

STUDY PROTOCOL

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Analysing the protection from respiratory tract infections and allergic diseases early in life by human milk components: the PRIMA birth cohort

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Abstract

Background: Many studies support the protective effect of breastfeeding on respiratory tract infections. Although infant formulas have been developed to provide adequate nutritional solutions, many components in human milk contributing to the protection of newborns and aiding immune development still need to be identified. In this paper we present the methodology of the “Protecting against Respiratory tract Infections through human Milk Analysis” (PRIMA) cohort, which is an observational, prospective and multi-centre birth cohort aiming to identify novel functions of components in human milk that are protective against respiratory tract infections and allergic diseases early in life.

Methods: For the PRIMA human milk cohort we aim to recruit 1000 mother–child pairs in the first month postpartum. At one week, one, three, and six months after birth, fresh human milk samples will be collected and processed. In order to identify protective components, the level of pathogen specific antibodies, T cell composition, Human milk oligosaccharides, as well as extracellular vesicles (EVs) will be analysed, in the milk samples in relation to clinical data which are collected using two-weekly parental questionnaires. The primary outcome of this study is the number of parent-reported medically attended respiratory infections. Secondary outcomes that will be measured are physician diagnosed (respiratory) infections and allergies during the first year of life.

Discussion: The PRIMA human milk cohort will be a large prospective healthy birth cohort in which we will use an integrated, multidisciplinary approach to identify the longitudinal effect human milk components that play a role in preventing (respiratory) infections and allergies during the first year of life. Ultimately, we believe that this study will provide novel insights into immunomodulatory components in human milk. This may allow for optimizing formula feeding for all non-breastfed infants.

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Keywords: Breastfeeding, Human milk, Respiratory tract infections, Allergies, Immune development, Antibodies, Human milk oligosaccharides, T cells, Extracellular vesicles, Biobank

Background

The burden of respiratory infections

Respiratory infections are one of the major causes of illness in infants, specifically during the first 5 years of life [1]. It was estimated for 2015 that 703,900 children under the age of five died worldwide because of lower respiratory infections [2]. Most respiratory tract infections in children are caused by viral infections, with respiratory syncytial virus (RSV), influenza virus (IFV), parainfluenza (PIV) and metapneumovirus (MPV) being the most commonly found viruses [3, 4]. Each year, about 41.3–112.0 per 1000 children under one year of age were admitted to the hospital for RSV in Europe [5]. This leads to about 28,000 infants requiring medical care for RSV bronchiolitis in the Netherlands [6, 7] of which approximately 2000 require hospitalization. Hospitalisation for respiratory tract infections not only leads to considerable costs, but is also associated with recurrent wheeze, asthma and long term impaired lung function [8–11].

Breastfeeding protects against infections

Breastfeeding clearly has several benefits for infants, such as the capacity to protect against the risk and impact of neonatal infections [12, 13]. For instance, infants that do not receive exclusive breastfeeding are at a higher risk of hospitalization in early life in relation to a wide range of common infections [14]. Exclusive breastfeeding until the age of four months followed by partial breastfeeding is associated with both reduction in gastrointestinal and respiratory infections [15, 16]. Infants admitted to the hospital with RSV infection are less likely to have been breastfed [10, 17]. In addition, in a recent meta-analysis, one out of the eight risk factors for RSV-induced acute lower respiratory infection in children was “no breastfeeding”, with an odds ratio of 2.24 (95% CI 1.56–3.20) [18]. A similar protective effect of breastfeeding was observed for other common respiratory pathogens like influenza and invasive pneumococcal disease [19, 20].

A meta-analysis strongly favoured breastfeeding over formula feeding to reduce the risk of gastrointestinal infections (i.e. 0.36 (95% confidence interval (CI) 0.3 to 0.41)) [12]. This finding was supported by another meta-analysis that described a higher infant mortality due to diarrheal disease in children who were not exclusively breastfed the first six months of life [21].

The protective effect of breastfeeding on the development of allergic disease is less well pronounced compared to infectious diseases. In a meta-analysis it was

found that breastfeeding protects from asthma at ages five to eighteen, eczema until the age of two and for allergic rhinitis until the age of five [22]. Similar results were seen in other studies, for instance the CHILd study that found that direct breastfeeding offers the best protection from asthma during the first three years of age [23, 24]. However, there are also other studies demonstrating that there is even an increased risk on (mild) allergic diseases when children are being breastfed [25–27]. It is particularly hard to evaluate the effect of breastfeeding on the development of allergic diseases, as different definitions are used for allergic disease and different comparisons in duration of lactation [28]. Since the composition of breastfeeding is variable, it may well be that only some components in breastfeeding are protective against allergic diseases and not breastfeeding itself [28]. This hypothesis is supported by the findings of Lodge et al. where groups of human milk oligosaccharide (HMOs) were associated with an increased or reduced change of allergic diseases development in infants [29]. This suggests that breastfeeding contains protective components that may have immunomodulatory capacity.

Protective components in breastfeeding

Several components within human milk have been linked to the risk of infection during the first year of infancy. Primarily, antibodies in human milk are acknowledged to play a pivotal role protecting infants against various pathogens. Yet, it is still unclear which antibody repertoire would be favourable and what the effect of antibodies is compared to other human milk components. Breakey et al. demonstrated that high IgA antibody titres are linked to a reduction in infectious episodes whereas Mazur et al. described that RSV-specific maternal in breastfeeding IgG but not IgA antibodies are linked to reduction in the risk of RSV infections [30, 31]. Next to pathogen-specific antibodies human milk contains many more components with a protective capacity. For instance, Ramani et al. showed that higher concentrations of the HMO 2'-fucosyl-lactose (2'-FL) was associated with symptomatic rotaviral infections whereas in another study the presence of 2'-FL was associated with relative protection from respiratory infections [32, 33]. Addition of specific prebiotic oligosaccharide mixtures to infant formulas was shown to decrease the proportion of infections and allergic diseases in children when compared to the standard infant formula [34–38]. In vitro data suggests

that HMOs exert their effects through various mechanisms. For instance, HMOs are known to influence interactions between immune cells (e.g. dendritic and T cells), stimulate mucosal barrier and immune system maturation, modulate immune responses following the formation of antigen–antibody complexes, and may affect the immune response following the formation of (endogenous) antibody-antigen complexes [39–44]. HMOs may also influence the gut microbiome of the infant. Breastfed children usually have a gut microbiome dominated by *Lactobacillus* species and bifidobacteria compared to infant formula fed children [45, 46]. Moreover, Ramani et al. found that LNT (Lacto-N-tetraose) and 6'SL (6'-Sialyllactose) were both associated with *Enterobacter* and *Klebsiella* species [32]. In addition to pathogen-specific antibodies and HMOs, human milk contains many more components, including immune cells and extracellular vesicles (EVs) [47–51]. Several miRNA detected in human milk EVs were immune-related miRNAs [52]. Activated and memory T cells seem to be enriched in early human milk samples and samples from infected mothers, T cells also seem to increase expression of immune proteins during maternal infection [53].

The PRIMA human milk cohort

Despite decades of development and improvement of infant formulas, formula fed children are still more susceptible to infections early in life [12]. It is largely unknown what impact the different human milk components have on immune development and how they reduce the susceptibility to infections and allergic diseases. Therefore, we initiated the PRIMA human milk cohort in which we will examine the components in human milk that may protect children from infections, in particular respiratory infections, and allergic diseases.

The added value of this cohort is its strong focus on infectious diseases and allergic disease combined with a detailed immunological analyses of the samples on various components (Additional file 2: supplemental protocol). Added to that, we will collect prospective data with longitudinal breastfeeding sampling in $n = 1000$ children, spread out over multiple winter seasons.

Methods

Objectives

Primary objective

To identify components in human milk associated with decreased risk of parent-reported medically attended respiratory tract infections (MARI) during the first year of life in healthy term breastfed infants.

Secondary objectives

- To identify which components, or combination of components, in human milk are protective against any parent-reported (medically attended) and physician-reported infections during the first year of life.
- To identify which components, or combination of components, in human milk are protective against the development of parent-reported, physician-reported allergic diseases during the first year of life.
- To identify (within a subgroup) which routes are involved in protection transferred from mother to child (human milk, cord blood and amniotic fluid) and are protective against any parent-reported, physician-reported infections/allergic diseases during the first year of life.
- To examine the underlying mechanism of action of the components that have been identified to be protective against MARI, other infections and/or allergic diseases.

Outcomes

Primary clinical endpoint

The number of parent-reported medically attended respiratory infections during the first year of life.

Secondary clinical endpoints

- The number of physician-reported medically attended respiratory infections during the first year of life.
- The number of other parent-reported infections during the first year of life.
- The number of parent-reported allergic diseases (e.g. atopic dermatitis and egg/cow's milk allergies) during the first year of life.
- The number of other physician-reported infections during the first year of life.
- The number of physician-reported allergies during the first year of life.
- The number of antibiotic courses during the first year of life

General study design

In this prospective, observational cohort we will recruit a total of 1000 mother–child pairs during the first month postpartum. The study population will consist of healthy mothers and children in the Utrecht region, the Netherlands, that already started breastfeeding. Human milk samples will be collected at one week, one month, three months and six months if possible (Fig. 1). These

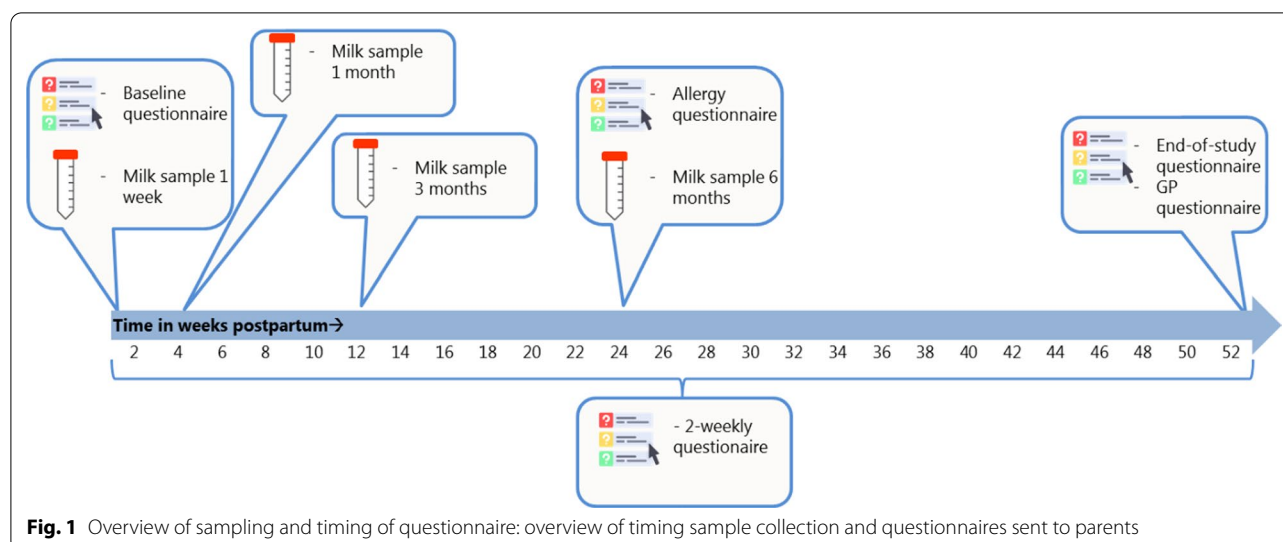


Fig. 1 Overview of sampling and timing of questionnaire: overview of timing sample collection and questionnaires sent to parents

timepoints span colostrum/transitional milk sample (within first week postpartum), early mature (one month sample) and late mature samples (three and six months) [54, 55]. During the first year of follow up, mothers will report their and their infant's susceptibility to infectious and allergic diseases by completing digital questionnaires sent by email every two weeks until the child reaches one year of age. After the infant has reached the age of one year, their general practitioner will be contacted to collect clinical data on infectious episodes, e.g. antibiotic use and sputum cultures during the first year of life (Additional file 1: Fig S1). In a nested subgroup ($n=20$), we will collect additional samples of maternal blood, cord blood, amniotic fluid and neonatal saliva and neonatal faecal samples. This subgroup will be used as a pilot to study the effect of all different routes through which mothers may transfer immune active components.

