STATISTICAL ANALYSIS PLAN

Protocol 2020/vers. 2

Nordic study on human milk <u>fort</u>ification in <u>e</u>xtremely preterm infants: a randomized controlled trial

SAP Version: **Date of Final Change:**

2019-06-28

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2020-06-22

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Published Date:_____

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Revision History

Version #	Date Effective	Page/Section/ Paragraph	Change From:	Change To:
2 (the changes were made before any data analyses was made)	2019-06-22	3.2.2.2 and 8.1.2.3 4.2.2 5		Added a secondary outcome: The composite of NEC and culture-proven sepsis: An infant should have had any of these two diagnoses to fulfil the criterion Vitamins and micronutrients will be analysed in a sample of infants with standard methods to evaluate if the different fortification may lead to different levels in blood. Added an exclusion criterium: Abdominal surgery before the time of inclusion Sample size estimation An estimation how many infants that would be needed for the composite of culture-proven sepsis and NEC has been added

2020-06-22

TABLE OF CONTENTS

1   LIST OF ABBREVIATIONS AND DEFINITIONS OF TERMS

AE= Adverse Event

AGA= Appropriate for Gestational Age

APGAR= Appearance, Pulse, Grimace, Activity, and Respiration

BPD=Bronchopulmonary dysplasia

CRF= Case Report Form

CRO=Contract research organisation

CRP= C-reactive protein

CLD= Chronic Lung Disease

CPAP=Continuous positive airway pressure

CSF= Cerebrospinal Fluid

CX= Culture

DC= Discharge

DOL= Day of Life

DSMB=Data and Safety Monitoring Board

EHM=Exclusively human milk

ELBW=Extremely low birth weight

ELGA=Extremely low gestational age

FOC= Frontal-Occipital Circumference

GCP= Good Clinical Practice

GW= Gestational Week

HC= Head Circumference

ICH= International Conference on Harmonisation

ICU= Intensive Care Unit

ID= Identification

IRDS= Infant Respiratory Distress Syndrome

IVH= Intraventricular haemorrhage

ITT= Intention-to-treat

LOS= Length of Stay

LPK= Leucocyte count

mg/L= milligrams per liter

NEC=Necrotizing enterocolitis

NICU=Neonatal Intensive Care Unit

NIPPV= Non Invasive Positive Pressure Ventilation

O2= Oxygen

PDA=Patent ductus arteriosus

PN= Parenteral Nutrition

PPA= Per Protocol analysis

PPROM=Preterm premature rupture of the membrane

PVL= Periventricular leucomalacia

REB=Research Ethic Board

ROP= Retinopathy of Prematurity

SAE= Serious Adverse Event

SCFA=Short Chain Fatty Acids

SGA= Small for Gestational Age

SIP=Spontaneous intestinal perforation

SUSAR= Suspected Unexpected Serious Adverse Reaction

TPK= Thrombocyte Count

TPN= Total parenteral nutrition

2   INTRODUCTION

2.1   PREFACE

Although the care of premature infants has improved dramatically during the last decades, still about 30% of the extremely low gestational age (ELGA born < gestational week 28+0) infants die – and severe infections and necrotizing enterocolitis (NEC) are common causes of death. There are clear links between nutrition and the risk of NEC, infection, neurological sequelae and mortality. A common cause of poor nutrition in this patient group is poor enteral feed tolerance due to immaturity and dysfunction of gastrointestinal tract – and thus ELGA infants usually require parenteral feeding though intravenous catheters that increase the risk of sepsis. Nutritional problems and their consequences are a major problem in neonatal care.

However, choice of diet has a major impact on morbidity and clinical course. Preterm infants receiving human milk have better feeding tolerance and lower incidence of NEC than those fed cow milk based preterm formula. Fortunately, because of the widespread availability of donor milk banks in Nordic countries, ELGA infants are generally receiving a base diet of human milk without the need for cow's milk-based formulas. Despite this, the incidence of NEC in ELGA infants is still as high as 12% according to the Swedish National Quality Register (www.snq.se). We hypothesize that a key reason for this is that even fully human milk fed ELGA infants have a significant intake of cow's milk protein. This is because human milk alone does not fully meet the nutritional requirements in ELGA infants, but needs to be supplemented with a nutrient fortifier – and such fortifiers available in Nordic Countries are based on cow's milk. Indeed, because breast milk protein levels are so low in relation to the needs of ELGA infants, such fortifiers often provide more cow's milk protein than the infant receives from an all-human milk diet.

$H^2MF^®$ (Prolacta Bioscience, CA, USA) is a human milk-based nutrient fortifier for preterm infants. Previous trials evaluating Prolacta fortifiers in extremely preterm infants receiving exclusive human milk diet suggest that exclusive human milk diet, devoid of cow milk-containing products, is associated with lower mortality and morbidity in these infants without compromising growth. Thus, such an exclusive human milk diet has been reported to reduce the incidence of NEC, sepsis, bronchopulmonary dysplasia (BPD) and retinopathy of the prematurity (ROP). No side effects have been reported. Still, there is no well powered trial in ELGA infants comparing a human milk-based fortifier with bovine protein-based fortifier in which both study groups receive an all-human breast milk-based diet. Such a trial is needed to provide evidence

that a human milk-based fortifier is superior to bovine-based one in ELBW infants also in centres with 100% coverage of breast milk donor banks. Moreover, due to the introduction of active intervention for infants also born before gestational week 25 during the last decade, a trial run in Nordic centres will have a predominance of infants born in gestational week 23-25, a patient population that could be expected to gain the most of a diet free from bovine protein. A significant positive result of this trial could easily be implemented in the NICUs, since ProlactH$_2$MF$^®$ already exists and has been used extensively - by around 1 in 4 level 3 NICUs in the US.

