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Efficacy of *Bifidobacterium animalis* subsp. lactis (BB-12), *B. infantis* and *Lactobacillus acidophilus* (La-5) probiotics to prevent gut dysbiosis in preterm infants of 28+0–32+6 weeks of gestation: a randomised, placebo-controlled, double-blind, multicentre trial: the PRIMAL Clinical Study Protocol

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ABSTRACT

Introduction The healthy 'eubiosis' microbiome in infancy is regarded as the microbiome derived from term, vaginally delivered, antibiotic free, breastfed infants at 4–6 months. Dysbiosis is regarded as a deviation from a healthy state with reduced microbial diversity and deficient capacity to control drug-resistant organisms. Preterm infants are highly sensitive to early gut dysbiosis. Latter has been associated with sepsis and necrotising enterocolitis, but may also contribute to long-term health problems. Probiotics hold promise to reduce the risk for adverse short-term outcomes but the evidence from clinical trials remains inconclusive and none has directly assessed the effects of probiotics on the microbiome at high resolution.

Methods and analysis A randomised, double blind, placebo-controlled study has been designed to assess the safety and efficacy of the probiotic mix of *Bifidobacterium animalis* subsp. lactis, *B. infantis* and *Lactobacillus acidophilus* in the prevention of gut dysbiosis in preterm infants between 28+0 and 32+6 weeks of gestation. The study is conducted in 18 German neonatal intensive care units. Between April 2018 and March 2020, 654 preterm infants of 28+0–32+6 weeks of gestation will be randomised in the first 48 hours of life to 28 days of once daily treatment with either probiotics or placebo. The efficacy endpoint is the prevention of gut dysbiosis at day 30 of life. A compound definition of gut dysbiosis is used: (1) colonisation with multidrug-resistant organisms or gram-negative bacteria with high epidemic potential or (2)

Strengths and limitations of this study

► This is a large randomised, placebo-controlled, double-blind, multicentre study in a diverse population of preterm infants of 28+0–32+6 weeks of gestation at risk for gut dysbiosis.

► Efficacy of *Bifidobacterium animalis* subsp. lactis, *B. infantis* and *Lactobacillus acidophilus* probiotics on gut dysbiosis is studied by high-resolution DNA sequencing of the microbiome and thorough clinical and immunological phenotyping of infants.

► The study design allows to control for inherent differences among neonatal intensive care units, such as their microbial environment, and for the maternal microbiome in a subset of infants.

► The time window of enrolment is restricted to the first 48 hours of life to assure that early development of the infant's microbiome is targeted.

► The definition of gut dysbiosis in preterm infants at day 28–30 of life is based on deviations compared with the microbiome of healthy term infants.

a significant deviation of the gut microbiota composition as compared with healthy term infants. Dysbiosis is determined by (1) conventional microbiological culture and (2) phylogenetic microbiome analysis by high-throughput 16S rRNA and metagenome sequencing. Persistence of dysbiosis will be assessed at 12-month follow-up

visits. Side effects and adverse events related to the intervention will be recorded. Key secondary endpoint(s) are putative consequences of dysbiosis. A subgroup of infants will be thoroughly phenotyped for immune parameters using chipcytometry.

Ethics and dissemination Ethics approval was obtained in all participating sites. Results of the trial will be published in peer-review journals, at scientific meetings, on the website (www.primal-study.de) and via social media of parent organisations.

Trial registration number DRKS00013197; Pre-results.

INTRODUCTION

It is a general assumption that the healthy 'gold-standard' microbiome (eubiosis) in infancy is derived from term, vaginally delivered, antibiotic-free, breastfed infants at 4–6 months. In the context of preterm infants, gut dysbiosis is defined as a microbiome deviation from the healthy state including reduced microbial diversity and deficient capacity to control the colonisation with multidrug-resistant organisms (MDROs) and gram-negative bacteria with high epidemic potential.[1] This definition used in our study is an approximation, as all preterm infants are exposed to factors impacting on microbiome development early in life, for example, delivery via caesarean section, formula feeding, exposure to hospital rather than maternal flora and antibiotics,[2–5] which can affect the gut microbiome in the long term.[6] The colonisation with *Bifidobacteria* and *Bacteroides* is delayed in preterm infants. Arboleya *et al* noted that faecal samples of preterm infants more frequently contain pathogenic species, such as *Clostridium difficile* or *Klebsiella pneumoniae*, as compared with term infants. The authors suggested that metabolic capacity is likely to be implicated in these changes, as indicated by the reduced levels of bioactive short-chain fatty acids in the microbiota of preterm infants compared with term infants.[7]

