Probiotics and the Neurodevelopment in the Premature Infant <32 Weeks Gestational Age and <1500g

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1 General Information

1.1 Identification of the Study

Title: Probiotics and the neurodevelopment of the very premature infant <32 weeks gestation and <1500g

1.2 Promotor

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Collaborators: Marta Astals[1], Vicente Andreu-Fernández[2,3], Elisabet Navarro-Tapia[2,3], Òscar García-Algar[1], Josep Figueras-Aloy[1]

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2 Justification

Introduction

Probiotics were defined by the WHO as those microorganisms that, when administered in adequate amounts, conferred a health benefit for the host[1]. This declaration empowered probiotics and paved the way for the research in this field, allowing the rise of a variety of studies in different fields, being the premature infant the population with great advances[2]. Some of these studies demonstrated that in the premature neonate there is a delay in microbiota acquisition, as well as a substantive difference in composition, presenting a decrease in *Lactobacillus* and *Bifidobacterium* (considered protective microorganisms) and a rise in *Enterobacteriaceae*, that contains pathogens such as *Escherichia coli* and *Klebsiella*[3]. Nevertheless, dysbiosis (disbalance between commensal flora and pathogenic flora that leads to pathology) could entail a dysregulation between proinflammatory factors and protective factors, putting the extreme premature infant at bigger risk of major complications and increasing the morality[4]. The study of the microbiota in the premature infant and the application of probiotics have encompassed different lines of research in dysbiosis (related to obesity, allergy and breathing problems in early stages of development[5]), however the most studied aspect has been the use of probiotics to prevent necrotising enterocolitis, one of the most severe disease a newborn infant can confront[-10]. Moreover, although there is still controversy, there has been a clear benefit in the prophylactic use of different strands of probiotics, specially combined, for the prevention and reduction of the incidence of necrotising enterocolitis[7,8]. Branching from here, we can also find the use of probiotics in prevention of late-onset sepsis, considered the main side effect of probiotics, and neonatal mortality, where evidence has also shown probiotics to have potential benefit, especially those probiotics containing genres *Bifidobacterium* and *Lactobacillus*[8]. For instance, in animal models *Bifidobacterium spp.* has been shown to have a possible antinflammatory effect through the production of cytokines and synthesis of Th2 lymphocites, as well as regulation IgE. Additionally, production of short chain fatty acids could help maintain intestinal barrier integrity[2].

Seeing these promising results, one has to ask if the effect of probiotics could extend beyond necrotising enterocolitis, late-onset sepsis and mortality, having other effects in the premature infant, specially those

more immature (<32 weeks gestational age) and very low birthweight (<1500g). A premature neonate is daily challenged by many complications and potential sequalae that will affect the rest of their lives. Between these we have the neurodevelopment with effects that extend beyond cerebral palsy, neurosensorial hearing loss and blindness[11].

Regarding neurodevelopment, a gut-brain axis has previously been described, connected through neural, endocrine, and immune pathways[12-14]. Microbiota with its ability to process indispensable metabolites, regulate pathogen microbiota and modulating the immune response, by for example reducing intestinal permeability to lipopolysaccharide (pro-inflammatory factor), could have a key role in the gut-brain axis[14]. For instance, substances such as brain derived neurotrophic factor, neutrophines o interleukin-6, implicated in neuroinflammation and neurodevelopment, could see themselves modified by changes in microbiota[16]. Nevertheless, the brain derived neurotrophic factor, nerve growth factor and neutrophines 3 and 4 promote neuronal survival and diminish apoptosis of central and peripheral nervous system neurones, being vital for the development of the pre and post-natal brain[17-18]. Despite everything, unfortunately there is a lack of published evidence, with only 4 studies analysing the effect of probiotics on the neurodevelopment of the premature new-born, without finding a benefit[19-22]. However, it is important to note that the assessment of neurodevelopment in those studies is limited around the 2 years of follow-up with the use of a Bayley test. Alas, studies such as the EPIcure[11] have shown that behavioural changes and impairment in neurodevelopment appear beyond the 2 years of age, even in adulthood, being some of these difficulties in learning, psychiatric disorders o attention deficit and hyperactivity disorder, difficult to detect at the age where the majority of the follow-up is discontinued[23,24].