The milk sample composition will be linked to clinical data in order to identify the components or combination of components associated with medically attended respiratory infections, other infections and/or allergies. The human milk samples will be stored in the biobank facility of the UMC Utrecht for future research, securing quality of sample storage. The study recruitment started in March 2019 and is still recruiting participants. The expected end date of recruitment will be in June 2023. Consequently the study will end one year later in June 2024. The study is registered in The Netherlands Trial Register (<https://www.trialregister.nl/>) under protocol number NL9056.

Study participants

Participants are recruited at the obstetric wards of the University Medical Centre Utrecht (tertiary centre) and

the Diaconessenhuis hospital Utrecht (secondary centre). Participants are recruited in two different ways. Mothers that are admitted to the obstetric wards of either hospitals, will be informed by their midwives about the study. Mothers are asked approval to share their contact details with the researchers of the PRIMA cohort. Mothers that gave consent to share their contact details are contacted consequently.

Mothers that give birth in the primary care birth centre of the University Medical Centre Utrecht will receive a letter from their midwives at discharge. This letter contains information about the study and the possibility to opt out to be contacted by the researchers. 48 h after discharge, researchers will receive only the contact details of those parents that did not indicate objection to be contacted.

Mothers are screened by eligibility by the researchers during the first phone call. Additional information about the study will be provided and whenever parents are willing to participate, they will be send the patient information folder and the first house visit to collect milk and informed consents are scheduled.

Inclusion is performed within the first week postpartum. An overview of inclusion criteria and study activities overview can be found in Table 1 and Additional file 1: Fig. S1, respectively.

Data collection

At inclusion, parents will fill out a baseline questionnaire to collect data on (Fig. 1):

- Perinatal and pregnancy health characteristics.
- Family demographics.
- Health status parents.

Table 1 Eligible criteria for the PRIMA human milk cohort

	Inclusion criteria	Exclusion criteria
Parents	All mother–child pairs that live in a proximity to Utrecht Parents currently breastfeeding and intending to continue breastfeeding until at least 3 months postpartum	Acquired or innate immune deficiencies Insufficient control of the Dutch language
Infant		Severe cardiac or pulmonary disorders or other severe organ diseases Severe prematurity defined as GA < 32 weeks

- Vaccination status of the mother.
After inclusion, participants will receive a questionnaire every 2 weeks collecting data about:
- Infectious episodes, allergic symptoms, feeding habits, day-care attendance and growth characteristics of infant.
- Doctor visits, diagnoses made and medication prescribed to infant.
- Medication use and health status mother
- Any changes in demographics.

At 6 and 12 months, an additional questionnaire will be added to the 2-weekly questionnaire to record any allergic disease manifestations (Fig. 1). This questionnaire is based on the EuroPrevall infant cohort questionnaires that were designed to investigate the prevalence of allergies in the European population [56]. At twelve months, the vaccination status of the infant, changes in household (e.g. number of children or pets) and more detailed information about any hospital admission of the infant are recorded (Fig. 1).

Sample processing

Samples are collected by participants at home, either by hand or by breast pump. Within 24 h, study personnel will collect the samples and will note time of expression, method of expression and whether milk was collected before, during or after a feeding or whether it was a whole feeding sample collected. Participants will store the milk samples in their own fridge until study personnel collects and transfers the samples on ice to the UMC Utrecht laboratory. After milk expression, milk is stored on ice or in the fridge until processing. We record what time the sample was expressed and whether it is a whole milk sample or milk collected only before or after a whole feeding. Within 24-h after collection, samples will be processed according to the protocol of Zonneveld et al., using 600 times gravity instead of 3000 times gravity to preserve living cells (Additional file 2: Fig. S2) [57]. After processing, milk supernatant and cream fractions will be stored in cryotubes at -80°C . Fresh cell fractions will be used for T cell assays. Antibody and HMO composition is measured in milk supernatant. Samples that will be used

for EV analysis, will be processed within 30 min after collection following the above mentioned protocol of Zonneveld et al. [57]. To ensure samples are processed within 30 min, only participants that live in close proximity to the lab and that had sufficient milk production from the first week postpartum are selected for EV isolation. Study personnel is present at moment of milk collection and transfer samples within 20 min at 37°C to our laboratory. Total time of transport is recorded for each sample.

Sample analysis

The milk samples collected in this cohort will be analysed for antibody and oligosaccharide composition (fraction D, Additional file 2: Fig. S2). EV and T cell composition will be analysed in subgroups. The HMO composition is determined by mass spectrometry (MS) based approaches. Recent advances in Liquid chromatography mass spectrometry (LC–MS) enables detection of a vast range of HMOs and to determine relative HMO concentration [58]. A recently published LC–MS assay has shown to successfully detect semi-quantitatively human milk tri-, tetra-, penta- and hexaoses in a single LC–MS assay run [58].

Next to HMOs, samples will be analysed for antibody composition using a bead-based multiplex assay. It has been previously shown that bead-based multiplex assays can reliably measure antibody titres against several pathogens in blood and are an effective means of assessing immunity in a population [59–63]. After optimization, we will use a bead-based multiplex to determine pathogen specific antibodies, e.g. Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), Cytomegalovirus (CMV), Bordetella pertussis, pneumococcus, RSV and influenza-specific antibodies [59–62, 64]. Additionally, we will also use a bead-based multiplex assay to determine the concentration of all human antibody isotypes and their subclasses [59–62]. Preliminary data showed that it is also feasible to use these assays in human milk samples, though we will have to optimise these multiplex assays further for the measurement of human milk antibody titres.

In a randomly selected subgroup, we will analyse T cell profiles and functional skewing with flow cytometry

as well as their transcriptome and T-cell receptor (TCR) repertoire using single-cell RNA/TCR- sequencing.

Statistical considerations

We base our sample size calculation on the variation in 2'-FL concentration, as this is one of the most abundant HMOs present in human milk [65]. The sample size of 1000 participants has an 80% power to detect a ratio of the means of 0.91 in those with vs. those without a parent-reported medically attended respiratory infection using a two-sided two-sample t-test, assuming that 10% of the cohort participants have a parent-reported medically attended respiratory infection ($N=100$) and that the 2'-FL HMO concentration in the population has a mean of 3 g/L, a standard deviation of 1 g/L, and a normal distribution [65]. The significance level (α) is 0.05. The sample size was calculated using Power Analysis for Sample Size (PASS) 2008.

The primary aim of this study is to identify components in human milk associated with decreased risk of parent-reported MARI. To this end we will analyse the association between 2'-FL concentration and the MARI during the first year of life using regression analysis. The possible association between 2'-FL concentrations and the number of parent-reported medically attended respiratory infections during the first year of life will be determined using multivariable Poisson regression analysis, adjusting for potential confounders. Potential confounders will be identified by a literature search, complemented by expert knowledge. We will analyze whether the moment of maternal atopy, birth month, duration of lactation postpartum, season, socio-economic status and ethnicity modify the effect of HMOs on the number of respiratory infections.

For our secondary analysis, we will analyse the relationship between HMOs (other than 2'-FL) or antibodies and MARI in a similar fashion as 2'-FL using regression analysis and Poisson analysis.

For the secondary analysis, the statistical analysis plan will be based on the cohort of 1000 mother child pairs, providing milk samples as well as consecutive questionnaire data related to parent-reported medically attended respiratory infection (MARI). Based on the parent reported respiratory tract infection symptoms, the number infectious episodes during the first year of life will be defined and separates the cohort into two groups (high frequency of infection vs low frequency of infection). We will analyze the relationship between HMOs and antibodies and MARI with a machine-learning based multivariate analysis. We will use recursive ensemble feature selection (REFS) algorithm [66] to reduce the variables to the minimum necessary set to differentiate between the two groups (signature). REFS has been applied

successfully to different types of complex biological data and has been shown to have better accuracy [67] than univariate methods and stable enough between datasets [68]. Finally, to validate our results we will use metrics such as area under the curve (AUC) and receiver operating characteristic (ROC) curve [69, 70] to validate the diagnostic prediction using the found signature. A similar approach can be followed regarding studying the relation between parent-reported, physician-reported allergic diseases during the first year of life (high frequency of allergy vs low frequency of allergy) and HMOs and antibodies in human milk.

In case there is loss to follow-up because parents stop to report symptoms, we will use the data retrieved from the general practitioner to estimate incidence of MARI. If this is not possible, we will impute the missing data using multiple imputation. We will impute missing data using multiple imputation.

Discussion

Respiratory infections are still a major cause of burden of disease and child death, RSV being the most common found pathogen [4, 17, 71]. In 2015, the worldwide death of 118,200 children under five years of age were attributed to RSV [72]. Protection from respiratory infections will not only lead to a reduction in childhood mortality or other complications, but protection from viral infections is also associated with reduction of other diseases, like recurrent wheeze during first year of life and even asthma [73, 74]. Infants can be protected by vaccinations either given early after birth or by maternal vaccination during pregnancy [75]. These vaccinations elicit a pathogen-specific immune response, offering protection to these specific pathogen only [75]. Currently, no effective (maternal) vaccination is available in the clinic to protect from RSV infections. An important and validated strategy to reduce the number of respiratory infections is breastfeeding [12, 17]. Despite all efforts taken, still, the number of children being exclusively breastfed for six months remains low in some regions. In 2006–2012, only an estimated 25% of infants in the World Health Organisation (WHO) European Region were exclusively breastfed for the first 6 months, which is far below the WHO recommendation of 6 months exclusive followed partial breastfeeding until the age of two.

Understanding the composition of breast milk in relation to disease susceptibility in early life will guide the development of much needed interventions for infants that cannot be exclusively breast fed. At the present moment, there is a lack in knowledge about the specific components or combination of components present in human milk that support the protective capacity against infections. In order to be able to improve safety and

efficacy of infant formulas, it first has to be established which components including their underlying mechanism of action in breastfeeding are essential to protect against infections.

The PRIMA human milk cohort is designed to study human milk components in a novel public–private collaboration. The PRIMA human milk cohort will be one of the largest cohorts combining both longitudinal human milk samples with prospective clinical data collection. This initiative will offer a unique possibility to add scientific understanding regarding the protective effect and influence of specific human milk derived components on the protection against respiratory infections and allergies early in life. Data and samples from this cohort will be used to study the underlying mechanism of several components in human milk which might lead to the development of new concepts/hypotheses for the management and therapy of immune related disorders such as allergies and infections.