2.2   PURPOSE OF THE ANALYSES

To evaluate if diet supplementation with the human milk-based nutrient fortifier H$_2$MF$^®$ reduces severe complications - NEC, sepsis, mortality - and improves enteral feeding tolerance as compared with bovine protein-based nutrient fortifier in extremely preterm infants receiving exclusive human breast milk (own mother´s milk and/or donor milk). Possible mechanisms underlying the effect of H$_2$MF$^®$ will be analysed in blood, urine and stool samples. The health economic impact of an implementation will also be evaluated.

3   STUDY OBJECTIVES AND ENDPOINTS

3.1   STUDY OBJECTIVES

3.1.1   Primary Objective

The primary objective is to evaluate if the supplementation of a human milk-based nutrient fortifier H$_2$MF$^®$ reduces the severe complications of NEC, sepsis and mortality as compared with bovine protein-based nutrient fortifier in extremely preterm infants receiving exclusive human breast milk (own mother´s milk and/or donor milk).

3.1.2   Secondary Objectives

Secondary objectives are to evaluate if H$_2$MF$^®$ supplementation improves enteral feeding tolerance and reduces other severe complications such as BPD, ROP and neurological impairment. Possible mechanisms underlying the effect of H$_2$MF$^®$ will be analysed in blood, stool, urine and breast milk samples. Health economic analyses will be made to evaluate the costs and benefits of an introduction of human milk-based fortifier in NICUs in the Nordic countries.

3.2   ENDPOINTS

3.2.1   Primary

The composite of NEC, culture-proven sepsis and mortality: An infant should have had any of these diagnoses to fulfil the criterion.

3.2.2   Secondary

3.2.2.1   *Food intolerance and growth*
1. Time to reach full enteral feeds: the day of life the infant has received at least 150 mL/kg enteral feeds.
2. Feeding interruption: number of days feedings held for ≥12 hours OR feeds reduced by >50% (ml/kg/d) not due to a clinical procedure or transitioning to the breast.
3. Parenteral nutrition: number of days of parenteral amino acid and/or lipid infusion. Only days when the enteral feed <150mL/kg/day should be included.
4. Gastric aspirates: ≥100% pre-feed volume (2 hours feeding volume if continuous feeding). Lower limit=2 ml/kg.
5. Stool frequency: number of stools per day.
6. Time to regain birth weight: first day of three success days when the infant weight is greater than birth weight. Indicate 0 days, if the infant never has a weight below birth weight.
7. Weight, height and head circumference at 7, 14, 21 and 28 days, the end of intervention (≤ 34+0), gestational week 36+0, at discharge from neonatal ward (or at gestational week 44+0, whatever comes first) and at 2 years of age (corrected) and 5.5 years of age (uncorrected). Standard deviation score (based on Niklasson[1] will be used in the calculations.

3.2.2.2   *Clinical variables for morbidity*
-Neonatal period:
1. Mortality
2. Necrotising enterocolitis (NEC): Bell´s stage II-III. The age (in days) of onset will be noted.
3. The composite of NEC and culture-proven sepsis: An infant should have had any of these two diagnoses to fulfil the criterion. Please see the definition of NEC above and sepsis below.
4. Spontaneous intestinal perforation (SIP: intestinal perforation without signs of intramural and/or portal gas and no signs of inflammation at surgery).
5. Abdominal surgery
6. Culture-proven sepsis: Positive blood, urine and/or CSF culture, clinical deterioration and laboratory inflammatory response*

*Laboratory inflammatory response (<u>one</u> of the following is needed):
1. LPK $\leq$ 5 or $\geq$ 20 (x10^9 cells/L)
2. TPK $\leq$ 100 (x10^9 cells/L)
3. CRP $\geq$ 15 mg/L

Culture proven sepsis should further be classified into early (<72 hours postpartum) or late (>72 hours postpartum) onset. Also, the age (in days) at onset will be noted.

7. Suspected sepsis, not culture-proven: clinical deterioration and laboratory inflammatory response* but negative blood culture. Age at onset will be noted.

The infant shall not get a diagnosis of sepsis or suspected sepsis if it has got a NEC diagnosis during the same period.

8. Pneumonia: Pathological X-ray confirmed by an independent radiologist, need of increased respiratory support/oxygen and laboratory inflammatory response*. Age at onset will be noted.
9. Bronchopulmonary dysplasia (BPD): Need of extra oxygen, CPAP or ventilator at gestational week 36+0.
10. Retinopathy of the prematurity (ROP): diagnosed by an independent ophthalmologist according to international classification. [37] Classified into stage I-V. The diagnosis is set after gestational week 42+0.
11. Intraventricular haemorrhage (IVH): classified into grade I-IV according to Papile[2]
12. Assessed by independent radiologist if the onset was after the intervention started. Age at onset will be noted.
13. Periventricular leukomalacia (PVL). Criteria according to de Vries[3] assessed by an independent radiologist.
14. Number of days with intensive care: need of respirator or CPAP until discharge (not later than gestational week 44+0).
15. Length of stay at the hospital: gestational week and day at discharge (not later than gestational week 44+0).
16. Length of need of feeding tube: gestational week and day when the infant does not need it anymore (not later than gestational week 44+0).

