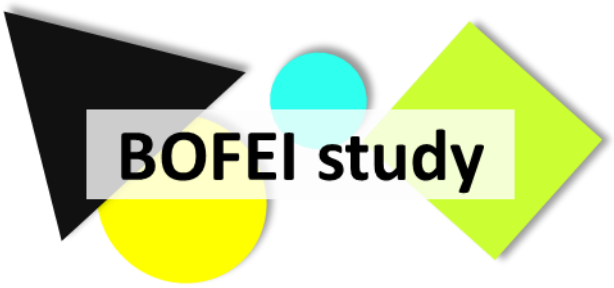

BOFEI study

Bovine Osteopontin for Elderly Immune Support

PROTOCOL TITLE 'Bovine Osteopontin for Elderly Immune Support'

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

Abbreviation	Definition
(S)AE	(Serious) Adverse Event
ABR	General Assessment and Registration form (ABR form), the application form that is required for submission to the accredited Ethics Committee; in Dutch: Algemeen Beoordelings- en Registratieformulier (ABR-formulier)
AE	Adverse Event
ALT	Alanine Aminotransferase
a.m.	In the morning
ALAT	Alanin amino transferase
AR	Adverse Reaction
AST	Aspartate Aminotransferase
BMI	Body Mass Index
CA	Competent Authority
CCMO	Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek
CFU	Colony Forming Units
CV	Curriculum Vitae
CTX-1	type I collagen cross-linked C-telopeptide
DNA	Deoxyribonucleic acid
DSS	Dextran Sodium Sulfate
EFSA	European Food Safety Authority
ELISA	Enzyme-linked ImmunoSorbent. Assay
EU	European Union
GCP	Good Clinical Practice
GDPR	General Data Protection Regulation; in Dutch: Algemene Verordening Gegevensbescherming (AVG)
GGT	Gamma-Glutamyl Transferase
GI	Gastrointestinal
IB	Investigator's Brochure
IC	Informed Consent
GRAS	Generally Recognized As Safe
h	Hour(s)
IFN	Interferon
(s)Ig	(secretory) Immunoglobulin
IL	Interleukin
IFNγ	Interferon gamma
IgA	Immunoglobulin A
IgM	Immunoglobulin M
IL	Interleukin

IMP	Investigational Medicinal Product
IMPD	Investigational Medicinal Product Dossier
LPS	Lipopolysaccharide
LMM	Linear Mixed Model
MEC	Medical ethical committee
METC	Medical research ethics committee (MREC); in Dutch: medisch-ethische toetsingscommissie (METC)
OPN	Osteopontin
PBMC	Peripheral Blood Mononuclear Cells
PFGE	Pulsed Field Gel Electrophoresis
P1NP	Procollagen-1 N-terminal propeptide
QPS	Qualified Presumption of Safety
MIP-1α	Macrophage Inflammatory Protein-1α
SPC	Summary of Product Characteristics; in Dutch: officiële productinformatie IB1-tekst
Sponsor	The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.
TLR	Toll Like Receptor
TLR4	Toll like receptor 4
TNFα	Tumour necrosis factor alpha
UAVG	Dutch Act on Implementation of the General Data Protection Regulation; in Dutch: Uitvoeringswet AVG
URTI	Upper Respiratory Tract Infection
U.S.	United States
V	Visit
wk	Week
WMO	Medical Research Involving Human Subjects Act; in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen

SUMMARY

Rationale:

Osteopontin (OPN) is a phosphorylated glycoprotein that is present in human milk and bovine milk. It has been shown to be involved in immune function. Arla Foods Ingredients has isolated a protein fraction from bovine milk which is rich in OPN, which is now commercially available as Lacprodan® OPN-10. In infants, supplementation with Lacprodan® OPN-10 is well tolerated, with clinically proven immunomodulatory outcomes such as downregulation of inflammatory cytokines and increases in T-cells and monocytes. Potential beneficial effects of Lacprodan® OPN-10 in adults and elderly have not yet been studied. During aging, the immune system undergoes profound decline, and specifically the responsiveness of the Th1-cell is attenuated. Given that OPN is a key factor in the Th1-response, this provides a potential for Lacprodan® OPN-10 to be a protein ingredient that is beneficial for immune health in both infants and the elderly. The current study design therefore aims to investigate the potential immune effects of Lacprodan® OPN-10 in elderly.

Objectives:

Primary objective:

Assess the percentage of responders to hepatitis B vaccination (i.e. attaining anti-hepatitis B antibody titres beyond 10 IU/L) in healthy elderly receiving a daily dose of Lacprodan® OPN-10, compared to a placebo product.

Secondary objectives:

- Compare the changes in serum anti-hepatitis B antibody titres between treatment groups
- Compare the change in circulating cytokines between treatment groups
- Quantify plasma levels of human and bovine OPN at baseline, and after intervention
- Determine whether intake of OPN-10 affects plasma levels of human OPN.
- Compare the change in serum LPS binding protein (LBP) between treatment groups
- Compare the incidence of infections during the trial, including specific upper and lower respiratory tract infections, between treatment groups
- Assess the safety of the selected intervention dose, including effects on markers of bone remodelling

Study design:

The study is designed as a double-blind, randomized, placebo-controlled trial, with two parallel treatment arms. After an 8-week intervention period, all subjects will receive a hepatitis B vaccination, at weeks 8, 10 and 12. Vaccination response will be measured at weeks 12 and 14. The intervention will be continued until the end of the study at week 14.

Study population:

Healthy, non-smoking men and women aged ≥60y and BMI 22-30, with anti-hepatitis B antibody titer ≤ 4 IU/L.

Intervention:
The active treatment consists of Lacprodan OPN-10, in the form of powder, that will be provided in 2 sachets in a dose of 2,5g/day for subjects weighing lower than 70 kg at screening, and a dose of 3,2g/day for subjects weighing 70 kg or more at screening. The sachets will be taken twice a day, with a meal.
The placebo treatment consist of powder in which Lacprodan® OPN-10 is replaced by maltodextrin, Total duration of the intervention is 14 weeks.

Main study parameters/endpoints:

- Anti-hepatitis B antibody titre
- Serum cytokines
- Plasma OPN (bOPN and hOPN)
- Serum LPS-binding protein (LBP)
- Infection incidence
- Clinical chemistry and hematology
- Serum P1NP and CTX1 (bone modelling markers)

Nature and extent of the burden and risks associated with participation, benefit and group relatedness:
The subjects will not benefit directly from participation in this study, apart from receiving a subject fee for their time investment plus reimbursement of traveling expenses.

Potential risks could be related to a) study product, b) study procedures or c) non-investigational product (hepatitis B vaccination).

Lacprodan® OPN-10 has a GRAS notification, based on the views of an independent Expert Panel and it is considered safe for the use by infants by the EFSA NDA Panel. Standard safety evaluations have shown no indications for potential risk involved with consumption. The dose administered in this study is 3.3% of the NOAEL calculated in safety studies.

The burden imposed by study procedures includes the daily intake of the study product, the (7) visits to the research location, the blood sampling (at 6 visits), faecal sample collection (3 times) and the vaccination injection (3 times). The collection of blood samples may produce discomfort or minor bleeding and the possibility of bruising at the site of the needle puncture. There is also a slight risk of infection at the site of the needle puncture. Side effects of hepatitis B vaccination that are reported to occur often or very often are: loss of appetite, irritability, headache, gastrointestinal symptoms (e.g., nausea, vomiting, diarrhea, abdominal pain), pain and redness at the injection site, fatigue, fever, malaise. Less common side effects include dizziness, myalgia, and influenza-like symptoms.
Overall, the risks associated with participation in this study are considered small.

1 INTRODUCTION AND RATIONALE

1.1 Osteopontin in infant formula

Osteopontin (OPN) is a highly phosphorylated glycoprotein that is present in human milk and bovine milk. OPN is involved in many physiological processes, such as for instance, biomineralization, bone and tissue remodelling, wound healing and immune function. It is expressed in various tissues and cell types, including epithelial cells and immune cells.