Abbreviations

EV: Extracellular vesicle; RSV: Respiratory syncytial virus; IFV: Influenza virus; PIV: Parainfluenza; MPV: Human metapneumovirus; CI: Confidence interval; HMO(S): Human milk oligosaccharide(s); IgA: Immunoglobulin A; IgG: Immunoglobulin G; 2'FL: 2'-Fucosyllactose; MARI: Medically attended respiratory infection; MS: Mass spectrometry; LC–MS: Liquid chromatography mass spectrometry; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; CMV: Cytomegalovirus; TCR: T-cell receptor; PASS: Power analysis for sample size; WHO: World Health Organisation; GA: Gestational age.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12879-022-07107-w>.

Additional file 1: Figure S1. Study activities overview: overview of all study activities. **Figure S2.** Sample processing in PRIMA human milk cohort: A) Human milk samples are centrifuged for 10 min at 600 g in order to remove the cream (layer on top, indicated in yellow) and cells (collected in pellet on the bottom, indicated in dark grey). B) Milk supernatant is collected by pipetting through cream layer without disturbing the cell pellet and subsequently transferred into a new tube. C) Collected 600 g supernatant is again centrifuged for another 10 min at 600 g. D) Milk supernatant is collected by pipetting through the residual cream and transferred to and aliquoted into clean cryotubes, and stored at -80 °C until further analysis. E) The cream layer is removed without disturbing the cellular pellet and transferred into cryotubes stored at -80 °C for further analysis. F) 20 mL PBS is added to rinse and cells are spun at 600 g for 10 min, after which the supernatant is discarded and cells are kept on ice until further processing for analysis G). Milk EVs are processed further according to the Zonneveld protocol at 3000 g.

Additional file 2. PRIMA research protocol METC Utrecht version 4A: this file contains the protocol including the novel additions for collection of dietary information from mother.

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Authors' contributions

AvS, KOR and LB wrote the manuscript. LB, JL and BvL supervised and initiated the project. KB and WdW supported resources for recruitment of study participants. AvS, SP, TL, FvW, MN, AH, CL, GdH, MvH, JG, LK, LV, CdT, AL, JL, BvL and LB were involved in setting up the methodology of the trial. AvS and MN performed validation experiments. All authors were involved in the revision of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

As the current manuscript describes the study protocol and no other data, we do not have any raw data to share at the moment.

Declarations

Ethics approval and consent to participate

The PRIMA cohort has been approved by the Medical Research Ethics Committee of the UMC Utrecht, the Netherlands (reference number: SL/

avd/20/500151, protocol ID: 20-578/X-D). All participating parents gave written consent to participate in this cohort.

Consent for publication

Not applicable.

Competing interests

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Figure S1:

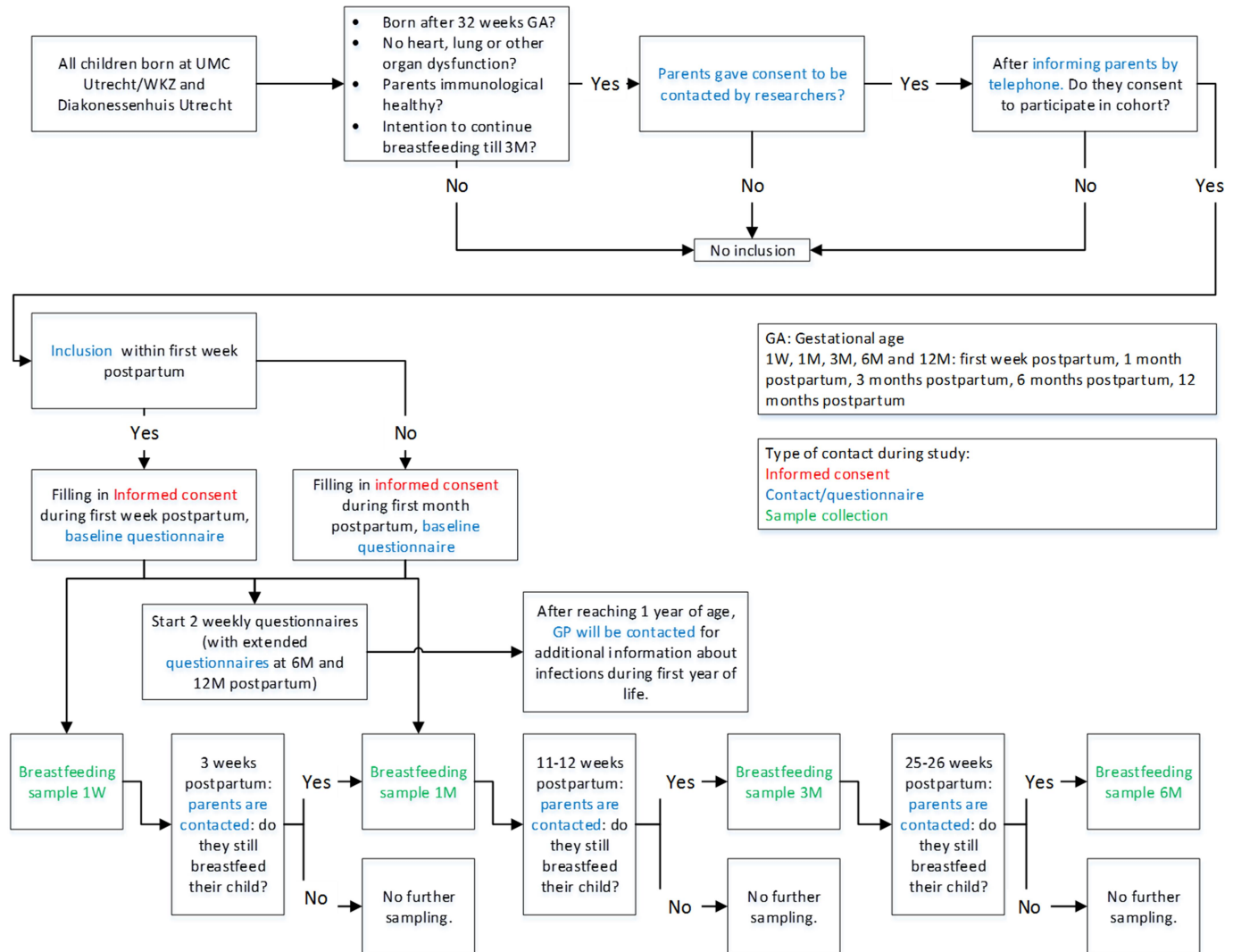


Figure S1. Study activities overview: overview of all study activities

Figure S2:

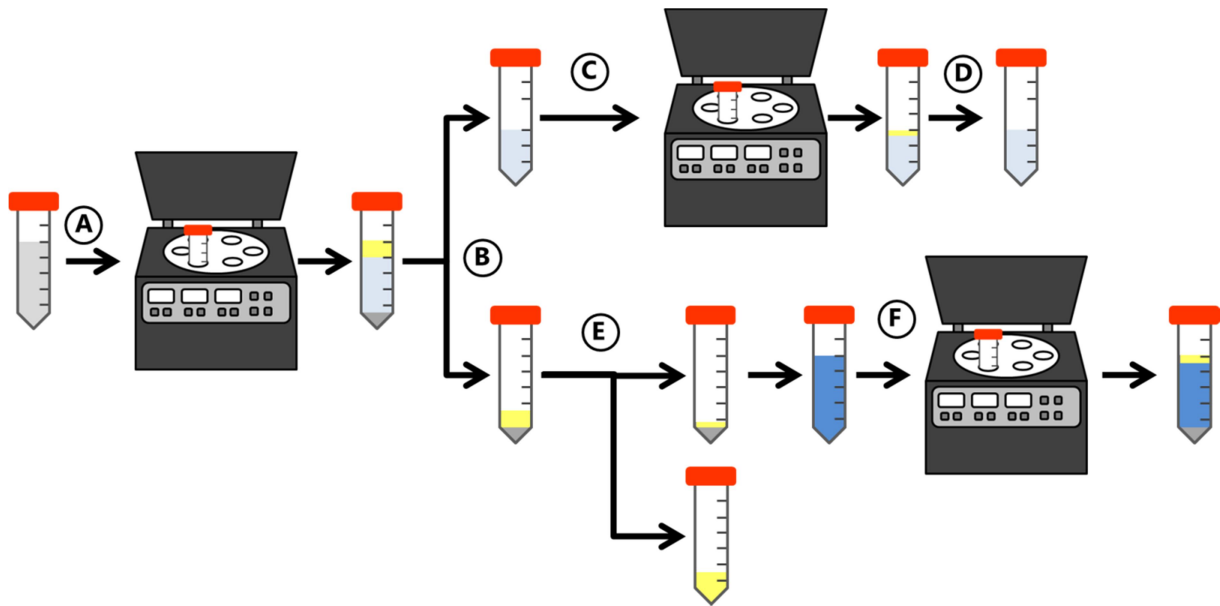


Figure S2. Sample processing in PRIMA human milk cohort: A) Human milk samples are centrifuged for 10 minutes at 600 g in order to remove the cream (layer on top, indicated in yellow) and cells (collected in pellet on the bottom, indicated in dark grey). B) Milk supernatant is collected by pipetting through cream layer without disturbing the cell pellet and subsequently transferred into a new tube. C) Collected 600 g supernatant is again centrifuged for another 10 minutes at 600 g. D) Milk supernatant is collected by pipetting through the residual cream and transferred to and aliquoted into clean cryotubes, and stored at -80°C until further analysis. E) The cream layer is removed without disturbing the cellular pellet and transferred into cryotubes stored at -80°C for further analysis. F) 20mL PBS is added to rinse and cells are spun at 600 g for 10 minutes, after which the supernatant is discarded and cells are kept on ice until further processing for analysis G). Milk EVs are processed further according to the Zonneveld protocol at 3000 g.

PRIMA RESEARCH PROTOCOL

**Protecting against Respiratory tract Infections through human Milk
Analysis**



PROTOCOL TITLE 'Protecting against Respiratory Infections through human Milk Analysis'

Protocol ID	PRIMA
Short title	Protecting against Respiratory Infections through human Milk Analysis
EudraCT number	<i>Not applicable</i>
Version 4A	
Date 8-7-2021	
Coordinating investigator/project leader	<i>A.H. Hellinga, PhD-student</i>
Principal investigator(s) (in Dutch: hoofdonderzoeker/ uitvoerder)	<i>Prof. L.J. Bont, MD Ph, PI RSV research group</i> <i>J.H.W. Leusen, PHD, PI immunotherapy research group</i> <i>B. Van't Land, PHD, PI immune development group</i>
Sponsor (in Dutch: verrichter/opdrachtgever)	<i>UMC Utrecht</i>
Subsidising party	<i>Nutricia Research bv.</i> <i>Regio Deal Foodvalley</i>
Independent expert (s)	<i>Bert Arets, MD PhD</i>
Laboratory sites	<i>Laboratory of translational immunology (LTI), dLAB, UMC Utrecht</i> <i>Nutricia Research bv</i> <i>Division of Pharmacology I Department of Pharmaceutical Sciences I Utrecht Institute for</i>

<i>Pharmaceutical Sciences</i>	
Pharmacy	<i>Not applicable</i>

PROTOCOL SIGNATURE SHEET

Name	Signature	Date
Sponsor or legal representative: <i>Management of dLAB:</i> <i>Prof. Dr. R.A.W. van Lier.</i> Head of Department: <i>Prof. Dr. L. Meyaard</i>		
Principal Investigator: <i>Prof. dr. L.J. Bont</i>		