2-year follow up
1. Weight, height and head circumference.
2. Need of feeding tube after discharge from the hospital at the neonatal period (not later than gestational week 44+0)
3. Extra nutritional support after discharge from the hospital at the neonatal period (not later than gestational week 44+0)

4. Neurocognitive development: Bayley III [4]
5. Cerebral palsy
6. Epilepsy
7. Squint and/or impaired vision
8. Impaired hearing
9. Need of extra oxygen and/or ventilatory support after discharge from the hospital at the neonatal period (not later than gestational week 44)
10. Wheeze and/or asthma
11. Severe infections after discharge from the neonatal unit (not later than gestational week 44).
12. Mortality: including cause of death.
13. Level of education of the parents
14. Family status
15. Day-care

5.5 year follow up
1. Weight, height and head circumference.
2. Neurocognitive development: Wechsler Preschool and Primary Scale of Intelligence IV (WPPSI-IV ™) and Movement ABC-2
3. Cerebral palsy
4. Epilepsy
5. Squint and/or impaired vision
6. Impaired hearing
7. Wheeze and/or asthma
8. Mortality: including cause of death.

3.2.2.3   *Laboratory variables*

Separate protocols for each laboratory analysis will be created. Time points of collection of samples are displayed in the Flow chart (paragraph 2).

1. Microbiology analyses: Bacterial DNA will be extracted from stool and breast milk and samples analysed with high-throughput next generation sequencing methods. Shotgun metagenomics will be performed to sequence the whole bacterial genome in order to analyse bacterial functional genes. Specific focus will be on genes for degradation of oligosaccharides, formation of short chain fatty acids (SFCA), vitamin production, intestinal barrier function and virulence factors. Because of the prospective design the analyses could be performed in samples collected before the onset of an outcome parameter- thereby providing evidence related to a cause-and-effect relationship. Gut

bacteria metabolites in faeces such as SCFA will be assessed with gas chromatography and be related to the microbiology analyses and the clinical outcome.

2. Immunological analyses in blood samples: Blood samples are collected in EDTA tubes for plasma and separate pre-prepared tubes for masscytometry of blood cells (CyTOF). The plasma and cells will be stored at -80°C. The purpose of the immunological analyses is not only to compare the active and control group to assess the effect on the immune system of human milk-based and bovine-based nutrient fortifier, respectively, but also to map the immune system in this extreme group of patients and to identify immunological markers associated with specific diseases such as NEC and BPD.

   1. T helper subsets (TH1, Th2, TH17, Treg), T cells subsets associated with the intestinal mucosa ($\gamma\delta$-T cells, MAIT cells) and neutrophils will be assessed using masscytometry.
   2. Levels of Anti- and proinflammatory cytokines (e.g. IL-10. IL-6, TNF-α) and chemokines (e.g. CXCL11, CCL18) as well as immune modulatory enzymes such as indoleamine 2,3-dioxygenase (IDO) are high enough in plasma to be measured even in these small infants. To use the plasma as efficiently as possible, it will be analysed using multiplex assays.

3. Growth factors and neurotransmitters in blood samples: The levels of growth factors such as IGF-1 and the associated IGFBP-3 will be analysed. Besides being important for growth, these factors are also important in the pathogenesis of several of the complications (ROP, BPD) under study in this project. Neurotransmitters, which affect gut motility and neurological development, have been associated with the gut microbiota and will also be analysed. Markers for CNS damage such as Neurofilament light protein will be analysed in plasma.

4. Lipidomic analyses will be performed in plasma samples and eyrthrocyte membranes to assess the effects of the different feeding regimens on lipid profiles

5. Metabolomic analyses will be performed in urine samples. Urine is collected using cotton wool (first urine after 8 am), centrifuged and divided to 2-3 aliquots of about 1 mL of urine and stored at -80°C. Low molecular weight metabolites (metabolome), intermediate or products of metabolism in human cells and gut bacteria, will be analysed with different techniques such as proton nuclear magnetic resonance spectroscopy (NMR), liquid chromatography (LC) and mass spectroscopy couple to gas chromatography (GC-MC). These methods allow us to prospectively identify potential biomarkers of malnutrition in ELGA infants and potential metabolic mechanism underlying the effect of human milk-based nutrient fortifiers.

2020-06-22

6. Proteomic analyses in breast milk samples after fortification from the mothers but also from the donor milk <u>that the infant receives at the day of sample collection</u> (at 7, 14, 21 and 28 days and w33 of life). Also, human milk oligosaccharides will be measured in the breast milk samples. The protein profile in breast milk samples and in H^2 MF and the cow milk protein-based fortifier will be related to feeding tolerance, NEC and other complications during the neonatal period in order to identify specific proteins or patterns of proteins important for the outcomes

7. Vitamins and micronutrients will be analysed in a sample of infants with standard methods to evaluate if the different fortification may lead to different levels in blood.

3.2.2.4   *Health economics*

The intervention is costly but may prevent important complications such as NEC, sepsis and food intolerance and may thereby reduce the overall cost of neonatal care. We will use level of care to calculate health care costs.

1. The number of days at each level of care will be recorded until discharge from the hospital (not longer than gestational week 44+0).
2. The cost will be calculated by multiplying the number of days at each level of care by the average cost.

3.2.3   **Associated predictor variables (covariates)**

1. Gender
2. Caesarean section
3. Multiple pregnancies.
4. Birth weight and height.
5. Small for gestational age (SGA): Birth weight <2 SD.
6. Maternal smoking during pregnancy.
7. Preeclampsia: diagnosis by the responsible obstetrician.
8. Chorioamnionitis: diagnosis by the responsible obstetrician.
9. Preterm premature rupture of the membranes (PPROM). Rupture $\geq$ 1 hour before contractions started.
10. Antenatal antibiotics: pertain the period of the mother´s actual attendance at the hospital.

