyrosine, alanine, taurine, aspartic acid, glutamic acid, hydroxyproline, glutamine, methionine, lysine and asparagine.

[73] Cytokines analyzed: IL-2, IL-6, IL-8, IL-10, IL-12, IL-15 and TNF-α.

[74] Analysis of covariance, a general linear model which blends ANOVA and regression.

Table 24. Head circumference, formula intake, and sleep time (Lönnerdal *et al.*, 2016).

Variable	F0	F65	F130	BF	*P*-value, all groups	*P*-value, formula *vs* breast fed	*P*-value, all formula groups
Head circumference (cm)							
1 mo	37.0±1.24	36.7±1.24	37.1±1.07	37.1±1.01	0.6154	0.3340	0.6423
2 mo	38.7±1.35	38.5±1.12	38.7±1.10	38.9±1.13	0.7826	0.4803	0.7489
3 mo	40.1±1.48	39.9±1.22	40.2±1.25	40.3±1.10	0.8203	0.4644	0.8205
4 mo	41.3±1.50	41.1±1.29	41.4±1.23	41.6±1.16	0.5045	0.2421	0.6041
5 mo	42.3±1.44	42.1±1.24	42.3±1.21	42.7±1.12	0.1831	0.0444	0.6503
6 mo	43.2±1.53	42.8±1.25	43.3±1.27	43.6±1.16	0.0981	0.0303	0.4183
Formula intake (mL/d)							
1 mo	795 ^{a}±164	787 ^{a}±152	838 ^{a}±159				0.1388
2 mo	877 ^{a}±139	834 ^{a}±142	867 ^{a}±140				0.1252
3 mo	878 ^{a}±143	843 ^{a}±113	857 ^{a}±128				0.2997
4 mo	864 ^{a}±141	858 ^{a}±126	862 ^{a}±154				0.9880
5 mo	896 ^{a}±157	833 ^{b}±112	872 ^{ab}±122				0.0218
6 mo	824 ^{ab}±127	792 ^{b}±126	849 ^{a}±126				0.0333
Sleep time (h)							
1 mo	17.7±2.25	17.7±2.10	17.4±2.43	17.4±1.90	0.6290	0.3332	0.6588
2 mo	16.6±1.95	16.1±2.10	16.1±2.32	15.7±1.83	0.1237	0.0614	0.3122
3 mo	15.6±1.86	15.2±1.75	15.4±1.91	15.0±1.69	0.2364	0.1044	0.4468
4 mo	14.7 ^{a}±1.72	14.6 ^{ab}±1.30	14.7 ^{a}±1.52	14.0 ^{b}±1.22	0.0170	0.0016	0.9382
5 mo	14.1±1.36	14±1.40	14.0±1.22	13.7±1.22	0.3816	0.0960	0.8729
6 mo	13.7±1.02	13.6±1.31	13.4±0.99	13.4±1.03	0.1996	0.1512	0.2742

BF = Breast fed; F0 = standard infant formula; F65 = formula with 65 mg/L OPN; F130 = formula with 130 mg/L OPN; (Radhakrishna *et al.*, 2010; Jones *et al.*, 2014); Different letter superscripts indicate $P<0.05$.

Table 25. Drop-out rates of infants during study (Lönnerdal *et al.*, 2016).

	F0	F65	F130	BF
Infants/group at study initiation ($n=$)	$n = 67$	$n = 67$	$n = 70$	$n = 75$
Drop-outs				
Follow-up: 1 month	$n = 0$	$n = 0$	$n = 0$	$n = 5$
Follow-up: 2 months	$n = 0$	$n = 1$	$n = 1$	$n = 6$
Follow-up: 3 months	$n = 0$	$n = 3$	$n = 6$	$n = 9$
Follow-up: 4 months	$n = 0$	$n = 3$	$n = 6$	$n = 13$
Follow-up: 5 months	$n = 1$	$n = 6$	$n = 7$	$n = 13$
Follow-up: 6 months	$n = 1$	$n = 6$	$n = 7$	$n = 14$
Infants completing all 1-6 months	$n = 66$	$n = 61$	$n = 63$	$n = 61$
Drop-out rate	2%	10%	11%	23%

Weight, length, head circumference measurements and Z-scores did not differ among the FF groups, although the BF infants had increased weight from 2 – 6 months of age and greater head circumference at five and six months of age (Table 26). The weight-for-height (WHZ) scores

were significantly increased in the FF groups fed OPN-10 ($P < 0.05$), but were not different from the higher WHZ score of the BF group, showing that consumption of OPN-10 increased the WHZ to levels comparable with a score obtained in the BF group.

Table 26. Body weight and height and z-scores (Lönnerdal *et al.*, 2016).

Variable	F0	F65	F130	BF	*P*-value, all groups	*P*-value, formula *vs* breast fed	*P*-value, all formula groups
Weight (g)							
Birth	3277±372	3210±399	3339±338	3329±394			
1 mo	4519±456	4531±475	4640±449	4639±495	0.5537	0.7088	0.3762
2 mo	5612±515	5591±512	5690±511	5844±574	0.1030	0.0140	0.9374
3 mo	6511±613	6485±561	6635±622	6770±689	0.2115	0.0462	0.7531
4 mo	7206±718	7158±687	7365±697	7555±743	0.0827	0.0197	0.5283
5 mo	7748±758	7732±680	7897±776	8154±802	0.0771	0.0133	0.7018
6 mo	8242±839	8186±723	8417±832	8600±857	0.1481	0.0468	0.4944
Length (cm)							
Birth	49.88±0.85	49.75±0.98	49.77±0.71	49.81±1.01			
1 mo	54.3±1.57	54.2±1.32	54.5±1.43	54.6±1.66	0.8491	0.4297	0.9161
2 mo	58.0±1.91	57.8±1.48	58.0±1.69	58.5±1.87	0.2790	0.0848	0.6616
3 mo	61.2±2.10	61.2±1.68	61.5±1.75	61.7±1.66	0.7769	0.3977	0.8256
4 mo	63.9±2.23	63.5±1.86	64.1±1.96	64.4±1.80	0.4943	0.2160	0.6382
5 mo	66.1±2.23	65.7±1.76	66.1±1.88	66.3±1.76	0.8218	0.6604	0.6940
6 mo	68.2±2.36	67.7±1.81	68.2±2.02	68.3±1.94	0.7800	0.6375	0.6435
WAZ							
1 mo	0.17±0.71	0.16±0.76	0.34±0.74	0.26±0.81	0.6655	0.8056	0.4706
2 mo	0.38±0.65	0.35±0.69	0.48±0.67	0.62±0.80	0.1548	0.0290	0.7955
3 mo	0.55±0.70	0.47±0.67	0.65±0.73	0.78±0.83	0.3487	0.0797	0.8854
4 mo	0.61±0.76	0.52±0.77	0.75±0.74	0.93±0.82	0.0948	0.0212	0.5754
5 mo	0.63±0.78	0.60±0.72	0.76±0.81	1.01±0.82	0.0755	0.0115	0.7724
6 mo	0.68±0.81	0.62±0.75	0.85±0.80	1.00±0.84	0.1906	0.0620	0.5202
HAZ							
1 mo	-0.09±0.82	-0.14±0.68	-0.01±0.75	-0.04±0.88	0.9761	0.7731	0.9399
2 mo	0.18±0.93	0.07±0.72	0.14±0.85	0.30±0.94	0.4485	0.2262	0.5676
3 mo	0.35±0.97	0.28±0.78	0.42±0.80	0.47±0.83	0.9240	0.4953	0.9951
4 mo	0.46±1.03	0.26±0.89	0.51±0.83	0.60±0.83	0.6196	0.3202	0.6663
5 mo	0.56±1.01	0.38±0.82	0.55±0.80	0.60±0.83	0.8790	0.7620	0.7465
6 mo	0.73±1.02	0.50±0.84	0.70±0.84	0.73±0.88	0.8230	0.9132	0.6375
WHZ							
1 mo	0.38±0.85	0.43±0.82	0.53±0.80	0.46±0.89	0.8582	0.7384	0.7242
2 mo	0.45±0.95	0.52±0.83	0.60±0.90	0.63±0.92	0.7037	0.4773	0.6322
3 mo	0.49±0.91	0.42±0.89	0.53±0.89	0.66±0.96	0.7092	0.2664	0.9238
4 mo	0.48±0.81	0.53±0.88	0.61±0.93	0.78±0.88	0.3169	0.0838	0.7686
5 mo	0.46^{b}±0.91	0.56^{ab}±0.78	0.65^{ab}±0.99	0.94^{a}±0.84	0.0359	0.0073	0.5223
6 mo	0.46±0.90	0.54±0.83	0.69±0.93	0.87±0.85	0.1071	0.0349	0.4467

BF = Breast fed; F0 = standard infant formula; F65 = formula with 65 mg/L OPN; F130 = formula with 130 mg/L OPN; HAZ = height-for-age z-score; WAZ = weight-for-age z-score; WHZ = weight-for-height z-score (Radhakrishna *et al.*, 2010; Jones *et al.*, 2014); Different letter superscripts indicate $P<0.05$.

Hematocrit values were increased in the BF group at 1 and 4 months of age, and RBC count was higher in the BF group at 1, 4 and 6 months of age in the FF groups ($P < 0.05$), although

hemoglobin concentrations did not differ among groups (Table 27).[75] When adjusted for CRP concentration, serum ferritin concentrations did not differ between BF and FF infants.[76] When analyzing for anemia (defining anemia as Hb < 105 g/L), 10%, 16%, 18% and 19% of the F0, F65, F130 and BF infants respectively, were anemic at four months and 18%, 9%, 9% and 6% were anemic at six months of age, respectively, with no significant differences between groups, although the BF group had significantly more anemic infants than the FF groups combined and evaluated at 6 months of age.

The formulas in this study were fortified with 7 mg iron (as $FeSO_4$)/L, as currently recommended and the level used in many European countries. No follow-up was conducted at twelve months of age, due to limited funding and the high degree of mobility of the study population. Analysis of the treatment of this population for anemia was beyond the scope of this study. The potential for infant anemia in China may be higher than expected due to maternal iron deficiency concurrent with breast feeding, which has been analyzed by others (Wang *et al.*, 2015; Zhao *et al.*, 2015).

Table 27. Red blood cells, hemoglobin, plasma ferritin, CRP and adjusted ferritin (Lönnerdal *et al.*, 2016).

Variable	F0	F65	F130	BF	*P*-value, all groups	*P*-value formula *vs.* breast fed	*P*-value, all formula groups
RBCs ($x10^6$)							
1 mo	3.21±0.38	3.31±0.48	3.27±0.46	3.40±0.41	0.1133	0.0432	0.3785
4 mo	4.14 ^{ab}±0.37	4.19 ^{ab}±0.42	4.12 ^{b}±0.36	4.31^{a}±0.38	0.0329	0.0078	0.4322
6 mo	4.41±0.34	4.41±0.38	4.39±0.33	4.54±0.51	0.2362	0.0405	0.9812
Hemoglobin (g/L)							
1 mo	107±10.8	109±12.5	108±12.8	111±11.0	0.1987	0.0785	0.4441
4 mo	114±8.75	114±8.59	112±7.85	114±9.23	0.5458	0.7591	0.3590
6 mo	117±9.23	115±9.13	116±7.25	115±11.9	0.4348	0.3109	0.4404
Ferritin (µg/mL)							
1 mo	303 ^{a}±137	284 ^{a}±115	295^{a}±148	191^{b}±101	<0.0001	<0.0001	0.7862
4 mo	44.0±44.1	52.6±46.6	62.5±50.9	53.8±64.9	0.2567	0.9640	0.1330
6 mo	43.8 ^{a}±62.2	23.3 ^{b}±29.1	40.3^{ab}±37.1	25.2 ^{ab}±25.3	0.0197	0.9669	0.0073
CRP (mg/L)							
4 mo	0.41^{b}±0.99	0.41 ^{b}±1.37	0.59 ^{b}±1.92	3.26^{a}±7.66	<0.0001	<0.0001	0.2118
6 mo	0.63^{ab}±1.82	0.67 ^{b}±2.17	0.54 ^{a}±1.08	1.29 ^{a}±2.29	0.0084	0.0790	0.0114
Adjusted Ferritin (µg/mL)							
4 mo	42.7±42.5	52.4±46.6	61.6±50.2	53.1±65.0	0.2205	0.9543	0.1110
6 mo	43.6 ^{a}±62.1	23.0^{b}±29.0	40.9 ^{a}±37.1	25.0^{ab}±24.9	0.0168	0.9458	0.0062

BF = Breast fed; CRP = C-reactive protein; F0 = standard infant formula; F65 = formula with 65 mg/L OPN; F130 = formula with 130 mg/L OPN; RBCs = red blood cells; Different letter superscripts indicate $P < 0.05$.