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LIST OF ABBREVIATIONS AND RELEVANT DEFINITIONS

ABR	General Assessment and Registration form (ABR form), the application form that is required for submission to the accredited Ethics Committee; in Dutch: Algemeen Beoordelings- en Registratieformulier (ABR-formulier)
AE	Adverse Event
AR	Adverse Reaction
CA	Competent Authority
CCMO	Central Committee on Research Involving Human Subjects; in Dutch: Centrale Commissie Mensgebonden Onderzoek
CV	Curriculum Vitae
DSMB	Data Safety Monitoring Board
EU	European Union
EudraCT	European drug regulatory affairs Clinical Trials
GCP	Good Clinical Practice
GDPR	General Data Protection Regulation; in Dutch: Algemene Verordening Gegevensbescherming (AVG)
IB	Investigator's Brochure
IC	Informed Consent
IMP	Investigational Medicinal Product
IMPD	Investigational Medicinal Product Dossier
METC	Medical research ethics committee (MREC); in Dutch: medisch-ethische toetsingscommissie (METC)
(S)AE	(Serious) Adverse Event
SPC	Summary of Product Characteristics; in Dutch: officiële productinformatie IB1-tekst
Sponsor	The sponsor is the party that commissions the organisation or performance of the research, for example a pharmaceutical company, academic hospital, scientific organisation or investigator. A party that provides funding for a study but does not commission it is not regarded as the sponsor, but referred to as a subsidising party.
SUSAR	Suspected Unexpected Serious Adverse Reaction
UAVG	Dutch Act on Implementation of the General Data Protection Regulation; in Dutch: Uitvoeringswet AVG
WMO	Medical Research Involving Human Subjects Act; in Dutch: Wet Medisch-wetenschappelijk Onderzoek met Mensen

SUMMARY

Rationale:

Study A:

Infants who are not breastfed have a higher risk of hospitalization in early life in relation to a wide range of common infections (Ajetunmobi2015). Exclusive breastfeeding until the age of 4 months followed by partial breastfeeding is associated with reduction in respiratory and gastrointestinal infections (Walker2015, Duijts2010). For example, infants admitted to hospital with respiratory syncytial virus (RSV) infection are less likely to have been breastfed (Ajetunmobi2015, Blanken 2018). This suggests that breast milk contains protective components against respiratory tract infections, which lack in infant formula. Although infant formula has been developed over many decades into adequate nutrition for those infants who cannot receive human milk, even modern infant formulas lack many components made by each mother for the immune imprinting of her baby, such as specific antibodies (based on the immunologic history of the mother) and human milk oligosaccharides (HMOS). The provision of secretory mixtures of HMOS, have been associated with direct protection against infections (Laucirica2017). The protective effects of breast milk are, in part, due to antibodies and in part due to other factors, including oligosaccharides. Identification and confirmation of the compounds and their mechanisms by which they offer protection against infections is critical to be able to design new and improved formula products. In addition, compounds, or combination of compounds, that seem to have a protective effect, could be used as novel strategies to treat pneumonia and other respiratory tract infections.

Study B:

There is a growing interest in the use of maternal vaccinations during pregnancy and several maternal vaccinations are at this moment studied, e.g. against *Bordetella pertussis*, influenza, group B streptococcus and RSV (Marchant2017, Abu Raya2017, Heath2017)). Already, a maternal vaccination against *Bordetella pertussis* is approved for vaccination during pregnancy and is currently implemented in the UK (Amirthalingam2016). From December 2019 onwards, all pregnant women in the Netherlands will be offered a *Bordetella pertussis* vaccination at 28 till 32 weeks gestational age (Rijksoverheid). Although it is generally believed that the protective effect of maternal vaccination is derived from placental transfer of antibodies, definitive evidence whether placental transfer or transfer of immunity through breastfeeding or amniotic fluid is absent (Marchant2017, Pou2019). Moreover, maternal vaccination studies in mice demonstrated that transfer of antibodies through breastfeeding and not placental transfer is of major importance to protect offspring (Gaillard2017, Caballero-Flores2019). Further mechanistic insights into maternal immunity transfer will be essential for further development of maternal vaccinations. Therefore we plan to perform a substudy (afterwards called study B) within the PRIMA human milk cohort. In this cohort (n=20) we will collect additional samples next to the breastfeeding samples and the questionnaires. We will sample cord blood, amniotic fluid, maternal blood sample and saliva samples of the participating children. Cord blood samples and the amniotic fluid samples will be compared to the composition of antibodies and HMOS in human milk, as it is known they contain HMOs and/or antibodies as well (Jacobino2016, Hirschmugl2019). The maternal blood samples will be used to observe the maternal response to *Bordetella pertussis* revaccination. Likewise, the maternal blood sample will serve as a golden standard to see if there is any kind of selection of antibody transfer through one of the three media (i.e. cord blood, human milk or amniotic fluid).

To evaluate the influence of each individual route of maternal immune transfer on immune development, we will also collect saliva samples. We have a particular interest in the development of the antibody repertoire in children enrolled in this substudy. To further evaluate the effect of the maternal *Bordetella pertussis* vaccination on the immune development in infants, saliva samples will be collected during the house visits (i.e. at 1 week, 1 month, 3, 6 and 12 months postpartum). Saliva samples have demonstrated to be a

reliable representation of antibody serum repertoire, and are considered less invasive than blood samples (Litt2006, Hetteger2019).

Study C:

The PRIMA cohort (i.e. study A) is performed to identify which components, or combination of components, in human milk are protective against respiratory tract infection in infants. The composition of these components in human milk is influenced by many factors. Some of these factors cannot be influenced, such as the origin of the mother, whereas other factors can be influenced, such as the diet of a lactating woman (Bravi et al., 2016; Munblit et al., 2014). As an example, an observational study with Finnish lactating women found that a breast milk sample 3 months postpartum of women who consumed fatty fish in the two days before sample collection had a 34% increase of omega-3 fatty acids, compared to breast milk of women who did not consume fatty fish (Mäkelä et al., 2013). For other components, such as HMO composition, the influence of the maternal diet has been studied very limited but the study of Quin and colleagues found a positive correlation between fruit intake and unsaturated fatty acids consumed on the day before breast milk sampling with various HMOs in breast milk (Quin et al., 2020). Even more importantly, although there are strong indications that the maternal diet influences the breast milk composition, it is not studied what the consequential effect maternal diet has on infants' health outcomes. However, it is known that the omega-3 polyunsaturated fatty acid docosahexaenoic acid (DHA) have an increased capacity to phagocytise and have an increased microbicidal activity (Hachey et al., 1989; Nasser et al., 2010). Also for the HMOs in breast milk, there is increasing attention for and evidence of their antimicrobial, antiviral and prebiotic capacity (Ayechu-Muruzabal et al., 2018; Morozov et al., 2018). The impact of this on the intestinal microbiota composition might also impact the development of respiratory tract infections and/or allergies via the gut-lung axis (Hilty et al., 2010; Kanmani et al., 2017; Remot et al., 2017; Schuijt et al., 2016). Together, this shows that there is a strong need to study the impact of the maternal diet on breast milk composition, and how this consequently influences the development of respiratory tract infections and/or allergies in the first year of life. Therefore, the cohort should be expanded with dietary assessment of the mother's diet, and sample infants' saliva and feces.

Objective:

Study A:

Main objective:

To identify which components, or combination of components, in human milk and cord blood that are protective against respiratory tract infection during the first year of life and to unravel their underlying mechanisms.

Secondary objective 1:

To identify which components, or combination of components, in human milk are protective against other infections during the first year of life and to unravel their underlying mechanisms.

Secondary objective 2:

To identify which components, or combination of components, in human milk are protective against the development of allergies during the first year of life and to unravel their underlying mechanisms.

Secondary objective 3:

To obtain insight in the gut discomfort and crying of infants in the first 6 months of life.

Study B:

Secondary objective 4:

To obtain insight in the transfer of maternal immunity to human milk, amniotic fluid and cord blood.

Study C:

Secondary objective 5:

To identify the long-term and short-term influence of the maternal diet on the breast milk composition.

Secondary objective 6:

Link the long-term and short-term influence of the maternal diet to the clinical outcome of respiratory tract infection and/or allergies during the first year of life.

Secondary objective 7:

To derive hypotheses about potential mechanisms if the maternal diet-influenced components in human milk are protective against respiratory tract infection and/or of allergies during the first year of life.

Secondary objective 8:

To identify the association between long-term and short-term influence of the maternal diet on the microbiota composition of a 3-month old infant, and its consequence for the risk of developing respiratory tract infections and/or allergies in the first year of life.

Secondary objective 9:

To identify the antibody repertoire and viral genome in saliva samples from 3 month-old

Secondary objective 10:

To link the antibody repertoire, viral genome load and possible other immune markers in saliva samples with feces microbiota composition, and how this relates to the risk of developing respiratory tract infections in the first year of life.

Study A:

Study design: Observational birth cohort study

Study population: healthy mother-child dyads recruited during the first month postpartum.

Intervention (if applicable): none.

Main study parameters/endpoints: The main study parameter is the number of MARI's (Medically Attended Respiratory tract Infections) during the first year of life. This will be measured using questionnaires that parents have to fill in every two weeks. In the same questionnaire, the gut discomfort and crying time of the infant is assessed for the first six months. During the 6-month and 12-month questionnaire, questions about allergy development are added to the questionnaire.

In addition to the questionnaire, we will also collect samples both from the mother as from the child.

Human milk samples will be collected from the mothers during the first week postpartum and at 1, 3 and 6 months postpartum. In human milk, we will measure the antibody titers (both isotype as well as pathogen specific), oligosaccharide composition and immune cell repertoire. A subgroup will be used to analyse extra cellular vesicles (n=15). Since the extracellular vesicle analyses requires bigger sample volumes (at least 5mL sample is required for vesicle analyses, preferably more), only mothers that donate a large amount of human milk at the first week postpartum will be analysed for extracellular vesicles.

Amniotic fluid and cord blood and maternal blood samples will be collected in a subgroup of 20 mother-child pairs. Cord blood and amniotic fluid will be collected within the first hours postpartum from the umbilicus. Maternal blood samples will be collected during the first week postpartum. Blood and amniotic fluid samples will be analysed on antibody, oligosaccharide and extracellular vesicle composition and immune cell repertoire

Study B:

Study design: Observational birth cohort study

Study population: healthy mother-child dyads recruited during pregnancy.

Intervention (if applicable): none.

Main study parameters/endpoints:

The main study parameter is the number of MARI's (Medically Attended Respiratory tract Infections) during the first year of life. This will be measured using questionnaires that parents have to fill in every two weeks.

Human milk samples will be collected from the mothers during the first week postpartum and at 1, 3 and 6 months postpartum. In human milk, we will measure the antibody titers (both

isotype as well as pathogen specific), oligosaccharide composition and immune cell repertoire. At 1 week, 1 month, 3 months, 6 months and 12 months postpartum saliva sample of the infants will be collected. Saliva samples will be analysed on antibody repertoire.

Additionally, amniotic fluid, cord blood, infant saliva and maternal blood samples will be collected. Cord blood and amniotic fluid will be collected within the first hours postpartum from the umbilicus. Maternal blood samples will be collected during the first week postpartum. Blood and amniotic fluid samples will be analysed on antibody, oligosaccharide and extracellular vesicle composition and immune cell repertoire.

Study C:

Study design: Observational birth cohort study

Study population: healthy mother-child dyads recruited during pregnancy.

Intervention (if applicable): none.

