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MELK study: an observational study on human milk composition and infant health determinants during the first year of life in a Dutch cohort

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ABSTRACT

Introduction Human milk plays a crucial role in infants' nutrition and immunity, with its composition being influenced by different factors. The role of maternal diet on human milk fatty acid composition, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), is well studied. Higher fish intake is associated with higher DHA levels in human milk, while fewer associations have been reported for EPA. Yet, maternal diet's impact on human milk composition for other nutrients is understudied, and variations in sampling protocols and study quality hinder definitive conclusions on its overall impact. Ethnicity may also impact milk composition, with different dietary habits intertwined with different genetic backgrounds, but also here research lacks standardised protocols. Our study aims to investigate maternal diet, ethnicity, and their association with human milk composition, along with their potential short-term associations with infant health and development, through detailed dietary assessment and by recruiting participants from diverse backgrounds. Understanding these associations could inform the development of tailored nutritional guidelines for mothers and infants, ultimately promoting optimal health and well-being for both. The study design, with its strict sampling procedure, can guide and inspire future studies.

Methods and analysis We will conduct an observational study involving 120 healthy mothers from three ethnic backgrounds (Chinese, Caucasian and Turkish), exclusively breastfeeding their 2-month-old infants. We will collect human milk samples at two time points and assess maternal dietary intake over 4 days. Additionally, we will collect data on various maternal and child characteristics, including maternal stress, socioeconomic status and health, and infant feeding, sleeping, crying, gastrointestinal health and developmental status.

Ethics and dissemination The study has been approved by the Medical Ethical Committee Oost-Nederland (NL79447.091.21), and all participants provided written informed consent before entering the study. Findings will be widely disseminated at international conferences and meetings including the annual Nutrition & Growth conference, ESPGHAN, and through publication in scientific peer-reviewed journals.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ Standardised milk sampling based on time postpartum, time of day and breast pump use is an important strength of this study.
⇒ Applying modern dietary assessment methods allows for real-time reporting of consumed food items, minimising recall bias.
⇒ Repeated food diaries and a food frequency questionnaire (FFQ) are integrated to study short-term and long-term dietary intake in relation to human milk composition.
⇒ Concurrent assessment of maternal diet and ethnicity allows for differentiation of their respective impacts.
⇒ Recruiting individuals from diverse ethnic background is challenging and requires innovative approaches.

Trial registration number ISRCTN registry (ISRCTN35735283).

INTRODUCTION

Human milk, together with recommended supplementations of vitamins D and K,[1] is the most complete and healthiest food choice for infants.[2] Accordingly, the WHO and the United Nations Children's Fund (UNICEF) recommend exclusive breastfeeding from the first hour after birth to the first 6 months of life, and continued breastfeeding with complementary foods up to 2 years of age.[3] Comparative studies between human milk-fed and formula-fed infants reveal that human milk provides protection against overweight/obesity and chronic diseases, such as type 2 diabetes, high cholesterol and high blood pressure,[4] and is associated with higher IQ outcomes and accelerated infant development.[5] However, while extensive research demonstrates the health advantages of human milk

over infant formula, the precise mechanisms underlying these benefits remain unknown. This is partly due to the variability in human milk composition attributed to factors such as lactation stage, time of day, breastfeeding pattern, season, gestational age, parity, infant sex, ethnic background and maternal dietary intake.[2 6] A deeper understanding of the variability in milk is crucial to further uncover the responsible pathways for human milk's health advantages which eventually also serve a variety of public health purposes including further optimisation of infant formula as well as establishing more specific dietary guidelines for lactating women. Besides those positive effects on infant health, breastfeeding also benefits mothers by being protective against breast and ovarian carcinoma and by increasing the duration of postpartum infertility.[7]

While the exact composition of human milk is thus highly variable, the macronutrient composition is thought to be rather stable in mature milk. Mature milk consists of about 6.7 to 7.8 g/dL lactose, 3.2–3.6 g/dL fat and 0.9–1.2 g/dL protein.[8] Human milk protein can be divided into caseins and whey proteins, with a wide array of different specific proteins. The most abundant proteins in human milk are β-casein, α-lactalbumin, lactoferrin, secretory immunoglobulin A (sIgA), lysozyme and serum albumin.[8] Several proteins from human milk prime the immune system, for example, sIgA transfers maternal immunity, while lactoferrin has bactericidal and antiviral activity and is thus protecting from pathogens.[9] Triglycerides, primarily composed of oleic acid, and palmitic acid, together with the most abundant essential fatty acid linoleic acid, constitute most human milk fat.[2] Human milk fatty acids, such as arachidonic acid (AA) and docosahexaenoic acid (DHA), also called the 'fish fatty acids', are thought to positively contribute to cognitive development.[6] Lactose is by far the most abundant carbohydrate in human milk, followed by human milk oligosaccharides (HMOs).[6] HMOs are non-digestible carbohydrates that fuel infant gut microbiota growth,[10] which is suggested to contribute to the prevention of neonatal diarrhoea, allergies and metabolic disorders,[6 11 12] and beneficially influence brain development.[13]