[75] Iron deficiency was defined by a ferritin value < 12 µg/L and adjusted for CRP concentration.

[76] Ferritin is an acute phase reactant that is known to increase due to infection, a response not directly related to iron status. CRP is also an acute phase reactant, and therefore adjusting ferritin concentrations for CRP concentrations allows for a correction for concurrent infection by measuring CRP.

In general, the plasma concentrations of essential and branched chain amino acids were similar among the FF groups when compared to the BF group (Table 28), although there were small, but statistically significant differences in threonine, glutamic acid, valine, methionine, lysine and leucine plasma concentrations between the FF and BF groups. These differences were not consistent between the different treatment groups, and were not consistent between the four- and six-month timepoints. The differnces in plasma amino acid levels may be due to the differences between FF and BF infants, as Axelsson *et al.* (1989) showed that FF infants having higher protein intakes compared to breast milk have differences in amino acid levels, when compared to BF infants. When comparing among n the FF groups, the F130 group had significantly ($P < 0.05$) lower plasma threonine concentrations than the F0 and F65 groups and lower ($P < 0.05$) valine concentrations than the F0 group. The plasma aspartic acid concentration was lower ($P < 0.05$) in the F65 group, compared to the F0 group, while increased ($P < 0.05$) levels of plasma serine and plasma asparagine concentrations were found in the F65 group. Compared to the F0 group, plasma glutamic acid concentration was significantly lower in the F65 and F130 groups, while plasma glycine, alanine and methionine concentrations were lower ($P < 0.05$) in the F130 group than in the F0 group. The F65 group had significantly ($P < 0.05$) higher plasma concentrations of asparagine and hydroxyproline, compared to the F130 group. The authors hypothesize that the higher amino acid concentrations in the plasma of the FF infants may be due to a higher protein intake. The increased threonine concentration in the plasma of the FF infants may be due to the threonine levels in the whey protein concentrate that was used by the authors to adjust the whey/casein formula ratio. The decreased ($P < 0.05$) plasma threonine concentration in the F130 group compared to the F65 group was not expected by the authors. The authors did not speculate on a reason for this slight, but significant change. Plasma levels of tryptophan and glutamine were slightly higher than other reported plasma levels in infants, but this slight increase is not considered a safety concern, as tryptophan is readily used by healthy infants (Nayak and Buttar, 2015) and glutamine is a major energy source for intestinal cells (Martin *et al.*, 2016). Infant plasma amino acid concentrations vary by growth stage and by diet; the plasma amino acid concentrations found in the Lönnerdal *et al.* (2016) study were consistent with the range of reference values for healthy infants of the same age (Haschke-Becher *et al.*, 2016) and with other studies (Lepage *et al.*, 1997; Davis *et al.*, 2007; Trabulsi *et al.*, 2010). Overall, the plasma amino acid concentration changes seen in the Lonnerdal *et al.* (2016) study were not considered an adverse event by the authors.

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Table 28. Plasma amino acids (µmol/mL) in the formula-fed and breast fed groups (means ± SD), $n = 20$ in each group (Lönnerdal *et al.*, 2016)

Variable	F0	F65	F130	BF	*P*-value, all groups	*P*-value formula *vs* breast fed	*P*-value, all formula groups
Taurine							
4 mo	0.100±0.020	0.095±0.034	0.11±0.04	0.10±0.04	0.5481	0.6456	0.3962
6 mo	0.10±0.03	0.087±0.051	0.091±0.025	0.086±0.027	0.0804	0.5056	0.0449
Aspartic acid							
4 mo	0.013 ^{a} ±0.004	0.010 ^{ab}±0.002	0.019 ^{ab}±0.024	0.011 ^{b} ±0.004	0.0057	0.0201	0.0272
6 mo	0.013 ^{a} ±0.005	0.010 ^{ab}±0.002	0.010 ^{a} ±0.003	0.008 ^{b} ±0.002	0.0001	0.0001	0.0318
Threonine							
4 mo	0.22 ^{a} ±0.05	0.22 ^{a} ±0.06	0.18 ^{a} ±0.04	0.14 ^{b} ±0.04	<0.0001	<0.0001	0.0289
6 mo	0.19 ^{ab}±0.05	0.23 ^{a} ±0.06	0.16 ^{bc}±0.05	0.13 ^{c} ±0.03	<0.0001	<0.0001	0.0003
Serine							
4 mo	0.16 ^{b} ±0.02	0.18 ^{a} ±0.02	0.15 ^{b} ±0.02	0.17 ^{ab}±0.02	0.0009	0.4095	0.0004
6 mo	0.15 ^{b} ±0.02	0.18 ^{a} ±0.02	0.15 ^{b} ±0.03	0.18 ^{a} ±0.02	0.0013	0.0313	0.0044
Glutamic acid							
4 mo	0.16 ^{a} ±0.02	0.15 ^{ab}±0.08	0.14 ^{ab}±0.04	0.13 ^{b} ±0.06	0.0186	0.0207	0.0890
6 mo	0.15 ^{a} ±0.03	0.11 ^{bc}±0.04	0.13 ^{b} ±0.02	0.10 ^{c} ±0.02	<0.0001	<0.0001	<.0001
Glutamine							
4 mo	0.80 ^{b} ±0.08	0.80 ^{b} ±0.19	0.75 ^{b} ±0.08	0.93 ^{a} ±0.11	<0.0001	<0.0001	0.1521
6 mo	0.75 ^{b} ±0.09	0.80 ^{ab}±0.14	0.78 ^{ab}±0.13	0.85 ^{a} ±0.11	0.0162	0.0057	0.2803
Glycine							
4 mo	0.22±0.03	0.22±0.03	0.21±0.02	0.21±0.02	0.0544	0.0935	0.0759
6 mo	0.23 ^{a} ±0.05	0.22 ^{a} ±0.04	0.19 ^{b} ±0.02	0.21 ^{ab}±0.03	0.0071	0.5915	0.0026
Alanine							
4 mo	0.43 ^{a} ±0.09	0.41 ^{ab}±0.07	0.37 ^{ab}±0.07	0.37 ^{b} ±0.07	0.0136	0.0261	0.0408
6 mo	0.41 ^{a} ±0.09	0.40 ^{ab}±0.06	0.34 ^{b} ±0.08	0.34 ^{b} ±0.05	0.0034	0.0231	0.0082
Valine							
4 mo	0.22 ^{a} ±0.04	0.19 ^{a} ±0.04	0.18 ^{a} ±0.04	0.15 ^{b} ±0.03	<0.0001	<0.0001	0.0395
6 mo	0.22 ^{a} ±0.04	0.23 ^{a} ±0.05	0.20 ^{a} ±0.04	0.16 ^{b} ±0.03	<0.0001	<0.0001	0.0993
Methionine							
4 mo	0.033 ^{a} ±0.005	0.032^{ab}±0.006	0.027 ^{b} ±0.005	0.027 ^{b} ±0.005	0.0006	0.0150	0.0019
6 mo	0.030 ^{a} ±0.006	0.032 ^{a} ±0.007	0.026 ^{b} ±0.006	0.023 ^{b} ±0.003	<0.0001	0.0002	0.0014
Isoleucine							
4 mo	0.084 ^{a} ±0.019	0.073 ^{a} ±0.025	0.068 ^{ab}±0.018	0.055 ^{b} ±0.013	0.0003	0.0002	0.0510

Table 28. Plasma amino acids (μmol/mL) in the formula-fed and breast fed groups (means ± SD), $n = 20$ in each group (Lönnerdal *et al.*, 2016)

Variable	F0	F65	F130	BF	*P*-value, all groups	*P*-value formula *vs* breast fed	*P*-value, all formula groups
6 mo	0.079[a] ±0.018	0.082 [a] ±0.023	0.072 [a] ±0.022	0.055[b] ±0.014	0.0002	<0.0001	0.2714
Leucine							
4 mo	0.13[a] ±0.02	0.12[ab] ±0.03	0.11 [ab] ±0.02	0.095[b] ±0.019	0.0017	0.0007	0.1198
6 mo	0.12[a] ±0.02	0.13[a] ±0.04	0.12 [a] ±0.03	0.096[b] ±0.020	0.0014	0.0001	0.5086
Tyrosine							
4 mo	0.077±0.015	0.079±0.015	0.075±0.013	0.074±0.014	0.7983	0.5964	0.6830
6 mo	0.075±0.017	0.078±0.014	0.072±0.017	0.070±0.010	0.5467	0.3749	0.4820
Phenylalanine							
4 mo	0.058±0.006	0.058±0.011	0.061±0.020	0.058±0.008	0.9701	0.6916	0.9572
6 mo	0.063±0.008	0.065±0.012	0.061±0.023	0.062±0.010	0.4139	0.7880	0.2448
Tryptophan							
4 mo	0.12 [a] ±0.02	0.11 [a] ±0.02	0.098[b] ±0.015	0.11[ab] ±0.02	0.0048	0.7796	0.0016
6 mo	0.11±0.02	0.10±0.02	0.10±0.02	0.11±0.01	0.4358	0.1728	0.6888
Lysine							
4 mo	0.22 [a] ±0.03	0.20 [ab] ±0.05	0.18 [b] ±0.03	0.15 [c] ±0.03	<0.0001	<0.0001	0.0161
6 mo	0.20 [a] ±0.04	0.20 [a] ±0.04	0.18 [ab] ±0.04	0.15 [b] ±0.02	0.0002	<0.0001	0.1205
Histidine							
4 mo	0.088[ab] ±0.010	0.094[a] ±0.009	0.085[b] ±0.007	0.087[ab] ±0.008	0.0141	0.5195	0.0058
6 mo	0.085[b] ±0.012	0.094[a] ±0.008	0.084[b] ±0.006	0.088 [ab] ±0.006	0.0011	0.8418	0.0004
Hydroxyproline							
4 mo	0.042[ab] ±0.004	0.044[a] ±0.005	0.038[b] ±0.008	0.044[ab] ±0.006	0.0219	0.2794	0.0160
6 mo	0.034±0.006	0.036±0.006	0.033±0.006	0.034 ±0.005	0.4858	0.9307	0.3022
Asparagine							
4 mo	0.033±0.008	0.042±0.015	0.034±0.010	0.039±0.012	0.0639	0.2528	0.0536
6 mo	0.028[b] ±0.006	0.055[a] ±0.020	0.033[b] ±0.011	0.044[a] ±0.015	<0.0001	0.0036	<0.0001
Proline							
4 mo	0.20[a] ±0.03	0.20[ab] ±0.03	0.18 [b] ±0.03	0.20[ab] ±0.05	0.0484	0.9058	0.0204
6 mo	0.20±0.04	0.20±0.03	0.18±0.04	0.19±0.05	0.2906	0.6771	0.1626

BF = breast fed; F0 = standard infant formula; F65 = formula with 65 mg/L OPN; F130 = formula with 130 mg/L OPN; mo = month; Different letter superscripts indicate $P<0.05$.

Plasma BUN concentrations were higher ($P < 0.05$) in the FF infants, compared to the BF infants, for the 1, 4 and 6 time points ($P < 0.05$; Table 29). Plasma insulin concentrations were variable, with the F65 groups having higher insulin concentrations than the F0 and BF groups at the 4- and 6-month time points, with no difference between FF and BF infants at the 1-month time point. The plasma insulin concentration in the F130 group was significantly higher than the F0 and BF groups at the 4-month time point ($P < 0.05$), but returned to the same ranges as the F0 and BF concentrations at the 6-month time point (Table 29). In addition, the sum of the plasma branched-chain amino acids (BCAAs) (*i.e.*, valine, leucine and isoleucine) was significantly ($P = 0.05$) higher in all FF infants, compared to the BF infants, but when compared among FF groups, the plasma branched-chain amino acids were lower in the F130 group compared to the F0 group ($P < 0.05$), approaching levels similar to those of the BF group (Table 29).