Arla Foods Ingredients (AFI) has isolated a protein fraction from bovine milk which is rich in OPN. This product, Lacprodan® OPN-10, has a protein content ranging between 86-90% (based on nitrogen (N) factor of 7.17), of which 95% is OPN. Several pre-clinical and one clinical study have been performed with this ingredient. The clinical study was performed in infants (Lonnerdal et al. 2016; West et al. 2017; Jiang et al. 2019). Lacprodan® OPN-10 in dosages of 65 mg/L or 130 mg/L was shown to be well tolerated in infants (Lonnerdal et al. 2016). In the same study, it was shown that OPN downregulates TNF-α in infants which had received infant milk formula with Lacprodan® OPN-10 when compared to infants who had received regular infant milk formula without Lacprodan® OPN-10. Although antibody response to tetanus vaccination was not increased (Lonnerdal et al. 2016), it was shown that infants which had received the formula with 130 mg/L Lacprodan OPN-10 had increased T-cell proportions compared with infants which had received infant milk formula with a lower dosage (65 mg/L) or the control formula (West et al. 2017). Furthermore, monocyte proportions were higher in the infants receiving Lacprodan OPN-10, when compared to the breast-fed reference group. Higher plasma levels of human OPN were observed in infants receiving the infant milk formula with bovine Lacprodan OPN-10 when compared to infants receiving the control formula, suggesting that bovine Lacprodan OPN-10 promotes the synthesis or secretion of endogenous human OPN (Jiang et al. 2019).

Potential immunomodulatory or other potentially beneficial physiological effects of Lacprodan OPN-10 in adults have not yet been studied. During aging, the immune system undergoes profound decline (Simon et al. 2015), and specifically the responsiveness of the Th1 cells is attenuated. Given that OPN is a key factor in the Th1-response, this provides a potential for Lacprodan® OPN-10 to be a protein ingredient that is beneficial for immune health also in the elderly. The current study design therefore aims to investigate the potential immune effects of Lacprodan OPN-10 in elderly.

1.2 Proposed mechanism of bovine versus human OPN

Osteopontin (hOPN) is a multifunctional protein involved in a wide range of bioactivities, including cell proliferation and differentiation, immunomodulatory functions, biomineralization, as well as myelination (Jiang et al. 2020). OPN exerts its multiple functions by binding to its receptors on cell membranes, activating various cell signalling pathways. It can be produced by a variety of cell types including epithelial cells and immune cells.

Human OPN exists in two forms: secreted OPN present in body fluids and intracellular OPN found in immune cells. Secreted OPN appears in most body fluids, such as milk, urine, blood, saliva, and bile.

hOPN is present at a high concentration in human milk, ~178 mg/L in colostrum and ~134 mg/L in transitional milk.

Bovine OPN (bOPN) has been shown to stimulate immune development in a similar way as endogenous OPN in young animals (Jiang et al. 2020), and LPS-injected piglets tended to have a lower incidence of diarrhoea after bOPN intake (Ren et al. 2019). In a study in piglets, an increased response to influenza vaccination has been observed after bOPN administration (Donovan 2012) Concentrations of bOPN in bovine milk and infant formula are markedly lower than in human milk, around 18 and 9 mg/L, respectively (Schack et al. 2009; Jiang et al. 2020).

No studies have been reported on the effect of oral ingestion of bOPN in adult humans. In adult mice, OPN intake reduced DSS induced weight loss and neutrophil activity (Kanwar et al. 2016).

The safety of Lacprodan® OPN-10 has been extensively evaluated (Kvistgaard et al. 2014; Matulka 2017; EFSA NDA Panel 2022) and there are no indications that adverse effects could occur. However, higher plasma levels of human OPN were observed in infants receiving infant milk formula with bovine Lacprodan® OPN-10 when compared to infants receiving a control formula (Jiang et al. 2019). It was postulated that small amounts of undigested bOPN are absorbed and, by similarities in amino acid sequences, promoted the synthesis or secretion of endogenous hOPN. The presence of high levels of hOPN is associated with inflammatory conditions, and cellular senescence and other potentially adverse effects. It is unknown how the administration of bOPN will affect the circulating levels of hOPN in elderly. This is one of the secondary research questions to be answered in this study.

The beneficial effects observed for bOPN consumption in infants and piglets lead us to hypothesize that bOPN will strengthen the immune response in elderly, without increasing markers of inflammation. Given the results of the piglet study (Donovan 2012) in which an increased antibody response to influenza vaccination was observed, we have chosen a vaccination response model to evaluate the potential immune modulating characteristics of bOPN.

1.3 Vaccination response model

In their Guidance on the substantiation of health claims (EFSA NDA Panel (EFSA Panel on Dietetic Products 2016), EFSA states that an increase in the number of responders to vaccination (i.e. attaining antibody titres beyond a cut-off value which is considered to protect against the infection) is an appropriate outcome variable for the scientific substantiation of claims related to immune defence against pathogens.

When administering a vaccination challenge, it is preferable to use a vaccine with no previous exposure. If previous exposure has taken place, which may occur in the case of an influenza vaccination, there is a risk of boosting the previous vaccination with the challenge, which has a different mechanism and has different kinetics.

Hepatitis B vaccination has been used previously to study the immune modulatory effects of a dietary intervention (Albers et al. 2003). In that study, the hepatitis B vaccination was used to mimic a primary viral infection capable of inducing both humoral and the cellular responses. Standard vaccination schedules yield protection rates of 94 – 99% (Coates et al. 2001), with lower responses in older persons (> 40 y). Albers et al (Albers et al. 2003) used an accelerated vaccination schedule to provide a suboptimal trigger to the immune system, reasoning that this would be more reflective of natural infections and would leave room for improvement of the response due to the supplementation with the dietary ingredient. The response rates observed were indeed lower than with standard vaccination schedules. For hepatitis B antibodies, a cut-off value has been agreed (>10 IU/L) that is considered to protect against infection.

1.4 Hypothesis

Based on the results of this hepatitis B vaccination study (Albers et al. 2003), we have designed a randomized controlled intervention study, with a suboptimal vaccination challenge, in a population of healthy elderly men and women, with no prior hepatitis B vaccination or infection.

We hypothesize that, in a group of elderly subjects receiving Lacprodan® OPN-10 in a dose of 40 mg/kg body weight per day, the protective anti-HepB antibody titre is attained in a statistically significantly higher % of subjects compared to subjects receiving a placebo product.

1.5 Secondary research questions

As mentioned in section 1.1, OPN has been shown to downregulate inflammatory cytokines (TNF-α) in infants (Lonnerdal et al. 2016). In a healthy adult population, it is not expected that the level of circulating cytokines would be affected by a dietary intervention. However, an effect might be observed in elderly, as the immune system in elderly in general functions less well and markers of inflammation (low-grade) are on average increased in elderly. The effect of OPN-10 on inflammatory cytokines will therefore be addressed in this study.

In addition, a specific form of hOPN has shown the capability to bind LPS and hence may potentially lower low-grade inflammation (Ge et al. 2014). Serum LPS-binding protein (LBP), considered a more stable marker of microbial translocation as compared to LPS, will be analysed to investigate this potential effect of OPN-10 intervention. In healthy older individuals, higher levels of LBP were found to be associated with worse physical function and inflammation (Stehle et al. 2012).

Human OPN influences bone homeostasis by inhibiting mineral deposition and stimulating osteoclast activity (Standal et al. 2004; Nagao et al. 2011), which potentially might result in lower bone mass or bone density. Markers of bone formation (P1NP) and resorption (CTX-1) will be assessed to monitor Lacprodan® OPN-10's effects on bone homeostasis (Szulc et al. 2017).

2 OBJECTIVES

2.1 Primary Objective:

To assess the percentage of responders to hepatitis B vaccination (i.e. attaining anti-hepatitis B antibody titres beyond 10 IU/L) in healthy elderly receiving a daily dose of Lacprodan® OPN-10, compared to a placebo product.