2020-06-22

11. Antenatal corticosteroids: the mother should have received at least 12 mg Betametasone. The corticosteroid prophylaxis is considered completed if the mother has received 2 doses at least 24 hours before delivery.
12. Born at level 1-2 NICUs.
13. Apgar score.
14. Surfactant-administration.
15. Intubation.
16. Infant respiratory distress syndrome (IRDS): Verified by X-ray
17. Respirator duration.
18. Patent ductus arteriosus (PDA): requiring medical or surgical treatment.
19. Antibiotics: name, treatment period and number of days.
20. Probiotics: name, treatment period and number of days
21. Opioids: name, treatment period and number of days
22. Gastric acid inhibitors: name, treatment period and number of days
23. Day of life when the supplementation of the study product was started
24. The amount of enteral feeds per day that the infants received when the supplementation of the study product was started
25. Number of days the infants has not received the study product
26. Number of days with intravenous line
27. Number of days with insulin treatment (and treatment period)
28. Number of days and doses of postnatal corticosteroids
29. Feeding regime: continuous or bolus.
30. Amount of nutrient protein fortifier per day
31. Amount of fat supplement per day
32. Total amount of protein, fat, carbohydrate, energy and micronutrient per day.
33. The relative amount of donor breast milk per day
34. Amount of extra enteral lipid
35. Breastfeeding: exclusive, partial.

3.2.4   Derived variables

The primary endpoint will be a composite of NEC, culture-proven sepsis and mortality: An infant should have had any of these diagnoses to fulfil the criterion.

4   STUDY METHODS

4.1   STUDY DESIGN

This is a randomised controlled multi-centre trial comparing diet supplementation with human breast milk-based nutrient fortifier $H^2MF^®$ and standard bovine protein-based nutrient fortifier in

2020-06-22

extremely preterm infants (born before gestational week 28+0) exclusively fed with human breast milk (own mother´s milk and/or donor milk). The infants will be randomised to receive either the human breast-milk based $H^2MF^®$ or the standard bovine protein-based nutrient fortifier before oral feeds have reached 100 ml/kg/day. If fortification with extra enteral lipids is needed during the intervention period, the infants receiving $H^2MF^®$ will be supplemented with the human milk-based Prolact $CR^®$, while the infants receiving standard bovine protein-based fortification will be supplemented with the standard lipid products used at the unit. The study subjects will be enrolled at level III NICUs. Only infants with a home clinic with the logistics to maintain the intervention until gestational week 34+0 will be included.

The intervention will continue until gestational week 34+0, but terminates earlier if the infant is transferred to a clinic without the logistics needed for the intervention, or if the clinic terminates the supplementation with donor breast milk earlier than gestational week 34+0 and the infant is not fed completely with mother´s own milk. After the intervention has ended there should be a transition period when the fortification of the breast milk during a 5-day period gradually goes from 100% of H^2 MF to 100% of standard bovine protein-based fortifier, if protein fortification still is needed. The infant must not be fed with formula during the intervention period. Thus, the interventions terminate when the infant receives the first dose of formula. A specific transition protocol is required in order to gradually wean the infant off the exclusive human milk diet.

Randomisation will be stratified by inclusion centre, gestational week (22+0-24+6 and 25+0-27+6), gender and parity. The allocation will be concealed before inclusion, but after randomisation the study is not blinded

The enrolled infants are characterized with clinical data including growth, feeding intolerance, use of enteral and parenteral nutrition, treatment, antibiotics and complications collected daily in a study specific case report form from birth until discharge from the hospital (not longer than gestational week 44+0). A follow up focusing on neurological development, growth and feeding problems will be performed at 2 and 5.5 years of age (corrected). Stool, urine, blood and breast milk samples are also collected for microbiology, metabolomic, proteomic and immunology analysis in order to study underlying mechanisms. Health economic analyses will be done to evaluate the costs and benefits of introducing a human milk-based fortifier into Nordic countries.

4.2   INCLUSION/EXCLUSION CRITERIA AND THE GENERAL PATIENT POPULATION

4.2.1   Inclusion criteria
1. Gestational age at birth 22+0-27+6: based on prenatal ultrasonography.
2. Enteral feeds < 100 mL/kg/day at the day of randomisation.
3. Written informed consent from the legal guardians of the infant.
4. The home clinic of the infant has the logistics of maintaining the intervention until gestational week 34+0

4.2.2   Exclusion Criteria
1. Lethal or complicated malformation known at the time of inclusion
2. Chromosomal anomalies known at the time of inclusion
3. No realistic hope for survival at the time of inclusion
4. Gastrointestinal malformation known at the time of inclusion
5. Abdominal surgery before the time of inclusion
6. Participation in another intervention trial aiming at having an effect on growth, nutrition, feeding intolerance or severe complications such as NEC and sepsis
7. Infants having nutrient fortifier or formula prior to randomisation

4.3   RANDOMISATION

This is a randomised trial. Randomisation will be based on the following stratification variables: primary enrolment site, gestational week (22+0-24+6 or 25+0-27+6), singleton/twin and gender. Because there are 4 stratification variables and not a particularly large study, an adaptive randomisation scheme will be used based on the method of minimisation. This will include a biased-coin randomisation scheme as needed in the adaptive scheme. [42] A specific computer program will be used to accomplish this scheme and the program will be carefully validated prior to incorporation in the study. The allocation will be concealed before inclusion, but after randomisation the study is not blinded.