Table 29. Plasma branched-chain amino acids (BCAAs), insulin and BUN in the formula-fed groups and the breast fed group (Lönnerdal *et al.*, 2016).

Variable	F0	F65	F130	BF	*P*-value, all groups	*P*-value formula *vs* breast fed	*P*-value, all formula groups
BCAAs (μmol/L)							
4 mo	0.43 ^{a}±0.08	0.38 ^{a}±0.09	0.36 ^{ab}±0.08	0.30 ^{b}±0.06	0.0001	<0.0001	0.0410
6 mo	0.42 ^{a}±0.08	0.43 ^{a}±0.11	0.39 ^{a}±0.09	0.31 ^{b}±0.06	<0.0001	<0.0001	0.1912
Insulin (μU/mL)							
1 mo	6.11 ^{a}±7.09	10.3 ^{a}±8.26	10.3 ^{a}±10.6	5.21 ^{a}±4.13	0.0353	0.1054	0.0466
4 mo	3.34 ^{b}±2.67	9.62 ^{a}±9.29	9.33 ^{a}±8.24	3.97 ^{b}±3.44	<0.0001	0.0035	<0.0001
6 mo	2.64 ^{b}±2.85	10.9 ^{a}±12.0	2.32 ^{b}±1.57	3.84 ^{b}±3.42	<0.0001	0.9938	<0.0001
BUN (mg/dL)							
1 mo	10.3 ^{a}±3.52	10.7 ^{a}±2.90	11.3 ^{a}±3.15	8.56 ^{b}±3.30	<0.0001	<0.0001	0.1332
4 mo	9.29 ^{ab}±3.24	8.46 ^{b}±2.98	10.5 ^{a}±3.82	5.95 ^{c}±2.90	<0.0001	<0.0001	0.0033
6 mo	9.06 ^{a}±3.28	8.35 ^{a}±2.66	9.46 ^{a}±3.49	6.50 ^{b}±2.62	<0.0001	<0.0001	0.2297

BF = breast fed; BUN = blood urea nitrogen; F0 = standard infant formula; F65 = formula with 65 mg/L OPN; F130 = formula with 130 mg/L OPN; mo = month; Different letter superscripts indicate $P<0.05$

The completion of a daily symptom diary by the parents throughout the study (from inclusion until six months of age) showed that parents of BF infants reported fewer episodes of pyrexia compared to parents of FF infants, but that the F0 group had significantly higher incidence (% of infants) and prevalence (*per* 100 days) of pyrexia than BF infants ($P < 0.05$). In contrast, F65 and F130-fed infants were not different from BF infants. The incidence and prevalence of pyrexia in the F65 and F130 groups was lower than the F0 group (by almost one-half in the F65 group), but did not reach statistical significance (Table 30). Prevalence of diarrhea, gastrointestinal problems, vomiting, infections, respiratory or skin problems did not differ among formula groups (Table 30). Parents of BF infants reported lower incidence and prevalence of crying or sleeplessness than FF parents, but FF groups did not differ ($P > 0.05$).

The infants were vaccinated against DPT (diphtheria, pertussis, tetanus) at four months of age as recommended by the Centers for Disease Control (CDC), then analyzed at six months of age for immunoglobulin G (IgG) antibodies against tetanus (Table 23). There was no significant difference between BF and FF infants, and although the F65 group had fewer antibodies than the F0 group, there was no significant difference between the F130 group and any other groups.

While there are statistical differences in the plasma amino acid concentrations among the FF groups and between the FF and BF groups, the data presented in this work do not indicate a toxicological response to the ingestion of OPN-10 at proposed or half the concentration proposed for addition to infant formula. This study shows that consumption of OPN-10 when added to formula at the recommended level of intake has no adverse effect on infants, and the authors concluded that "formulas with added bovine OPN were well tolerated and resulted in satisfactory growth" relative to National Center for Health Statistics data (Lönnerdal *et al.*, 2016).

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Table 30. Incidence or prevalence of adverse events in formula-fed infants vs. breast fed infants (Lönnerdal *et al.*, 2016).

Variable	F0	F65	F130	BF	*P*-value, all groups	*P*-value formula *vs* breast fed	*P*-value, all formula groups
Diarrhea (18-day prevalence)#	2.67±7.33	1.08±3.65	1.83±5.49	3.77±10.2	0.2659	0.1833	0.3326
Vomiting (18-day prevalence)	14.3±22.8	11.9±19.2	10.2±18.9	11.0±18.3	0.5526	0.3542	0.5313
Incidence of low appetite (% of mo)	3.48±10.7	3.98±9.66	3.57±7.97	1.87±7.46	0.2487	0.0657	0.6958
Incidence of eye problems (% of mo)	0.25±2.04	0	0	0.44±2.70	0.3648	0.1379	0.6209
Incidence of pyrexia (% of mo)	8.21^{a}±11.7	4.03^{ab}±7.82	5.48 ^{ab}±10.1	3.20 ^{b}±7.28	0.0139	0.0269	0.0541
Incidence of crying or sleeplessness (% of mo)	14.7^{a} ±22.6	13.7^{a} ±19.9	13.1^{a} ±21.8	6.93^{b} ±15.6	0.0887	0.0131	0.8197
Incidence of gastric problems (% of mo)	8.51±14.1	7.71±13.1	6.05±10.5	5.87±12.3	0.4074	0.2143	0.4958
Incidence of infections (% of mo)	2.99±7.64	3.98±10.1	2.86±6.93	2.00±8.20	0.3450	0.0841	0.8414
Incidence of respiratory problems (% of mo)	15.5±20.8	18.0±22.7	11.9±17.3	12.4±17.1	0.3505	0.4362	0.2576
Incidence of skin problems (% of mo)	12.9±21.9	12.7±18.4	13.1±21.0	14.6±23.4	0.9791	0.7302	0.9664
Prevalence of low appetite (*per* 100 d)	0.36±1.12	0.42±1.14	0.43±1.33	0.21±0.90	0.2159	0.0667	0.5830
Prevalence of eye problems (*per* 100 d)	0.008±0.067	0± 0	0± 0	0.067±0.464	0.3372	0.1095	0.6739
Prevalence of pyrexia (*per* 100 d)	0.59 ^{a}±1.17	0.33 ^{ab}±0.70	0.42^{ab}±0.84	0.21 ^{b}±0.51	0.0241	0.0141	0.1761
Prevalence of crying or sleeplessness (*per* 100 d)	1.49 ^{a} ±3.12	1.51 ^{a} ±2.67	2.03 ^{a} ±5.76	0.72 ^{b} ±2.57	0.0861	0.0134	0.7712
Prevalence of gastric problems (*per* 100 d)	1.56±3.45	1.33±2.97	1.03±2.41	0.85±1.93	0.4105	0.1601	0.6230
Prevalence of infections (*per* 100 d)	0.35±1.08	0.70±2.32	0.42±1.87	0.75±3.59	0.4870	0.1511	0.8261
Prevalence of respiratory problems (*per* 100 d)	2.68±4.66	2.69±3.75	2.20±3.67	2.71±7.66	0.7148	0.5664	0.5918
Prevalence of skin problems (*per* 100 d)	3.44±8.91	3.07±5.98	4.10±10.3	5.48±12.3	0.9442	0.5796	0.9664

BF = breast fed; d = day; F0 = standard infant formula; F65 = formula with 65 mg/L OPN; F130 = formula with 130 mg/L OPN mo = month; Different letter superscripts indicate $P<0.05$; #Parents were asked about what happened the previous 3 days in each of the 6 monthly questionnaires. Thus, 18 days of data were available for each subject.

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7. EVALUATION

To demonstrate that OPN-10, a bovine whey-isolated ingredient composed primarily of osteopontin (OPN), a phosphoprotein secreted into milk by mammals but found at low levels in infant formula, is safe under its intended conditions of use, the safety of the intake of OPN-10 resulting from its consumption in infant formula and toddler drinks has been established. The conclusion of GRAS status is based on the following:

- OPN-10 is a food ingredient isolated from bovine whey, which provides infants and toddlers with a source of OPN. OPN-10 contains greater than 78% protein (when evaluated as N*6.38). OPN contains less than 1% lactose and fat.

- Production of OPN-10 starts with the isolation of pasteurized whey from bovine milk, which then undergoes isolation and ultrafiltration steps to concentrate the OPN. The concentrated extract is then heat-treated and spray dried to form OPN-10.

- OPN-10 specifications are set to ensure a food-grade ingredient and five batches of OPN-10 consistently met standards and limits on potential contaminants appropriate for food-grade ingredients. OPN-10 is stable at ambient temperatures for up to three (3) years and is stable as an added ingredient to infant formula for a minimum of three months.

- OPN-10 is to be added to cow's milk-based infant formula such that the overall consumption of OPN as an ingredient in term nonexempt cow's milk-based infant formula. The Estimated Daily Intake (EDI) will be approximately 23 mg OPN/kg BW/day, the level found in human breast milk. The mean and 90th percentile estimated daily intake of OPN-10 at the suggested level of addition to infant formula is 24.8 and 39.5 mg/kg BW/day, respectively, based on the average body weight of one-month-old infants. The EDI is below the Acceptable Daily Intake (ADI) of 50 mg/kg bw/day. OPN-10 is also to be added to cow's milk-based powdered beverages targeted for children 1 – 3 years of age, such that consumption of OPN by toddlers is over three-fold lower than that consumed by infants, on a body weight basis. Bovine osteopontin is added to infant formulas in North and South East Asia and China at levels ranging from 21 to 110 mg/L of formula.

- An acute oral dose of OPN at approximately 2500 mg/kg BW to OPN-deficient, 3-week-old and ten-week-old mice was well tolerated, resulting in plasma levels of OPN at up to 3092 ng/ml one hour post dose in the three-week-old mice and up to approximately 5000 ng/ml in the 10-week-old mice four hours post dose, then decreasing to negligible levels by 24 hours post dose in both age groups.

- A published 13-week repeat dose toxicity study in male and female rats, conducted according to OECD guidelines, evaluated the toxicity of OPN-10 at 0, 0.5, 1 and 2% of the diet (providing an average intake of 0, 301, 596 and 1208 mg/kg BW/day OPN-10 by male rats and 0, 322, 637 and 1272 mg/kg BW/day OPN-10 by female rats, respectively) for 91 days. The male rats consumed an average of 0, 223.0, 441.6 and 895.1 mg OPN/kg BW/day and the female rats consumed an average of 0, 238.6, 472.0 and 942.6 mg OPN/kg BW/day, respectively. There were no significant differences in mean body weight between treatment groups and the control group. The only significant change in hematological parameters was an increase in mean

MCHC for 0.5% and 1.0% male dose groups. There were no significant changes in clinical chemistry parameters for the male dose groups, compared to the control group, while only the 1.0% female dose group had a significant increase in albumin/globulin ratio. There were non-dose-dependent changes in urine volume and density in the male treatment groups, and a significant increase in urinary protein in the 0.5% male dose group. There were no significant differences in absolute and relative organ weights in the male dose groups, whereas the female rats had a significant trend to increased kidney weight, significant only for the relative kidney weight of the 1.0% dose group. The absolute and relative thymus weight showed a significant trend for increasing weight, but no statistically significant difference among groups was found. None of these changes was considered dose-related by the authors, and there were no histopathological findings considered dose-related. The authors concluded a NOAEL of 1208 and 1272 mg/kg BW/day OPN-10 in this repeat-dose study, corresponding to 895.1 and 942.6 mg OPN/kg BW/day, in male and female rats, respectively.

- A published teratogenicity study was conducted in which pregnant rats (n = 14/group) were fed OPN-10 at 0, 630, 1250 and 2500 mg/kg BW/day *via* gavage from Days 7 – 16 of gestation. The rats were bled and euthanized on Day 20 of gestation, and the numbers of implantations, live, dead and absorbed fetuses were recorded. The fetuses were examined for abnormalities and one-half dissected for visceral examination and the other half was evaluated for skeletal abnormalities. OPN-10 had no adverse effects on the reproduction of the dams on body weight, pregnancy rate, number of live fetuses, fetuses *per* litter, absorbed or dead fetuses, or fetal body weight or length. There were no significant effects on gross, visceral, or skeletal abnormalities found in the treatment groups, compared to the control group. This study indicated that OPN-10 had no adverse effect on fetal formation. The NOAEL for this study is stated at 2500 mg/kg BW/day and the ADI was calculated at 50 mg/kg bw/day.