2.2 Secondary Objectives:

- Compare the change in serum anti-hepatitis B antibody titres from baseline (V1) to 14 days after the second vaccination (V6) between treatment groups
- Compare the change in serum anti-hepatitis B antibody titres from baseline (V1) to 14 days after the third vaccination (V7) between treatment groups
- Compare the change in circulating cytokines from baseline to 8 weeks intervention (V4) and at the end of the study (V7) between treatment groups
- Quantify plasma levels of hOPN and bOPN at baseline, and at 4 weeks and 8 weeks intervention
- Determine whether intake of OPN-10 affects plasma levels of hOPN.
- Compare the change in serum LPS binding protein (LBP) from baseline to 8 weeks intervention between treatment groups
- Compare the incidence of self-reported URTI or LRTI during the trial, between treatment groups using weekly reports of symptoms related to upper and lower respiratory tract infectionsAssess the safety of the selected intervention dose, including effects on markers of bone remodelling

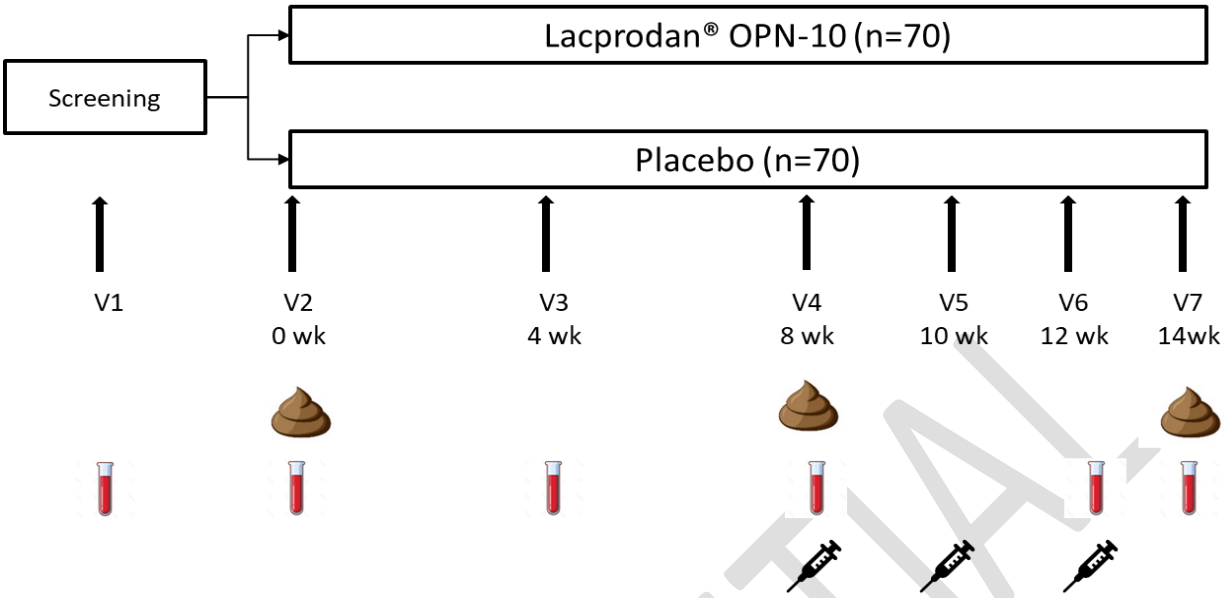
2.3 Exploratory Objectives:

- Compare the change from baseline in plasma brain-derived neurotrophic factor (BDNF) after 8 weeks intervention between treatment groups (optional)
- Compare the change in faecal microbiota composition after 8 and 14 weeks intervention between treatment groups (optional)

3 STUDY DESIGN

The study is designed as a double-blind, randomized, placebo-controlled trial, with two treatment arms.

After an 8-week intervention period, all subjects will receive a hepatitis B vaccination, administered by intramuscular injection, at weeks 8, 10 and 12. Vaccination response will be measured at weeks 12 and 14. The intervention will be continued until the end of the study at week 14.



The study will be conducted at the clinical research facilities of NIZO in Ede and EBMR in Almere.. For the clinical conduct within these facilities, EB Medical Research (Almere) will be responsible. The responsibilities of EB Medical Research include the recruitment and screening of study subjects, conduct of the study visits and medical supervision.

The schedule of assessments at the different visits is as follows:

	V1	V2	V3	V4	V5	V6	V7
	Screening	Week 0	Week 4	Week 8	Week 10	Week 12	Week 14
		Day 0	Day 28	Day 56	Day 70	Day 84	Day 98
Screening	x						
Eligibility check	x						
Clinical conduct							
Randomization		x					
Study product supply		x		x	x	x	
HepB Vaccination				x	x	x	
Blood sample collection		*x*	*x*	*x*		*x*	*x*
Serum anti HB titer	*x*					*x*	*x*
Serum chemistry & hematology	*x*	*x*	*x*	*x*			
Plasma circulating cytokines		*x*		*x*			*x*
Plasma/serum P1NP		*x*		*x*			
Plasma/serum CTX-1		*x*		*x*			
Plasma levels of OPN		*x*	*x*	*x*			
Serum LPS binding protein (LBP)		*x*		*x*			
Faecal sample collection		*x*		*x*			x
OPTIONAL BDNF		*x*		*x*			
AE registration		x	x	x	x	x	x

4 STUDY POPULATION

4.1 Population (base)

The study population will consist of healthy, non-smoking men and women aged ≥60y and BMI 22-30, with anti-hepatitis B antibody titer ≤ 4 IU/L. Subjects will be recruited from the general healthy population in the Netherlands, primarily from the local region around the study location (Ede).

4.2 Inclusion criteria

In order to be eligible to participate in this study, a subject must meet all of the following criteria:

Substantial

1. Age ≥60 and healthy
2. Self-reported regular Dutch eating habits as assessed by questionnaire (3 main meals per day)
3. Anti hepatitis B antibody titer ≤ 4 IU/L
4. Non-smokers (ex-smokers can participate)
5. BMI ≥22 and ≤30
6. In good health as assessed during screening, and the medical investigator's professional judgment
7. Adherence to habitual diet, no changes during study period
8. Signed informed consent

Procedural:

9. Ability to follow Dutch verbal and written instructions
10. Willing to accept disclosure of the financial benefit of participation in the study to the authorities concerned
11. Willing to accept use of all encoded data, including publication, and the confidential use and storage of all data for at least 15 years
12. Willing to comply with study procedures, including intake of study products and collection of stool and blood samples
13. Willingness to give up blood donation starting at screening and during the entire study

4.3 Exclusion criteria

A potential subject who meets any of the following criteria will be excluded from participation in this study:

Substantial

1. Prior HB vaccination or infection
2. Any vaccination in the past month or any scheduled vaccination during the study period
3. Acute infection in the past month

4. Treatment with oral antibiotics within 2 months of the start of the study,
5. Serious progressive disease or non-stabilized chronic illness (e.g., diabetes mellitus, cardiac insufficiency, respiratory insufficiency, cancer, chronic kidney or liver disease)
6. History of cancer
7. Gastrointestinal disorders (e.g., inflammatory bowel disease)
8. Immunodeficiency or autoimmune disorder
9. Use of immunosuppressive drugs (e.g. cyclosporine, azathioprine, systemic corticosteroids, antibodies)
10. Allergy or hypersensitivity to milk proteins, or lactose intolerance
11. Unexplained weight loss or weight gain of > 3 kg in the 3 months prior to pre-study screening
12. Evidence of current excessive alcohol consumption (>4 consumptions/day or >20 consumptions/week) or drug (ab)use
13. Mental status that is incompatible with the proper conduct of the study
14.

Procedural:

15. Not having a general practitioner, not allowing disclosure of participation to the general practitioner or not allow to inform the general practitioner about abnormal results.
16. Participation in any clinical trial including blood sampling and/or administration of substances starting 1 month prior to study start and during the entire study.
17. Personnel of NIZO, EBMR or AFI, their partner and their first- and second-degree relatives.

4.4 Sample size calculation

Sample size calculation was based on a publication by Albers et al (Albers et al. 2003). In this study (with another nutritional intervention), seroconversion after hepatitis B vaccination in the placebo group was 33%, and it was 63% in the active group. For the present study, we assumed seroconversion of 25% in the placebo group. To be able to detect a doubling of seroconversion (to 50%) in the active group, a sample size of 58 subjects per group was calculated (Sample Size Calculator (clincalc.com)), with an alpha of 0.05 and a power of 0.8. Taking into account a drop-out rate of ~15%, we propose to include 70 subjects per group.

5 TREATMENT OF SUBJECTS

5.1 Investigational product/treatment

The active intervention group will receive Lacprodan® OPN-10, as a food supplement. The placebo group will receive a supplement in which Lacprodan® OPN-10 is replaced by maltodextrin, but indistinguishable in appearance and taste.

5.2 Use of co-intervention / restrictions

Subjects will be instructed to maintain their habitual lifestyle. Regular use of food supplements can be part of such lifestyle, but any changes in dietary pattern during the study period should be discussed with the study staff first.

With respect to the test days the following restrictions need to be met:

- No alcohol consumption on the day prior to the visits
- On the visit days, the subjects visit the research facility after an overnight fast (no eating or drinking after 21.00h (except for water)

6 INVESTIGATIONAL PRODUCT

6.1 Name and description of investigational product(s)

Lacprodan® OPN-10 is an osteopontin (OPN)-based milk-derived protein product isolated from bovine whey and produced by commonly used ultrafiltration and ion exchange chromatography (IEC) processes in the whey industry to fractionate whey. The Lacprodan® OPN-10 batch used for this study has protein content of 79.6% based on conventional calculations (N*6.38) and 89.5% protein based on appropriate correction factors (N*7.17) included in calculations for this ingredient. OPN comprises > 99.5% of the total protein.