There is an urgent need for a more in-depth analysis of gut dysbiosis in a clinical study context of highly vulnerable preterm infants. Gut dysbiosis, that is, lower abundance of *Bifidobacteria* and higher abundance of *Gammaproteobacteria*, was found to precede the development of sepsis and necrotising enterocolitis (NEC).[8–10] It has been hypothesised that dysbiosis contributes to immunological dysregulation and sustained inflammation. Both are very probable causes of long-term health problems in preterm infants, including chronic lung disease, growth failure, the metabolic syndrome and an adverse neurobehavioural and cognitive outcome.[11 12] Well defined 'risk' and 'resilience' microbiome patterns may eventually serve as biomarkers or targets for modifications.

Several approaches to modify gut dysbiosis in adults, such as selective decontamination, use of prophylactic antibiotics (eg, colistin) and faecal transplantation[13–15] are not feasible for preterm infants, in particular for ethical and safety concerns. Instead, probiotics with bacteria that excel as gut colonisers of breast milk-fed infants,[16] are highly attractive agents to foster the early microbiome establishment.[17] For instance, a probiotic supplementation may restore the microbiome of antibiotic-treated

or caesarean-born term infants.[5] Numerous studies on the therapeutic effects of probiotics in preterm infants (PIPS) have been performed. However, the results remain inconclusive due to a high variability in study protocols, target populations, probiotic formulations (eg, strain composition and inclusion of single vs multiple strains) and endpoints. The majority of studies have focused on short-term endpoints, in particular NEC and sepsis.[18–24] Meta-analyses have found a benefit for preterm infants regarding the risk of adverse short-term outcome.[19 25] In contrast, the largest randomised controlled trial (RCT) to date, the PIPS study using Bifidobacterium brev probiotics, did not find any benefit for preterm infants.[24] However, controversy remains regarding the analysis and interpretation of the findings.[26] The scientific uncertainty in regard to the efficacy and safety of probiotics is reflected in their heterogeneous use in medical practice. Prophylaxis with *B. longum and infantis/L. acidophilus* has routinely been adopted by several European neonatal intensive care units (NICUs), for example, in Austria,[22] the Netherlands[23] and Germany.[20 27] In contrast, the Norwegian community of neonatologists has stopped this approach after occurrence of sporadic probiotic strain sepsis cases,[28] and NICUs in the USA are still reluctant to implement probiotic prophylaxis strategies in preterm infants.[28] As of yet, mechanistic data on how probiotics exert a potentially beneficial effect in preterm infants are lacking and no study has directly assessed the effects of probiotics on the microbiome at high resolution. Furthermore, the efficacy of probiotics might depend on the gut microbiome composition when the probiotics are administered ('baseline'). Potentially influencing factors, such as maternal microbiome/metabolome,[29 30] the perinatal exposure to antibiotics and the nutritional context (eg, human milk oligosaccharides)[31] were hardly considered in previous studies. Hence, an adequately powered RCT using a well-defined probiotic strain mix and validated clinical and microbiological outcome measures in preterm infants at risk of microbiome-related sequels will improve clarity in this field.

Here, we report on the methodology of a large, double blind, RCT using *B. animalis* subsp. lactis and *infantis and L. acidophilus* in preterm infants born between 28+0 and 32+6 weeks of gestation (German Clinical Trial Register: DRKS00013197). The study is funded by the German Ministry of Research and Education (01GL1746B). The specific aims of this RCT are (1) to evaluate if *B. animalis* subsp. lactis *and infantis and L. acidophilus* reduce the risk of gut dysbiosis compared with placebo and (2) to determine the safety profile of this probiotic mix. We hypothesise that the administration of probiotics will be associated with a reduction in gut dysbiosis, fewer infections and an improved metabolism, that is, growth rates more frequently in line with age-based percentiles. We also hypothesise that probiotics will not be associated with serious adverse events (SAEs). This RCT is the core study of our PRIMAL consortium, which more broadly investigates the interaction between gut microbiome

and immunity on a cellular level at the beginning of life.