- Rhesus monkey infants were fed OPN-10 for three months at a concentration to provide 125 mg OPN/L formula and were compared to groups fed commercial formula and maternal milk. There were no differences in body weight, crown-rump length or BMD between the monkeys fed formula (FF) and those fed formula with added OPN-10 (OPN) stated in this published study. The infant monkeys consumed the same amount of OPN-10 (on a body weight basis) as would human infants on the same formula. There were no differences between FF and OPN groups for FFM, MCV, WBC, differential WBC, RBC, hemoglobin and hematocrit levels. There were no significant differences in overall growth, hematological parameters and lean and fat body mass endpoints between commercial formula-fed infant monkeys and infant monkeys consuming formula containing OPN-10 providing 125 mg OPN/L formula.

- OPN-10 was not genotoxic when evaluated in a published reverse bacterial mutation assay in *S. typhimurium* and *E. coli* tester strains at up to 5000 µg/plate, either with or without incubation with S9 metabolic activation mix. OPN-10 was also found to lack the ability to produce structural chromosomal aberrations in human peripheral blood lymphocytes, either in the presence or absence of S9 metabolic activation, when evaluated according to OECD guidelines at concentrations up to 5000 µg/ml. When evaluated in the *in vivo* mouse micronucleus assay for chromosomal or mitotic apparatus damage due to OPN-10 ingestion, administration of 2000 mg/kg BW OPN (2300 mg/kg OPN-10) did not induce micronuclei formation in the bone marrow PCE and was not cytotoxic to the developing cells, and did not induce structural and/or numerical chromosomal damage in the immature erythrocytes.

- A double-blind, randomized clinical trial was conducted in infants 1 – 6 months of age, in which the infants consumed commercial infant formula with OPN-10 at 65 mg/L (F65) or 130 mg/L (F130), compared with infants that were breast fed and infants that consumed only the commercial formula. The infants in this published study were evaluated for changes in body weight, length and head circumference, a daily symptom diary and provided information on stool consistency and frequency and disease symptoms. There were no differences in body weight, height, head circumference and sleep time between formula fed groups, with only a transient decrease in formula intake at five months of age for the group consuming 65 mg OPN-10/L formula. Blood samples obtained at 1, 4 and 6 months of age showed a decrease in ferritin in the F65 group at six months of age, compared to the control formula fed group, but this was not significantly different from breast fed infants and was no longer significant when adjusted for CRP concentration. The CRP level was increased in the F65 group compared with the F130 group at six months of age, but was not significantly different from the formula fed control group. Plasma BUN concentrations were significantly higher in the FF groups when compared to the BF group, for the 1, 4 and 6 month time points, but the OPN-10-fed infant BUN levels were not significantly different from the formula-fed control group. Plasma insulin levels varied between groups during the study, with increased insulin levels in the F65 group at the four- and six-month time points, but the F130 group had increased insulin levels only at the four-month time point, with both F65 and F130 groups insulin levels returning to control levels at the six-month time point. There were no significant differences between formula groups for the prevalence of diarrhea, GI problems, vomiting, infections, respiratory or skin problems.

In summary, information available on the production, components, safety and intended use levels of Lacprodan® OPN-10 support the safety-in-use of Lacprodan® OPN-10 at an anticipated upper consumption of 160 mg/kg bw/day in infants consuming milk-based formula, and an anticipated upper consumption of 39.5 mg/kg bw/day in young children ages 1 – 3 when consuming milk-based follow-on drinks.

The following pages provide the signed Expert Panel conclusion on the GRAS status of Lacprodan® OPN-10 at the levels of consumption when used as intended in milk-based infant formula and milk-based beverages for young children ages 1 – 3.

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- The Acceptable Daily Intake (ADI), is 50 mg/kg bw/day, based on NOAEL data from the lack of toxicologically significant effects at the highest doses evaluated in the preclinical and clinical studies conducted with the test substance.

The safety of OPN-10 has been evaluated as described in this dossier, when used as a direct food ingredient to increase the total intake of OPN, such that total daily consumption of OPN-10 from cow's milk-based infant formula (which includes follow-on formula specifically formulated for infants 6-12 months of age) and cow's milk-based powdered beverages targeted for children 1-3 years of age, is up to 39 mg OPN-10/kg BW/day (160 mg OPN-10/L infant formula). In particular, the Expert Panel has evaluated the proposed use of OPN-10 in term cow's milk-based infant formula and has concluded that such use results in an estimated intake that is GRAS.

It is our opinion that other experts qualified by scientific training and experience to evaluate the safety of food and food ingredients would concur with these conclusions.

10. SIGNATURES

(b) (6)

9 Jan 2017

Roger Clemens, DrPH.
VP, Polyscience Consulting

Date

Eric L. Lien, Ph.D.
Adjunct Full Professor
Department of Food Science and Human Nutrition
University of Illinois

Date

Robert Nicolosi, Ph.D.
RJ Nicolosi, LLC

Date

Kelly Tappenden, Ph.D., R.D.
Scientific Consultant

Date

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10. SIGNATURES

Roger Clemens, DrPH.
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Date

June 10, 2017

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University of Illinois

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Date

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6/24/17
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8. List of Supporting Data

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List of Corroborative Information

ARLA. (2015) Stability of Lacprodan® OPN-10 (Lacprodan® LPN-10). (Personal Communication)

ARLA. (2016) Statement on AFI monitoring of contaminants. (Personal Communication)

ARLA (2017) Dossier in Support of the Generally Recognized as Safe (GRAS) status of Lacprodan® OPN-10 as a Food Ingredient in Infant formula and Toddler beverages.

Kvistgaard, A. S. (2016) Study data. (Personal Communication)

Sorensen, E. S. (2016) Comparison of human osteopontin ELISAs. (Personal Communication)

Appendix I

Individual lot analysis for OPN-10 specifications.

Analysis	Unit	Specification	(b)(4)	[redacted]	[redacted]	[redacted]	[redacted]	Average	Range
Chemicals and Specifications									
Protein as is (N x 6.38)	min. %	78	78.3	79.46	78.74	78.3	79.19	78.798	78.3 - 79.46
Protein as is (N x 7.17)	min. %	86	88	89.3	88.5	88	89	88.56	88-89.3
OPN as % of protein	min. %	95	97.2	98.3	> 99.9	>99.5	>99.5	97.75	97.2 - >99.9
Lactose	max %	1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Fat	max %	1	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Ash	max %	11	9.7	9.2	9.3	9.5	9.5	9.44	9.2-9.7
Moisture	max %	5.5	4.1	4.1	4.8	5	4.5	4.5	4.1- 5
Minerals									
Sodium	%	0.5-1.5	1.27	1.17	1.07	1.21	1.22	1.188	1.07 - 1.27
Phosphorus	%	1.4-2.2	1.95	1.86	1.84	1.85	1.84	1.868	1.84 - 1.95
Chloride	max	0.2	0.04	0.04	0.04	<0.05	0.1	0.055	.04 - 0.1
Calcium	%	1.6 - 2.7	2.22	2.2	2.37	2.34	2.34	2.294	2.2 - 2.37
Nutritional Data									
Energy	KJ/ Kcal	1338/ 319							
Physical Specification									
Scorched Particles		Disc A		Disc A	Disc A	Disc A	Disc A	Disc A	
Solubility Index	max	1 ml		1 ml		<0.1		< 1 ml	
Microbiological Specifications									
Total Plate Count	Max CFU/g	5000	<1000	<1000	<1000	<1000	<1000		
Bacillus cereus	Max CFU/g	50	40	<10	<10	<10	<10		
Enterobacteriaceae	Max CFU/g	10	<10	<10	<10	<10	<10		
Staphylococcus aureus	CFU	Absent in 1 g	Absent	Absent	Absent	Absent	Absent		
Yeast / Mold	CFU/g	Max 100 CFU/g	<10	<10	<10	<10	<10		
Salmonella	CFU/g	Absent in 125g	Absent	Absent	Absent	Absent	Absent		
C.Sakazaki##	CFU/g	Absent in 10g	Absent	Absent					
Heavy Metals#									
Arsenic	mg/ Kg	< 0.5	<0.01	<0.01	<0.1				
Cadmium	mg/ Kg	< 0.05	<0.01	<0.01	<0.01				
Lead	µg/ Kg	< 50.0	14	13	16				
Mercury	mg/ Kg	< 0.05	<0.005	<0.005	<0.005				

CFU = colony forming unit; #Heavy metal analysis will only be conducted on an annual basis; ##*C. sakazakii* analysis will be conducted on an intermittent basis.

Appendix II

Infant formulas (milk-based or not made with soy or other plant-based material) from NHANES 2011-2012*

Description	Amount of OPN-10 (mg/g)
Infant formula, NFS	0.160
Similac Expert Care Alimentum, infant formula, NS as to form	0.160
Similac Expert Care Alimentum, infant formula, ready-to-feed	0.160
Similac Expert Care Alimentum, infant formula, prepared from powder, made with water, NFS	0.160
Similac Expert Care Alimentum, infant formula, prepared from powder, made with tap water	0.160
Similac Expert Care Alimentum, infant formula, prepared from powder, made with plain bottled water	0.160
Similac Expert Care Alimentum, infant formula, prepared from powder, made with baby water	0.160
Similac Advance, infant formula, NS as to form	0.160
Similac Advance, infant formula, ready-to-feed	0.160
Similac Advance, infant formula, prepared from liquid concentrate, made with water, NFS	0.160
Similac Advance, infant formula, prepared from powder, made with water, NFS	0.160
Similac Advance, infant formula, prepared from liquid concentrate, made with tap water	0.160
Similac Advance, infant formula, prepared from liquid concentrate, made with plain bottled water	0.160
Similac Advance, infant formula, prepared from liquid concentrate, made with baby water	0.160
Similac Advance, infant formula, prepared from powder, made with tap water	0.160
Similac Advance, infant formula, prepared from powder, made with plain bottled water	0.160
Similac Advance, infant formula, prepared from powder, made with baby water	0.160
Similac Advance Organic, infant formula, NS as to form	0.160
Similac Advance Organic, infant formula, ready-to-feed	0.160
Similac Advance Organic, infant formula, prepared from powder, made with water, NFS	0.160
Similac Advance Organic, infant formula, prepared from powder, made with tap water	0.160
Similac Advance Organic, infant formula, prepared from powder, made with plain bottled water	0.160
Similac Advance Organic, infant formula, prepared from powder, made with baby water	0.160
Similac Sensitive, infant formula, NS as to form	0.160
Similac Sensitive, infant formula, ready-to-feed	0.160
Similac Sensitive, infant formula, prepared from liquid concentrate, made with water, NFS	0.160
Similac Sensitive, infant formula, prepared from powder, made with water, NFS	0.160
Similac Sensitive, infant formula, prepared from liquid concentrate, made with tap water	0.160
Similac Sensitive, infant formula, prepared from liquid concentrate, made with plain bottled water	0.160
Similac Sensitive, infant formula, prepared from liquid concentrate, made with baby water	0.160
Similac Sensitive, infant formula, prepared from powder, made with tap water	0.160

Infant formulas (milk-based or not made with soy or other plant-based material) from NHANES 2011-2012*