When the infant fulfils the requirement of inclusion (please see section 9.1.1), a web-based randomisation service centre will be used: Randomize.net (Interrand Inc., Ottawa, Ontario, Canada). The patient identification number will be the consecutive number in the identification list at the study site, while the randomisation service centre will to which study group the infant shall be allocated. It is the patient identification number that should be used in the CRF and on

samples etc. The allocation should be indicated in the identification list and also in the CRF immediately. To confirm eligibility inclusion and exclusion criteria will be checked for, including signed parental consent, before randomisation is done.

Twins will be stratified for as indicated above. They will be randomised together. Thus, they will get the same randomisation number and also the same nutritional protocol. Twin number one will get the suffix A and number 2 the suffix B (e.g. 101 A and 101 B). There will also be stratification for gender. Because of limitations of randomisation/stratification procedure, a twin pair will be considered having the same gender when the randomisation is made. Thus, enter the gender of the first-born twin in the program.

4.4   STUDY SCHEDULE

Table 1 gives the schedule of visits and evaluations.

Table 1

Time point	Inclusion					Inter-vention ends		Neonatal follow up ends	Follow up at age of	
									2y	5.5y
Day of life	<100 ml/kg/d	7 d ± 2d	14 d ± 2d	21 d ± 2d	28 d ± 2d					
Gestational week						-w34+0	w36+0	-w44+0		
Informed consent	X									
Incl.-/exclusion criteria	X									
Background history	X									
Randomisation	X									
Intervention	‖==================================‖									
Feeding intolerance	‖==================================‖									
NEC, sepsis, mortality		‖===‖								

2020-06-22

Neurological impairment*									X	X
Antibiotics and concomitant medication	X	X	X	X	X	w34+0	X	X		
Weight**		X	X	X	X	w34+0	X	X	X	X
Height/head circumf.**		7d	14d	21d	28d	w34+0	X	X	X	X
Blood sample (if available)		X	X		X	w33+0-6				
Urine sample (if available)		X	X	X	X	w33+0-6				
Stool sample (if available)		X	X	X	X	w33+0-6				
Breast milk (if available)		X	X							
X	X	w33+0-6	X				X	X		
X	X	X								

5　　　SAMPLE SIZE

The incidence of the primary outcome, the composite of NEC, culture-proven sepsis and mortality, was 47.4% (1153/2428) in extremely preterm infants in 2007-2014 according to the Swedish Neonatal Quality Register (www.snq.se). Only infants surviving more than 3 days were included in this estimation since the inclusion will take place after 3 days of life. The NEC incidence (incl. NEC cases that also had a late onset sepsis episode) was 12.6% (305/2428). The sepsis incidence (without NEC) was 29.7% (720/2428), and the mortality incidence (without sepsis and NEC) was 5.3% (128/2428). The reduction of sepsis in the exclusive human milk fed group using Prolacta human milk-based products in a previous trial was 37.2%. With a similar effect on sepsis there would be a reduction to 20.9% (507/2428) in the $H^2MF^{®}$ group in our proposed trial (incl. 42 sepsis cases from the NEC with sepsis group above because as we estimate a 60% reduction of NEC, please see below). Since there is not any previous trial with 100% coverage of breast milk it is difficult to estimate the effect on NEC from previous trials.

2020-06-22

However, a conservative estimation would be a 60% reduction of the NEC incidence to 5.4 % (131/2428), as compared to US studies cited above where the fall in NEC was around 75%. Since many deaths may reflect undiagnosed NEC and sepsis episodes, mortality is estimated to be reduced by 25% to 4.0% (96/2428). Thus, the incidence of the composite outcome is estimated to be reduced to 28.0% (680/2428). With at least 101 infants in each group a reduction from 47% in the control to 28% in the active group would be detected at a 5% level of significance and with 80% power using a large sample Gaussian approximation to the comparison of two binomial proportions (using a pooled estimate of variance). Thus, the aim would be to enrol a total of 222 extremely premature infants in the trial to allow for an approximate 10% dropout rate.

However, because there are uncertainties in the effect size presupposed here, the study will be conditioned power in the following sense. An evaluation of the overall rate of the primary clinical endpoint of NEC/sepsis/death will be made prior to the formal analysis of that aspect of the study data in order to determine whether the trial sample size should be re-evaluated and increased in order to continue study enrolment. This evaluation will be based on the methodology suggested by Gould *et al.* [5]. Let σ be the pooled rate of this study endpoint in the two arms, i.e. $\sigma = (p_{control} + p_{human\ fortifier\ group})/2$, where p is the incidence of the primary study endpoint in the respective groups. Let σ^* be the same value, but based on some assumed rates for these rates from the beginning of the trial. Then the following sample size multiplier is determined:

$$f = [\sigma(1-\sigma)]/\sigma^*(1-\sigma^*)$$

If f is greater than 1, then the new sample size, n_1, is determined as f x n, where n is the original sample size (per group) of 101. If f is less than one, then the study may be analysed at that point for the primary clinical endpoints NEC/sepsis/death. Note that an independent statistician not associated with the study conduct will perform this conditional analysis. An independent statistician will make a sample size re-estimation when 150 infants have been included. Thus, the definitive sample size might be increased (never decreased) based on this interim analysis.

Theoretically, if the effect is very low, the calculation according to Gould will suggest a very high number of infants needed. Thus, there is a need of a clinically relevant upper limit of the inclusion. With a conservative estimation of a 50% NEC reduction in the H^{2}MF® group, a reduction of the composite outcome from 47% in the control and 31% in the active would be detected with at a 5% level and 80% power with 145 infants in each group. Based on that estimation, the upper limit of included infants would be 322 to allow for an approximate 10% dropout rate.