METHODS AND ANALYSIS

Overview

This is a double-blind, multicentre clinical RCT to evaluate both short-term and long-term efficacy and safety of *L. acidophilus* and *B.* spp in preterm infants. Between 1 April 2018 and 31 March 2020 preterm infants born between 28+0 and 32+6 weeks of gestation are assessed for eligibility, approached for informed consent and randomised in the first 48 hours to receive placebo or probiotics. Study drug or placebo will be administered once daily for 28 days after randomisation. Physicians, nursing staff, parents and all study personnel are blind to the intervention. The study is conducted in compliance with the Declaration of Helsinki, the current revision of International Conference on Harmonisation (ICH) Topic E6, the Guidelines for Good Clinical Practice (GCP) and according to current Consolidated Standards of Reporting Trials (CONSORT) and Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) recommendations.[32 33]

Setting

Patients are being recruited at 18 German Children's Hospitals with a tertiary-level NICU, including the University Children's Hospitals in Bochum, Bonn, Cologne, Essen, Freiburg, Hannover, Heidelberg, Homburg, Jena, Lübeck and Tübingen, and the regional Children's Hospitals in Aschaffenburg-Alzenau, Bremen, Hamburg-Wilhelmstift, St. Vincenz Paderborn, Rostock-Klinikum Südstadt, Schwerin and Wiesbaden.

Each centre has a solid research infrastructure. Seventeen centres belong to the German Neonatal Network (GNN).[20 27] The Centre for Clinical Studies (CCS) (ZKS) and the Institute of Medical Biometry and Statistics (IMBS) at the University of Lübeck are responsible for data management and data analysis. An independent data safety monitoring board (DSMB) composed of a patient representative, a statistician and two paediatricians with long-standing experience in clinical trials in neonatology and paediatric infectious diseases convenes on a regular basis to review the enrolment, the study procedures, completion of the case report forms (CRFs), data quality, lost to follow-up and interim safety and efficacy results.

Inclusion criteria and rationale

1. Patient's parents or appropriate legal guardians have been informed about the study procedures and interventions and have given written informed consent.
2. Female or male preterm infants born between 28+0 and 32+6 gestational weeks of any ethnic background and admitted to the listed study sites within the first 48 hours of life.

We have selected the specific group of infants born between 28+0 and 32+6 weeks for several reasons.

Extremely preterm infants <28 weeks of gestation have a high risk of short-term morbidities, which might interfere with analysis on host-microbial interactions. Biomaterials in these infants are very limited. Moreover, very few neonatologists in Germany would agree to participate in a clinical RCT on the efficacy of probiotics in infants <28 weeks of gestation. Approximately 70% of very low birthweight infants born in GNN centres are currently supplemented with probiotics as a medical standard to prevent NEC.[20] The incidence of SAEs and AEs in preterm infants between 28+0 and 32+6 gestational weeks is much lower as compared with infants <28 weeks (eg, NEC, 28+0–32+6 weeks ≤1%[27]). In addition, our cohort allows to study a subgroup of patients, who are not exposed to antibiotics (≈20% of our target population[27]), which is a rarity in infants <28 gestational weeks. Furthermore, preterm infants between 28+0 and 32+6 gestational weeks are preferable to near-term or term infants, since they are cared for under highly controlled conditions in NICUs and usually stay in hospital until day 28. In contrast, term infants are discharged early and follow-up is difficult to establish. Moreover, the rate of dysbiosis is higher in preterm infants compared with term born infants, therefore, the effect of the intervention is expected to be more distinct in this group of patients.

Exclusion criteria and rationale

We exclude infants with malformations that are not compatible with survival beyond the first 48 hours of life or that severely affect the gastrointestinal tract. The definition for the latter is preclusion of appropriate enteral feeding, requirement of surgery in the neonatal period or NEC Bell's stage ≥1B.

Implementation

Eligible patients are identified by an approved study investigator (SI), who maintains a log of all screened preterm infants that is available for later monitoring. The SI discusses the background and objectives of the study with the caregivers of the patient and gives information on procedures and interventions. If written informed consent is given, the SI assigns a patient identification number, randomises the patient, collects baseline demographic variables and completes the according CRF.

Participant allocation

Participants are randomised to probiotics or placebo in a 1:1 ratio in the first 48 hours after birth. Randomisation is organised centrally by the ZKS Lübeck using standard operating procedures (SOPs) to guarantee concealment of allocation. Block randomisation is used with randomly varying block length and with stratification by centre, gender and gestational age. Genders are divided into two blocks of gestational age: (1) 28+0 to 30+6 and (2) 31+0 to 32+6. The lists are sent to the pharmacy of the University Medical Centre of Lübeck that prepares study treatment boxes for all patients and study sites according to the randomisation schedule. The boxes are consecutively

numbered according to the randomisation schedule and sent to the study centres according to requirements. Blinding information is supplied in case unblinding is deemed necessary for medical reasons, such as severe AEs (death, NEC). The study medication, that is, probiotics and placebo, is uniformly packaged and probiotics are taken from a single batch.