Similac Sensitive, infant formula, prepared from powder, made with plain bottled water	0.160
Similac Sensitive, infant formula, prepared from powder, made with baby water	0.160
Similac Sensitive for Spit-Up, infant formula, NS as to form	0.160
Similac Sensitive for Spit-Up, infant formula, ready-to-feed	0.160
Similac Sensitive for Spit-Up, infant formula, prepared from powder, made with water, NFS	0.160
Similac Sensitive for Spit-Up, infant formula, prepared from powder, made with tap water	0.160
Similac Sensitive for Spit-Up, infant formula, prepared from powder, made with plain bottled water	0.160
Similac Sensitive for Spit-Up, infant formula, prepared from powder, made with baby water	0.160
Similac Go and Grow, infant formula, NS as to form	0.160
Similac Go and Grow, infant formula, prepared from powder, made with water, NFS	0.160
Similac Go and Grow, infant formula, prepared from powder, made with tap water	0.160
Similac Go and Grow, infant formula, prepared from powder, made with plain bottled water	0.160
Similac Go and Grow, infant formula, prepared from powder, made with baby water	0.160
Enfamil PREMIUM Newborn, infant formula, NS as to form	0.160
Enfamil PREMIUM Newborn, infant formula, ready-to-feed	0.160
Enfamil PREMIUM Newborn, infant formula, prepared from powder, made with water, NFS	0.160
Enfamil PREMIUM Newborn, infant formula, prepared from powder, made with tap water	0.160
Enfamil PREMIUM Newborn, infant formula, prepared from powder, made with plain bottled water	0.160
Enfamil PREMIUM Newborn, infant formula, prepared from powder, made with baby water	0.160
Enfamil PREMIUM Infant, infant formula, NS as to form	0.160
Enfamil PREMIUM Infant, infant formula, ready-to-feed	0.160
Enfamil PREMIUM Infant, infant formula, prepared from liquid concentrate, made with water, NFS	0.160
Enfamil PREMIUM Infant, infant formula, prepared from liquid concentrate, made with tap water	0.160
Enfamil PREMIUM Infant, infant formula, prepared from liquid concentrate, made with plain bottled water	0.160
Enfamil PREMIUM Infant, infant formula, prepared from liquid concentrate, made with baby water	0.160
Enfamil PREMIUM Infant, infant formula, prepared from powder, made with water, NFS	0.160
Enfamil PREMIUM Infant, infant formula, prepared from powder, made with tap water	0.160
Enfamil PREMIUM Infant, infant formula, prepared from powder, made with plain bottled water	0.160
Enfamil PREMIUM Infant, infant formula, prepared from powder, made with baby water	0.160
Enfamil PREMIUM LIPIL, infant formula, NS as to form	0.160
Enfamil PREMIUM LIPIL, infant formula, prepared from liquid concentrate, made with water, NFS	0.160
Enfamil PREMIUM LIPIL, infant formula, prepared from powder, made with water, NFS	0.160
Enfamil PREMIUM LIPIL, infant formula, prepared from liquid concentrate, made with tap water	0.160
Enfamil PREMIUM LIPIL, infant formula, prepared from liquid concentrate, made with plain bottled water	0.160

Infant formulas (milk-based or not made with soy or other plant-based material) from NHANES 2011-2012*

Enfamil PREMIUM LIPIL, infant formula, prepared from liquid concentrate, made with baby water	0.160
Enfamil PREMIUM LIPIL, infant formula, prepared from powder, made with tap water	0.160
Enfamil PREMIUM LIPIL, infant formula, prepared from powder, made with plain bottled water	0.160
Enfamil PREMIUM LIPIL, infant formula, prepared from powder, made with baby water	0.160
Enfamil LIPIL, infant formula, NS as to form	0.160
Enfamil LIPIL, infant formula, ready-to-feed	0.160
Enfamil LIPIL, infant formula, prepared from liquid concentrate, made with water, NFS	0.160
Enfamil LIPIL, infant formula, prepared from powder, made with water, NFS	0.160
Enfamil LIPIL, infant formula, prepared from liquid concentrate, made with tap water	0.160
Enfamil LIPIL, infant formula, prepared from liquid concentrate, made with plain bottled water	0.160
Enfamil LIPIL, infant formula, prepared from liquid concentrate, made with baby water	0.160
Enfamil LIPIL, infant formula, prepared from powder, made with tap water	0.160
Enfamil LIPIL, infant formula, prepared from powder, made with plain bottled water	0.160
Enfamil LIPIL, infant formula, prepared from powder, made with baby water	0.160
Enfamil A.R. Lipil, infant formula, NS as to form	0.160
Enfamil A.R. Lipil, infant formula, ready-to-feed	0.160
Enfamil A.R. LIPIL, infant formula, prepared from powder, made with water, NFS	0.160
Enfamil A.R. LIPIL, infant formula, prepared from powder, made with tap water	0.160
Enfamil EnfaCare LIPIL, infant formula, NS as to form	0.160
Enfamil EnfaCare LIPIL, infant formula, ready-to-feed	0.160
Enfamil EnfaCare LIPIL, infant formula, prepared from powder, made with water, NFS	0.160
Enfamil A.R. LIPIL, infant formula, prepared from powder, made with plain bottled water	0.160
Enfamil A.R. LIPIL, infant formula, prepared from powder, made with baby water	0.160
Enfamil Gentlease LIPIL, infant formula, NS as to form	0.160
Enfamil Gentlease LIPIL, infant formula, ready-to-feed	0.160
Enfamil Gentlease LIPIL, infant formula, prepared from powder, made with water, NFS	0.160
Enfamil EnfaCare LIPIL, infant formula, prepared from powder, made with tap water	0.160
Enfamil EnfaCare LIPIL, infant formula, prepared from powder, made with plain bottled water	0.160
Enfamil EnfaCare LIPIL, infant formula, prepared from powder, made with baby water	0.160
Enfamil Gentlease LIPIL, infant formula, prepared from powder, made with tap water	0.160
Enfamil Gentlease LIPIL, infant formula, prepared from powder, made with plain bottled water	0.160
Enfamil Gentlease LIPIL, infant formula, prepared from powder, made with baby water	0.160
Enfamil Enfagrow PREMIUM Next Step LIPIL, infant formula, NS as to form	0.160
Enfamil Enfagrow PREMIUM Next Step LIPIL, infant formula, ready-to-feed	0.160
Enfamil Enfagrow PREMIUM Next Step LIPIL, infant formula, prepared from powder, made with water, NFS	0.160

Infant formulas (milk-based or not made with soy or other plant-based material) from NHANES 2011-2012*

Enfamil Enfagrow PREMIUM Next Step LIPIL, infant formula, prepared from powder, made with tap water	0.160
Enfamil Enfagrow PREMIUM Next Step LIPIL, infant formula, prepared from powder, made with plain bottled water	0.160
Enfamil Enfagrow PREMIUM Next Step LIPIL, infant formula, prepared from powder, made with baby water	0.160
Enfamil Gentlease Next Step LIPIL, infant formula, NS as to form	0.160
Enfamil Gentlease Next Step LIPIL, infant formula, prepared from powder, made with water, NFS	0.160
Enfamil Gentlease Next Step LIPIL, infant formula, prepared from powder, made with tap water	0.160
Enfamil Gentlease Next Step LIPIL, infant formula, prepared from powder, made with plain bottled water	0.160
Enfamil Gentlease Next Step LIPIL, infant formula, prepared from powder, made with baby water	0.160
Pediasure, infant formula, NS as to form	0.160
Pediasure, infant formula, ready-to-feed	0.160
Pediasure Fiber, infant formula, NS as to form	0.160
Pediasure Fiber, infant formula, ready-to-feed	0.160
Gerber Good Start Gentle Plus, infant formula, NS as to form	0.160
Gerber Good Start Gentle Plus, infant formula, ready-to-feed	0.160
Gerber Good Start Gentle Plus, infant formula, prepared from liquid concentrate, made with water, NFS	0.160
Gerber Good Start Gentle Plus, infant formula, prepared from powder, made with water, NFS	0.160
Gerber Good Start Gentle Plus, infant formula, prepared from liquid concentrate, made with tap water	0.160
Gerber Good Start Gentle Plus, infant formula, prepared from liquid concentrate, made with plain bottled water	0.160
Gerber Good Start Gentle Plus, infant formula, prepared from liquid concentrate, made with baby water	0.160
Gerber Good Start Gentle Plus, infant formula, prepared from powder, made with tap water	0.160
Gerber Good Start Gentle Plus, infant formula, prepared from powder, made with plain bottled water	0.160
Gerber Good Start Gentle Plus, infant formula, prepared from powder, made with baby water	0.160
Gerber Good Start Protect Plus, infant formula, NS as to form	0.160
Gerber Good Start Protect Plus, infant formula, prepared from powder, made with water, NFS	0.160
Gerber Good Start Protect Plus, infant formula, prepared from powder, made with tap water	0.160
Gerber Good Start Protect Plus, infant formula, prepared from powder, made with plain bottled water	0.160
Gerber Good Start Protect Plus, infant formula, prepared from powder, made with baby water	0.160
Gerber Good Start 2 Gentle Plus, infant formula, NS as to form	0.160
Gerber Good Start 2 Gentle Plus, infant formula, prepared from powder, made with water, NFS	0.160

Infant formulas (milk-based or not made with soy or other plant-based material) from NHANES 2011-2012*

Gerber Good Start 2 Gentle Plus, infant formula, prepared from powder, made with tap water	0.160
Gerber Good Start 2 Gentle Plus, infant formula, prepared from powder, made with plain bottled water	0.160
Gerber Good Start 2 Gentle Plus, infant formula, prepared from powder, made with baby water	0.160
Gerber Good Start 2 Protect Plus, infant formula, NS as to form	0.160
Gerber Good Start 2 Protect Plus, infant formula, prepared from powder, made with water, NFS	0.160
Gerber Good Start 2 Protect Plus, infant formula, prepared from powder, made with tap water	0.160
Gerber Good Start 2 Protect Plus, infant formula, prepared from powder, made with plain bottled water	0.160
Gerber Good Start 2 Protect Plus, infant formula, prepared from powder, made with baby water	0.160
America's Store Brand, infant formula, NS as to form	0.160
America's Store Brand, infant formula, prepared from liquid concentrate, made with water, NFS	0.160
America's Store Brand, infant formula, prepared from powder, made with water, NFS	0.160
America's Store Brand, infant formula, ready-to-feed	0.160
America's Store Brand, infant formula, prepared from liquid concentrate, made with tap water	0.160
America's Store Brand, infant formula, prepared from liquid concentrate, made with plain bottled water	0.160
America's Store Brand, infant formula, prepared from liquid concentrate, made with baby water	0.160
America's Store Brand, infant formula, prepared from powder, made with tap water	0.160
America's Store Brand, infant formula, prepared from powder, made with plain bottled water	0.160
America's Store Brand, infant formula, prepared from powder, made with baby water	0.160
Similac Expert Care for Diarrhea, infant formula, NS as to form	0.160
Similac Expert Care for Diarrhea, infant formula, ready-to-feed	0.160
Enfamil Nutramigen LIPIL, infant formula, NS as to form	0.160
Enfamil Nutramigen LIPIL, infant formula, ready-to-feed	0.160
Enfamil Nutramigen LIPIL, infant formula, prepared from liquid concentrate, made with water, NFS	0.160
Enfamil Nutramigen LIPIL, infant formula, prepared from powder, made with water, NFS	0.160
Enfamil Nutramigen LIPIL, infant formula, prepared from liquid concentrate, made with tap water	0.160
Enfamil Nutramigen LIPIL, infant formula, prepared from liquid concentrate, made with plain bottled water	0.160
Enfamil Nutramigen LIPIL, infant formula, prepared from liquid concentrate, made with baby water	0.160
Enfamil Nutramigen LIPIL, infant formula, prepared from powder, made with tap water	0.160
Enfamil Nutramigen LIPIL, infant formula, prepared from powder, made with plain bottled water	0.160
Enfamil Nutramigen LIPIL, infant formula, prepared from powder, made with baby water	0.160
Enfamil Nutramigen AA LIPIL, infant formula, NS as to form	0.160
Enfamil Nutramigen AA LIPIL, infant formula, prepared from powder, made with water, NFS	0.160

Infant formulas (milk-based or not made with soy or other plant-based material) from NHANES 2011-2012*

Enfamil Nutramigen AA LIPIL, infant formula, prepared from powder, made with tap water	0.160
Enfamil Nutramigen AA LIPIL, infant formula, prepared from powder, made with plain bottled water	0.160
Enfamil Nutramigen AA LIPIL, infant formula, prepared from powder, made with baby water	0.160
Enfamil Pregestimil LIPIL, infant formula, NS as to form	0.160
Enfamil Pregestimil LIPIL, infant formula, ready-to-feed	0.160
Enfamil Pregestimil LIPIL, infant formula, prepared from powder, made with water, NFS	0.160
Enfmail Pregestimil LIPIL, infant formula, prepared from powder, made with tap water	0.160
Enfmail Pregestimil LIPIL, infant formula, prepared from powder, made with plain bottled water	0.160
Enfmail Pregestimil LIPIL, infant formula, prepared from powder, made with baby water	0.160

Infant formulas obtained from NHANES 2011-2012 food survey that do not indicate production from soy or other plant-based materials. NFS = not further specified; NS = not specified.