Hypothesis

1. The prophylactic use of a combination of probiotics containing *Bifidobacterium bifidum* and *Lactobacillus acidophilus* in premature infants <32 weeks gestation and <1500g could contribute to improving neurodevelopment at 2 years follow-up. Said improvement will be even more evident at 6 years of age, as well as entailing changes in biomarker patterns of neuroplasticity and neuroinflammation at 6 years of age.
2. The said combination of probiotics will contribute to reducing necrotising enterocolitis, late-onset sepsis, intraventricular haemorrhage, and neonatal mortality. Additionally, there will be a reduction in allergy and breathing disorders in early infancy.

Task distribution

Neonatology (BCNatal Hospital Clinic)

- Dr Benjamin J Baucells: information of study, recruitment, informed consent. Data collection and sampling. Coordination of neuropsychology team and laboratory. Interviews with families. Maintenance of database and support to rest of researchers. Elaboration of future manuscripts and statistical analysis of data.
- Dr Georgia Sebastiani: information of study, recruitment, and informed consent. Data collection and sampling. Coordination and supervision of the project and neuropsychology and laboratory teams. Maintenance of database. Supervision and writing of future manuscripts.
- Ms Marta Astals: information of the study. Neuropsychological assessment of patients at 2 and 6 years of age. Performance of semi structured interviews to families. Coordination and collaboration with other psychologists attached to the Neonataology Services of BCNatal Hospital Clinic.
- Dr Òscar García-Algar: review of protocols and research. Supervision of project and achievement of objectives. Supervision and writing future manuscripts. Coordination of research teams. Support to other teams and services.

- Dr Josep Figueras-Aloy: review of protocols and research. Supervision of project and achievement of objectives. Data quality assessment and statistical data analysis. Supervision and writing of future manuscripts.

Laboratory (IDIBAPS-GRIE):

- Vicente Andreu-Fernández: processing and collection of biological samples. Analysis of biomarkers of neuroinflammation and neurodevelopment with kits based on Luminex Multiplex technology of R&D. Analysis of intestinal permeability with ELISA kit of LifeSpan BioSciences (LS-F55511) in serum. Database management of recruited cohorts.
- Elisabet Navarro-Tapia: processing and collection of biological samples. Analysis of biomarkers of neuroinflammation and neurodevelopment with kits based on Luminex Multiplex technology of R&D. Analysis of intestinal permeability with ELISA kit of LifeSpan BioSciences (LS-F55511) in serum. Database management of recruited cohorts.

2.1 Relevant references

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3 Objectives and purpose of the study

The objective of the study is to determine the effect of the combination of 2 probiotics (*Bifidobacterium bifidum* NCDO 2203 y *Lactobacillus acidophilus* NCDO 1748) in the neurodevelopment and neuroimage of extreme premature infants less than 32 weeks gestation and under 1500g at 2 years of age, and the neurodevelopment and neuronal plasticity biomarkers at 6 years of age.

Secondarily the incidence of necrotizing enterocolitis, late-onset sepsis, intraventricular haemorrhage and mortality of premature infants will also be assessed comparing those neonates that received probiotics from those that did not. Moreover, growth curves will be considered to determine the moment of "catch-up" (moment where the infant returns to his correspondent growth curves) between the two groups, and to elucidate whether there is any effect from the moment of "catch-up" and neurodevelopment. Finally, there will be a comparison between obesity, asthma, and respiratory disease at 6 years of age.

On the other hand, at 6 years of age there will be an assessment of neurodevelopment biomarkers and neuroplasticity in blood serum to determine changes in expression linked to probiotic administration, and therefore contribute to the enforcement of the existence of a brain-gut axis and it's importance in neurodevelopment.

3.1 Main and secondary variables

- Gestational age, weight, length and head circumference at birth
- Sex
- Multiple gestation