Intervention and comparator

The probiotic formulation (verum), consisting of B. species and *L. acidophilus* corresponds to the formulation that has been most commonly used among the participating study sites in the past.[20] The active intervention is provided once daily in single dose capsules. Each dose contains 1.5×10^9 colony forming units (CFUs) of each of the strains: *Lactobacillus acidophilus, Bifidobacterium animalis* subsp. lactis *and B. infantis.* The placebo is corn starch provided as powder in similar colour and odour in identical capsules as it was used in the PIPS study.[24] Both products are packaged into boxes, which contain capsules for a complete course for one infant. The boxes are stored in a fridge at 4°C until usage. After inclusion of a patient, the boxes remain at the bed site at room temperature. Verum or placebo is given as soon as possible after randomisation, immediately after start of enteral feeding usually within the first 24 hours after birth. The content of one capsule is dissolved in milk/formula or glucose solution and administered orally or per nasogastric tube. The study sites are encouraged to preferably feed colostrum or breast milk. An intervention is defined as successful even when the capsule content is only partially administered. Treatment is continued for 28 days or until hospital discharge (whatever is earlier). A record of doses omitted will be kept. All aspects of clinical management, including discontinuation of the study medication due to medical reasons, are at the discretion of the attending neonatologists. They should follow local, national and international guidelines.

Probiotic quality control

Samples of all batches of the probiotics are tested every 6 months for microbiological quality (PRIMAL Faecal Core Centre in Mainz, FCCM), as dose decay has been an issue in a previous RCTs.[24 34] Probiotic formulations and placebo are 16S rRNA sequenced for baseline content.

Sample collection

After informed consent, the first two study investigations are performed by the attending physicians of the study site after randomisation (baseline, day 1–3 of life; time point (1) and 28 days after the start of intervention or discharge (day 28–31 of life, at least 14 days of intervention; time point (2), while the infants are cared under controlled conditions in neonatal units. The third assessment is performed at 12 months (time point 3) corrected age at the study site by the PRIMAL study team from the lead site at the University of Lübeck or the local principal investigators. The flow chart is described in online supplementary figure 1.

AFor microbiome analysis stool samples are collected. At the time points (1) and (2), three faecal aliquots are obtained from one sample. Aliquot 1 (room air) is sent to the local microbiological laboratory for microbiological culture and identification of

► Multidrug-resistant strains (multiresistant gram-negative bacteria (MRGN); 2 MRGN; 3 MRGN; 4 MRGN; methicillin-resistant *Staphylococcus aureus.*
► Pathogens without resistance characteristics but highly epidemic potential for outbreaks (*Serratia* spp; *Pseudomonas* spp; *Klebsiella* spp; *Enterobacter* spp).

This procedure is in line with the German screening recommendations according to the Kommission für Krankenhaushygiene und Infektionsprävention (KRINKO).[35] The results of the screening at time point 2 will be documented on the CRF-2 for the microbiological definition of 'gut dysbiosis'. Aliquot 2 and 3 are uniformly packed and frozen according to protocol and stored at −80°C, until the aliquots are sent to the PRIMAL FCCM for an independent microbiological analysis according to KRINKO from previously frozen material. For a nested-subgroup study of n=120 infants (n=60 verum, n=60 placebo) with complete faecal sample sets of time points 1–3, the FCCM receives a maternal stool sample for analysis taken at any time after birth during hospital stay. Preparation of stool samples, DNA extraction and microbiome sequencing is performed using two approaches: (1) Amplicon-based sequencing of the V4 region of the bacterial 16S rRNA genes (Illumina MiSeq in Mainz); (2) Whole genome shotgun metagenomic sequencing of a subset of the samples and their respective mother samples (Illumina HiSeq 2000/2500 at the European Molecular Biology Laboratory (EMBL) Genomics Core Facility in Heidelberg). Using the marker-based microbiome profiles and additional metagenomic information, the genetic and phylogenetic composition of the gut flora is profiled and assessed using publicly available databases like the Comprehensive Antibiotic Resistance Database, ResFam antibiotic resistance gene database, 16S rRNA databases (SILVA) and the Kyoto Encyclopaedia of Genes and Genomes Database.