Despite the importance of a healthy diet for postpartum recovery and infant nutrition,[14 15] only recently the work on specific guidelines for lactating mothers has started.[16] One of the challenges is quantifying the impact of maternal diet on human milk composition.[17 18] A 2023 systematic review noted weak evidence in this area due to underpowered studies, non-standardised sampling protocols and unaccounted-for confounders.[18] Thus far, fat and fatty acids are most widely studied and are most affected by maternal diet, with, for example, maternal fish intake positively associated with milk DHA content.[18] However, more studies assessing maternal diet and human milk carbohydrates, proteins, vitamins and minerals are urgently needed to draw conclusions on the importance of the maternal diet for human milk composition and ultimately its impact on infant health.

Moreover, while recognising human milk as the healthiest infant food choice, not all mothers breastfeed. To illustrate, breastfeeding rates decline significantly in the Netherlands in the first months, with only 31% of infants being exclusively breastfed at the third month and 16% at the sixth month postpartum.[19] This highlights the need of exploring the underlying causes to better support women during lactation,[20] as well as the pressing need for effective alternatives to human milk for mothers unable to exclusively breastfeed. Infant formula composition in the Netherlands adheres to stringent European regulations informed by scientific reports, primarily targeted at 'infants living in Europe'.[21 22] New insights into factors such as age, sex and genetic background could lead to more tailored guidelines, accommodating individual variability. While previous research has explored ethnicity's impact on human milk composition, especially in specific fatty acids, studies vary in methods and lack detailed maternal information.[23 24] Limited studies directly compare populations using consistent sampling methods, identifying variations in fatty acids, protein and carbohydrate composition.[25–27] Besides, most studies fail to consider differences in maternal diet, leaving the separate impacts of ethnic background and diet unclear.

The MELK study will investigate associations of maternal diet and ethnicity with human milk composition, employing rigorous inclusion criteria and standardised sampling procedures to yield valuable data for nutritional guidelines and infant formula enhancement. Given its strong connection with infant well-being, this study also aims to assess potential associations between human milk composition and infant health and development, including weight and length, sleeping, crying, and feeding behaviour and developmental milestones, as well as GI health and gut microbiota composition.

METHODS AND ANALYSIS

Study design

This observational study aims to recruit 120 healthy lactating women living in the Netherlands between July 2022 and December 2024. Final measurements are expected to take place by February 2025. Mothers will be recruited from all 12 provinces of the country. Mothers enter the study during the first 3 weeks of the second postpartum month. Data collection is scheduled over 5 study days within a 4 week period (figure 1). On the first day, participants complete a questionnaire on general characteristics and one 24-hour food record. On the second day, mothers collect a 20 mL milk sample from a complete expression from a chosen breast during their first morning feeding, which will be labelled and stored in the household fridge. Mothers also complete the second food record and provide a 24-hour urine sample. Moreover, mothers collect an infant faecal sample at the latest by the third day, which will be labelled and stored in the household freezer. On the third day, mothers collect another 20 mL milk sample during their morning feed.