Lacprodan® OPN-10 is a proprietary food ingredient produced and sold by AFI containing OPN isolated from bovine whey processed under current Good Manufacturing Practices (cGMP). Further detailed information about the identity of the investigational product can be found in the notification of GRAS status for Lacprodan OPN-10 (Matulka 2017; EFSA NDA Panel 2022).

Table 1. Chemical composition of Lacprodan® OPN-10

Chemical composition (%)	Lacprodan® OPN-10
Protein (Nx6.38)	79.6
Protein (Nx7.17)	89.5
OPN of total protein	>99.5
Lactose	0.1

Fat	0.2
Ash	9.4
Calcium	2.49

Table 2. Main ingredient composition of the intervention products, per 1000 mg

Ingredient	Lacprodan® OPN-10 sachets	Placebo sachets
Lacprodan OPN-10	1400 (<70kg) or 1800 mg (>70 kg) (~12,70 or 16,40% of total weight)	0
Of which OPN	~89% is OPN	0
Maltodextrin	~78,00-82,00% (depends on amount of OPN-10)	94,55 %
Lemon flavour	2,10%	2,10%
Sucralose	0,17%	0,17%
Citric Acid Monohydrate	2,80%	2,80%
Colour yellow	0,38%	0,38%
Antifoam (for fast foam knockdown in protein shake)	0,04%	0%

6.2 Summary of findings from non-clinical studies

Results from *in vitro* and *in vivo* safety evaluations have been published (Kvistgaard et al. 2014). In vitro genotoxicity tests conducted according to accepted guidelines at up to 5000 µg/plate OPN failed to induce genetic mutations in *Salmonella typhimurium* strains and *Escherichia coli* strain and did not induce chromosomal aberrations or cytotoxicity in human lymphocytes. Administration of an acute dose of Lacprodan® OPN-10 (2300 mg/kg body weight) to male and female mice did not induce chromosomal damage or mitotic apparatus damage to erythroblasts from bone marrow. Lacprodan® OPN-10 was evaluated in a 13-week oral toxicity study in which rats were fed diets containing 0.5%, 1.0% and 2.0% Lacprodan® OPN-10. No test-article-related clinical observations or toxicological effects on body or organ weights, food consumption, ophthalmic effects, locomotor activity, hematology, clinical chemistry, urinalysis, or pathology were identified. In a teratogenicity study, administration of Lacprodan® OPN-10 up to 2500 mg/kg bw/day via gavage to pregnant rats had no effect on dams or pups. The No Observed Adverse Effect Level (NOAEL) for Lacprodan® OPN-10 in the 13-week toxicity study was 2.0% of the diet (equivalent to 1208 mg/kg bw/day in male rats and 1272 mg/kg bw/day in female rats).

OPN has been closely associated with 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. 2009). Nau et al. (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, Chupp 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 (Matulka 2017).

In young animals, bOPN has been shown to stimulate immune development in a similar way as endogenous OPN (Jiang & Lonnerdal 2020), and LPS-injected piglets tended to have a lower incidence of diarrhoea after bovine OPN intake (Ren, Hui et al. 2019). In a study in piglets, an increased response to influenza vaccination has been observed after bovine OPN administration (Donovan 2017).

6.3 Summary of findings from clinical studies

Clinical studies with OPN-10 have been performed in infants. Lacprodan® OPN-10 in dosages of 65 mg/L or 130 mg/L was shown to be well tolerated in infants (Lonnerdal, Kvistgaard et al. 2016). In the same study, it was shown that OPN downregulates inflammatory cytokines (TNF-α) in infants which had received infant milk formula with Lacprodan® OPN-10 when compared to infants who had received regular infant milk formula without Lacprodan® OPN-10 (West, Kvistgaard et al. 2017). All infants were vaccinated with a trivalent vaccine (diphtheria, pertussis, tetanus) at 4 months of age. The antibody titre (IgG) against tetanus was measured at 6 months of age (Lonnerdal, Kvistgaard et al. 2016). There was no significant difference between breastfed and formula-fed infants, but among the formula groups, the mid-dose group had fewer antibodies than the low-dose group. There were no statistically significant differences between the high-dose group and any of the other groups.
It was shown that the infants which had received the infant formula with 130 mg/L Lacprodan® OPN-10 had increased T-cell proportions compared with infants which had received infant milk formula with a lower dosage (65 mg/L) or a control formula without Lacprodan® OPN-10 (West et al. 2017). Furthermore, monocyte proportions were higher in the infants receiving Lacprodan® OPN-10, when compared to the breast-fed reference group. Higher plasma levels of human OPN were observed in infants receiving infant milk formula with bovine Lacprodan® OPN-10 when compared to infants receiving a control formula (Jiang and Lonnerdal 2019), suggesting that bovine Lacprodan® OPN-10 promotes the synthesis or secretion of endogenous human OPN. They attributed this to small amounts of undigested bOPN being absorbed stimulating endogenous OPN production by similarities in the amino acid sequences. This is consistent with findings in animal studies with both young (Jiang, Prell et al. 2019) and adult (Ge, Lu et al 2013) mice.

Effects of Lacprodan OPN-10 in adults have not yet been studied.

6.4 Summary of known and potential risks and benefits

Recently, the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) published an opinion on bovine milk osteopontin as a novel food, in which they conclude that the currently available scientific data do not raise safety concerns (EFSA NDA Panel 2022).
In 2017, a GRAS notification (Matulka 2017) has been submitted to the FDA for the use of Lacprodan® OPN-10 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. The GRAS status was based on the views of an independent Expert Panel.
Standard safety evaluations have shown no indications for potential risk involved with consumption. Potential benefits in the general adult population have not been studied so far.

6.5 Description and justification of route of administration and dosage

The intervention product has been developed as a food supplement or nutritional ingredient. Therefore, the appropriate route of administration is by ingestion, supplementing the normal diet. The product will be supplied in two different dosages: 2.5 g/day for subjects weighing lower than 70 kg at screening, and a dose of 3,20 g/day for subjects weighing 70 kg or more at screening.

The estimated intake of OPN-10 in infants, based on 160 mg OPN-10/L infant formula, is 24.8 mg/kg BW/day for infants at the 50th percentile, and 39.5 mg/kg BW/day for infants at the 90th percentile. The influenza vaccination study in piglets (Donovan 2012) provided an intake of 50.4 mg/kg BW/day. No data are available from any study in adult human subjects. The selected dosages result in an intake of 50 mg per kg BW for subjects weighing 50 kg, and 35,6 mg/kg BW for subjects weighing 105 kg, which is at the high end of the regular intake in infants, via OPN-10 supplemented infant formula, but still within the safety range.

6.6 Dosages, dosage modifications and method of administration

OPN will be administered in the form of sachets. The individual dose is dependent on the body weight at screening, with a dose of 2.5g/day for subjects weighing lower than 70 kg at screening, and a dose of 3,20 g/day for subjects weighing 70 kg or more at screening. Each participant will consume 2 sachets of study product per day. The dosage will not be adjusted in case a weight change occurs during the study.

Table 3. Calculation of individual dose of OPN with 2 sachets per day

Weight of subject at screening (kg)	Daily intake (grams)	Amount per sachet	Actual dose (mg/kg BW)
50	2,5	1,25	50,0
55	2,5	1,25	45,5
60	2,5	1,25	41,7

65	2,5	1,25	38,5
70	3,2	1,6	45,7
75	3,2	1,6	42,7
80	3,2	1,6	40,0
85	3,2	1,6	37,6
90	3,2	1,6	35,6
95	3,2	1,6	33,7
100	3,2	1,6	32,0
105	3,2	1,6	30,5

The sachets will be taken in 2 servings per day: 1 sachet in the morning at breakfast, and 1 sachet in the evening at dinner. In case of a missed dose, subject can consume the missed sachets at a later timepoint on the same day.

6.7 Preparation and labelling of Investigational Product

Test and placebo products will be delivered to NIZO separately by the sponsor and will be stored at the recommended conditions.

On the label of the bulk package, the following information will be provided by the sponsor:
Bulk packaging coming from sponsor

- Company name sponsor
- Project number of the study (xxx)
- Manufacturing batch number
- Number of carton boxes with 30 sachets
- Expiry date
- The text “for nutrition trial use only”

An example of the label of the bulk package is included in section D3 of this submission.