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October 20, 2017

Nadine Bewry, Ph.D., MPH
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Re: Questions concerning GRAS notification (GRN) 000716

Dear Dr. Bewry,

We received a request from you for Burdock Group to provide responses to several different questions posed by your division concerning the notification of the conclusion of GRAS status for bovine whey-derived osteopontin (designated as GRN 000716). The specific questions you have provided are indicated below, numbered and in italics; we have addressed your questions as indicated by a "REPLY" statement following the numbered question.

Please let me know if you need any additional information for this notification of GRAS status.

Sincerely,

Ray A. Matulka, Ph.D.
Director of Toxicology
Burdock Group

Chemistry

1. The notifier describes a nitrogen quantification method to quantify the protein content of their ingredient. However, the notifier also described other methods extensively within the notice, including the ELISA method.

Please clarify what methods were used to quantify the protein content of their ingredient.

REPLY: The total nitrogen content of the Lacprodan OPN-10 raw material is determined under ISO 8968-3/ IDF 20-3, the determination of nitrogen content in milk and milk products. Two different factors were used, either nitrogen multiplied by a factor of 6.38, commonly used to determine protein levels in dairy raw materials (WHO-FAO, 2008), or nitrogen multiplied by a factor of 7.17, a more accurate factor based on the purity of the ingredient, its post translational modifications and other factors (to accurately reflect protein content in raw material contributed by OPN) to determine the protein content of the ingredient (de Boer, 2014). The Lacprodan OPN-10 specifications lists the total nitrogen content calculated with the factor of 6.38.

Toxicology

2. On page 16, Table 4, the notifier states that the predicted bovine whey-derived osteopontin (OPN) exposure to infants <1 month of age from the intended use at the 90th percentile is 39.5 mg/kg bw/day. On Pg. 86, the notifier states that the Acceptable Daily Intake (ADI) for bovine whey-derived OPN is 50 mg/kg bw/day based on No-Observed-Adverse-Effect-Level (NOAEL) of 2500 mg/kg bw/day from a published teratogenicity study in rats. However, traditionally, the safety factor for interspecies and intraspecies extrapolations using rodent studies is 100 (Benford, 2000). Thus, the NOAEL of 2500 mg/kg bw/day would be extrapolated to ADI of 25 mg/kg bw/day.

Please provide a rationale for:
- *Why the safety factor of 50, instead of 100, is appropriate.*
- *Why the estimated exposure of 39.5 mg/kg bw/day at the 90th percentile in a sensitive and vulnerable population is not a safety concern.*

REPLY: The OPN molecule contained in the Lacprodan OPN-10 product is substantially similar to the OPN molecule that is naturally found in human breast milk, and is being added to infant formula at a level not exceeding that found in breast milk (Schack et al., 2009). The NOAEL from the 90-day toxicity study was 2000 mg/kg bw/day, the highest dose evaluated, and the NOAEL of 2500 mg/kg bw/day from the published teratogenicity study was also the highest dose evaluated in the study (Kvistgaard et al., 2014). The evaluation by the Expert Panel was that, based on the lack of toxicologically relevant adverse events in any of the safety studies conducted on OPN-10, the level of estimated intake at 50 mg/kg bw/day was safe for the intended consumers (i.e., infants). In addition, OPN from bovine sources has been consumed for centuries at low levels when contained in dairy products.

Further, the estimated exposure of 39.5 mg/kg bw/day at the 90th percentile is not a safety concern for the same reasons that were provided above, as the safety studies did not indicate adverse effects at any level, and the amount of OPN from OPN-10 when added to infant formula is at or below the levels of OPN found in human breast milk as cited in the published literature (Schack et al., 2009).

Lastly, with regard to the 100-fold safety factor, this is an arbitrary, one-size-fits-all rule of thumb and not part of any regulation pertaining to a GRAS conclusion. Publications on determining the safety of novel foods and food ingredients stresses that safety assessments for such products should take account of the characteristics of the individual ingredient, including in the assessment an understanding of the origin, production, compositional analysis, nutritional characteristics, previous human exposure and anticipated use of the product (Edwards, 2005; Jones, 2007). The information indicated above and provided in the notification confirms that a 100-fold safety factor is not necessary for Lacprodan OPN-10. The 100-fold safety factor, is however, recommended in U.S. regulations, but specifically for food and color additive petitions and, is not required for a conclusion of GRAS status. In this instance, OPN-10, under the intended conditions of use, was concluded, among qualified experts, to be safe under the conditions of its intended use, and to meet the statutory and regulatory safety standard of "a reasonable certainty of no harm" and, is therefore GRAS.

3. OPN is similar to lactoferrin in that they both possess immunomodulatory bioactive properties. It has been previously reported that lactoferrin binds OPN at approximately 3:1 ratio (Yamniuk et al., 2009). Lactoferrin is considered lower in non-supplemented infant formulas compared to breastmilk.

Given that many infant formulas do not supplement the formula with lactoferrin to levels normally observed in breastmilk, please provide a rationale as to why increasing the levels of OPN does not negatively impact the bioavailability of lactoferrin in bovine whey-derived OPN-supplemented infant formulas.

REPLY: Yamniuk et al. (2009) described the in vitro binding of osteopontin to lactoferrin in titration experiments performed in HEPES buffers. The pH conditions under which the experiments are performed are not given in the paper, but HEPES buffer is usually used at neutral pH (6-8). The highly acidic environment of the stomach is expected to protonate acidic groups of the amino acid side chains in osteopontin and hence, eliminates potential electrostatic interactions the protein may have with other proteins, including lactoferrin. Likewise, the action of pepsin in the stomach on both lactoferrin and osteopontin is also expected to contribute to dissociation of whatever complexes they may have formed. It is therefore highly unlikely that osteopontin influences the bioavailability of lactoferrin in milk or infant formulas in any significant way.

In general, in vitro studies of protein-protein interactions can only to a limited extent be used as indications for binding under in vivo conditions, especially at very high protein concentrations as in milk. There are few proteins from milk that have been shown, under carefully designed in vitro conditions, can interact with each other. Examples of this include lactoferrin electrostatic binding to milk beta-lactoglobulin and of lactoferrin binding to albumin (Lampreave et al., 1990); both of which are present in milk and infant formulas in significantly higher concentrations than OPN. Even if such a complex between OPN and lactoferrin may be found in vitro, the low pH of the stomach and bile emulsifiers and pancreatic enzymes make it very unlikely that it would even exist in vivo. It is also noted that Yamniuk et al. (2009) concluded that a complex between OPN and lactoferrin would likely be considered a benefit to the consumption of both OPN and lactoferrin, not a detriment; the authors suggested that "OPN may act as a carrier protein for LF [lactoferrin] in milk".

4. Estimation of the level of human OPN (hOPN) in breastmilk was based on a single study (Schack et al., 2009) of 29 samples from Denmark, a country considered to have relatively homogeneous population (Athanasiadis et al., 2016). As stated by the study authors as well as the notifier, there is also a considerable large variation in the level of OPN detected.

Please address the following:
• Given the difference in demographics between nursing mothers in Denmark and the United States as well as the existence of large variations obtained from a small sample size, elaborate on why ~138 mg/L of OPN was chosen with respect to its level being generally recognized as safe. In your answer, elaborate on why the concentration of OPN (i.e. mg/L of breastmilk) was chosen rather than %OPN/total protein in breastmilk for the estimation of appropriate amount of OPN to be added to infant formula.
• Given that one of the components in your safety narrative relies on the assumption that ~138 mg/L of OPN is the "normal" level of OPN found in all breastmilk across demographics and days post- parturition, it appears that the reliability of this information is vital to your assessment. If this is not the case, please elaborate.

REPLY: While the presence of OPN in milk was initially reported in a published paper in 1989 (Senger et al), there have been few published papers since that time that determine the concentration of OPN in human breastmilk. Among those that analyzed human breast milk levels of OPN, Schack et al. (2009) utilized a method that has since been evaluated as being specific for human OPN and accounted for potential confounding factors in the quantification of OPN in breast milk. Schack et al. (2009) provided the concentration of OPN in human and bovine milk in both a "mg/L" and "%OPN/total protein" basis, but conducted most comparisons with infant formula utilizing the "mg/L" data. Utilizing the "mg/L" dataset, along with the amount of formula consumed per day allowed for a more direct comparison with available consumption data and safety study intake data. In addition, previous notifications of GRAS status indicated the addition of an infant formula ingredient on a "mg/100 ml formula" basis (Morinaga, 2014) and therefore considered acceptable for analysis of an infant formula ingredient.

Recently, a multicenter study comprising 629 mothers from China, Denmark, Japan and South Korea was conducted. The data from the study is not published, but may be viewed as corroborative and will soon be submitted for publication. In brief, the median OPN content across sites, based on the first sample from each of the 629 mothers was 157.00 mg/L (IQR 95.40-229.50, min-max 2.19-474.84). Based on the first sample from the 495 mothers with a corresponding protein concentration available, the median OPN concentration was 172.04 mg/L (IQR 114.36-240.76, min-max 11.99-474.84), and the median OPN/protein% was 1.79% (IQR 1.25-2.56, min-max 0.14-16.47).

Some variation among study sites was observed. In China the median OPN concentration was 266.22 mg/L, (IQR 210.82-323.92, min-max 100.52-455.68), corresponding to 2.69% (IQR 2.18-3.58, min-max 0.84-16.47) of the protein concentration. In South Korea the mean OPN concentration was 216.20 mg/L (IQR 160.56-268.80, min-max 35.56-474.84), corresponding to 1.76% (IQR 1.27-2.09, min-max 0.27-3.52) of the protein concentration (high protein concentrations were observed in the South Korean milk). In the Japanese milk the median OPN concentration was 185.00 mg/L (IQR 151.00-229.50, min-max 60.00-358.00), corresponding to 2.39% (IQR 1.77-2.90, min-max 0.71-6.24) of the protein concentration. In the Danish breast

milk the median OPN concentration was 99.68 mg/L (IQR 67.45-149.10, min-max 2.19-355.40). Regarding the 185 samples with enough material for macronutrients analysis, the OPN concentration was 107.40 mg/L (IQR 68.19-156.20, min-max 11.99-355.40), corresponding to 1.32% (IQR 0.88-1.71, min-max 0.14-8.70) of the protein concentration.

Variation in the OPN content do exists among mothers and among different geographical populations. As the Danish mothers' milk have the lowest OPN level among the populations analyzed, it reasonable to claim that a level of 138 mg/L can be regarded as safe. The data from the Asian populations indicate that much higher levels could also be regarded as normal and safe.

Several studies have shown that the composition of human milk vary geographically. Recently, it was shown that human milk oligosaccharide concentrations and profiles varied extensively among milk samples from 11 international cohorts from four different continents (McGuire et al., 2017). In an older study, the content of the bioactive milk protein lactoferrin was found to be significantly higher in milk from Ethiopian than Swedish mothers (Lönnerdal et al., 1976). Likewise, a recent study showed that the levels of both lactoferrin and lactadherin in breastmilk of both Indian and South African women were significantly higher than those from women in the United States (Moon et al., 2013).

The soon to be published data provided to Arla reported the content of OPN in breast milk that was analyzed from 629 mothers from China, Denmark, Japan and South Korea (Table 1). This multicenter study obtained a total of 829 breast milk samples from the subjects (521 mothers provided one sample, 16 provided 2 samples, and 92 mother delivered 3 samples at different visits). This corroborative data found that, across all sites and when delivering the first sample, the median OPN content was 157 mg OPN/L breast milk, which is slightly higher than the conservative 138 mg/L published by Schack et al (2009). The data also showed that there was a decrease in the OPN concentration with increasing infant age, but this inverse relationship was evident only within the first three months of life.