Main study parameters/endpoints:

The main study parameters/endpoints of study A will also account for sub-study C. Additionally, a main study parameter is the diet of lactating women. The dietary pattern (long-term) will be assessed by a food frequency questionnaire (FFQ) at 2 month and 4 month post-partum. Additionally, we will assess the short-term influence of the diet on breast milk composition by a 24-h recall on the day before the collection of a 1-month and 3-month human milk sample.

In addition to the questionnaires, we will also collect samples both from the mother (human milk) as from the child (saliva and feces):

In the collected human milk samples (1 week, 1 month, 3 and 6 months postpartum) we will perform additional analyses, such as to measure fatty acid composition, protein and amino acid composition, fat- and water-soluble vitamins and other fibres (than HMOs).

The feces will be collected from infants at 3 months postpartum. The feces will be analysed for microbial composition.

The saliva will be collected from infants at 3 months postpartum. The saliva will be analysed for the antibody repertoire and viral genome.

Nature and extent of the burden and risks associated with participation, benefit and group relatedness:

Study A, B and C:

Breast milk is collected by pump or by manual expression. In case women who do not intend to use a pump, will use a pump, this may result in some discomfort. Burden and risks of expressing milk: expressing milk may cause some discomfort. No specific risks are reported with expressing breastmilk.

Number, timing and amount of samples: We will collect four samples: during the first week post-partum, 1 month, 3 months and 6 months postpartum. We will to collect 5-10mL for each sample.

Questionnaires to assess the MARIs have to be filled in during the trial: 27 (baseline and afterwards 26 questionnaires every 2 weeks (including the longer questionnaires at 6 and 12 months post-partum)). Estimated total time spend on questionnaire is 2-3 hours per participating mother-child dyad spread over 1 year.

Study B:

Burden and risk of cord blood samples: Since blood will be collected from the cord blood, we expect no burden or risks arising from cord blood sampling.

Burden and risk of amniotic fluid samples: Since amniotic fluid will be collected in a non-invasive way, we expect no burden or risks arising from amniotic fluidsampling.

Maternal blood sampling: Maternal blood will be collected through a venipuncture. Although considered safe, rarely phlebitis, extravasation of blood, bruising and hematoma forming following venipuncture have been reported. Likewise a venipuncture might cause discomfort (i.e. pain during venepuncture or from the arising hematoma or bruises).

Burden and risk of saliva samples: Since saliva will be collected in a non-invasive way, we expect no burden or risks arising from saliva sampling. A similar methods of saliva collection is used in the SInFoNIA study (NL65543.041.18).

Burden and risk of amniotic fluid samples: No risk or burden to be expected.

Study C:

Dietary assessment

The FFQs have to be filled in around 2 months and 4 months postpartum. The 24h-recall is filled in at 1 month and 3 month postpartum. Estimated total time spend on dietary assessment questionnaire is 3 hours per participating mother-child dyad spread over 3 months.

Feces sampling

Feces will be samples by the parent from the diaper of the infant. Since feces will be collected in a non-invasive way, we expect no burden or risks arising from feces sampling.

Saliva sampling

No burden or risk due to saliva sampling expected, as discussed for study B.

1. INTRODUCTION AND RATIONALE

Study A:

Respiratory infection is one of the major causes of illness in infants. For example, RSV infects virtually every child before the age of 2 years. Each year, about 28,000 infants require medical care for RSV bronchiolitis in the Netherlands (Yu2013, Isaacs2013) of which approximately 2000 require hospitalisation with costs of €2000–€4000 per patient (Meijboom2012, Bos2007, Jansen2007). Within a recent meta-analysis, one of the 8 risk factors for RSV-induced acute lower respiratory infection in children was “no breastfeeding”, with odds ratio of 2.24 (95% CI 1.56-3.20) (Rietveld2004). This clearly indicates that currently, infant formula does not offer the same level of protection to RSV as breastfeeding does. In 2006–2012, only an estimated 25% of infants in the WHO European Region were exclusively breastfed for the first 6 months, which is far below the global recommendation. Even though the rate of early initiation of breastfeeding is high in some countries, exclusive breastfeeding rates drop rapidly between 4 and 6 months of age and are very low at 6 months, subscribing the clear need for improvement of infant formula. Previously conducted research shows that apart from antibodies, other components in human protects children form infectious disease. Particularly research in human oligosaccharide shows that this may also be an important component in human milk that modulates infectious disease (Ramani2018).

Additionally, oligosaccharides seem to modulate the immune system as well and even seem to have an effect on the development of allergies in children. Children that received an infant formula enriched with the scGOS/lcFOS oligosaccharide mixture developed fewer allergies and eczema during the first year of life, compared to children that received infant formulas without scGOS/lcFOS(Arslanoglu2012).

It has been postulated that HMOS mediate protection against infections and allergies through two (possibly three) mechanisms. HMOS are supposed to have direct pathogen inhibiting effects. Duska et al. showed that some HMOS have a direct RSV-inhibiting effect. (Duska-

McEwen2014). In addition, the potential of both neutral (LnNt) and acidic HMOS to effectively inhibit infection by specific pathogens is shown (Duska-McEwen2014). Mechanisms by which these specific HMOs exert their anti-infective properties are by inhibiting virus binding to the host cells and/or blocking virus entry into the cell or blocking viral replication within the cell. In addition to direct blockage of viral infection by mimicking viral receptors, indirect microbial and immune modulation by HMOS have been hypothesized.

In addition, immune modulatory effects have been demonstrated for 2'FL, suggesting an additional function of specific oligosaccharides (Goldman2007, Koning2015, Erney2000). HMOS have been postulated to modulate TLR signaling on immune cells and induce regulatory responses (Kulinich2016, He2016). For instance, 2-FL modulates CD14 expression in human enterocytes, thereby attenuating LPS-induced inflammation in vitro (He2016)). Moreover, it has nicely been shown that the addition of human milk as well as human milk oligosaccharides interact directly with the immune system in particular with DCs, through DC-Sign (Koning2015)). Siglecs and related glycan-binding proteins are essential in immune responses and immune regulation (Shang2013), providing ways for improvement of DC development. DCs are key in the identification of pathogens and directing the immune response towards effective immunity, including T cell and B cell responses. Because TLRs and C-type lectins are vital in pathogen recognition and immune modulation (essential during development of the immune system in early life) it seems likely that via multiple direct and indirect pathways specific HMOS contribute to the development of a balanced immune system, hence protection towards infections. Therefore, we postulate that HMOS are involved in regulation of mucosal immune function and this will be investigated further in the present proposal.

In addition, the role of microbial modulation of specific HMOS structures have been subject of extensive studies. More specifically, Maternal fucosyltransferase 2 status has been suggested to affect the gut bifidobacterial communities of breastfed infants (Puccio2017). In addition, Utilization of major fucosylated and sialylated human milk oligosaccharides by isolated human gut microbes have been shown (Thurl2010). Its correlation towards respiratory infection susceptibility however remains to be established.

Aside from the HMOS and antibodies, other components may play an important role as well in early life immune development. That is why we aim to build up a biobank to analyse additional components in the future. Components that may be of interest are specific fatty acids and cytokines. For instance, presence of IL-2 and IL-13 in human milk are associated with a reduction of eczema in children (Munblit2017). Additionally, the presence of IL-13 in colostrum is associated with a lower incidence of food allergies (Munblit2017). Hernell et al. showed that addition of specific fats (retrieved from cow's milk) to plant-based infant formulas reduced the amount of infections in children (Hernell2016).

The components that we will identify to have a protective effect, we will further analyse in our laboratories to unravel their underlying mechanism. Not only will this aid to improve infant formulas, but this will also lead to new therapeutic strategies to treat (respiratory tract) infections and allergies.

Combined, this collaboration will lead to new insights in the components of breastmilk (including macro and micro nutrients, proteins, peptides, lipids, amino-acids, but specifically HMOS) and the role they play in providing protection to infants. More knowledge regarding by which mechanism the specific HMOS structures can provide protection towards specific pathogens, will help to further improve infant formulas to prevent infections and allergies

Study B:

In the sub study we will also collect additional samples to research what the influence of breastfeeding is on immune development, compared to other routes that are enrolled in the transfer of maternal immunity to the neonate. This will be done in a subgroup of 20 mother-

child dyads. The samples that we will collect are cord blood samples, a maternal blood sample and an amniotic fluid sample. Jacobino et al. showed that antibodies retrieved from human amniotic fluid protected mice pups against RSV-infections (Jacobino2016). Very little is known about the protective value antibody titers in cord blood. Active placental transport of maternal antibodies to the neonatal blood has been described, but little is known about the effectiveness of these antibodies (Kohler1966). To obtain more insight in the effect of the antibodies that are being transferred by breastfeeding, we will compare the effect of breastfeeding to the antibodies in cord blood and amniotic fluid. To evaluate the effect of the maternal Bordetella pertussis vaccination on (pertussis-specific) immune development in children, we will also collect saliva samples at 1 week, 1 month, 3 months, 6 months and 12 months postpartum. Saliva samples offer an ideal method to evaluate the (Bordetella-pertussis specific) immune development during the first year of life since saliva antibody titers correlate well with serum titers but saliva samples are considered to be non-invasive.

Study C:

In an additional sub-study (afterwards called study C) we would like to expand the current study design to assess the role of the maternal diet and infants' intestinal microbial development on the risk of developing respiratory tract infections in the first year of life.

Breast milk is a very variable body fluid influenced by many maternal and environmental factors (Munblit et al., 2014). The maternal diet during lactation is one of the factors for which there are strong suggestions that it influences breast milk composition (Bravi et al., 2016). A Finnish observational cohort study with normal weight (n=49) and overweight (n=51) women found an association of a 34% percent increase of n-3 fatty acids (FA) in a 3 month postpartum-breast milk sample for women who consumed fatty fish in the two days before the sample collection. The same study found a significantly increased n-6 FA concentration in breast milk of women who consumed vegetable-oil-based-spreads on a regular basis. Consumption of high-fat dairy products was associated with a significantly increased concentration of short-chain fatty acids (SCFAs) (Mäkelä et al., 2013).

As elaborately discussed in the background for study A, breastfeeding protects against respiratory tract infections in infants, including RSV infection. Apart from antibodies and HMOs, also FAs have antiviral and -microbial properties. Especially medium chain saturated, such as lauric acid (C12:0), and long chain unsaturated FAs, such as linoleic acid (C18:2) appear to be potent in affecting the lipid coating of viruses (Isaacs, 2005; Thormar et al., 1987). In addition, macrophages exposed to omega-3 FA docosahexaenoic acid (DHA) (C22:6) and omega-6 FA arachidonic acid (C20:4) have an increased capacity to phagocytise and have an increased microbicidal activity. Endogenous fatty acid production by the mammary gland is limited to a chain length of 14 (*de novo* FAs), therefore, the long chain FAs are derived from the diet (Hachey et al., 1989; Nasser et al., 2010), indicating the relevance of the maternal diet in relation to risk of RTIs for infants.

Very limited research has been performed focusing at the association between maternal diet and HMO composition in breast milk. A prospective observational birth cohort did not find a correlation between maternal diet quality and total HMO concentration nor for individual HMOs (after correction for multiple comparisons) in breast milk (Azad et al., 2018). However, the study of Azad and colleagues assessed the maternal diet by means of a food frequency questionnaire (FFQ) during late pregnancy, while the breast milk sample was collected 3-4 months postpartum. Another prospective cohort did find a positive correlation between fruit intake and unsaturated fatty acids consumed on the day before breast milk sampling, with various (sulfonated) HMOs in breast milk. Even more, monosaccharide 5-N-glycolyl-neuraminic acid (Neu5Gc) was identified in all breast milk samples. Notably, this monosaccharide cannot be synthesized endogenously and is diet-derived (e.g. from red meat) (Quin et al., 2020).