Multivariate statistical models are created to identify and assess clusters and differences in the microbiome profiles of the study samples and integrate the metadata and clinical data collected at the study sites. Comparisons of the study samples and their respective mother samples are performed to investigate the potential impact of the vertical microbial transfer from mother to child. At the 12-month follow-up visit, a stool sample of the infant is collected according to a home stool collection protocol which is similar to the collection and storage procedure at time points 1 and 2. The protocol consists of providing the family with a specimen collection kit and an insulated envelope. The samples should be as fresh as possible before follow-up visit, transported with cool packs and immediately frozen at −80°C on arrival at the

study site. The aliquots are sent to FCCM and microbiome sequencing will be performed.

For immunophenotyping and experimental subprojects of the PRIMAL consortium, blood samples including plasma aliquots are collected at time points 1 to 3 at selected study sites (n=250, n=125 verum, n=125 placebo).

For immuno-phenotyping, the peripheral blood mononuclear cells from whole blood are separated by ficoll density centrifugation, resuspended in wash buffer, and then pipetted into ZellSafe chips, using a standardised protocol. The chips are stored at 4°C until shipment to the PRIMAL Immunological Core Centre (ICC) in Hannover for analysis of cellular markers of T-cells, B-cells, granulocytes, monocytes, NK-cells and stem cells.

Outcome measures

The efficacy endpoint is the rate of gut dysbiosis at day 28–30 of life. The compound definition of gut dysbiosis is based on (1) the guideline definitions of the KRINKO[26]: Colonisation with MDRO or bacteria with epidemic potential (*Enterobacter* spp, *Klebsiella* spp, *Serratia* spp, *Pseudomonas* spp) as detected by microbiological culture (primary endpoint) or/and (2) significant deviations from the microbiome composition of healthy term infants (proportions of bacterial phyla, reduced diversity or specific features, such as increased virulence capacity and blooms of specific pathogens; secondary endpoint).[36] Gut dysbiosis in preterm infants is assessed through comparison with published and upcoming large-scale metagenome and 16S datasets describing the developmental trajectories in term infants, such that the variability of these trajectories will define a range of eubiotic infant gut states.[37 38] The latter is determined by analysis of all samples by resolution at the genus level (16S rRNA MiSeq sequencing). In detail, faecal samples will be collected, frozen at −80°C and sent by the various participating PRIMAL centres under standardised conditions, then stored frozen in Mainz until use. DNA will be extracted using a suitable kit (Nextera XT DNA Library Preparation Kit). DNA preparations will be subjected to amplicon PCR starting with primer pair sequences for the V3 and V4 rRNA region. Before loading pooled and finally cleaned samples to the next-generation sequencing (NGS) system dual indices and sequencing adapters will be attached during the index PCR step (Nextera XT Index Kit). For bioinformatic evaluation of the sequencing data, NGS system will be used, which classifies the generated V3 and V4 rRNA sequences by comparison to the Greengenes database (http://greengenes.lbl.gov/). Classification of reads will result in quantification of the sample composition at as specific a taxonomic level as is possible, usually at least phylum, class, order, family and in some cases also genus and species. Quality control measures will be implemented covering the NGS procedure itself, but also the preparation and processing of the library. Further analysis will be carried out using the LotuS, MOCAT or QIIME bioinformatics systems. Alternative bioinformatic

tools (like SIAMCAT, ANCOM) will be further considered as needed in order to test the hypothesis of this study.

Metagenomic shotgun sequencing of stool samples will be executed on an Illumina HiSeq system to allow for discrimination of individual species and strain populations, also in respect to transmission from the mother.[5] Samples will be paired-end sequenced (depth 5 Gbp). Metagenomic sequencing data will be processed using a previously published computational pipeline, with the results analysed by bioinformaticians at EMBL. Briefly, the reads will be aligned to reference databases using the MOCAT pipeline, drawing on the CARD and ResFams antibiotic resistance gene databases, virulence factors as annotated in PATRIC and for metabolic profiling, using to the Kyoto Encyclopaedia of Genes and Genomes database. Resulting taxonomic and functional profiles infants receiving verum vs placebo will be further analysed using univariate and multivariate statistics, association networks and machine learning techniques. We will be able to observe differential dysbiosis, in particular concerning four clinically central aspects of the gut microbiome: (1) loss/survival of keystone taxa, (2) loss/retention of ecological diversity, (3) shifts in metabolic and functional capacity (eg, increased abundance of virulence and antibiotic resistance genes) and (4) blooms of pathogens/pathobionts. All microbiome data will be integrated with clinical metadata and immunological data at EMBL and the CCS in Lübeck. Key secondary efficacy endpoint(s) are clinical or laboratory signs related to infection, immunity and metabolism. In particular, these are frequency of blood culture proven sepsis and clinical sepsis during the primary hospital stay; postnatal exposure to antibiotics, defined as daily doses of antibiotic treatment per 1000 patient days and number of antibiotic cycles (as surrogate parameter for infection episodes); infectious episodes in the first year of life, for example, otitis media, upper respiratory tract infections, bronchitis, gastrointestinal infections; number of antibiotic courses, as assessed by patient's diary, telephone interviews and follow-up at 12 months of age; wheezing episodes and atopic dermatitis as assessed by patient's diary, telephone interviews and follow-up at 12 months of age; leucocyte subset distribution and marker expression; growth and nutritional aspects, for example, weight gain per day, velocity of growth for head circumference, body length; number of days to achieve full enteral feeding, number of episodes to discontinue feeds >12 hours, sources of feeding (human milk, formula) and body weight, length, head circumference and blood pressure at 12 months of age.