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Table 1. Maternal and infant characteristics, absolute (mg/L) and relative (%) OPN concentration

	China	Denmark	Japan	South Korea
Period of collection	February 2016 – October 2016	May 2012 – February 2014	February 2015 – September 2016	March 2017 – April 2017
Mothers (*N*)	76	318	118	117
Number of samples (*n*)	225	318	169	117
Number of visits	1-3	1	1-3	1
Samples collected (*n*)				
Visit 1	75	318	79	117
Visit 2	76	-	69	-
Visit 3	74	-	21	-
Number of mothers with				
One sample	1[1]	318	85[6]	117
Two samples	1[2]	-	15[7]	-
Three samples	74	-	18	-
Infant age (weeks), median (IQR)				
Visit 1	4.29[3]	17.4 (14.9-19.3)	8.0 (6.0-9.1)	3.9 (3.0-4.9)
Visit 2	8.58	-	16.9 (14.1-18.9)	-
Visit 3	12.87	-	25.1 (22.7-26.3)	-
Maternal age (years), mean ± SD (min-max)				
Visit 1	29.8 ± 3.6 (20.33-41.41)	31.4 ± 4.0 (21.73-43.43)	32.6 ± 4.2 (24.12-41.87)	32.2 ± 3.6 (22.28-42.24)
Visit 2	29.9 ± 3.6 (20.40-41.49)	-	31.6 ± 4.2 (21.55-42.02)[8]	-
Visit 3	29.9 ± 3.6 (20.53-41.58)	-	33.5 ± 4.3 (26.69-42.19)	-
OPN (mg/L), median (IQR)				
Visit 1	266.24 (212.72-325.52)	99.68 (67.45-149.10)	182.50 (151.00-225.00)	216.20 (160.56-268.80)
Visit 2	195.20 (147.50-237.46)	-	169.50 (118.00-229.50)	-
Visit 3	175.92 (117.44-210.40)	-	119.00 (81.50-198.00)	-
OPN/protein (%), median (IQR)				
Visit 1	2.72 (2.18-3.58)[4]	1.32 (0.88-1.71)[5]	2.39 (1.75-2.81)	1.76 (1.27-2.09)
Visit 2	2.24 (1.60-3.34)	-	2.20 (1.50-2.80)	-
Visit 3	2.05 (1.54-2.72)	-	1.80 (1.06-2.50)	-

1) At visit 1; 2) At visits 1 and 2; 3) Corresponding to day 30, 60 and 90 respectively; 4) $n = 74$ due to macronutrients analysis device breakdown; 5) $n = 185$ due to lack of sample material; 6) At visit 1, $n = 46$; at visit 2, $n = 39$; 7) At visits 1 and 2, $n = 12$; at visits 1 and 3, $n = 3$; 8) Lower than the previous due to a number of mothers providing their only sample at the second visit

5. Although ELISA quantitation described in Nagatomo et al. (2004) may be considered an overestimation, it appears that majority of hOPN in whey protein (presumably from crude preparations) in transitional and mature human milk is in the full-length form as assayed by Western blotting analysis using 10A16 monoclonal antibody (Fig. 2 of the manuscript). In fact, Bissonnette et al. (2012) confirmed the absence of cleaved hOPN form in breastmilk. However, the purified bovine whey-derived OPN in the notice consists mainly of cleaved peptides (80% C-terminal truncated vs. 20% full-length, pg. 9 of notice). Furthermore, as stated by Christensen and Sorensen (2014), "... the cleavage pattern observed for hOPN in milk is not necessarily identical to that for bOPN ... [k]knowledge of the exact cleavage sites is important, as ***small differences in the C-terminal of the fragments*** *may have significant effects on the interaction between these and integrins. (emphasis added)"*

Please discuss why the potential differences in the proportion of full-length vs. cleaved peptide(s) between hOPN in human milk and bovine OPN (bOPN) in bovine milk are not a safety concern.

REPLY: We have reached out to Dr. Sorensen (Aarhus University, Denmark) who has +20 years of experiences with human and bovine milk OPN and has published extensively on OPN and other milk constituents, who has stated the following:

> we do always observe several OPN fragments when analyzing human milk by SDS-PAGE or Western blotting. The degree of fragmentation is subject to large variation among individual mothers, which is most likely a reflection of the activities of proteases that cleave OPN in the most susceptible region around the thrombin/plasmin cleavage site. The staining and migration of the OPN fragments varies significantly from system to system and this could be some of the explanation to why some articles describes human milk OPN as less fragmented or not fragmented.
>
> To emphasize the relatively rare occurrences of mothers milk without OPN fragmentation, we have a very few milk donors that we have designated "super-moms", to reflect that they have very little fragmentation of their milk OPN (approx. 90% full length OPN). Milk from these donors is used to purify the pure full length OPN form, which is used for structural OPN studies in our laboratory.

In Christensen et al. (2010), the fragmentation of OPN in human milk is thoroughly characterized by Western blotting, reverse-phase HPLC and mass spectrometry (MS) identification of sites of cleavage. The Western blotting (using polyclonal antibodies which recognizes several different epitopes and modification variants of OPN) of human milk OPN purified from pooled donor milk from several mothers showed significant fragmentation of OPN (Fig 1 in the Christensen article, provided below).

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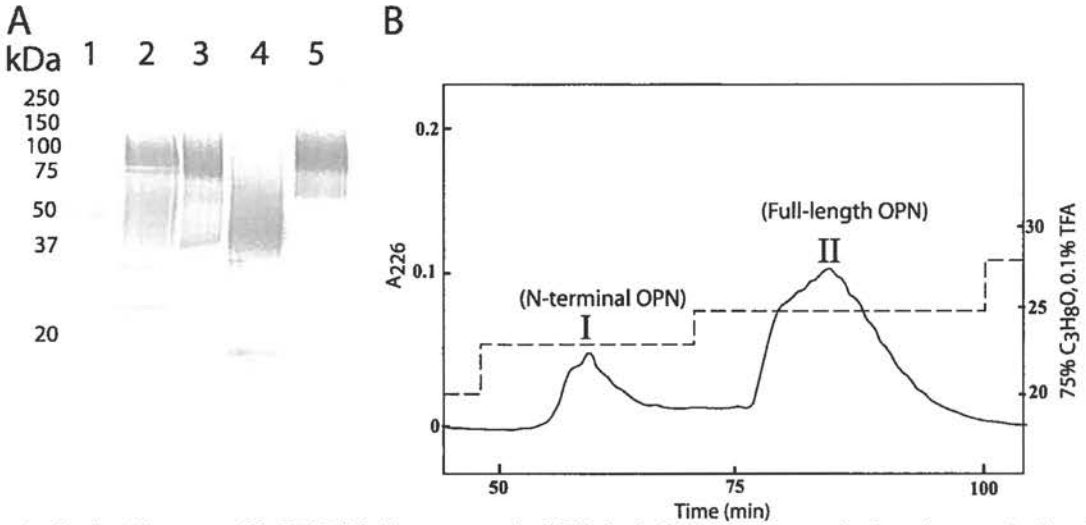

Figure 1. Analysis of human milk OPN (Christensen et al., 2010a). A, Western blot analysis using a polyclonal OPN antibody. Lane 1, molecular mass standards. Lane 2, skim milk. Lane 3, OPN purified from milk. Lane 4, fraction I from B. Lane 5, fraction II from B. B, RP-HPLC of purified human milk OPN.

The fragmented forms of human OPN were purified and characterized and six sites of cleavage were determined in the region close to the OPN integrin binding sites in OPN. Hence, it is clear that several truncated OPN forms exists in human milk. In Christensen and Sørensen (2014) the cleavage forms of OPN in bovine milk were determined and though they are not exactly the same as those observed in the human milk OPN, they are all located in the same region within a couple of amino acids from each other. A 100% match in proteolytic cleavage between species cannot be expected, as there are also large individual variations. Much of the variation in the cleavage sites is most likely due to trimming of the C-terminal by carboxypeptidases in the milk.

The antibodies used by Nagatomo et al. (2004) for Western blotting are the same antibodies used in the IBL OPN ELISA, which has been shown to overestimate the amount of OPN in milk quite significantly. This overestimation is most likely due to incorrect recognition of the OPN in milk and/or the OPN standard. Hence, it is also questionable whether the antibodies recognize the OPN milk forms correctly and quantitatively in Western analyses. It is not clear from the article how many individual mothers actually contributed to the milk, and how and why they use milk whey protein for Western analyses is also unclear. The normal procedure would be to apply a few microliter of fresh skimmed milk to the gel. The preparation of the milk whey protein (and perhaps drying?) could potentially include steps that resulted in a loss of some of the fragments of OPN. Though, I would claim that fragmentation is actually seen in Fig 2 in Nagatomo et al (2004). The "smear" observed to migrate at 50 kDa and faster in panel (a) represents N-terminal OPN fragments. In panel (b) using the 10A16 antibody this staining is not observed as this antibody recognizes the epitope KSKKFRRPDIQYPDATDE, present in the C-terminal part of OPN. In this panel C-terminal fragments are seen migrating at 33 kDa. These fragments are not observed in panel (a) as the antibody used here is O-17 which recognizes an N-terminal epitope in OPN. So in conclusion, Nagatomo et al. (2014) actually nicely show that human milk OPN is indeed fragmented.

In Bissonnette et al. (2012) it is noted that human milk OPN is not fragmented and no truncated form is present in milk. This conclusion is based on Western of a single human milk sample (the origin of this is not stated in the article) and of a commercial human OPN sample. This commercial human OPN is a recombinant protein, which of course is not fragmented. Based

on the current knowledge, it is known that in the majority of women OPN in milk is fragmented (except for a few super moms) and also cleavage patterns between bovine and humans are substantially similar.

6. On page 23, paragraph 4, and page 57, paragraph 3, there are blank parentheses after the citations.

Please indicate whether this is a typo or missing references.

REPLY: The blank parentheses after the citations were typographical errors. Please disregard.

7. On page 79, in discussing findings of Lonnerdal et al. (2016), the notifier states: "The decrease ($P<0.05$) plasma threonine concentration in the F130 group compared to the F65 group was not expected by the authors. The authors did not speculate on a reason for this slight, but significant change."
Since the GRAS conclusion is made by the notifier, not the study authors, please clarify whether this "slight, but significant change" is a safety concern.

REPLY: Formula-fed infants generally have considerably higher plasma threonine concentrations (due to the high protein content and threonine-rich whey protein) than breast fed infants (Sandstrom et al 2008; Haschke-Becher et al., 2016). It is generally considered that lowering the plasma concentrations of formula-fed infants, making them more similar to breast-fed infants, would be beneficial. The Lonnerdal et al (2016) reported that the F130 dose group had plasma threonine levels (at 6 months) similar to plasma threonine levels found in the breast fed infants. Achieving infant plasma amino acid levels similar to levels found in breast milk-fed infants is considered optimal, as breast milk is considered the gold standard of infant nutrition. Thus, this decrease in plasma threonine levels in the F130 dose group is NOT a safety concern.

8. On page 22, in discussing the association of variant splice forms of OPN to cancer, the notifier states that the OPN-a form, a full-length native OPN present in human bovine milk, "has never been associated with such malignant properties."

However, FDA's literature search has identified two published reports (Blasberg et al., 2010; Hao et al., 2017) in which OPN-a form has been associated with non-small cell lung cancer:
Blasberg et al. concludes:
"OPNa overexpression was associated with increased bovine capillary endothelial tubule length and vascular endothelial growth factor secretion … These findings may lead to therapeutic strategies for selective isoform inhibition in non-small cell lung cancer."
Hao et al. state:
"Collectively, our results have clearly demonstrated the clinical value of OPN-a in human non-small cell lung cancer as a potential target for therapy and a potential prognostic factor. The study has also revealed the importance of OPN-a in the aggressiveness of lung cancer cells with a particular relevance to bone metastasis related cell function of lung cancer cells."

Please provide a brief explanation of why this information does not impact the notifier's safety assessment.

