NEOINFLAM:

Circulating markers in preterm infants with perinatal and neonatal inflammation: An observational study

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List of Abbreviations

BPD	Bronchopulmonary Dysplasia
CRF	Case Report Form
CRP	C-Reactive Protein
LC-MS	Liquid Chromatography-Mass Spectrometry
LOS	Late-Onset Sepsis
PHI	Protected Health Information
NEC	Necrotizing Enterocolitis
NETs	Neutrophil Extracellular Traps
NICU	Neonatal Intensive Care Unit
PROM	Premature rupture of membranes
RDS	Respiratory Distress Syndrome
ROC	Receiver Operating Characteristic

Protocol title: Circulating markers in preterm infants with perinatal and neonatal inflammation: An observational study (NEOINFLAM)

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Study Summary

Title	Circulating markers in preterm infants with perinatal and neonatal inflammation: an observational study (NEOINFLAM)
Short Title	Markers for perinatal and neonatal inflammation
Study type	Observational study
Study Duration	18 months (October 2017-June 2019)
Study Center(s)	Single Center- Neonatal department, Bao'an Maternal and Child Health Hospital, Shenzhen, Guangdong, China
Objectives	The main objective of the study is to identify new circulating markers associated with early life inflammatory conditions in preterm infants including chorioamnionitis, late-onset sepsis and necrotizing enterocolitis in preterm infants during the first four weeks of life. The aim is to better understand these conditions, and more long-term, to provide earlier and better diagnosis.
Number of Subjects	200
Diagnosis and Main Inclusion Criteria	Inclusion criteria: preterm infants (< 32 weeks of gestation) Main diagnosis before and during the study: chorioamnionitis, late onset-sepsis and necrotizing enterocolitis

1 Introduction

This document is a protocol for a human research study. This study is to be conducted according to Chinese medical standards, applicable government regulations and Institutional research policies and procedures.

1.1 Background

Every year, approximately 15 million infants are born preterm (< 37 weeks of gestation) and 1 million die (1). Preterm infants, especially those are born < 32 weeks of gestation, have immature gastrointestinal tract and innate immune system, which lead to high risks of development of necrotizing enterocolitis (NEC, a devastating intestinal disease affecting 7% preterm infants) and early life systemic infection (late-onset sepsis, LOS, up to 20-40% all hospitalized infants) (1,2). Those infectious diseases may lead to further long-term complications such as neurodevelopmental delay (1). NEC is very often related to LOS, possibly because an abnormal gut bacterial colonization and high gut permeability allow bacterial translocation (3). Conversely, a systemic inflammatory insult may predispose to NEC via impaired gut motility and immunity. As a result, in clinical trials, diagnostic markers are often used for combined LOS and/or NEC. Despite tremendous efforts to develop diagnostic markers for LOS and NEC, none of them are specific enough for clinical use (4). The current "gold standard" of blood culturing for diagnosis of LOS has many highly false negatives and is time-consuming whereas patients may develop septic shock and die shortly after clinical presentation. Ng et al performed a proteomic study to characterize protein biomarkers for LOS and NEC, but their work was exploratory without detailed focus on the mechanism based on an conventional LC-MS protocols (4). Recently, new mechanisms have been suggested to play role in the pathogenesis of adult sepsis such as circulating neutrophil extracellular traps (NETs), microparticles and miRNA but they have not been characterized in neonatal diseases (5,6).

During pregnancy and labor, 20% of term and up to 60% of preterm deliveries show signs of maternal/prenatal inflammation (7), chorioamnionitis, which is the most important risk factor for preterm birth. Prenatal inflammation is associated with postnatal gut and systemic inflammation in newborn infants (8) and this may affect immune development in several ways. However, no study has yet investigated whether parameters related to postnatal immune development in preterm infants are affected by chorioamnionitis, and how immune parameters in infants with chorioamnionitis are associated with postnatal infections. Further, it is unclear how important the progression of enteral feeding with milk (mother´s own milk, donor milk or formula) is for the postnatal NEC and LOS sensitivity. We speculate that early feeding with an optimal milk diet may help to support both gut and systemic immunity development, and thereby increase the resistance against NEC and LOS.

To improve the understanding of NEC and LOS, and their diagnostic criteria, and the postnatal immune development after chorioamnionitis, we investigate temporal blood samples in n=200 preterm infants. We apply proteomics and several immune assays to plasma, as well as whole blood transcriptomics/epigenetics to blood collected from preterm infants with and without NEC, LOS and/or CA. The aim is to identify new proteins and transcriptomic/epigenetic parameters that are related to the mechanisms of NEC, LOS and CA. These biological markers may help to provide possible new diagnostic markers for neonatal LOS and NEC, with and

without maternal chorioamnionitis and with/without difficulties in establishing normal enteral feeding.