An estimation how many infants that would be needed for the composite of culture-proven sepsis and NEC has also been made, since this secondary outcome could be of special interest, as it is not affected by mortality rates. The incidence of this outcome was 42.2% (1045/2428) and could be estimated to be reduced to 26.2% (638 /2428) based on the background data described above.

With at least 142 infants in each group, a reduction from 42% in the control to 26% in the active group would be detected at a 5% level of significance and with 80% power. Thus, with a dropout rate of 10% in total 312 infants would be needed for this outcome. A similar evaluation according to Gould *et al.* as described above will also be made for this outcome after 150 infants have completed the study in order to determine whether the trial sample size should be re-evaluated and increased.

6   GENERAL CONSIDERATIONS

6.1   TIMING OF ANALYSES

There will be an analysis of the neonatal period at the conclusion of the enrolment of the subjects and the collection of the study endpoints. Additional analyses will be performed at 2 years and 5.5 years of follow-up.

6.2   ANALYSIS POPULATIONS

The primary basis for all analyses of the clinical outcome will use the intent-to-treat paradigm. This means that in this approach all randomised participants are included in the analysis regardless of their disposition in the study (including whether they actually adhered to the randomized nutritional protocol). In addition, a per protocol analysis will be conducted that will only include patients who appropriately complete the intervention. A further stratification in per protocol analysis will be if the onset of an outcome was before or after the start of the intervention.

6.3   MISSING DATA

No imputation of missing values will be performed. If there is no data on the primary endpoint because the subject terminated early, then we will assume that they had the endpoint.

6.4   INTERIM ANALYSES AND DATA MONITORING

Interim analysis will be carried out for safety analyses, please see section 13.4 in the study protocol. Also, it will be made for the final sample size calculation, please see 5. The interim analyses will be made by an independent statistician.

An independent DSMB, consisting of at least three members with at least one specialist in Neonatology and one Biostatistician, will have regular meetings to make interim safety analyses. Such an interim analysis shall be made after 50, 100 and 150 completed CRFs until discharge

2020-06-22

from the hospital. If any of the SAE is significantly more common (p<0.05) in the active than the control group, the DSMB will bring in all medical data on infants affected by this specific SAE. The DSMB will then assess the causality between the use of $H^2MF^{®}$ and the specific SAE in the affected infants. Based on this analysis the DSMB will decide if the trial can continue or not after consultation with the co-ordinating principal investigator and the sponsor. The DSMB may decide that it is ethically correct to pursue if the positive effects of the active intervention outweigh the SAE.

In case of a very strong effect of the active treatment, the study can be terminated prematurely based on a decision of the sponsor and the DSMB, if the primary outcome is significantly lower (with a significance level <0.001) in the $H^2MF^{®}$ than in the standard fortification group in the interim analysis made after 150 infants have been included. If the significant level is $\geq$0.001 the study enrolment will continue.

7 SUMMARY OF STUDY DATA

Descriptive statistics will be computed for all quantitative outcome variables: mean ± standard deviation and median ± interquartile range. For qualitative (categorical) outcomes, proportions and/or percentages will be computed. All summary tables will be structured with a column for each study arm.

7.1 SUBJECT DISPOSITION

A tabulation of subject disposition will be presented by study arm and overall, including the number screened, the number dosed at each level, the number for primary analysis, the number that withdrew prior to completing therapy, and reasons for withdrawal.

7.2 PROTOCOL DEVIATIONS

Protocol violations will be listed by patient.

7.3 DEMOGRAPHIC AND BASELINE VARIABLES

Patient distribution across demographic and baseline characteristics will be tabulated and presented by treatment arm and overall.

Comparability of demographic and baseline characteristics will be evaluated between the active and control groups. Comparisons will use a Wilcoxon rank sum test for quantitative data or a chi-square test for homogeneity (or Fisher's exact test if any expected value is less than 5) for categorical data.

2020-06-22

The following demographic and baseline characteristics will be evaluated:

1) Birth weight (gm)

2) Length (cm)

3) Head circumference at birth (cm)

4) Gestational age (Weeks+days)

5) Gender

6) 1 minute APGAR

7) 5 minute APGAR

8) 10 minute APGAR

9) Mode of delivery (c-section or vaginal)

10) Multiple birth (Yes/No)

        o If yes, number of infants

11) Born at hospital with level 1-2 NICU (Yes/No)

12) Gravida

13) Number of children mother as given birth to including this one

14) Maternal smoking during pregnancy (Yes/No)

15) Premature prolonged rupture of membranes $\geq$ 1 hour before contractions began (Yes/No)

16) Mother diagnosed with chorioamnionitis (Yes/No)

17) Mother diagnosed with preeclampsia (Yes/No)

18) Antenatal antibiotics during mother's hospital stay (Yes/No)

19) Number of 12mg doses of betamethasone >24 hours before delivery (Two, One, None)

20) IRDS verified by x-ray (Yes/No)

21) Surfactant (Yes/No)

22) IVH (Yes/No)

        o If yes, highest grade (I-IV)

23) Ductus (PDA treated) (Yes/No)

24) Culture proven sepsis (Yes/No)

25) Jaundice treated (Yes/No)

26) Other

27) Mechanical ventilator (Yes/No)

28) Need of extra oxygen (Yes/No)

29) NPPV and/or CPAP (Yes/No)

8   ENDPOINT EVALUATION

8.1.1   Primary Endpoint

8.1.1.1   *Occurrence of either NEC or sepsis and/or whether the infant dies*

As this is a binary outcome variable, the two groups will be compared using the chi-square test for homogeneity.