The products will be packaged in carton boxes. All boxes will contain 30 sachets. The sachets will be marked with one dot (.) for the sachets with a level of 1,25g/sachet and with two dots (..) for the sachets with a level of 1,6 g/sachet. Each carton box will be delivered to NIZO with the following information:

- Best before [date]
- Random 5-digit code (see section 8.2)
- Number of sachets

NIZO will add an additional label to the carton boxes with the following:

- Study name
- Subject number
- “To be used in study weeks: …”
- The text “for nutrition trial use only” (in Dutch)

For the active treatment, 3 random 5-digit codes will be used; for the placebo treatment, 3 other random 5-digit codes will be used. Each carton box will be coded with one of the three applicable codes.

For each subject, 2-weekly packages will be prepared by NIZO. Labelling of these packages with the individual subject codes and weeks will be performed by NIZO. After arrival of the bulk package at NIZO, the NIZO study team will be responsible for labelling of the 2-weekly doses of the study products.

The label that will be used on the 2-weekly package for the subjects ('Voorbeeldetiket D3') will contain the following information:

- Study name and project number
- Subject number
- Best before [date]
- Random 5-digit code (see section 8.2)
- The text "for nutrition trial use only" (in Dutch)
- Information for use
- Information about storage conditions
- Contact details of the researcher

6.8 Investigational product accountability

The sponsor is responsible for transportation of products to NIZO study location by means of a courier. NIZO is responsible for overall IP accountability and storage at the recommended conditions. EB Medical Research is responsible for providing subjects at Visits 2, 3, 4, 5 and 6 with sufficient study product until the next visit, according to the randomization schedule. EB Medical Research is responsible for subject IP accountability.

At each of the visits 2 to 6, each subject will receive one or more carton boxes, with at least the number of sachets required until the next visit, and instruction on the number of sachets to take each day. At each of the visits 3 to 7, subjects have return the empty carton boxes and the carton boxes with the unused sachets of the previous study weeks. The leftover sachets will be counted by the EBMR study team to check compliance. At the end of the study, leftover sachets will be returned to the sponsor.

7 NON-INVESTIGATIONAL PRODUCT

7.1 Name and description of non-investigational product

The non-investigational product in this study is the registered Engerix-B® vaccine. For details on the product composition we refer to the Summary of Product Characteristics (SPC), h17316_smpc.pdf (geneesmiddeleninformatiebank.nl).

7.2 Summary of findings from non-clinical studies

See SPC, h17316_smpc.pdf (geneesmiddeleninformatiebank.nl)

7.3 Summary of findings from clinical studies

Engerix-B induces the development of specific humoral antibodies to hepatitis B antigens. Anti-HB antibodies ≥ 10 IE/l provide protection against infection with hepatitis B virus.

7.4 Summary of known and potential risks and benefits

The safety profile for Engerix-B is described in the SPC. Side effects that are reported to occur often or very often are: loss of appetite, irritability, headache, gastrointestinal symptoms (e.g., nausea, vomiting, diarrhea, abdominal pain), pain and redness at the injection site, fatigue, fever, malaise. Less common side effects include dizziness, myalgia, and influenza-like symptoms.
The benefit of the vaccination is the protection against hepatitis B virus infection. However, due to the accelerated schedule, seroprotection will be achieved for about 75% of the participants (h17316_smpc.pdf (geneesmiddeleninformatiebank.nl).

7.5 Description and justification of route of administration and dosage

The vaccination will be administered by a 1 ml intramuscular injection, delivering 20 microgram of hepatitis B antigen. This is the standard injection dose (Engerix-B 20 microgram/1 ml, suspensie voor injectie | Geneesmiddeleninformatiebank | College ter Beoordeling van Geneesmiddelen).

7.6 Dosages, dose modifications and method of administration

Vaccinations will be given according to an accelerated schedule, on days 57, 71 and 85 (all +/- 1 day) of the study (day 1 being the date of V2). Here an accelerated schedule is used to provide a suboptimal trigger to the immune system, reasoning that this will be more reflective of natural infections and will leave room for improvement of the response due to the supplementation with Lacprodan® OPN-10. At the end of the study, the anti-HepB titer will be assessed, and the vaccination coverage will be known. Subjects with an insufficient coverage will be informed that for full coverage a fourth vaccination can be obtained 12 months after the first vaccination, through the study team.

7.7 Preparation and labelling of Non Investigational Medicinal Product

The vaccine must be stored at a temperature of 2°C – 8°C (not in the freezer). Before use, the vaccine must be shaken, after which it will get somewhat cloudy. A visual check must be done on strange particles or an abnormal appearance. After opening of the package, the vaccine must be administered immediately.

7.8 Non-investigational product accountability

The vaccine will be ordered from the pharmacy and stored in a locked box until use under the prescribed conditions (refrigerator, 2-8°C). Only the medical investigator and the study coordinator

will have access to the box. If any material is left after completion of the study, this will be returned to the pharmacy.

8 METHODS

8.1 Study parameters/endpoints

Details of sample handling and analytical methods will be described in a separate Lab Manual, to be completed before study start.

8.1.1 Main study parameter/endpoint

Primary outcome is the percentage of responders to hepatitis B vaccination. A "responder" is defined as a subject attaining anti-hepatitis B antibody titres >10 IU/L at Visit 7 (2 weeks after the third vaccination). Antibody titres will be determined with ELISA.

8.1.2 Secondary study parameters/endpoints

- Change in serum anti-hepatitis B antibody titres from baseline (V1) to 14 days after the second vaccination (V6) and 14 days after the third vaccination (V7)
- Change in circulating cytokines from baseline to 8 weeks intervention (V4) and to the end of the study (V7). The analyses will be performed at NIZO with a multiplex assay in duplicate. Results will be displayed in pg/ml (dependent on the actual outcomes). In principle, the following set of cytokines will be analysed; however, the final choice of the most relevant cytokines may be slightly different, depending on the outcomes of ongoing analytical validation experiments.

    - Interleukin 6 (IL-6)
    - Interleukin 10 (IL-10)
    - Interferon ɣ (IFNɣ)
    - Tumour necrosis factor α (TNFα)
    - Chemokine (C-C motif) ligand 3 (CCL-3)
    - Interleukin 15 (IL-15)
    - Interleukin 17a (IL-17a)
    - Interleukin 4 (IL-4)
    - Interleukin 2 (IL-2)
    - Interleukin 2RA (IL-2RA)

- Change in serum levels of P1NP and CTX-1 (as markers of bone formation and bone resorption) from baseline to 8 weeks intervention. Analyses will be performed by an external routine lab.
- Change in plasma levels of hOPN and bOPN from baseline to 4 weeks and 8 weeks intervention. hOPN and bOPN will be analysed at NIZO with an ELISA assay in duplicate. Results will be displayed in pg/ml (dependent on the actual outcomes).

- Change in serum LPS binding protein (LBP) from baseline to 8 weeks intervention. Serum LBP will be analysed at NIZO with an ELISA assay in duplicate.
- Incidence of self-reported URTI or LRTI during the trial, including weekly reports of symptoms related to upper and lower respiratory tract infections
- Safety monitoring by routine assays for hematology and serum clinical chemistry. Analyses will be performed by an external routine lab.

Table 4 Blood parameters for safety monitoring:

Clinical chemistry	Method	Unit
Serum alkaline phosphatase	Photometry	U/l
Serum alanine aminotransferase (ALAT)	Photometry	U/l
Serum aspartate aminotransferase (ASAT)	Photometry	U/l
Serum triacylglycerols	Photometry	mg/dl
Serum total cholesterol	Photometry	mg/dl
Serum total bilirubin	Photometry	mg/dl
Serum urea	Photometry	mg/dl
Serum Creatinine	Photometry	mg/dl
Serum hs C-reactive protein	Nephelometry	mg/dl
Serum albumin	Nephelometry	mg/dl
Blood Glucose (Sodium Fluoride tubes, NaF)	Photometry	mg/dl
Hematology		
Red blood cell (RBC) count	Flow cytometry	10^12/L
White blood cell (WBC) count	Flow cytometry	10^9/L
Hemoglobin	Photometry	g/L
Hematocrit	Photometry	L/L
Platelet	Flow cytometry	10^9/L
Differential leukocyte count	Flow cytometry	%

8.1.3 Other study parameters (exploratory)

- Change in plasma brain-derived neurotrophic factor (BDNF) from baseline to 8 weeks intervention (optional)
- Change in faecal microbiota composition after 8 and 14 weeks intervention (analysis depending on results for primary and secondary outcomes)

8.2 Randomisation, blinding and treatment allocation

To ensure blinding of the NIZO study team, an independent person in the AFI study team will be responsible for assigning a random 5-digit number code to the products. The number code is printed on the label of each carton box. Per treatment 3 different, 5 digits, codes will be used. The NIZO and EBMR teams, laboratory personnel and participants will be unaware of the coding until all data analyses are finished and until after the blind data review meeting. Only in a case of emergency possibly related to treatment, the Principal Investigator can request the independent party to open a sealed de-blinding envelope containing information to check the type of treatment of (a) particular subject(s) without disclosure of the other treatments.