- Gestational pathology (diabetes, preeclampsia, maternal sepsis, placental disease, in vitro fecundation)
- IUGR and degree of IUGR
- Inborn vs Outborn
- Apgar at 5 and 10 minutes
- Antenatal steroids
- Type of delivery (vaginal vs caesarean)
- Coriamnionitis
- Type of feed (breastfeeding, donor milk or formula)
- Duration of breastfeeding
- Necrotising enterocolitis, early and late-onset sepsis, mortality
- Antibiotic use beyond 48 hours of life
- Days of parenteral nutrition
- Days of enteral nutrition
- Days of invasive ventilation, days of non-invasive ventilation, days of oxygen
- Days of stay in intensive care
- Chronic lung disease
- Intraventricular haemorrhage (I-IV)
- Periventricular leukomalacia
- Cranial ultrasound result
- Retinopathy of prematurity
- Neurosensorial hearing loss
- Cerebral palsy
- Colic in early infancy
- Bayley score at 2 years of age
- MRI at 2-3 years of age
- Growth chart
- Moment of “catch-up”
- Childhood diseases (asthma, atopic skin, hospital admissions, allergy,…)
- Need for antibiotics or probiotics during childhood >48 hours
- Weight and height at the assessment
- WISC-V at 6 years
- CBCL 6-18 (Child Behaviour Checklist 6-18)
- BRIEF-2
- Mother tongue
- Parental age
- Cultural level of parents
- Ethnical origin
- Parents work
- Kind of family (single parent, …)
- Social services requirements
- Need to attend early stimulation centre

4 Study design

Quasi-experimental, sequential, unicentric, cohort study with the performance of neuropsychological tests and biomarkers of neurodevelopment, inflammation, and intestinal permeability.

We estimate an initial phase of 2 years with a comparison at 6 years of age (4 years after start of trial).

5 Enrolment of participants

Infants born below 32 weeks gestational age and birth weight under 1500g cared for at BCNatal Hospital Clínic (tertiary neonatal unit) in Barcelona between January 2014 and December 2019. There will be a 12 month wash out period (year 2017) to alleviate cross contamination of probiotics.

Participants will be contacted via phone (parent/legal guardian) for those that consented to be included for a 6-year assessment and blood sampling. In those cases where contact via phone has not been possible, contact to reference Paediatrician will be sought.

5.1 Inclusion criteria

Extreme premature infants <32 weeks and <1500g, cared at BCNatal Hospital Clinic during years 2014-2019. Participants will have to survive beyond 7 days of life (moment where the probiotic intervention will be given). Recruitment will start from January 2014 and will last until December 2019.

5.2 Exclusion criteria

All neonates presenting with suspected congenital anomalies, inborn errors of metabolism, or genetic defects will be excluded. Infants with a suspected syndrome, or who had suffered events beyond the neonatal period, not related to prematurity, that could entail impairment in neurodevelopment (severe cranioencephalic trauma, oncological process, meningitis, or exposure to toxic substances) will also be excluded.

6 Treatment and study calendar

Intervention

Daily dose of 6x109 UFC Infloran® -Berne, Switzerland- (Bifidobacterium bifidum NCDO 2203 and Lactobacillus acidophilus NCDO 1748) from 7 days of life until reaching a postmenstrual age of 34 weeks or discharge. The probiotic mixture will be provided in capsules, opened, dissolved in water and given orally or via nasogastric tube, according to the feeding regime of each neonate (breast milk, donor milk or formula). Where concerns for NEC or LOS are present, probiotics will be stopped and reinstated once enteral feeds are recommenced.

Evaluation of effect of intervention

At 24 months corrected age there will be a Bayley-III scale test in an extensive examination performed by independent assessors, blinded to group allocation. After this evaluation, patients will be divided into four categories according to their degree of neurodevelopment: survival without neurodevelopment impairment (normal neurodevelopment), mild impairment, moderate impairment, or severe impairment26. Mild impairment will be considered if they have muscle tone changes, impaired fine or gross motor coordination, Bayley scale score between 71-84, moderate behaviour disorders or mild visual

disability. Moderate impairment will be diagnosed when suffering from spastic diplegia, hemiplegia, seizures (non-febrile), Bayley scores between 50-70, severe behaviour disorders, moderate visual disability or mild-moderate hypoacusis. Severe impairment will be attributed to subjects with spastic quadriplegia, choreoathetosis, ataxia, Bayley score <50, blindness or severe hypoacusis.

Posteriorly, at 6 years of age, we will assess the presence of cerebral palsy, learning difficulties, behavioural disorders (autism spectrum, attention deficiency and hyperactivity disorder) and scores in the tests WISC-V, CBCL 6-18 and BRIEF-2. We will also perform a semi-structured interview to parents or legal guardians to determine the degree of impairment of the child, that might have not been previously included.