Additionally, the primary endpoint will be evaluated using a multivariate adjustment model using logistic regression that will include appropriately selected covariates.

Potential covariates will include variables such as birth weight, gestational age, AGA versus SGA and other clinical variables as described in Section 3.2.3. A univariate screening process will be employed to determine which covariates demonstrate the best correlation with the outcome.

8.1.2   Secondary Endpoints

Neonatal period:

There are a number of secondary outcome variables (described in Section 3.2.2). Quantitative variables will be compared between the study groups using the Wilcoxon rank-sum test or the Poisson means test (using the large sample z-approximation).

Right-censored time-to-event data (e.g. NICU or hospitalization time) will be evaluated by the method of Kaplan and Meier and compared between the groups using the log-rank test. A censoring event would be a patient dropout of the study

In addition, secondary analyses will involve the use of various covariates that will be incorporated into the evaluation through the use of generalized linear models using the

2020-06-22

appropriate link function for linear, logistic, or Poisson regression, or Cox proportional-hazards model for right-censored data.

Potential covariates will include variables such as birth weight, gestational age, AGA versus SGA and other clinical variables as described in Section 3.2.3. A univariate screening process will be employed to determine which covariates demonstrate the best correlation with the outcomes.

Qualitative variables such as the occurrence of BPD, ROP and IVH will be compared between the groups using the chi-square test for homogeneity (or Fisher's exact test for small [<5] expected values).

The number of days using TPN will be evaluated using the method of Ghandehari *et al.* [6]

The sections below contain information on specific endpoints for each type of endpoint.

8.1.2.1   *Quantitative Endpoints*

Quantitative endpoints will be compared between the study groups using the Wilcoxon rank-sum test. Data that are counts will be compared using the Poisson means test (using the large sample z-approximation). Covariates will be incorporated into the analytical model using a multiple linear regression or, for counts, Poisson regression.

These endpoints include the following:

- number of days feedings held for ≥12 hours OR feeds reduced by >50% (ml/kg/d)

- number of days of parenteral amino acid and/or lipid infusion. Only days when the enteral feed <150mL/kg/day should be included.

- Gastric aspirates: ≥100% pre-feed volume (2 hours feeding volume if continuous feeding). Lower limit=2 ml/kg.

- Number of stools per day

- Weight, height and head circumference at 7, 14, 21 and 28 days, the end of intervention (≤ 34+0), gestational week 36+0, at discharge from neonatal ward (or at gestational week 44+0, whatever comes first) and at 2 years of age (corrected) and 5.5 years of age (uncorrected). The standard deviation score (based on Niklasson [1] will be used in the calculations. A general linear mixed model (GLMM) will be used to evaluate the data over time between the two groups and a univariate Wilcoxon rank sum test can be used to evaluate change from baseline at various time points to compare the two groups. In addition, the velocity of change from baseline to discharge, say, will be computed with weight velocity calculated in g/kg/day (by the Patel[7] method), and length and head

2020-06-22

circumference velocity in cm/week. These results can be compared between the study arms using a Wilcoxon rank sum test.

8.1.2.2 Right-censored time-to-event data

The Kaplan/Meier method will be used to model the time to event curves and a log rank test will compare groups. Additionally, a Cox proportional hazards regression will be utilized to evaluate the effects of selected variables.

Length of need for a feeding tube may consist of intervals. These days will be combined as described in Ghandehari *et al.* [6]

- Time to reach full enteral feeds: the day of life the infant has received at least 150 mL/kg enteral feeds.
- Time to regain birth weight: first day of three success days when the infant weight is greater than birth weight. Indicate 0 days, if the infant never has a weight below birth weight.
- Number of days with intensive care: need of respirator or CPAP until discharge (not later than gestational week 44+0).
- Length of stay at the hospital: gestational week and day at discharge (not later than gestational week 44+0).
- Length of need of feeding tube: gestational week and day when the infant does not need it anymore (not later than gestational week 44+0).

8.1.2.3 Qualitative Endpoints

Qualitative variables such as the occurrence of BPD, ROP and IVH will be compared between the groups using the chi-square test for homogeneity. A logistic regression model will be utilized for binary outcome variables to evaluate the effects of covariates as described above. For non-binary outcome variables, (e.g., intraventricular haemorrhage (IVH): classified into grade I-IV), a polytomous logistic regression will be performed. The qualitative variables include:

- Mortality

- Necrotising enterocolitis (NEC): Bell´s stage II-III

2020-06-22

- The composite of NEC and culture-proven sepsis: An infant should have had any of these two diagnoses to fulfil the criterion.

- Spontaneous intestinal perforation (SIP)

- Abdominal surgery

- Culture-proven sepsis

- Suspected sepsis, not culture-proven

- Pneumonia

- Bronchopulmonary dysplasia (BPD)

- Retinopathy of the prematurity (ROP)

- Intraventricular haemorrhage (IVH): classified into grade I-IV

- Periventricular leukomalacia (PVL)

8.1.3 Laboratory variables

- Microbiology
    - Bacterial DNA
    - Gas chromatography

- Immunological analyses in blood samples
    - T-helper subsets (TH1, TH2, TH17, Treg)
    - Levels of anti- and pro-inflammatory cytokines (e.g. IL-10, IL-6, TNF-α)
    - Levels of anti- and pro-inflammatory cytokines (e.g. CXCL11 and CCL18)
    - Immune modulatory enzymes (Indoleamine 2,3 Dioxygenase (IDO))

- Growth factors and neurotransmitters in blood samples
    - Levels of growth factors such as IGF-1 and the associated IGFBP-3
    - Neurotransmitters
    - Markers for CNS damage such as Neurofilament light protein (in plasma)

- Lipodemic analyses in plasma and erythrocyte membranes to assess the effects of the different feeding regimens on lipid proteins.