Allocation will be stratified for gender and age.

8.3 Study procedures

8.3.1 Recruitment

Candidates from the Netherlands will be recruited by Link2Trials B.V. (Haarlem) with support of EB Medical Research. Subjects will be recruited by advertisements in regional newspapers and on social media, and, if needed, by advertisements in local newspapers and by posters mounted in public buildings. Candidate matching the main in and exclusion criteria as indicated during recruitment, will be contacted for a registration call after which they will receive information of the study. Candidates will be invited for an information meeting or online meeting (via Teams) with the medical investigator or one of the delegated research team members. The medical investigator or team member will explain the background, objectives and study procedures. During this meeting, subjects have the possibility to ask questions about the study. When candidates indicate to be interested in participation they are invited to sign the informed consent form. After the consent form has been signed, a health questionnaire will be distributed and the subjects will be asked to complete the questionnaire. Subjects who are willing to participate and are considered to be healthy as assessed by the medical investigator on the basis of the health questionnaire, will be invited for the screening visit.

8.3.2 Screening visit (visit 1)

All study visits are scheduled to take place at NIZO, Kernhemseweg 2 in Ede or at EB Medical research, Louis Armstrongweg 88 in Almere.

The subjects will have a pre-study screening (V1) within 2-4 weeks before visit 2, the start of the intervention period.

During the screening visit, a blood sample will be drawn, for the determination of anti-hepatitis B antibody titer, and for a routine safety panel (clinical chemistry and hematology, as listed under 8.1.2). Body weight and height will be measured. A subject's eligibility will be assessed based on the

lifestyle and health questionnaire, BMI, antibody titer, clinical chemistry and hematology. The medical investigator will inform the subjects about their eligibility in writing. In case of a surplus of eligible participants, random allocation will determine which of these participants will be able to join the study.

During the screening visit, verbal instructions will be provided about the collection of fecal samples. All screened subjects will receive a fecal collection kit before visit 2. This kit will also contain written instructions about the collection of fecal samples.

8.3.3 Study Visits

Visit 2

After the screening visit and assessment of eligibility, subjects will be invited for the baseline visit (visit 2, week 0). All subjects will be asked to collect a fecal sample on the day before the visit (with a maximum time window of 48 hours before the visit). Furthermore, all subjects are asked to not eat or drink anything, except water after 21:00 on the day before the visit, to not consume alcoholic beverages on the day before the visit, and to not perform intense physical exercise. After arrival in fasted state,

- Overall wellbeing will be checked, including questions on common infectious diseases
- Compliance with study restrictions will be checked
- Fecal samples, collected at home and brought to the site by the participant, will be taken in
- Fasting blood samples will be drawn (before 12 a.m.) – see sampling schedule in section 8.3.4
- Test products will be handed out for a 4-week period (until V3).

The duration of visit 2 will be about 30 minutes for each subject.

Visit 3

All subjects are asked to not eat or drink anything, except water after 21:00 on the day before the visit, to not consume alcoholic beverages on the day before the visit, and to not perform intense physical exercise. After arrival in fasted state,

- Overall wellbeing will be checked, including questions on common infectious diseases
- Compliance with study restrictions will be checked
- Fasting blood samples will be drawn (before 12 a.m.) – see sampling schedule in section 8.3.4
- Test products will be handed out for a 4-week period (until V4).
- A fecal collection kit will be handed out for fecal collection the day before V4.

The duration of visit 3 will be about 20-30 minutes for each subject.

Visit 4

All subjects are asked to not eat or drink anything, except water after 21:00 on the day before the visit, to not consume alcoholic beverages on the day before the visit, and to not perform intense physical exercise. After arrival in fasted state,

- Overall wellbeing will be checked, including questions on common infectious diseases
- Compliance with study restrictions will be checked
- Fecal samples, collected at home and brought to the site by the participant, will be taken in
- Fasting blood samples will be drawn (before 12 a.m.) – see sampling schedule in section 8.3.4
- After blood collection, the hepatitis B vaccine will be administered by intramuscular injection
- Test products will be handed out for a 2-week period (until V5).

The duration of visit 4 will be about 30-45 minutes for each subject.

Visit 5

All subjects are asked to not consume alcoholic beverages on the day before the visit, and to not perform intense physical exercise. After arrival in the morning,

- Overall wellbeing will be checked, including questions on common infectious diseases
- Compliance with study restrictions will be checked
- The hepatitis B vaccine will be administered by intramuscular injection
- Test products will be handed out for a 2-week period (until V6).

The duration of visit 5 will be about 20-30 minutes for each subject.

Visit 6

All subjects are asked to not eat or drink anything, except water after 21:00 on the day before the visit, to not consume alcoholic beverages on the day before the visit, and to not perform intense physical exercise. After arrival in fasted state,

- Overall wellbeing will be checked, including questions on common infectious diseases
- Compliance with study restrictions will be checked
- Fasting blood samples will be drawn (before 12 a.m.) – see sampling schedule in section 8.3.4
- After blood collection, the hepatitis B vaccine will be administered by intramuscular injection
- Test products will be handed out for a 2-week period (until V7).
- A fecal collection kit will be handed out for fecal collection the day before V7.

The duration of visit 6 will be about 30-45 minutes for each subject.

Visit 7

All subjects are asked to not eat or drink anything, except water after 21:00 on the day before the visit, to not consume alcoholic beverages on the day before the visit, and to not perform intense physical excercise. After arrival in fasted state,

- Overall wellbeing will be checked, including questions on common infectious diseases
- Compliance with study restrictions will be checked
- Fecal samples, collected at home and brought to the site by the participant, will be taken in
- Fasting blood samples will be drawn (before 12 a.m.) – see sampling schedule in section 8.3.4

The duration of visit 7 will be about 20-30 minutes for each subject.

8.3.4 Blood collection

The amount of blood collected amounts to max 22 ml per visit (see table 5).

Table 5. Maximum quantities of blood collected at visits

			v1	v2	v3	v4	v5	v6	v7	
			Screening	Wk0	Wk4	Wk8	Wk10	Wk12	Wk14	
	Tube	ml								
Chemistry	**Serum tube 1**	8	8	8	8	8				32
P1NP				*		*				0
Anti-HB titer	**Serum tube 2**	3	*					3	3	6
Serum LPS binding protein (LBP) and Cytokines	**Serum tube 3**	4		4		4			4	12
Cytokines				**		**				0
Serum LPS binding protein (LBP)				**		**				0
Glucose	**NAF bloed**	2	2	2	2	2				8
Hematology and CTX	**EDTA plasma tube 1**	4	4	4	4	4				16
Hematology			***	***	***	***				0
CTX (beta crosslabs)				***		***				0
OPN and BDNF	**EDTA plasma tube 2**	4		4	4	4				12
Plasma levels of OPN				****	****	****				0
OPTIONAL BDNF				****		****				0
Total			14	22	18	22	0	3	7	86

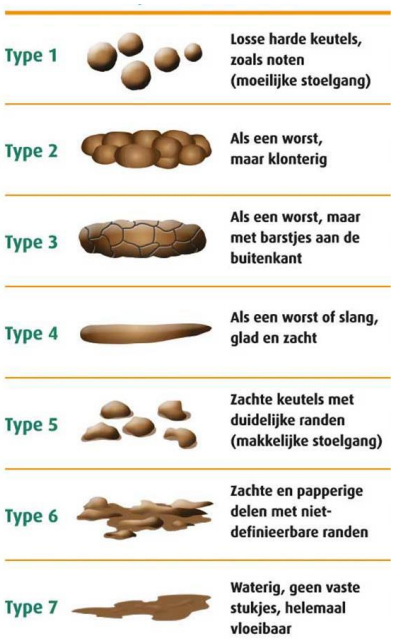
* Part of serum tube 1, ** Part of serum tube 2, *** Part of EDTA plasma tube 1, **** Part of EDTA plasma tube 2

Blood tubes for clinical chemistry and hematology, glucose, P1NP and CTX-1 will be sent to a routine lab for analysis after drawing. Blood for the other analysis in serum will be collected and centrifuged after clotting. The serum will be frozen in aliquots of 200µl and one rest volume.
Blood drawn for plasma will be centrifuged and the plasma will stored in aliquots of 200µl for the analysis of OPN and BDNF.