HMOs are of increasing interest because of their antimicrobial and antiviral capacity on one hand, and prebiotic capability on the other hand. HMOs prevent viruses to adhere to epithelial surfaces, by mimicking viral receptors or block receptors on epithelial cells (Morozov et al., 2018). Their prebiotic effect is achieved by the fact that specific strains of the commensal species *Bifidobacteria* and *Bacteroides* are able to metabolize HMOs and therefore are in favour in competition with strains unable to metabolize HMOs, such as *E. coli* and *Enterococci* (Ayeche-Muruzabal et al., 2018).

Accumulating evidence shows that the intestinal microbial composition mediates the lung microbiota composition, also referred to as the gut-lung axis. The presence of microbiota in the respiratory tract, and more importantly the significant role it has in the pathogenesis of respiratory tract infections, is only studied for about a decade. Schuijt and colleagues performed a mice study for which mice were treated with antibiotics, after which *S. pneumonia* was administered intranasally. It was found that the antibiotic-treated mice had a.o. an increased mortality, inflammatory markers and an increased bacteria outgrowth. The significant role of the intestinal microbiota specifically was shown by normalizing the bacterial counts and IL-10 and TNF- α levels in the respiratory tract after fecal microbiome transplantation (FMT) (Schuijt et al., 2016). A different mice study of Kanmani and colleagues showed the relevance of the lung microbiota in the context of respiratory tract infections. Mice were intranasally administrated with *Corynebacterium pseudodiphtheriticum*, a bacterium which is a commensal in the respiratory tract microbiota. The priming appeared to reduce the susceptibility to a primary RSV infection by modulating the toll-like receptor 3-mediated antiviral inflammatory response (Kanmani et al., 2017). The microbiota community present in the lung also seems to be relevant for the development of asthma. Comparison of the lung microbiota of both asthmatic adults and children shows that there was an increased presence of pathogenic Proteobacteria compared to the healthy controls (Hilty et al., 2010). Remot and colleagues performed a study with mice, for which they isolated several strains from the lung microbiota of healthy mice. The authors showed that intranasal administration of isolated strain to neonatal mice influenced the asthmatic immune response towards a house dust mite challenge (Remot et al., 2017).

Considering the influence of HMOs on the intestinal microbial development in infants, and the careful associations between intestinal microbial composition and risk of developing RTIs and/or asthma, the pathway from maternal diet to breast milk composition to intestinal composition to eventually risk of developing RTIs and/or allergies is very relevant.

Taking together, although there are strong suggestions for the influence of maternal diet on breast milk composition, the scarcity of high quality studies and diversity in study design restrain the ability to draw strong conclusions. In addition, there is a lack of studies linking the maternal diet and associated breast milk composition to the gut health of the infant resulting in protection against respiratory infections and/or the development of allergies. There is a vast need for an observational study with a bigger sample size, which is also based on a power calculation (Bravi et al., 2016). Therefore, we plan to perform a substudy within the PRIMA human milk cohort. In this cohort (n=250), we will additionally assess the maternal diet, and collect feces and saliva of the infant. This study design allows to not only link the short- and long-term influence of maternal diet to breast milk composition, but also the clinical health outcomes in infants, with Medically Attended Respiratory tract Infections during the first year of life as main study parameter. Even more, the influence of the maternal diet and an infant's antibody repertoire on intestinal microbial composition, and subsequently on the risk of developing RTIs is explored.

2. OBJECTIVES

Study A:

Primary objective:

To measure to what extent 2'FL human milk oligosaccharides are protective against medically attended respiratory tract infection during the first year of life.

Secondary objective 1:

To identify which components, or combination of components, in human milk are protective against any parent-reported, physician reported infections during the first year of life.

Secondary objective 2:

To identify which components, or combination of components, in human milk are protective against the development of parent-reported, physician reported allergies during the first year of life and.

Secondary objective 3:

To identify which immunological components (antibodies, immune cells) in human milk, cord blood and amniotic fluid are protective against any parent-reported, physician reported infections during the first year of life.

Secondary objective 4:

To obtain insight in the gut discomfort and crying of infants in the first 6 months of life.

Study B:

Secondary objective 5:

To describe antibody concentrations in compartments relevant to materno-fetal antibody transfer. We will define the association between maternal serum, breast milk antibodies, cord blood, amniotic and infant antibodies over time. This study is performed while maternal pertussis vaccination is introduced. We will therefore analyse total and pertussis-specific antibody concentrations.

Study C:

Secondary objective 6:

To identify the long-term and short-term influence of the maternal diet on the breast milk composition.

Secondary objective 7:

Link the long-term and short-term influence of the maternal diet to the clinical outcome of respiratory tract infection during the first year of life.

Secondary objective 8:

To derive hypotheses about potential mechanisms if the maternal diet-influenced components in human milk are protective against respiratory tract infection during the first year of life.

Secondary objective 9:

To identify the association between long-term and short-term influence of the maternal diet on the microbiota composition of a 3-month old infant, and its consequence for the risk of developing respiratory tract infections in the first year of life.

Secondary objective 10:

To identify the antibody repertoire and viral genome in saliva samples from 3 month-old infants.

Secondary objective 11:

To link the antibody repertoire in saliva samples with antibody repertoire in feces and microbiota composition, and how this relates to the risk of developing respiratory tract infections in the first year of life.

3. STUDY DESIGN

Study A

This cohort concerns a prospective observational cohort study. A retrospective collection of data collection or sample collection would not be useful for this cohort. Retrospective data collection will decrease the validity of data collected from parents through questionnaires. Moreover, since the composition of breast milk differs in time postpartum, samples have to be collected at certain time points in order to elucidate its precise and unique composition.

Study B

Saliva samples will be collected at 1 week, 1 month, 3 and 6 months because of pragmatic reasons. The 12 months sample will be collected since the last vaccination during the first year of life against *Bordetella pertussis* is offered at 11 months (the next vaccination is offered at 4 years in the Netherlands) (Rijksvaccinatieprogramma).

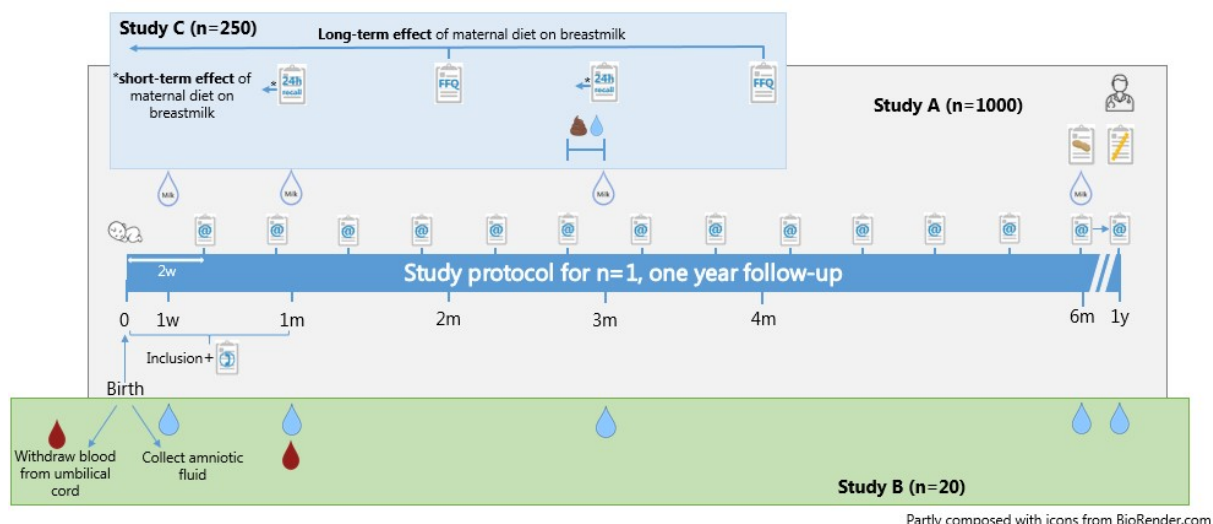
Study C

A 24h recall is filled in on the day of collection of the 1 and 3 month breast milk sample, to determine the short-term effect of maternal diet on breast milk composition. The FFQ is filled in at 2 and 4 months, to determine the long-term effect (dietary pattern) on breast milk composition. The 4 month FFQ is a repetitive measurement to evaluate whether the maternal diet has changed since 2 months, and therefore also can be linked to the 3 month breast milk sample. A change in dietary pattern is conceivable because, for example, the mother has stopped breastfeeding and/or maternity leave from work has ended.

Feces and saliva are collected at 3 months because at this time point the effect of the breast milk composition is expected to be more significant than at 1 month or 1 week postpartum. The 3 month time point is also more suitable than the 6 month time point because by that time, infants may have received solid foods aside of breast milk and/or infant formula.