The main safety outcome will be NEC or invasive infection with identification of probiotic bacteria in sterile fluids. Based on our experience in more than 10 000 treated infants born in GNN centres, where probiotics similar to the study medication are frequently used and infections with probiotic strains have not been observed, the safety of the intervention can be expected to be very high. In our target population, we expect an NEC rate of 0.8%, clinical sepsis rate of 17.8% and culture-proven

sepsis rate of 7.1%. All study physicians are asked to report complications of preterm birth (pneumothorax, intraventricular haemorrhage, death) regardless of the relation to the intervention. AE analysis follows the guidelines of Common Terminology of Adverse Events for assessment of attribution, toxicity grading scale and criteria for patient withdrawal. The secondary safety outcome is the presence of potential side effects such as gastrointestinal intolerance, blood in stool and abdominal pain. We acknowledge, however, that symptoms potentially related to the study drug may be difficult to be distinguished from those related to immaturity of the preterm gut. The study physicians will complete the respective forms for all AEs, identified during the primary stay in hospital and send it to the ZKS and the study centre in Lübeck.

Data collection

Standardised CRFs are used to record information on the patient's health at the time of birth (CRF-1; baseline), at day 28 after start of intervention (CRF-2; primary endpoint), at discharge (CRF-3), at the 6-month interview (CRF-4) and 12-month follow-up (CRF-5). Data collected at the follow-ups are collected to measure long-term outcomes. The forms are filled in manually by SIs in the study sites and are sent to the clinical project management (CPM) after the patients have been discharged.

CRF-1 includes information on the birth, causes of preterm birth, age, weight, head circumference and length, bonding, antenatal treatments with antibiotics and on the mother (diet, habits, body weight).

CRF-2 includes information on dysbiosis, growth parameters and detailed medical information on the treatment with antibiotics, invasive measures, feeding and on transfers to different hospital wards. Information on the use of mother's own milk, donor breastmilk and formula in this population of preterm infants in the study NICUs will be collected.

CRF-3 records growth parameters and details on feeding, pathogens in the event of sepsis and on complications.

CFR-4 data are collected in a telephone interview. Therefore, all caregivers will receive a diary at discharge to document infections, on medical consultations or hospital stays, antibiotic treatments, vaccinations and feeding. The interviews are coordinated by the CPM.

CRF-5 data are obtained during the 1-year follow-up examination, which is coordinated by the CPM and performed at the study site. Next to details concerning age, weight and size, feeding, faecal sample collection will be performed to assess the sustainability of microbiome patterns throughout infancy.

Data handling and monitoring

The CRFs of the study sites are sent to the CPM. There, patient data are saved for further contacts of the families. All data will be sent completely pseudonymised to the ZKS/IMBS Lübeck, where incoming data are entered into the central research database.

As personal data of the patients will be saved separately on a different server than the clinical database, data of individual participants cannot be 'reconstructed' by data mining or similar procedures. The procedures will closely follow the regulations as specified in the German data protection law. Data transfer between CCS Lübeck, the platform for microbiome analysis (FCCM/EMBL) and immune phenotyping (ICC Hannover/Homburg) is organised by a working group of platform members. The CPM will be responsible for maximising the output of PRIMAL clinical study. The steering group will also be contacted by external researchers who are interested in exploitation of the data.