- Metabolomic analyses in urine.

2020-06-22

- Proteomic and oligosaccharide analyses in breast milk samples

- Vitamins and micronutrients in blood samples

These variables are exploratory in nature and so will be summarized by descriptive statistics (mean/SD or median/IQR) for each group.

8.1.4   Health economics

The number of days at each level of care (as defined below in Table 2) will be recorded until discharge from the hospital (not longer than gestational week 44+0). The cost will be calculated by multiplying the number of days at each level of care by the average cost. The investigators will provide the calculations.

Table 2: Classification of level of care

0	**Infants who are not discharged but spend the whole day at home (cared for by home care team or only phone contact with neonatal unit) are cared for by home care team or who are on "leave"**
1	**Infants who do not require continuous monitoring by health care staff.**
2	**Infants who require continuous monitoring by staff and/or monitoring equipment**
3	**Low level intensive care: Infants who require continuous monitoring and CPAP treatment or central venous access.**
4	**Medium level intensive care: Infants who require continuous monitoring by intensive care staff**
5	**High level intensive care: Infants who require continuous monitoring by more than one intensive care staff**

Two year and 5.5 year follow-up

Section 3.2.2.2 lists the endpoints collected at these visits. Quantitative endpoints will be analysed using the methods described in Section 8.1.2.1. Qualitative endpoints will be analysed utilizing methods in Section 8.1.2.3

2020-06-22

9 SAFETY ANALYSES

9.1.1 Adverse Events

Treatment emergent adverse event data (with a start date on or after randomization) will be combined from several CRF pages: Infection, NEC, Clinical Condition from Birth to Discharge, and Adverse Events.

Tabulations will be presented by event and seriousness (SAE vs. non-SAE) and will be compared between treatment arms.

Suspected unexpected serious adverse reactions (SUSAR) will be tabulated separately. These events will be recorded on a separate SUSAR form.

A listing of all treatment emergent adverse events will also be presented. The information for each event may differ somewhat based on the CRF page in which the data were captured.

9.1.2 Concomitant Medications

Tabulations of major classifications of medications (antibiotic, fungicide, hydrocortisone, corticosteroids, gastric acid inhibitors, insulin, opioids, naloxone, probiotics) will be presented for each treatment arm.

Additionally, a listing of all medications recorded will be included.

9.1.3 Concomitant Conditions

Any adverse event recorded as described in 9.1.1 that has a start date prior to randomization will be tabulated by event type and treatment arm.

Additionally, a listing of all concomitant conditions will be presented.

10 TECHNICAL DETAILS

11 CHANGES IN CONDUCT OF STUDY OR TO PLANNED ANALYSES FROM PROTOCOL

Deviations from the statistical analyses outlined in this plan will be indicated; any further modifications would be noted in the final statistical analyses.

2020-06-22

12   REFERENCES

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13   LISTING OF TABLES AND LISTINGS

Tables

1. Summary of Subject Disposition

2. Summary of Demographics and Baseline Characteristics

3. Summary of Pregnancy Characteristics

4. Summary of Pre-Study Conditions

5. Summary of Intervention

6. Summary of NEC, culture-proven sepsis and mortality

7. Multivariate Analysis of NEC, culture-proven sepsis and mortality with selected covariates

8. Summary of food intolerance and growth – Neonatal period

9. Multivariate analysis of food intolerance and growth with selected covariates – neonatal period

10. Summary of Clinical variables for morbidity – Neonatal period

2020-06-22

11. Multivariate Analysis of clinical variables for morbidity with selected covariates – neonatal period

2 Year Follow-up

12. Weight, height and head circumference – 2 year follow-up

13. Multivariate analysis of weight, height and head circumference with selected covariates

14. Neonatal interventions up to 44 weeks

15. Multivariate analysis of neonatal interventions with selected covariates

16. Summary of neurological outcomes – 2 year follow-up

17. Multivariate analysis of neurological outcomes with selected covariates.

18. Morbidity/Mortality

19. Social Variables

5.5 Year Follow-up

20. Weight, height and head circumference – 5.5 year follow-up

21. Multivariate analysis of weight, height and head circumference

22. Summary of neurological outcomes – 5.5 year follow-up

23. Multivariate analysis of neurological outcomes with selected covariates.

24. Morbidity/Mortality 5.5 year follow-up

Laboratory analyses

25. Microbiology analyses

26. Immunological analyses in blood samples

27. Growth factors and neurotransmitters in blood samples

28. Lipidomic analyses

29. Metabolomic analyses – Urine

30. Proteomic analyses in breast milk

Health Economics

31. Level of care costs

Safety

32. Treatment emergent adverse events

33. Serious treatment emergent adverse events

34. Suspected unexpected serious adverse events

35. Concomitant medications

36. Concomitant medical conditions

Two year follow-up

37. Summary of findings at 2 years by treatment arm

Five year follow-up

38. Summary of findings at 5.5 years by treatment arm

Listings

1. Subject disposition

2. Protocol violations

3. Adverse events

4. Concomitant medications

5. Concomitant conditions