8.3.5 Fecal collection

Subjects are asked to collect spot fecal samples within 24h before visits 2, 4 and 7, resulting in 3 fecal spot samples. The maximum time window for the fecal samples is 48h before the visit. All material and instructions to collect a spot fecal sample will be provided to the subjects. Subjects are asked to collect their stool using the Fecesvanger (picture below).

Subjects need to collect approximately 3 times 1 - 2 grams of stool. This will be stored for potential exploratory analyses related to changes in fecal microbiota composition and/or functionality.
A collection vial with spoon will be provided to the subjects, to collect the spot sample. The rest of the stool is flushed away. Subsequently, the stool sample needs to be frozen at -20˚C in the subject's home freezer. For this, an envelope and materials for transport will be provided to prevent the sample from thawing during transport. Also reserve materials will be provided, in case not enough material can be collected during a first attempt.
In relation to the collected sample, consistency must be scored by the participant, by means of the 7-point Bristol Stool Scale (see below).



8.3.6 Questionnaires

Subjects will receive weekly questionnaires in which symptoms related to upper or lower respiratory infections will be recorded. These questionnaires will be completed online, via subject's own smartphone or computer/laptop.

8.4 Withdrawal of individual subjects

Subjects can leave the study at any time for any reason if they wish to do so without any consequences. The investigator can decide to withdraw a subject from the study for urgent medical reasons.

8.5 Replacement of individual subjects after withdrawal

If subjects withdraw before visit 2, subjects will be replaced. Subjects not completing the study for any reason will be considered as drop-outs. Drop-out rate should not exceed 25% of enrolled subjects, a minimum of 105 subjects should complete the trial. Only when the drop-out rate exceeds 25%, drop-outs will be replaced to achieve the minimum number of subjects to complete the trial.

8.6 Follow-up of subjects withdrawn from treatment

After possible withdrawal, no follow-up of participants will take place, except in cases of withdrawal for medical reasons.

8.7 Premature termination of the study

The study will only be terminated if an acute serious adverse event (SAE) occurs which in the opinion of the responsible medical investigator may be related to the test product. In case of premature termination of the study by the investigators, the subjects will be informed of the reason for termination and will be paid pro rata for the part of the study that has already been performed.

9 SAFETY REPORTING

9.1 Temporary halt for reasons of subject safety

In accordance with section 10, subsection 4, of the WMO, the sponsor will suspend the study if there is sufficient ground that continuation of the study will jeopardise subject health or safety. The sponsor will notify the METC without undue delay of a temporary halt including the reason for such an action. The study will be suspended pending a further positive decision by the METC. The investigator will take care that all subjects are kept informed.

9.2 AEs and SAEs

9.2.1 Adverse events (AEs)

Adverse events are defined as any undesirable experience occurring to a subject during the study, whether or not considered related to [the investigational product / trial procedure/ the experimental intervention]. All adverse events reported spontaneously by the subject or observed by the investigator or his staff will be recorded.

9.2.2 Serious adverse events (SAEs)

A serious adverse event is any untoward medical occurrence or effect that

- results in death;
- is life threatening (at the time of the event);
- requires hospitalisation or prolongation of existing inpatients' hospitalisation;
- results in persistent or significant disability or incapacity;
- is a congenital anomaly or birth defect; or
- any other important medical event that did not result in any of the outcomes listed above due to medical or surgical intervention but could have been based upon appropriate judgement by the investigator.

An elective hospital admission will not be considered as a serious adverse event.

The investigator will report all SAEs to the sponsor without undue delay after obtaining knowledge of the events. The sponsor will report the SAEs through the web portal *ToetsingOnline* to the METC that approved the protocol, within 7 days of first knowledge for SAEs that result in death or are life threatening followed by a period of maximum of 8 days to complete the initial preliminary report. All

other SAEs will be reported within a period of maximum 15 days after the sponsor has first knowledge of the serious adverse events.

9.3 Follow-up of adverse events

All AEs will be followed until they have abated, or until a stable situation has been reached. Depending on the event, follow up may require additional tests or medical procedures as indicated, and/or referral to the general physician or a medical specialist.
SAEs need to be reported till end of study within the Netherlands, as defined in the protocol.

10 STATISTICAL ANALYSIS

A detailed Statistical Analysis Plan (SAP) will be prepared and signed-off by an independent statistician, ultimately before database lock.

Descriptive information: all parameters studied will be described as mean and standard deviation when normal distribution has been ascertained. When the data are not normally distributed, descriptive values will be represented by median and interquartile range. In all cases the number of observations per parameter will be provided.

Throughout the study a p-value of lower than 0.05 will be considered to detect a statistically relevant difference, applying two-sided testing.

In the SAP, the intention-to-treat (ITT) population and the per protocol (PP) population will be defined. In a blind data review meeting the criteria for excluding specific subjects from the PP population will be applied.

10.1 Primary study parameter(s)

Subjects will be classified as responders and non-responders for antibody production. Responders are subjects with an anti HB antibody titer of ≥10 IU/L, non-responders have a titer < 10 IU/L. The difference in number of responders for hepatitis B-specific antibody production will be analyzed by Fisher's exact test.

10.2 Secondary study parameter(s)

The data will be analyzed with Linear Mixed Models (LMMs). LMMs model data from repeated measurements (where measurements from the same individual cannot be assumed to be independent) by including a separate model intercept for each longitudinal study unit (i.e. individual). Treatment group and timepoint will be included as fixed effects, while the study participant will be included as a random effect. Additionally, covariates will also be included as fixed effects: study location, age, gender, baseline value of the outcome parameter. An interaction term

between the effect of time and the effect of treatment groups will also be included in order to assess whether the outcome in question is differentially affected by the Lacprodan® OPN-10 intervention compared to placebo.

Model goodness-of-fit will be assessed and model assumptions (linearity, homoscedasticity, linearity of residuals, non-collinearity of parameters) will be checked.
In cases where the outcome distribution shows departure from normality, the outcome parameter of the LMM will be log-transformed.

10.3 Interim analysis

Not applicable.

11 ETHICAL CONSIDERATIONS

11.1 Regulation statement

Subjects will only participate in the study upon receiving written informed consent. The study will be conducted according to the principles of the Declaration of Helsinki latest version (Fortaleza, Brazil, October 2013), and according to the Dutch Medical Research involving Human Subjects Act.

11.2 Recruitment and consent

Candidates from the Netherlands will be recruited by Link2Trials B.V. (Haarlem), with support of EB Medical Research. Subjects will be recruited by advertisements in regional newspapers and on social media, and, if needed, by advertisements in local newspapers. Candidate subjects will receive written information of the study (see Section E METC dossier). Texts for the various recruitment materials (advertisements, letters, website etc) are supplied as part of the dossier in Section E.

The planning of the study execution will be fixed when recruitment starts. Therefore, it is important to recruit all 140 subjects within the available recruitment period. In case recruitment lags strongly behind expectations, a finder's fee will be introduced: if candidates are able to arouse interest of other subjects to participate in the study, they will receive €45.= for each subject that is randomized.

Link2Trials B.V. will use a pre-selection questionnaire based on a selection of major in- and exclusion criteria, to restrict the number of unnecessary screenings. Candidate subjects will receive written information of the study and will be invited for an information meeting or online meeting (via Teams) with the delegated member of the study team. They will explain the background, objectives, and study procedures. During this meeting, subjects have the possibility to ask questions about the study. It is expected that, based on general information and main selection criteria, about 336 candidates will be invited for the information call.

After having been informed about the study, and questions have been answered, subjects who agree to participate will be asked to sign two copies of the informed consent form, with one copy for the subjects, and one copy for the study team.

After signing the informed consent, a subject's eligibility will be assessed based on his anti-hepatitis B antibody titer, clinical chemistry and hematology results, the lifestyle and health questionnaire, and assessment of body weight and height. If a subject is eligible, the medical investigator will inform him in writing whether he/she is invited to participate or is not selected. If a subject is not eligible, the medical investigator will inform him in writing.

Subjects that fulfil all eligibility criteria will be included in the study. In case of a surplus of eligible participants, random allocation will determine which of these participants will be able to join the study.

The medical investigator will inform in writing the general practitioner of each subject. who has signed the informed consent form and is selected to participate in the study, on study participation. Any new significant findings reported in scientific literature that might affect the subject's participation in the study will be communicated to the subject.

11.3 Benefits and risks assessment, group relatedness

Benefits: The subjects will not benefit directly from participation in this study. A subject fee of 750 euro including reimbursement of traveling expenses (10 euro per visit) will be provided.