Data dissemination

The dissemination of progress in PRIMAL rests on four columns: (1) The PRIMAL website (www.primal-studie.de), which depicts researchers, projects, publications, webcasts, allows for direct interaction with interested patients, professionals and the media, and is linked to social media activities of patient organisations; (2) a newsletter, which will be sent quarterly to all centres in order to motivate participating physicians and to address topics of discussion for the regular study meetings; (3) presentation at meetings, which will allow for discussions with the scientific community; (4) publications, that is, reports on methodology and on various outcome variables will be published in peer-reviewed journals. The patient organisations European Foundation for the Care of Newborn Infants and the Bundesverband 'Das frühgeborene Kind' e.V. have also expressed their interest in contributing to dissemination of results (eg, through electronic newsletter and social media channels).

Patient and public involvement

The development of the research question and outcome measures and the design of this study were discussed with patient organisations beforehand. The data safety monitoring involves parents' representatives. The results will be disseminated in a deidentified fashion to study participants via different media as stated above. A potential burden of intervention will not be measures by patients or parents but by thorough safety measures of the study team as stated below.

Quality assurance and safety

During the clinical trial, quality control and assurance is ensured through on-site monitoring, auditing and, if applicable, through supervision by the authorities. All investigators agree that the monitor visits the clinical centre before (pretrial visit) during and after completion of the study in order to ensure that the study is conducted, recorded and reported according to the protocol, the SOPs, the GCP and the applicable regulatory requirements. The monitor provides each site with a written report, and sites have been required to respond to queries and resolve problems. In addition to these routine monitoring procedures, audits—by the sponsor or by

authorities—will be conducted in the framework of the auditing system in accordance with ICH-GCP guidelines. In the context of an audit, the planning, conduction and analysis of a clinical trial will be analysed for compliance with the ICH guidelines. This will address, whether data handling, the organisational structure of the study centre, and the original documents are in accordance with the agreement between the sponsor and the study board. It will be the primary goal of the auditing to ensure that all of the data required for the interim and final analysis can be properly extracted from the files.

SOPs on the administration of probiotics/placebo are shared among all study centres including at site training before start of enrolment. Central reporting of SAEs is provided. An independent DSMB (see the 'Setting' section) is established. It is the obligation of the DSMB to monitor the course of the study, and if necessary, to give recommendations to the study administration for discontinuation or modification of the study. The primary principles are the ethical and safety aspects concerning the patients. The DSMB examines, whether the continuation of the study can be ethically justified, whether the safety of the patients is ensured, and whether the progress of the study is acceptable. For this, the DSMB has to be informed about adherence to protocol, patient recruitment and any observed AEs. The DSMB receives the corresponding reports in due time before the planned interim analysis. The composition and responsibilities of the DSMB, the structure and procedures of its meetings, and its relationship to other key study team members are laid down in a separate DSMB file.

Stopping rules

Any patient may be withdrawn from the study at any time, at the request of parents, for any reason, specified or unspecified, and without penalty or loss of benefits to which the patient is otherwise entitled. Patients, who are withdrawn from the study, will not be allowed to re-enter later. Infants may have to be excluded from the study during the course of the study in case of any of the following events: an interruption of enteral or oral feeding for more than 72 hours caused by severe gastrointestinal disorders, major gastrointestinal surgery or multiorgan dysfunction. The responsible investigator has the right to discontinue the study in infants that experience one or several of the following incidents (1) AEs which do not allow any further treatment with the study medication, (2) unacceptable study conduction when balancing risk and benefit, (3) technical-logistic problems. The study sites are instructed to prematurely discontinue the study only, if substantial problems occur. All patients should be followed up and documented after discontinuation of the treatment, in order to record the data that is required in accordance with the intention-to-treat (ITT) principle.

The coordinating investigator will be informed immediately, should ethical or safety concerns occur at any study site. The trial will be stopped at any time, if this is recommended by the DSMB, based on severe safety concerns.

Furthermore, the DSMB will rule on the completion or discontinuation of the study in the interim analysis.

The coordinating investigator is authorised to exclude single centres in case of (1) inadequate recruitment, (2) insufficient quality of data or (3) other problems making the continuation of the study at that centre impossible.