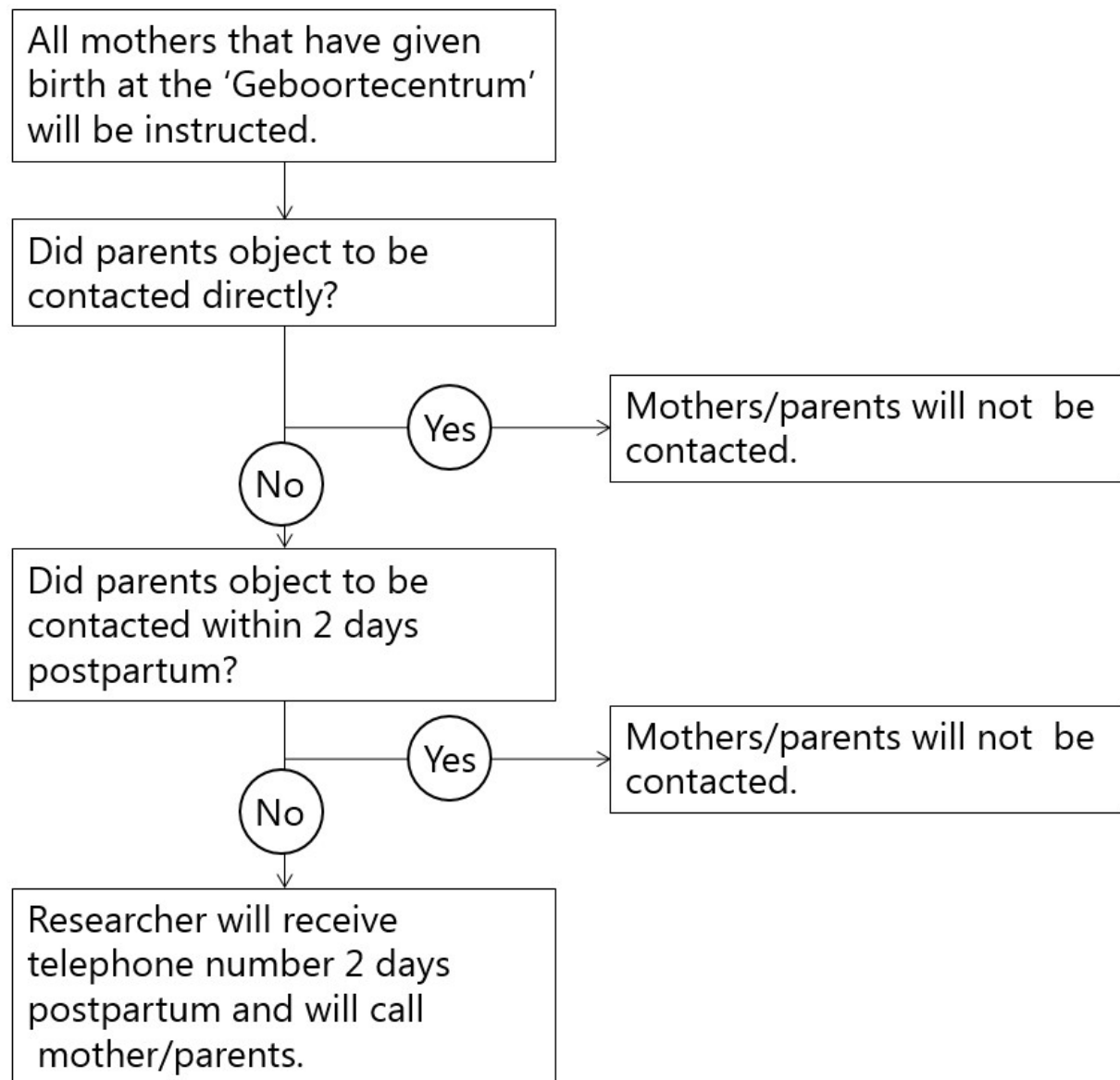
4. STUDY POPULATION

4.1 Population (base)



Participants will mainly be recruited from the obstetric wards at the Wilhelmina Children's Hospital (WKZ) and Diaconessenhuis (Utrecht, the Netherlands).

As women who delivered without complications, may leave the hospital, we will approach these women who delivered at WKZ as well. This letter is added to the discharge letter/package in an envelope with a bright coloured label. The recruitment is further explained in paragraph 8.2. and depicted in the flowchart below:



The researchers will report to METC Utrecht the amount of parents that were not properly informed about the research by healthcare workers after contacting the first 50 mothers.

At the time an initiation visit will be planned, we will already send the informed consent and Patient Information Letter via e-mail.

4.2 Inclusion criteria

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

- All mother-child dyads that live in a proximity to Utrecht (because of logistical issues) and/or gave birth at the WKZ or Diakonessenhuis.
- Parents should have the intention to continue breastfeeding until at least 3 months postpartum.

4.3 Exclusion criteria

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

- Concerning the child:

- (inborn) severe cardiac or pulmonary disorders or other severe organ diseases
- Extreme prematurity (defined as GA<32 weeks).
- Concerning parents:
 - Acquired or innate immune deficiencies (excluding asthma, eczema and allergies).
 - The (lactating) mother receives medication or has a physical condition which forms a contraindication for breastfeeding.
 - Insufficient control of the Dutch language (i.e. at least B1 level).

4.4 Sample size calculation

Study A:

A sample size of 1000 participants has an 80% power to detect a ratio of the means of 0.91 in those with vs. those without a medically attended LRTI using a two-sided two-sample t-test, assuming that 10% of the cohort participants have a medically attended LRTI (N=100) and that the 2'FL HMO concentration in the population has a mean of 3g/L, a standard deviation of 1g/L, and a normal distribution (Donovan2016). The significance level (alpha) is 0.05. The sample size was calculated using Power Analysis for Sample Size (PASS) 2008.

Study B: .

Group sample sizes of 9 and 9 achieve 82% power to detect a difference of 88.3 between the null hypothesis that both group means are 100.7 and the alternative hypothesis that the mean of group 2 is 12.4 with estimated group standard deviations of 80.0 and 18.0 and with a significance level (alpha) of 0.05 using a two-sided two-sample t-test (Maertens2016).

Study C:

From the questionnaires that will be used "24h recall" has the largest number of variables at 216 variables. Although, the number of necessary examples depend on the data itself, considering algorithms such as support vector machines will be used for the classification, we need at least the same number of examples as variables. This is because we will try to solve a system of linear equations, and to avoid an ill-conditioned system we need to have 1:1 relationship between variables and samples (Smola and Schölkopf, 2004). In addition, as we will divide our analysis into training and testing, we need an extra 10% of samples for testing and considering a measurement error of 5% puts the total of samples at 246.1 $\approx$ 247 (upper limit). Thus, we need at least 247 samples for the analysis of the dataset.

5. METHODS

5.1 Study parameters/endpoints

5.1.1 Main study parameter/endpoint

- Parent-reported medically attended respiratory tract infection during the first year of life.

5.1.2 Secondary study parameters/endpoints (if applicable)

Study A:

- Other infections during the first year of life (non-MARI respiratory tract infections but also gastro-intestinal infections).
- Allergies developed during first year of life.

- Gut discomfort of the infant during the first 6 months of life.
- Breast milk composition: antibody repertoire, oligosaccharides, immune cell repertoire (n=1000), extracellular vesicles (n=15).

Study B:

- Antibody repertoire in amniotic fluid, cord blood, maternal blood and infant's saliva.
- Oligosaccharide, extracellular vesicles, antibody and immune cell repertoire in blood and amniotic fluid.

Study C:

- Diet(ary pattern) of the lactating mother.
- Fatty acid, protein, amino acid, fat- and water soluble vitamins and fibres composition in breast milk.
- Microbial composition of infant's feces.
- Antibody repertoire and viral genome of infant's saliva.

5.1.3 Other study parameters (if applicable)

Other parameters that will be measured are:

- Complications and medication use during pregnancy and during labour.
 - Birth characteristics (e.g. birth weight, Apgar score)
 - Feeding habits (e.g. high allergenic food during pregnancy and first feeding of child)
 - Household characteristics (e.g. age and amount of other children in household, pets etc.)
 - Smoking habits of parents
 - General information of parents (age, genetic background, education level, presence of (chronic) diseases and allergic status)
 - Vaccination status mother
- See for all questions the baseline and 2-weekly questionnaire.

5.2 Study procedures

Breast milk sampling:

Women who participate in this study are asked to express and collect a whole milk sample from both breasts. The sample will be mixed by gently tilting the flask. After mixing, 5-10mL of the whole sample will be stored in a 50mL Falcon tube in the fridge. Parents are asked to note the date, time of expression and storage temperature on the tube. Samples will be handed in to the researcher during a house visit. The researcher will note the moment (1 week, 1, 3 or 6 month postpartum) and type (fore-, hind- or whole milk) of breast milk sample. The researcher will transport the sample to the UMC Utrecht. (Miller2013) The optimal processing conditions to fractionate the samples were tested in a pilot, see our SOP for more details about the processing of human milk samples before storage.

Study C:

For the breast milk sampling from mothers participating in study C, mothers are asked to collect a breast milk sample on the morning of collection before breakfast. This is asked because the assessment for the short-term influence of the maternal diet (24h recall) concerns the day before collection.

Cord blood sampling:

Cord blood is sampled from the umbilical vein, following the delivery of the child. First the umbilical has to be double clamped 3-5cm from the umbilicus to transect the umbilical cord. The child will consequently be removed from the field. The cord will now be prepared for blood sampling using a povidone iodine applicator to sterilize the umbilicus. Next, the needle will be inserted in the umbilical vein, standard blood sampling tubes will be used to collect the cord blood. The blood can be collected both *in utero* or *ex utero*. Meaning the venipuncture can be performed after or before the placental delivery. (Amson2015)

Amniotic fluid collection:

After rupturing the amniotic sac, amniotic fluid will be collected (regular procedure at the obstetric ward). The amniotic fluid will be stored at room temperature until retrieved by the researchers. For the study, about 200mL cord blood will be collected. 100ml will be used for cell analysis. About 9 vials with 1×10^7 cells each will be stored in the biobank, the other 1×10^7 cells will directly be used for FACS analysis. The remaining 100ml will be used to obtain serum. We will use about 1-2mL of serum to measure antibodies and HMOS, the remaining serum will be stored at the biobank facility of the UMC Utrecht.

Venipuncture:

Maternal blood samples will preferably be collected during the first house visit and not at delivery. We will collect venous blood samples from the median cubital vein. We will analyse these blood samples on various components. The antibody composition will be performed in the group of Jeanette Leusen (LTI, UMC Utrecht) using ELISA or multiplex approaches. Oligosaccharide composition will be measured in the group of Belinda van 't Land (LTI UMC Utrecht and Nutricia). Immune cell repertoire will be analysed in the group of Jeanette Leusen and/or Femke van Wijk (LTI, UMC Utrecht) using flow cytometry. Extracellular vesicles will be measured at the group of Marca Wauben (Faculty of veterinary medicine, Utrecht University). We will collect about 70mL blood. We will collect five 10mL sodium-heparin tubes, of which one will be used for FACs analysis. The other tubes will be stored at the biobank. We will also collect two 10mL serum tubes. 1-2mL of the serum will be used for antibody and HMOS analysis, the remaining serum will be stored at the biobank facility of the UMC Utrecht.

Saliva sampling:

Study B:

Saliva samples will be collected during the first week postpartum, at 1, 3, 6 and 12 months postpartum. Whenever children are already spilling saliva, this can be collected directly with a saliva swab (i.e. a soft inert polymer swab that will take up the saliva). When children are not spilling saliva, the saliva swab can also be introduced gently into the mouth in a similar way as a pacifier. The child has to suck or chew on the saliva swab for several seconds before the swab will be retrieved. The saliva swab will afterwards be stored in a collection tube on ice for transportation to the lab. At the lab, saliva will be removed from the swab by using a centrifuge. The collected saliva will be aliquoted in several tubes, maximal 900uL per tube. In case of lower amounts of saliva yields, tubes will contain less the 900uL, this will be logged for future sample usage.

Study C:

Saliva will be collected by a parent in the week before the 3 month sample collection. Saliva will be sampled with Micro•SAL™ for Children according to manufacturer's protocol. The pad of the Collector will be placed in the mouth of the infant until the pad is saturated (60-90 sec.). The saturated collector will be pushed into the compression tube, which is firmly connected with its base into an Eppendorf tube.

The Eppendorf tube with saliva sample will be stored at -20°C in the freezer at the participant's home, until collection by the researcher.

Questionnaires:

Study A:

Data will be collected using questionnaires and by interviewing general practitioners and/or pharmacists.

Questionnaires will be sent to parents using Castor EDC. Parents will receive a baseline questionnaire at moment of entering the study, which will cost them about 20-30 minutes to fill in. Consecutively they will receive every 2 weeks a short questionnaire to monitor infectious episodes, feeding habits, gut discomfort and crying of the infant and to screen for any infectious episodes in mothers, which takes 1-2 minutes each to fill in. The 2 weekly questionnaire will be expanded to a longer list at 6 months and 12 months postpartum to screen children for allergies developed during the first year. Parents will receive questionnaires through email.

Study C:

The questionnaires for the dietary assessment will be sent via e-mail. Parents receive login information for the tool which is reached via a website page. The FFQ will be sent at 2 month and 4 month postpartum and will take 45-60 min. to fill in each. The 24h recall will be sent at 1 month and 3 month postpartum and will take 20-30 min. fill in each.

5.3 Withdrawal of individual subjects

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

5.3.1 Specific criteria for withdrawal (if applicable)

Only when not one breast milk sample is collected that is stored on the proper conditions (i.e. stored in the fridge or on ice after collection), participants can be withdrawn from the study

5.4 Replacement of individual subjects after withdrawal

A new mother-child dyad will be recruited to replace a withdrawn dyads when withdrawal occurs before the first sample is taken.