1.2 Investigational Agent

Not applicable

2 Study Objectives

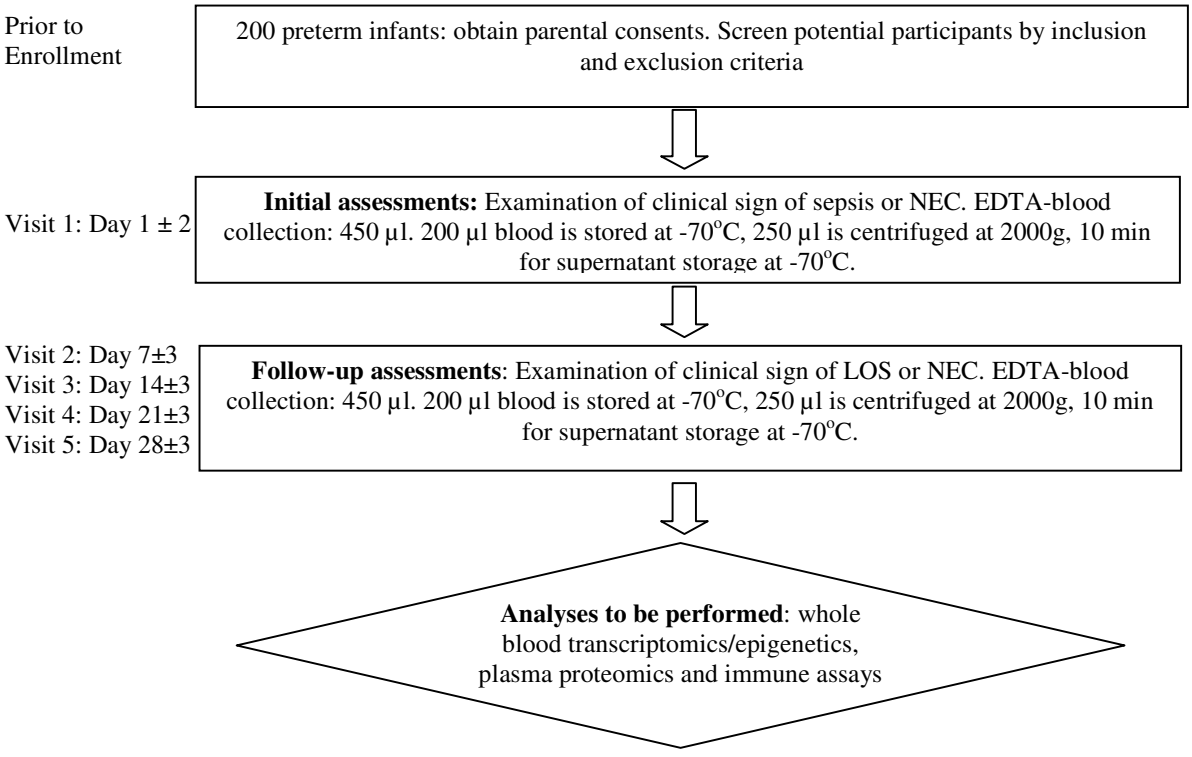
Primary objective: To characterize circulating markers associated with the early life complications including chorioamnionitis, LOS and NEC in preterm infants during the first four weeks of life.

Secondary objective: To characterize how different feeding regimes and diets may affect these markers during the first four weeks of life in preterm infants

3 Study Design

3.1 General Design

- This observational prospective cohort study includes 200 preterm infants, born < 32 weeks of gestation, at Neonatal Department, Bao'an Maternal and Child Health Hospital, Shenzhen, Guangdong, China.
- Patients, who fulfill inclusion criteria, will be recruited following the parental consent. The recruitment stops when the sample size reaches 200. Expected study duration: January 2017-June 2018.
- Patients will follow usual clinical procedures at the hospital. Patient groups will be categorized based on clinical records: infants born without and with clinical chorioamnionitis, infants without or with at least one episode of late-onset sepsis (LOS) and/or necrotizing enterocolitis (NEC).
- Enrollment and sampling are performed as indicated in the diagram below. Blood sampling is performed every week from birth until 4 weeks of age, or until discharge



3.2 Primary Study Endpoints

- Circulating markers in temporal postnatal blood samples associated with chorioamniotis.

- Circulating markers in temporal postnatal blood samples associated with prognosis and/or diagnosis of neonatal LOS and/or NEC

3.3 Secondary Study Endpoints

- Circulating markers in temporal blood samples associated with feeding regimes and diets: major amount of mother's own milk (>=50% of feeding times during the first two weeks of life as mother's own milk) or minor amount of mother's own milk (< 50% of feeding boluses during the first two weeks of life as mother's own milk).