Proposed sample size/power calculations

The sample size calculation is based on the primary endpoint gut dysbiosis. In a preliminary evaluation of microbiological screening data, we found a risk of gut dysbiosis of 7.5% on day 28 of life in infants, who received probiotics, as compared with 15% in infants without probiotic prophylaxis. A group sequential plan will be used with interim analysis at 50% information time, a one-sided $\alpha=0.01$ at the interim, and a futility stop of $\alpha_0=0.7$. This corresponds to an acceptance bound of -0.524 and a rejection bound of 2.326 at the interim analysis. The rejection bound at the final analysis has been set at 2.075 (one-sided $\alpha=0.019$). An adaptation of the number of interim analyses or the total sample size will be investigated using the conditional power approach. Stopping or adaptation will require recommendations by both the DSMB and the steering committee of the trial. In order to achieve a conservative sample size estimate, the additional power obtained from multiple offspring in a pregnancy has been neglected. The interim analysis will be performed with 161 infants per group, that is, 322 infants for the interim analysis, in total. For the final analysis, 327 infants need to be analysed per group to achieve 80% power, using the one-sided 0.019 test level, (continuity-corrected X^2). Accordingly, the final analysis will be performed after randomisation of at least 654 infants, that is, if 327 infants in each group have been recruited. With the effect size used for sample size calculations, the power for stopping the trial for efficacy reasons at the interim analysis is 38%.

Statistical analysis

The primary analysis will be conducted with the full analysis set using the ITT principle. A non-linear-mixed effect model with logit link will be estimated with gender as a fixed effect, and study site and gestational age as random effects in the primary analysis. The test of the treatment effect will be based on the Wald test for the log OR. Corresponding CIs will be estimated. For patients not completing the treatment as described in the study protocol, multiple imputations will be employed to address the primary endpoint. Sensitivity analyses will be performed based on the per protocol population of patients. Secondary endpoints will be analysed with the same type of non-linear-mixed effect model as applied to the primary endpoint.

Safety analyses will be performed for patients, who received at least one dose of verum/placebo. AEs and SAEs will be tabulated. Corresponding 95% CIs will be estimated if possible. Details of the statistical analysis will be fixed in a statistical analysis plan, which will be finalised by the trial statistician before randomisation of the

last patient. Subgroup analysis by study centre, gender, exposure to antibiotics and gestational age are planned and will be considered on an exploratory basis. Further exploratory analysis and modelling will be undertaken by integration of data from other PRIMAL subprojects. The statistis will be performed by the IMBS, Lübeck.

ETHICS AND DISSEMINATION

All investigators have agreed on sharing of data and biomaterials. Authorship of resulting manuscripts will be based on guidelines of the International Committee of Medical Journal Editors. All the important modifications and amendments will be communicated to the involved parties. All SIs will have access to the final data set of the trial. The results of our trial will be published in peer-review journals, presented at scientific meetings and disseminated through the website of our PRIMAL consortium (www.primal-study.de) and via social media of parent organisations.

DISCUSSION

This is the first large-scale trial to assess the role of probiotics in the prevention of early gut dysbiosis in vulnerable preterm infants. We propose to improve clinical outcomes in preterm infants by modifying the early microbiome-immunity interaction through a biologically plausible mechanism, the administration of potentially microbiome-stabilising bacteria. Probiotics are widely used in extremely preterm infants as a prophylaxis for NEC. However, the results of several high-quality studies using different formulations remain inconclusive. In addition, long-term costs and benefits of probiotics are unclear. Thus, the key question in the PRIMAL clinical trial is whether probiotics reduce the development of gut dysbiosis in preterm infants. The second major question is whether probiotics promote the development of antimicrobial immunity, both with respect to molecular and clinical endpoints. Several elements that were not adequately addressed in previous studies will be incorporated into this RCT: (1) microbiome studies with high-resolution sequencing tools in a multicentre network, (2) sequencing of the maternal microbiome and (3) thorough immunological phenotyping. If probiotics are found to prevent gut dysbiosis and improve clinical outcomes, this may result in a change in nutritional strategies for preterm infants. It is particularly valuable to study the efficacy of PIPS, because they are exposed to many factors which can lead to dysbiosis. Preventive measures in the early neonatal period may have a lifelong impact. Previous observational studies indicate that probiotics are safe and well tolerated in an adequate clinical setting.[22] The administration of placebo as comparator is necessary to clarify efficacy and safety of probiotics. Bias will be limited by strict adherence to current CONSORT and SPIRIT recommendations and blinding of participants, families, healthcare providers, data collectors, outcome adjudicators and data analysts. The verum and placebo are prepared at the University of Lübeck pharmacy to guarantee similarity in colour, texture, odour and taste. To address a potential confounding effect due to poor compliance and non-random loss of participants, we will perform an ITT analysis. Several cointerventions, such as feeding, antibiotics and invasive measures are recorded to adjust for potential confounding effects. In summary, the PRIMAL clinical study is a unique opportunity to evaluate the efficacy of probiotics for preterm infants and is independent from the influence and commercial interests of the pharmaceutical industry.

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