5.5 Follow-up of subjects withdrawn from treatment

na.

5.6 Premature termination of the study

na.

6. SAFETY REPORTING

na

7. STATISTICAL ANALYSIS

The section on statistical analysis only describes how the quantitative analyses will be done, answering the primary and secondary research objectives.

7.1 Primary study parameter(s)

The association between 2'FL concentrations and the number of medically attended respiratory tract infections during the first year of life will be determined using Poisson regression analysis, adjusted for potential confounders. Potential confounders will be identified by a literature search, complemented by expert knowledge. One such potential

confounder is like 3'SL that is both suggested to exert a direct antiviral effect as well as to be associated with a shorter lactation duration (Azad2011,; Laucirica2017, Pandey2018).

We will also explore whether the moment of lactation postpartum, season and ethnicity modify the effect of 2'FL on the number of respiratory tract infections. Additionally we will search for combinations of components that have a protective effect, by incorporating combinations together with their interactions (e.g. seasonality, ethnicity of mother and time postpartum that sample is collected).

7.2 Secondary study parameter(s)

The association between 2'FL concentrations and the number of allergies and total number of infections during first year of life will be analysed in a similar fashion as for the primary outcomes.

The 24h recalls filled in by the mother, will be checked on missing data in the same week by trained staff. When missing data is expected, for example 1 slice of bread but 5 portions of bread toppings are noted, the researcher or research nurse will contact the participant. Notes from the participants will also be processed by the trained staff.

7.3 Other study parameters

See 10.2. and 10.3.

7.4 Missing data

In case there is lost to follow-up because parents stop to report the questionnaires, we will use the data retrieved from the general practitioner to estimate the amount of medically attended respiratory tract infections. If this is not possible for the amount of respiratory tract infections, we will impute the missing data.

8. ETHICAL CONSIDERATIONS

8.1 Regulation statement

The study will be conducted according to the principles of the Declaration of Helsinki (version, date, see for the most recent version: www.wma.net) and in accordance with the Medical Research Involving Human Subjects Act (WMO) and the International Code of Marketing of Breast milk Substitutes.

8.2 Recruitment and consent

There are three ways by which the initial contact is made:

1. (research) nurses, researchers or midwives of the WKZ or Diakonessenhuis have direct contact with possible participants. This can be on the obstetric ward of WKZ and Diakonessenhuis (researcher, nurses, midwives or other healthcare workers working at the ward) or at home (during a visit of Kraambied-Nazorg, specific midwives that pay house visits after women gave birth at the WKZ). (research) nurses and midwives will provide information and ask whether they may be contacted by the research team. If so, the (research) nurses and midwives share contact information with the research team.

2. All women that will give labour at the UMC Utrecht will receive a letter at discharge from the hospital, to inform them that they will be contacted by researchers of the PRIMA study. This letter is added to the discharge letter/package in a bright coloured envelope. The following short narrative is added to the discharge package for the healthcare worker who will hand over this package: “In de komende dagen zouden onderzoekers u graag willen bellen of u wilt deelnemen aan een belangrijk onderzoek over borstvoeding. Vindt u dat goed? Zij zullen u inhoudelijk verder informeren. **Als u niet wilt worden benaderd kunt u dat nu aangeven, of binnen 48 uur via email of telefoon.** Graag de voor- en achternaam van uw kind en/of de moeder van het kind hierbij vermelden. Het e-mailadres en telefoonnummer vindt u in de envelop.”

English translation: “In the following days researcher would like to call you about an important study on breastfeeding. They will further inform you about the study. **If you prefer not to be contacted about this study, please indicate this now or within 48 hours via email or by telephone.** Please mention the first and family name of your child and/or the mother of the child. The email address and telephone number can be found in the orange envelope.”

As soon as parents directly have objections to be contacted, the healthcare worker will inform the researcher by email or telephone.

Whenever no objection is registered, the researchers will receive the telephone number of these mothers after 2 days postpartum. The researchers will inform parents about the procedure how and why their telephone numbers were retrieved during the first phone call.

Additionally, the researchers will register the amount of parents that indicate (not) to be informed. The researchers will use this as feedback to the healthcare workers at the obstetric ward.

The researchers will report to the METC Utrecht the amount of parents that were not properly informed about the research by healthcare workers after contacting the first 50 mothers.

3. Advertisement at the UMC Utrecht corporate website.

If parents indicate interest in the trial, additional information will be send through email (i.e. the research folder, IC and patient information folder will be sent). If necessary, an additional phone call will be planned to give parents enough time to decide whether they would like to participate. If parents indicate they would like to participate, a first visit will be planned. First visits occur within 24 hours after the first contact and within the first week postpartum. The baseline questionnaire will be filled in during the initiation visit.

During the initiation visit, parents will have time to ask questions (if not done earlier) and the informed consent will be signed. Directly afterwards, human milk samples will be collected.

8.3 Objection by minors or incapacitated subjects (if applicable)

This is not applicable to our study, since the only samples that will be collected from children, will be cord blood. Also the parents will fill in the questionnaires, not the child.

8.4 Benefits and risks assessment, group relatedness

Benefits: No benefits are to be expected from participating in this trial.

Risk for children:

Risk for the children is supposed to be minimal. Only a data breach forms a possible risk to them, but this will be adequately avoided using our Data Management Plan (see for more information the Data Management Plan). There is no risk for the child associated to the collection of neonatal blood from the placenta after birth or with saliva sample

collection. We assess that these risk are minimal, and since we will reduce this risk by our DMP, we deem this risks as acceptable.

Risk for mothers:

We also estimate that the risk for participating mothers is minimal. Risks associated with expressing milk are: pain during expression and concerns about milk production. Pain during expressing milk is mainly attributable to wrong techniques or a non-fitting pumping device. This can be overcome by using a properly fitting pump or by manual expression of milk. Also a data breach forms a possible risk to them, but this will be adequately avoided using our Data Management Plan (see for more information the Data Management Plan). There is a minimal risk associated with the venipuncture. Although considered safe, rarely phlebitis, extravasation of blood, bruising and hematoma forming following venipuncture have been reported. However, since complications from venipunctures and expressing milk are rare, and we will decrease the risk of a data breach as best as we can with our DMP, we assess these risks for mothers acceptable. No risk is associated with the collection of amniotic fluid.

8.5 Compensation for injury

Since no therapeutic intervention is investigated and there is a minimal risk for participants, we request dispensation for a liability insurance.

8.6 Incentives (if applicable)

Participants will not receive any special incentives, compensation or treatment for participation in the study.

9. ADMINISTRATIVE ASPECTS, MONITORING AND PUBLICATION

9.1 Handling and storage of data and documents

Data, samples and documents will be stored using a specific code. This code for samples consists of: study ID (i.e. UMCUPRIMA) an anonymised participant number (e.g. for participant 1 this will be 10001), moment of sampling (e.g. week 1 is W1) and sample number including type of sample (W for water fraction, F for fat and C for cellular fraction). Combining this codes will result in the following; for example participant 1 donated milk during the first 1 week postpartum and we will label a tube with the first part of the water fraction, the code will be: UMCUPRIMA.10001.W1.W01.

The coding system for neonatal blood samples from the placenta will be composed as follows: it consists of study ID (i.e. UMCUPRIMA) an anonymised participant number (e.g. for participant 1 this will be 10001) (this number will be similar to the milk sample number), indication it is neonatal blood (BLOOD.NEO) In case a mother gives labour to a twin, the N will be followed by either a 1 or 2. The 1 and 2 should correspond to the castor ID's that will be used for sending the questionnaires.

The coding system for maternal blood samples will be composed as follows: it consists of study ID (i.e. UMCUPRIMA) an anonymised participant number (e.g. for participant 1 this will be 10001) (this number will be similar to the milk sample number), indication it is maternal blood (BLOOD.MAT).

The coding system for amniotic fluid samples will be composed as follows: it consists of study ID (i.e. UMCUPRIMA) an anonymised participant number (e.g. for participant 1 this will be 10001) (this number will be similar to the milk sample number), indication it is maternal blood (AMNIOTICF.).

The coding system for infant saliva samples will be composed as follows: it consists of study ID (i.e. UMCUPRIMA) an anonymised participant number (e.g. for participant 1 this will be 10001), indication infant saliva sample (SALIV.INF.), moment of sampling (e.g. week 1 is W1) and number of tube (e.g. the first tube is T1). Combining this codes will result in the following: for example the first tube of saliva sample of participant 1 that donated saliva during the first 1 week postpartum, the code will be: UMCUPRIMA.10001.SALIV.INF.W1.T1.

The coding system for infant feces samples will be composed as follows: it consists of the study ID (i.e. UMCUPRIMA), an anonymised participant number (e.g. for participant 1 this will be 10001), indication infant feces sample (FECES.INF.), moment of sampling (e.g. week 1 is W1) and number of tube (e.g. the first tube is T1). Combining this codes will result in the following for the example of the first tube of saliva sample of participant 1 that donated saliva 3 month postpartum, the code will be: UMCUPRIMA.10001.FECES.INF.M3.T1

The codes being used in Castor EDC will be numerical and will be generated by castor itself.

The key for the code(s) will be safeguarded by the Data manager. Only the data manager(s) have access to the key at any time. Researchers that work with personal data only have limited access to this key. Personal data researchers need to be able to contact participants for follow up samples and for house visits. It may happen that a researcher that currently is performing an analysis on the data, simultaneously need access to the personal data as well. In this case, the research data will maximally be pseudonomised as possible by an independent data manager to reduce the risk that these data can be traced back to personal data.

The key(s) will be stored digitally in a folder from the research Folder Structure that only is accessible for datamanager(s). This will be stored in a Research Folder Structure according to the protocol on Connect (version 1.2. January 20, 2019) Multiple datamanager(s) have access to the key(s) in order to avoid loss of the key.

For additional information about data storage, protection and safety, we refer to the data Management Plan. The data and human material will be stored for unlimited time.

9.2 Monitoring and Quality Assurance

Monitoring of the study will take place at three time point: at initiation of the study, one midterm monitor visit and one close-out monitor visit. See for more details document 'K6 Monitoringplan'.

9.3 Amendments

Amendments are changes made to the research after a favourable opinion by the accredited METC has been given.

All substantial amendments will be notified to the METC and to the competent authority.

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

9.4 Annual progress report

The sponsor/investigator will submit a summary of the progress of the trial to the accredited METC once a year. Information will be provided on the date of inclusion of the first subject, numbers of subjects included and numbers of subjects that have completed

the trial, serious adverse events/ serious adverse reactions, other problems, and amendments.

9.5 Temporary halt and (prematurely) end of study report

The investigator/sponsor will notify the accredited METC of the end of the study within a period of 8 weeks. The end of the study is defined as the last patient's last visit.

The sponsor will notify the METC immediately of a temporary halt of the study, including the reason of such an action.

In case the study is ended prematurely, the sponsor will notify the accredited METC within 15 days, including the reasons for the premature termination.

Within one year after the end of the study, the investigator/sponsor will submit a final study report with the results of the study, including any publications/abstracts of the study, to the accredited METC.

9.6 Public disclosure and publication policy

All data arising from this study will be published. However, for patenting some components found to have a protective effect, Nutricia Research bv. reserved the right to delay publication for 3 months maximum.

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