3.4 Safety Endpoints

This is an observational study so safety endpoints are not applicable

4 Subject Selection and Withdrawal

4.1 Inclusion Criteria

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

- Born at neonatal department, Bao'an Maternal and Child Health Hospital
- Gestational age < complete 32 weeks
- Parenteral consent is signed by at least one of the two parents

4.2 Exclusion Criteria

Subjects who meet any of the following criteria will be excluded from the participation of the study:

- Major congenital abnormalities or birth defects
- Serious perinatal complications such as serious asphyxia or meconium aspiration syndrome.
- Life expectancy < 48 hours

4.3 Subject Recruitment and Screening

When infants are admitted to the Neonatal department, parents are informed about this observational study and the possibility to participate by the principal investigator. Signed parenteral consent is required to enter the study. Subjects should be recruited and the first blood sampling will be performed within the first 48 h of life.

4.4 Withdrawal of Subjects

A subject will be withdrawn from the study in one of the following conditions:

- The consent is withdrawn by parents prior to the expected completion of the patient
- The principal investigator reserves the right during the study to withdraw a patient from the study if the patient's conditions are evaluated to be not suitable for blood sampling. In this occasion, another patient needs to be recruited to replace the drop-out patient.
- Any infants that die before the completion of 4 postnatal weeks and are not diagnosed with LOS, NEC or chorioamnionitis beforehand will be withdrawn from the study. In this occasion, another patient needs to be recruited to replace the drop-out patient.

5 Study Procedures

Care of patients follows standard procedures established by the local hospital. Blood samples will be taken according to the study design. At each blood sampling period, the study investigator will evaluate the suitable blood volume to be taken, based on body weight, health status and other guidelines from the hospitals.

Feeding regime follows the practice at the local NICU: mother's own milk is the first priority, followed by donor milk and infant formula. For each time of feeding, the following details are noted in the feeding CRF: time, type of feeding (mother's milk, donor milk, formula), feeding volume, gastric residual from previous feeding. For feeding intolerance, any interventions such as aspiration, decreased the amount of feeding or stop feeding are noted in the comments section of the CRF.

5.1 Diagnosis of chorioamnionitis, LOS and NEC

Once the patient is recruited, information about diagnosis of chorioamnionitis is collected and filled in the CRF. Clinical chorioamnionitis is defined in pregnant women with fever ($\geq 38^{o}C$), high heart rate ($\geq$100/min for mothers and $\geq$160/min for fetuses), as well as when plasma CRP and blood leukocyte counts $\geq$ 20 mg/L and 12,000 leukocytes/mm^3. Histological chorioamnionitis is confirmed after histologic examination of the fetal membranes.

LOS is defined as sepsis occurring after 72 hours after birth. Clinical diagnosis of LOS is defined when the patient shows two or more of the following criteria: white blood cell count < $5x10^9$ cells/L or >$20x10^9$/L, total platelet count < $100x10^9$cells/L, CRP > 10 mg/L, procalcitonin > 2 ng/mL. Culture-proven sepsis is defined as clinical sepsis with positive blood bacterial culture result.

Diagnosis of NEC is based on the conventional Bell system (IA, IB, IIA, IIB, IIIA and IIIB). Medical NEC is defined when an infant is diagnosed at stage IA to IIB. When an infants is classified with Bell stage III A or B (advanced NEC and bowel perforation), surgical interventions is required (surgical NEC).

5.2 Information in CRF

All collected data from clinical records will be recorded in the study CRF and feeding CRF by the study investigator at the hospital, including:

- Patient ID number
- Gestational age at birth
- Birth weight and gender
- Type of delivery: C-section or vaginal birth
- Diagnosis of chorioamnionitis:
  - Clinical or histological,
  - Gestational age at diagnosis,
  - Temperature, heart rate, plasma CRP and blood leukocyte counts of mothers at diagnosis
- Diagnosis of LOS and/or NEC:
  - Day of life at diagnosis,
  - Plasma CRP, total leukocyte counts
  - Clinical or culture-proven LOS
  - For culture-proven LOS: identified bacteria
  - Medical or surgical NEC

- Time of blood sampling and volume of blood samples
- Feeding details (in the Feeding CRF)
- Other notes: incidence of RDS, BPD, pneumonia

5.3 Sample selection, analysis and comparison

At the end of the study, the selection of appropriate samples for analysis will be conducted by all investigators of the study. Samples included in the analysis are those collected from birth until the disease diagnosis or completion of 4 postnatal weeks. In case of clinical diagnosis of LOS and/or NEC before completion of 4 weeks, further routine sample collection after diagnosis is continued if feasible.

Temporal samples will be analyzed to perform three separate comparisons:

- Infants without versus with LOS and/or NEC
- Infants without versus with clinical/histological chorioamnionitis
- Infants with major or minor amount of mother's own milk during the first two weeks of life (≥ or < 50% of feeding times as complete mother's own milk).