On the other hand, at 6 years of age we will proceed to sampling of blood plasma for the study of different biomarkers related to neurodevelopment, inflammation, and intestinal permeability. We will analyse the following: NeuN, Doblecortina, GFAP, GDNF, Ki67, Nrf2, BDNF, NGF, neurotrofina-1 (NT-1), neurotrofina-3 (NT-3), neurotrofina-4 (NT-4), DYRK1A, HIF1α, S100B i GSK3B. Regarding inflammation we will assess IL1B, IL6, IL8, IL10, IL12, TNF- α. We will finally determine presence of lipopolysaccharide in plasma with an ELISA.

7 Statistics

Considering an alpha risk of $\alpha = 0.05$ and a beta risk of $\beta = 0.2$ in a bilateral contrast, a minimum of 90 subjects in each group were required to detect a statistically significant difference between groups, where for the control group the proportion of some neurodevelopmental alteration was expected to be at least 0.4 and for the group treated with probiotics at least 0.2.

7.2 Statistical analysis

IBM SPSS Statistics 27.0.1.0 will be used for the statistical analysis. Non-parametrical analysis with a double-sided Mann-Whitney U test will be calculated for all continuous variables, whilst for categorical variables the chi-square test will be applied. In all cases, 0.05 will be considered the threshold of statistical significance. The relative risk (RR) and 95% confidence intervals will be determined for dichotomous variables. When encountering significant differences, the number needed to treat (NNT) will be analysed.

8 Ethics

The study will be in accordance with the Declaration of Helsinki (Fortaleza Version, Brazil October 2013) and will follow the legal requirements to fulfil the Spanish Biomedical Law of 14/2007. All patients, parents or legal guardians will be sought for informed consent.

9 Data processing and confidentiality

Treatment, communication and cease of data of personal character will be following the EU Ruling 2016/679 of the European Parliament and the Counsel of 27th April 2016, referring to protection of physical people and the treatment of personal data and the free circulation of data, being legally binding on the 25th of May 2018. The legal base of use of data will be in accordance to this act, established in the article 9 of EU 2016/679 Ruling.

All collected data will be deidentified and allocated a specific code, that will not allow any recognition of participants. Only the main promotor and collaborators will have access to data and will be allowed to enter the clinical history of patients for the purpose of this study.

Only legal requirement of medical emergency will allow the disclosure of any data related to participants of the study.

There will, however, be access upon request of non-identified data to Health Authorities, Ethics Committee and authorised personnel, for the purpose of quality assessment and study evaluation. Nevertheless, this will follow the existing legal frame and always guaranteeing confidentiality.

If data is to leave the EU, whether it is to entities related to the Hospital where patient was cared for, o third healthcare parties or collaborators, data will be protected via contracts or other established mechanisms to comply with EU legal requirements to data protection.

As the study promotors we agree to treat data in accordance to EU Ruling 2016/679, and therefore register all activities undertaken with the data and an assessment of data security and risks of the treatment of data.

Participants will be allowed to access, modify, oppose and cancel data (supress in the new ruling) and limit collected data as well as its processing. They will also be allowed to request copies or cession to a third party. In order to fulfil with their rights they will have to contact the Main promotor or the Delegate of Data Protection at Hospital Clinic I Pronvicial de Barcelona (protecciodades@clinic.cat). If they are not satisfied with the process they may contact the Data Protection Agency.

If a patient opts out of the study, their data will not be deleted to guarantee the validity of the research and fulfil their requirements with the healthcare authorities and pharmaceutical authorities.

The main promotor is obliged to preserve all collected data for the study for at least 5 years after the finalisation of the study. Posteriorly, personal information will only be stored by the Healthcare centre for healthcare purposes and the main researcher only if the patient has authorised such treatment, always in accordance to existing law.

10 Biological sampling and processing

We will proceed to collect 2 mL of blood plasma for the study of plasticity, inflammation and permeability biomarkers.

They will be analysed at the laboratory of Institut d'Investigacions Biomèdiques Agustí Pi I Sunyer (IDIBAPS) by Dr Vicente Andreu-Fernández and Elisabet Navarro-Tapia.

After study completion all samples will be destroyed appropriately.

11 Funding

We will proceed to apply to national and international grants to achieve necessary funding (estimated 25,500€).

Researchers will receive no payments and all budget will be destined to pay for material and processing of samples, with some budget allocated to scientific sharing.

12 Publication policy

All results will be published in recognised peer review journals. Additionally, all participants will be entitled to dispose of their personal results for their convenience and discretion.