Risks: The risks associated with participation in this study are considered small. Potential risks could be related to a) study product, b) study procedures or c) non-investigational product (hepatitis B vaccination).
Recently, the EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA) published an opinion on bovine milk OPN as a novel food, in which they conclude that the available scientific data do not raise safety concerns (EFSA NDA Panel 2022).
Lacprodan® OPN-10 has a GRAS notification (Matulka 2017), based on the views of an independent Expert Panel. Standard safety evaluations have shown no indications for potential risk involved with consumption. The dose administered in this study is 3.3% of the NOAEL calculated in safety studies.
The burden imposed by study procedures includes the daily intake of the study product, the visits to the research location, the blood sampling and faecal sample collection. The collection of blood samples may produce discomfort or minor bleeding and the possibility of bruising at the site of the needle puncture. There is also a slight risk of infection at the site of the needle puncture. Side effects of vaccination with Engerix-B have been summarized in section 7.4.

11.4 Compensation for injury

The CRO has a liability insurance which is in accordance with article 7 of the WMO.

The CRO has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO). This insurance provides cover for damage to research subjects through injury or death caused by the study.

This insurance provides cover for damage to research subjects through injury or death caused by the study.

1. € 650.000,- (i.e. six hundred and fifty thousand Euro) for death or injury for each subject who participates in the Research;
2. € 5.000.000,-- (i.e. five million Euro) for death or injury for all subjects who participate in the Research;

The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study.

11.5 Incentives

Subjects will receive a subject fee of €750,- euro after full completion of the study, which will be subject to taxes. For each visit there a compensation for travel expenses of €10,- per visit is included. If subjects end participation earlier, they will receive only part of the financial reward. The reward is intended to compensate the subjects for their time spent on study activities, e.g. collection of the biological samples. Subjects will be informed about payment criteria orally and in writing.

Subjects who attended the screening visit, but were not eligible for the study will receive only part of the total subject fee (compensation of €60,-). Subjects who decide not to participate will also receive part of the total subject fee (compensation of €60,-).

Subjects who dropped out during the study will be paid a sum in accordance with study participation:

- After V2 € 160,00
- After V3 € 260,00
- After V4 € 380,00
- After V5 € 500,00
- After V6 € 620,00
- After V7 € 750,00

These fees will be paid If the subject ends his/her participation, or if participation is ended by

a. The medical investigator on medical grounds.
b. The principal investigator, based on deviations in the study conduct outside the participant's responsibility (to be judged by the project leader)
c. Premature discontinuation by the sponsor (AFI) or NIZO food research
d. The subject due to circumstances beyond his control.

No fee will be paid if the project leader prematurely ends the subject's participation on grounds of not complying with the rules and regulations of the study or misconduct.

12 ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

12.1 Handling and storage of data and documents

All data will be handled confidentially and in compliance with the Dutch Personal Data Protection Act. Only coded data will be used, without any reference to subjects' identity. A subject identification code list will be used to link the data to the subject. The code will not bebased on the subject initials and birth date. During the conduct of the study, the key to the code will be safeguarded by the study coordinator of EB Medical. The principal investigator and the project manager will have access to the code during the study. After completion of the study, the key to the code will be kept at NIZO in a locked cabinet.
After signing the informed consent form, subjects will be allocated to a pre-entry number consisting of the study code (223, followed by a slash ("/"), followed by a 3-digit number starting at 501 (e.g. 223/501). After inclusion of all subjects, subjects will be allocated to an entry number. Entry numbers will consist of the study code 223, followed by a slash ("/"), followed by a 3-digit number starting at 001 (e.g. 223/001 till 223/140).

Biological samples collected in-study will be coded with the study code (223), entry number and a visit time code. For fecal sample collection the subjects have to record the time and date of collection on the label.
Registration and research results are kept at NIZO food research, Department of Health. Biological samples obtained in the "BOFEI" study will be stored under appropriate conditions for a minimum of 2 years, and a maximum of 5 years to enable ad hoc retrospective research in the future, e.g. for new biomarker validation. In all study reports, only group averages or anonymized data will be presented.

12.2 Monitoring and Quality Assurance

Online monitoring will be performed by representatives from AFI who are not directly involved with the study execution. Development of the monitoring plan (with review from NIZO) and execution of the online monitoring will be the responsibility of AFI. The general purpose of monitoring is to review the compliance with the clinical trial protocol and applicable regulations to ensure that the integrity of the data is maintained with regard to accuracy and completeness and will protect the rights and well-being of the subjects involved in the study.

12.3 Amendments

A 'substantial amendment' is defined as an amendment to the terms of the METC application, or to the protocol or any other supporting documentation, that is likely to affect one of the following to a significant degree:

- the safety or physical or mental integrity of the subjects of the trial;
- the scientific value of the trial;
- the conduct or management of the trial; or
- the quality or safety of any intervention used in the trial.

All substantial amendments will be notified to the METC.

Non-substantial amendments will not be notified to the METC but will be recorded and filed by the sponsor.

12.4 Annual progress report

Not applicable.

12.5 Temporary halt and (prematurely) end of study report

The sponsor will notify the METC of the end of the study within a period of 90 days. The end of the study is defined as the last patient's last visit.

The sponsor will notify the METC immediately of a temporary halt of the study, including the reason of such an action.
In case the study is ended prematurely, the sponsor will notify the METC and the competent authority within 15 days, including the reasons for the premature termination.
Within one year after the end of the study, the investigator will submit a final study report with the results of the study, including any publications/abstracts of the study, to the METC.

12.6 Public disclosure and publication policy

The study protocol will be registered at a primary registry in the WHO registry network, https://www.isrctn.com/, after approval from the accredited METC is received.
Policy concerning the public disclosure of the study and its results has been determined in mutual agreement between the sponsor and NIZO food research and is specified in the Clinical Trial Agreement. Neither party will unreasonably withhold the public disclosure of study results.

Public disclosure may include:

- Summary of results in an online clinical trial register;
- Oral or poster presentation at conferences, symposia or other public meetings;
- Or full publication in peer-reviewed scientific journals.

13 STRUCTURED RISK ANALYSIS

13.1 Potential issues of concern

a. Level of knowledge about mechanism of action
Overall, there is no single known specific function of bOPN. bOPN has been closely associated with functions involving the immune response (see section 6.2). Few data are available from adult animal models, while, to the best of our knowledge, no data from adult clinical studies have been reported.

b. Previous exposure of human beings with the test product(s) and/or products with a similar biological mechanism
A clinical study has been performed with the test product, in 2 different dosages, in infants from 1 to 6 months of age (see section 6.3). The product was well tolerated, potential beneficial effects were observed. The available study results have supported a positive evaluation of the EFSA NDA Panel of the safety of bOPN as a Novel Food (EFSA NDA Panel 2022).

c. Can the primary or secondary mechanism be induced in animals and/or in *ex-vivo* human cell material?
The whole organism is needed to induce the expected effects, and this study is designed to investigate efficacy in relation to vaccination response, in addition to investigating mechanistic aspects. It is not known yet which are the primary or secondary mechanisms, although it is hypothesized that modulation of the inflammatory response plays an important role.

d. Selectivity of the mechanism to target tissue in animals and/or human beings
Effects on immune cells and inflammatory markers have previously been observed in animal models and/or healthy infants.

e. Analysis of potential effect
The dose of Lacprodan® OPN-10 is based on the doses that have been used in the infant clinical trial, and are far below the NOAEL that has been determined based on toxicological studies.
Because this is the first study with Lacprodan® OPN-10 in adults, potential adverse effects will be monitored and changes in standard clinical chemistry and hematology will be assessed, with particular attention for inflammatory markers.
As human OPN (with a different amino acid sequence compared to bovine OPN) has been found to be associated with some negative health effects, including increased bone resorption, levels of human OPN and markers of bone formation and bone resorption will also be monitored during the study.

f. Pharmacokinetic considerations
Not applicable

g. Study population

The study population consists of healthy elderly ≥65 years.

h. Interaction with other products
Not applicable

i. Predictability of effect
Based on the pig study with influenza vaccination, we expect to find an increased number of subjects attaining a protective level of anti-hepatitis B antibodies. However, in the clinical study in infants, no effects were observed on antibody titer response to tetanus vaccination.

j. Can effects be managed?
Short-term adverse effects will be monitored by analysing the clinical chemistry parameters and blood cells at baseline and after 4 and 8 weeks intervention, immediately after the visit. If unexpected changes are observed with clinical relevance, the MI may decide to stop participation for individual subjects. Any effects that might occur will be reversible after stopping the intervention.

13.2 Synthesis

In our opinion, the risks for participation in this study are small, and we have made every effort to minimize potential risks. Therefore, we feel that the remaining risks are acceptable and do not outweigh the scientific and social relevance of this study.

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