Plasma proteomic study will be prioritized and will include as many samples as possible, followed by validation with absolute quantification of potential proteins using immune assays. The type of other -omic analysis for plasma (miRNA profiling) whole blood (transcriptomics or epigenetics) as well as sample size will be decided depending on the results of proteomic study.

6 Statistical Plan

6.1 Sample Size Determination

Based on the practical issues of admission rate of preterm infants and capacity of temporal blood sampling at the Neonatal Department, we anticipate to include 200 preterm infants (< 32 weeks of gestation) with estimated rate of clinical chorioamnionitis of 15-20% (for overall preterm cases, 40-70% of premature PROM cases), and clinical LOS and/or NEC of 20-30%.

6.2 Statistical Methods

Infant grouping will be categorized based on the defined endpoints to compare: 1. Infants with vs. without chorioamnionitis; 2. Infants with vs. without LOS and/or NEC; 3. Infants with vs. without major mother's own milk feeding. Temporal data obtained from –omic analyses of blood and plasma samples collected every week will be analyzed by multivariate analysis with control for false discovery rates. Other data obtained from immune assays will be analyzed by univariate analysis. A panel of potential biomarkers will be identified by regression algorithm together with ROC analysis.

7 Safety and Adverse Events

This is an observational study so safety reporting will not be applicable

7.1 Definitions

7.2 Recording of Adverse Events

7.3 Reporting of Serious Adverse Events

7.3.1 Study Sponsor Notification by Investigator

7.3.2 Notification to Research Institute by Investigator

7.4 Unblinding Procedures

7.5 Stopping Rules

7.6 Medical Monitoring

7.6.1 Internal Data and Safety Monitoring Board

7.6.2 Independent Data and Safety Monitoring Board

8 Data Handling and Record Keeping

8.1 Confidentiality

Information about study subjects will be kept confidential. In the event that a subject revokes authorization to collect or use protected health information (PHI), the investigator, by regulation, retains the ability to use all information collected prior to the revocation of subject authorization. For subjects that have revoked authorization to collect or use PHI, attempts should be made to obtain permission to collect at least vital status (i.e. that the subject is alive) at the end of their scheduled study period.

8.2 Handling and storage of data and documents

All personal data will be coded by serial numbers. Only the investigators will have the access to the key. Statistics will be performed with the coded numbers. Published data from this study cannot be traced to a specific infant.

All data requested on the case report form (CRF) must be recorded. All missing data must be explained. If a space on the CRF is left blank because the procedure was not done, write “N/D”. If the item is not applicable to the individual case, write “N/A”. If any entry error has been made, to correct such an error, draw a single straight line through the incorrect entry and enter the correct data above it. All such changes must be initialed and dated.

8.3 Records Retention

All coded data will be stored in a security place for a minimum of ten years.

9 Ethical Considerations

This study is to be conducted according to applicable government guidelines and regulations and Institutional research policies and procedures.

This protocol and any amendments will be submitted to the Ethical Committee at Bao'an Maternal and Child Hospital, Shenzhen for formal approval of the study conduct. The decision of the Ethical Committee concerning the conduct of the study will be made in writing to the investigators.

All subjects for this study will be provided a consent form in Chinese describing this study and providing sufficient information for subjects to make an informed decision about their participation in this study. The consent form will be submitted with the protocol for review and approval by the Ethical Committee for the study. The formal consent of a subject must be obtained before that subject is submitted to any study procedure. This consent form must be signed by the subject or legally acceptable surrogate, and the investigator-designated research professional obtaining the consent.

Blood samples obtained for this cohort are left-over sample materials from routine clinical blood sampling for screening and diagnosis in preterm infants at the local hospital (Neonatal Department, Bao'an Maternal and Child Health Hospital, Shenzhen). Therefore, there are no potential harmful effects of blood sampling to the participating infants.

10 Study Finances

10.1 Funding Source

The analytical part of the study will be partly funded by NEOCOL research grant by Innovation Fund Denmark (Prof. Per T. Sangild as the grant holder). Additional funding will be searched locally in Shenzhen by MD Ping Zhou.

10.2 Conflict of Interest

All investigators declare no potential conflicts of interest

10.3 Subject Stipends or Payments

Not applicable

11 Publication Plan

The data are considered joint ownership between the study collaborators and Bao'an Maternal and Child Health Hospital, Shenzhen. All results derived from this study will be published jointly between these two institutions in pediatrics, nutrition or immunology journals.

Neither the complete nor any part of the results of the study carried out under this protocol will be published or passed on to any third party without the consent of all the study investigators. Any investigator involved with this study is obliged to provide the principal investigator with complete test results and all data derived from the study.

12 References

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13 Appendices

- Parental Consent Form (in Chinese)
- CRF (both English and Chinese)