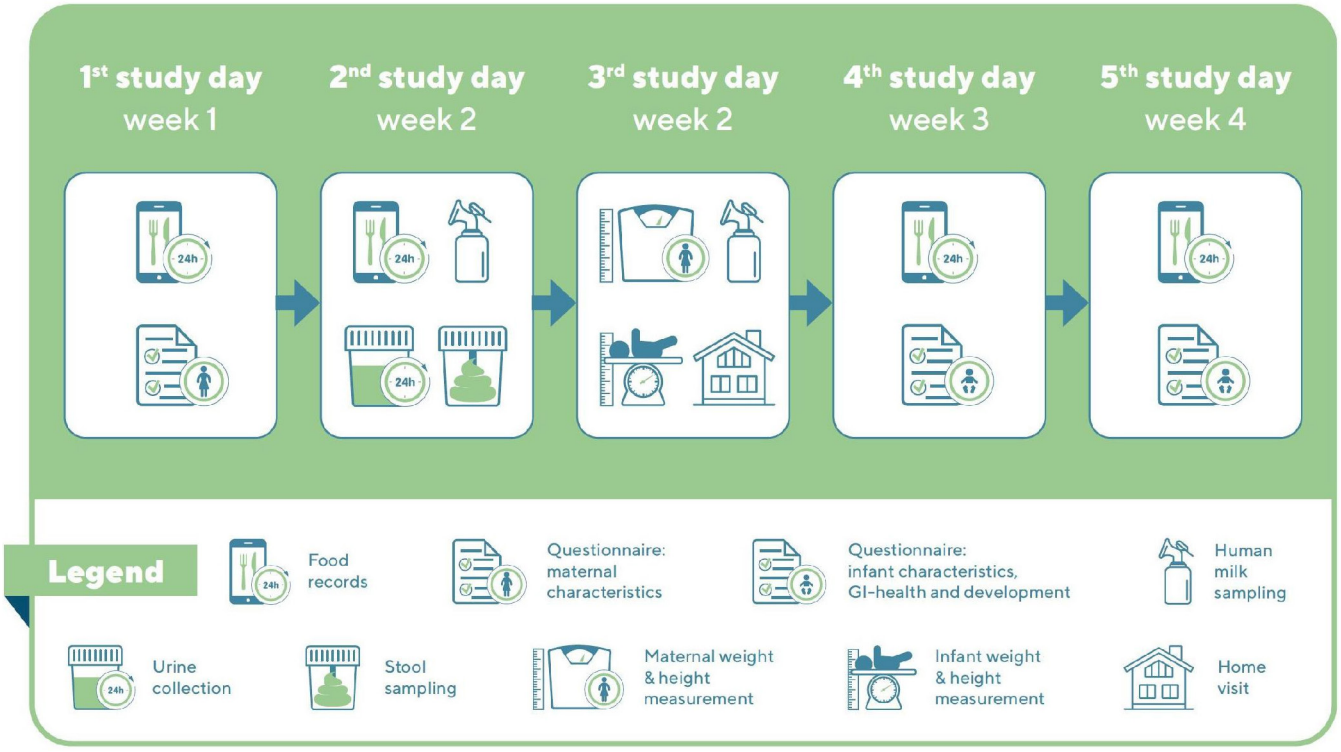

Figure 1 Study days in the MELK study.

The third study day is the home visit that includes weight and height measurements for both mothers and infants and collection of the milk, urine and stool samples. On the fourth day, a weekend day, the mothers complete questionnaires on infant characteristics and infant gastrointestinal (GI) health, along with a shortened food frequency questionnaire (FFQ) that is aimed to capture less frequently consumed food items. Participants also complete the third 24-hour food record. The fifth study day involves completing a questionnaire on infant development, and the final food record. Together, the four food records provide an estimate of maternal habitual dietary intake.

Participants

We aim to recruit 120 women in their second month postpartum: 100 participants of Caucasian, 10 of Chinese and 10 of Turkish ethnicity. The recruitment focused on mothers of Turkish background as they represent the second-largest ethnic group in the Netherlands. The Chinese group is selected to enable comparisons with parallel studies on Chinese mothers conducted in China. Women are approached via midwifes and maternity care specialists during the final trimester of their pregnancy or the first month postpartum using information flyers. Additionally, (social) media advertisements are posted. Interested women receive further information on the study purpose and design via telephone or an online meeting after initial contact. Recruitment of Turkish and Chinese mothers proved challenging so far. To address this, we translated information material into Chinese and Turkish, offered assistance in filling in questionnaires and enlisted women from these communities, to reach out to relevant groups, such as women associations at Mosques, mother-baby groups, Islamic maternity care, Chinese schools and Chinese and Turkish social media platforms. Recruitment efforts also included distributing flyers in Chinese and Turkish neighbourhoods, grocery stores and other public places, as well as discussing communication and cultural differences.

Inclusion criteria

Participants are eligible to join the study if they are 18 years or older, have a prepregnancy BMI between 18.5 and 24.9 kg/m^2, are at least 6 months pregnant on enrolment, are able to provide a human milk sample between the fourth and eighth week postpartum to ensure milk is mature,[1] are exclusively breastfeeding at enrolment and are planning to exclusively feed their own breast milk during the time of the study (excluding any medically necessary formula given in the first week after delivery), and provide written consent. If an infant received infant formula for medical reasons, an oral inquiry was done to assess reason and quantity of formula provided, to judge eligibility. Additionally, participants must belong to one of the three specified ethnicities, determined by the country of birth of both the participant and of their parents, following Dutch standard indicators for ethnic origin,[28] which aligns with the indicators of 'first generation migration background' and 'second generation migration background' in the municipality registries.[29] Infant inclusion criteria include being delivered full term (at 37–42 weeks of gestation), being apparently healthy with no diagnosed (chronic) illnesses and having a birth weight of at least

2.5 kg. Additionally, infants had to be vaginally delivered, and not treated with antibiotics. Vaginal delivery plays a key role in priming the gut microbiota by transferring maternal microbiota to the infant, while antibiotics can disrupt this balance. To minimise confounding factors, we excluded infants who received antibiotics, allowing us to study associations between human milk and the gut microbiota.