REPLY: Blasberg et al (2010) analyzes the expression of the three genetic isoforms of OPN in non-small cell lung cancer cells and immortalized bronchial epithelial cells. In these in vitro experiments using cell lines, they find that the OPN-a isoform is expressed by the cancer cells and that OPN-a overexpression is associated with events that could be involved in tumorigenic events, such as angiogenic properties. By overexpressing the OPN isoforms the authors intentionally create unnatural conditions, and since OPN is involved in numerous physiological processes, including tissue transformation and growth, it is not surprising that overexpression of OPN increased VEGF expression. The new finding of the paper is that the authors have analyzed the three isoforms and now report different capabilities of those in their in vitro assay. As the title states this study concerns lung cancer osteopontin, which is expressed by the lung cancer cells. It is a mechanistic in vitro study and there is absolutely no evidence that ingestion of milk osteopontin should in any way be correlated with development or progression on non-small cell lung cancer cells.

Hao et al. (2017) claims that OPN-a could be used as a prognostic marker for non-small cell lung cancer. This is not to be disputed, as numerous biomarkers have been suggested for such tumor site biomarkers. This does not mean that OPN-a causes or promotes the cancer, simply that the protein is upregulated during tumorigenic events. OPN is also upregulated under infections (as part of the immunological process), bone growth and fractures (during bone remodeling), in the growing fetus blood and many other events in which the body is undergoing traumas, transformations and growth.

Hao et al. (2017) also show that OPN is capable of binding the αVβ3 integrin and links this to bone metastatic events. The αVβ3 integrin is one of the most prominent and studied receptors for OPN, so it is well known that OPN binds to this receptor, though, OPN also binds this receptor under normal non-malignant conditions, such as when the osteoclasts use this receptor to anchor to the mineralized matrix of bone, via OPN, during bone remodeling processes. Use of OPN as a prognostic marker for some cancer types does not imply that OPN is causing cancer, but implies that the processes involved in cancer progression could use endogenously expressed OPN forms as a mediator molecule in some of the cellular processes, like cell anchoring. Another "role" of OPN in many cancers (like it is in infections and inflammations) is that OPN is actually part of the immune response to the cancer, as OPN has been shown to be expressed by immune cells and to take part in the cellular immune response (Brown, 2012).

Overall, the in vitro studies of Hao *et al.* (2017) and Blasberg *et al.* (2010) using OPN expressed by cell lines do not in any way impact the safety assessment of milk OPN intended for ingestion.

Additional Questions and Comments

1. On page 18, the notifier states, "*... would only be used in the wet blending-spray drying process of the production of infant formula, where ingredients are blended in water, homogenized, pumped to a heat exchanger for pasteurization, and then spray dried into a powdered product; for full- or near-full-term infants...*"

Please clarify the meaning of "near-full term infants." The notifier states that this ingredient is not intended for use in products that are preterm focused or exempt.

REPLY: OPN-10 is not intended for use in products that are preterm focused or exempt. The use of the term "near-full term infants" should be disregarded, as this term had been obtained from previous publications discussing infants and does not fit current definitions of "full-term infants. Please disregard use of the word "near" in this context.

*2. On page 5 (A.2), the notifier states "OPN-10 contains at least 78% protein (N*6.38), greater than 95% of which is bovine whey-isolated OPN."*

Please clarify what is the other 5% of protein.

REPLY: The enriched protein in Lacprodan OPN-10 is predominantly OPN as can be seen from the attached chromatogram (APPENDIX 1). We conservatively wrote the specification that the raw material contains greater than 95% OPN to account for any batch to batch variation and analytical uncertainty. Our production batches show greater 98 to 99% purity. Due to high purity observed, we have not taken the OPN fraction and conducted Mass Spectrometry analysis to see other minor proteins. A very small fragment of peptides of OPN or other dairy proteins cannot be ruled out. Pre-clinical safety (Kvistgaard et al 2014) trials and a clinical trial (Lonnerdal et al 2016) have been conducted with the Lacprodan OPN-10 material that includes any possible minor proteins that may be present in Lacprodan OPN-10.

3. On page 5, the second paragraph, the notifier states that "...OPN is safe for human consumption as a food ingredient in term nonexempt milk-based infant formula (which includes formula for infants 6-12 month of age)..."

Please clarify whether the ingredient will be added to non-exempt term infant formula for infants 0-12 months of age or only to non-exempt term formula for infants 6-12 months of age.

REPLY: The ingredient is to be added to non-exempt term infant formula for infants 0-12 months of age.

4. On page 45, first paragraph: Some of the cited references do not appear to support the statements in this paragraph. The Greer reference only concerns premature infants; there is no information in this reference that addresses the amount of human milk that a term infant will consume daily. The information on the American Academy of Pediatrics (accessed September 1, 2015) website does not support the information provided in this paragraph. Additionally, we are unable to find the stated information in the US Environmental Protection Agency 2011 reference. The Butte 2005 reference appears valid.

Please provide an accurate statement on the daily consumption of infant formula/human milk for term infants with appropriate references.

REPLY: The estimated daily consumption of OPN-10 was obtained utilizing the NHANES 2011-2012 national food survey data set. This information was the basis of the amount of OPN-10 that would be consumed when added to infant formula (mg/day). To determine "mg/kg bw/day", the information obtained from the EPA (2011) reference (Chapter 8; Body Weight Studies) was utilized. The website link to Chapter 8 of the EPA (2011) reference, which contains the infant body weight information, is provided as follows:
https://cfpub.epa.gov/si/si_public_file_download.cfm?p_download_id=526169

Daily consumption of infant formula/human milk has been evaluated by Hester *et al.* (2012), who found (through a systematic review of the literature) that "although variation in breast milk intakes was apparent during the first few days of life, "breast milk intake tended to increase from 21.5±0 4.2 mL/day on day 1 to 495.3±33.4 mL/day on day 7 to 673.6±29 mL/day after 14 days." When analyzing formula consumption, Hester *et al.* (2012) reported that "infant formula intake increased from 170.5 mL/day on day 1 to 265.0±67.7 mL/day on day 2 and to 761.8±18 mL/day after 14 days". The United Kingdom's Scientific Advisory Committee on Nutrition (SCAN, 2011) has published dietary reference values for energy, which when using an average energy content of infant formula (EU Directive, 2006), an estimated amount of infant formula (Table 2) was calculated (First Steps Nutrition Trust, 2017). The resources provided here, when used in determining the consumption of Lacprodan OPN-10 by term infants, indicates similar Lacprodan OPN-10 intake levels to the data provided by the NHANES consumption assessment (e.g., addition of Lacprodan OPN-10 at 160 mg/L formula consumed by the highest consumers/body weight (boys 1 month of age) would result in consumption of OPN-10 at 31.04 mg/kg/day; 160mg/L * 0.194 L/kg/day).

Table 2. Estimated amounts of infant formula required, using energy requirements from the SACN report Dietary Reference Values for Energy (2011).

Age (months)	Median weight (boys)	Energy requirements (kcal)	EAR (kcal/kg/day)	mL formula/day	mL/kg/day
1	4.47	563	126	866	194
2	5.56	646	116	992	178
3	6.37	657	103	1,010	159
4	9.0	607	87	934	133
5	7.51	639	85	982	131
6	7.93	665	84	1,023	129
Age (months)	**Median weight (girls)**				
1	4.19	515	123	792	189
2	5.13	589	115	741	144
3	5.84	600	103	923	158
4	6.42	573	89	881	137
5	6.9	601	87	924	134
6	7.3	623	85	958	131

50th percentile weight for age from the UK-WHO charts
*Energy content of infant formula assumed to be 65 kcal/100 ml, the middle of the range stipulated in the EU Directive (2006)

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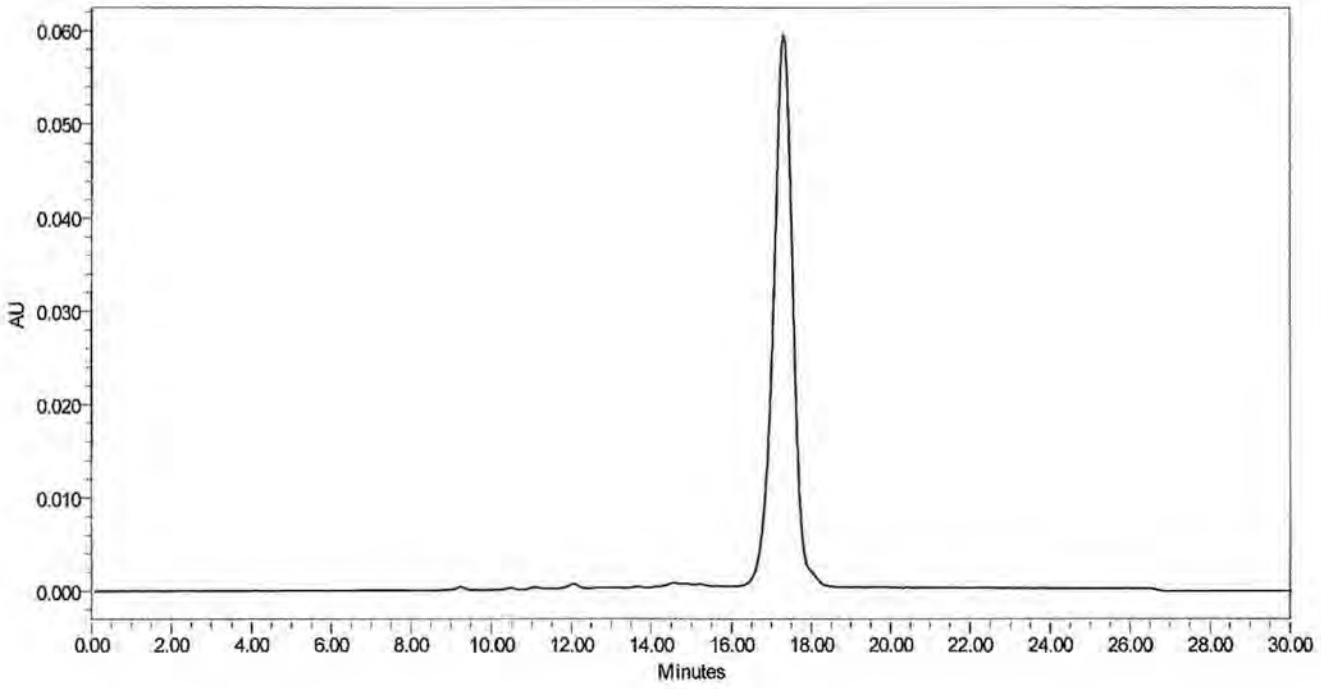
APPENDIX I. Chromatogram of Lacprodan OPN-10 purity (please see following page).

Empower 2 software

Default Individual Report

SAMPLE INFORMATION

Sample Name:	K. H	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	OPN210917SNS
Vial:	13	Acq. Method Set:	OPN271010
Injection #:	1	Processing Method	****
Injection Volume:	25.00 ul	Channel Name:	****
Run Time:	30.0 Minutes	Proc. Chnl. Descr.:	****
Date Acquired:	9/22/2017 6:55:52 AM CEST		
Date Processed:	****		



Error Log

Basic LC Peaks Table group contains information that doesn't match the data being reported.

From:	Ray Matulka
To:	Bewry, Nadine
Cc:	Carrie Kennedy
Subject:	RE: GRN 000716 (bovine whey-derived osteopontin (bOPN)): Meeting memorandum
Date:	Thursday, December 21, 2017 12:10:34 PM
Attachments:	image008.png

Dear Dr. Bewry,

We have reviewed the memorandum that you provided yesterday, and the client has requested that a statement made within the memorandum be modified, as we do not feel that the statement completely reflects the information that has been provided within the notification documents.

The statement that requires clarification is the following:

"There is no evidence provided in GRN 000716 or in the October 20, 2017 amendment to GRN 000716 that infants have been safety exposed to higher levels of bOPN from the intended use."

We believe that the 6-month clinical trial conducted by Lonnerdal et al (2016) that was referenced in GRN 0000716 provides growth (i.e., anthropometry), formula intake and adverse event evaluations, consistent with evaluating safety of infants. This study indicates no serious adverse events when infants consume bOPN (i.e., Lacprodan OPN-10, the new ingredient) at levels at the intended use level.

We would appreciate it that the above-stated sentence be removed from the memorandum, as we do not feel that it accurately reflects the scientific research that has been completed on OPN-10, and the lack of concern from the pediatric community on the results provided in this peer-reviewed, published (Journal of Pediatric Gastroenterology and Nutrition) clinical trial.

Please contact me if you have any questions concerning this request.

Sincerely,

Ray A. Matulka, Ph.D.
Director of Toxicology

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