Exclusion criteria

Participants are excluded if they are expecting or have given birth to twins, unable to breastfeed, gave infant formula after the first week of life, currently following a weight-loss diet, diagnosed with a gastrointestinal disease, or unable to read and/or understand Dutch or English.

Sample collection and processing

Milk samples

Participating mothers will collect milk samples according to the study schedule (figure 1). Despite limited day-to-day variation in macronutrient composition,[30] two human milk samples will be taken on two consecutive days to account for possible day-to-day variation in micronutrients and other compounds. Milk samples will be manually pumped between 6:00 and 8:00 in the morning to minimise compositional differences due to circadian rhythm.[31] Given intrafeeding variation,[32 33] a full expression best reflects infant nutrient intake. Mothers will follow a standardised pump cleaning protocol and wash hands and nipples before collection. Participants will fully empty the chosen breast, slowly shake the obtained sample and provide at least 20 mL of milk into a 50 mL transparent Greiner plastic culture tube. The remaining milk can be fed to the infant. Tube labels will include sampling time, breast side, expressed total volume of milk and details of the previous feeding before collection (ie, timing and chosen breast(s)). Milk samples will be stored in participants' household fridges for a maximum of 32 hours until collected during the home visit on study day 3 (see figure 1) and transported on ice to the laboratory. On arrival, samples will be processed according to protocol: each milk sample is mixed, and 7 mL of whole milk samples is transferred to biobank tubes for storage in the biobank until analysis. If participants provide more than 20 mL, additional whole milk samples are stored. The remaining milk is being centrifuged at 3000 rpm for 15 min at 2°C to separate fat and skim milk, which are further separated by centrifuging a second time at 3000 rpm for 15 min at 2°C, and separately stored in the biobank. All samples will be kept frozen at −80°C until analysis (see figure 2).

Human milk protein analysis

Skim milk obtained from the previously described method, containing milk proteins, will be analysed for protein composition using liquid chromatography (LC). This method follows protocols similar to those for bovine milk protein analysis.[34] Briefly, the skim milk sample will be combined with a buffer containing Bis-Tris, urea, sodium citrate and DTT. The mixture will then be filtered and injected into LC for separation and analysis. Four major human milk proteins, namely αS1-casein, β-casein, lactoferrin and α-lactalbumin will be identified and semiquantified.

Human milk fatty acids compositional analysis

Milk fat will be obtained by centrifugation as previously described. The analysis of fatty acid composition will follow the ISO 16958:2015 procedure.[35] Briefly, the fat sample will be directly transesterified, and the resultant Fatty acid methyl esters (FAMEs) will be subjected to gas chromatography (GC) for separation and analysis.[36] Fatty acid identification will be based on comparing retention times with known standards, and quantification will be performed using specific response factors for each fatty acid.

24-hour urine samples

Participants will provide a 24-hour urine sample to verify self-reported food records for protein and to measure potassium and sodium excretion. Urine collection starts after their first morning void and lasts until the same time the following day. Participants will follow a standardised collection protocol and receive containers with preservatives. Urine containers will be kept in a cool place or fridge. Urine completeness will be assessed by a urine diary where participants track their start and end of urination, along with any comments.[37] Empty and full container weights are recorded before and after collection by the investigator. In the lab, urine is mixed and transferred into biobank tubes for storage at −80°C until analysis. Urine analysis will follow the methods of NQplus.[37] Total 24-hour urinary nitrogen excretion will be quantified using the Kjeldahl technique with a Foss Kjeltec 2300 analyser. Urinary sodium and potassium concentrations will be measured using an ion-selective electrode on a Roche 917 analyzer (Roche Diagnostics). Total 24-hour sodium and potassium excretions will be calculated by multiplying the total weight of collected urine by the respective sodium or potassium concentration and then dividing by 0.86 for sodium and 0.81 for potassium, reflecting the assumed percentage of intake excreted in urine. Urinary protein will be calculated using the formula 6.25 × (urinary nitrogen/0.81), which accounts for faecal and skin losses, estimated to be approximately 19%.

Stool samples of infants

Participants receive a Sarstedt AG & Co. KG transparent and sterile, stool test tubes with an attached spoon, and instructions to collect a sample from their infant's diaper, noting the date and time on the label before storing it in the household freezer. During the home visit, research staff will collect the sample and transport it to the lab on dry ice for storage at −80°C until analysis. 16S rRNA gene amplicon sequencing will be used to assess the microbiota

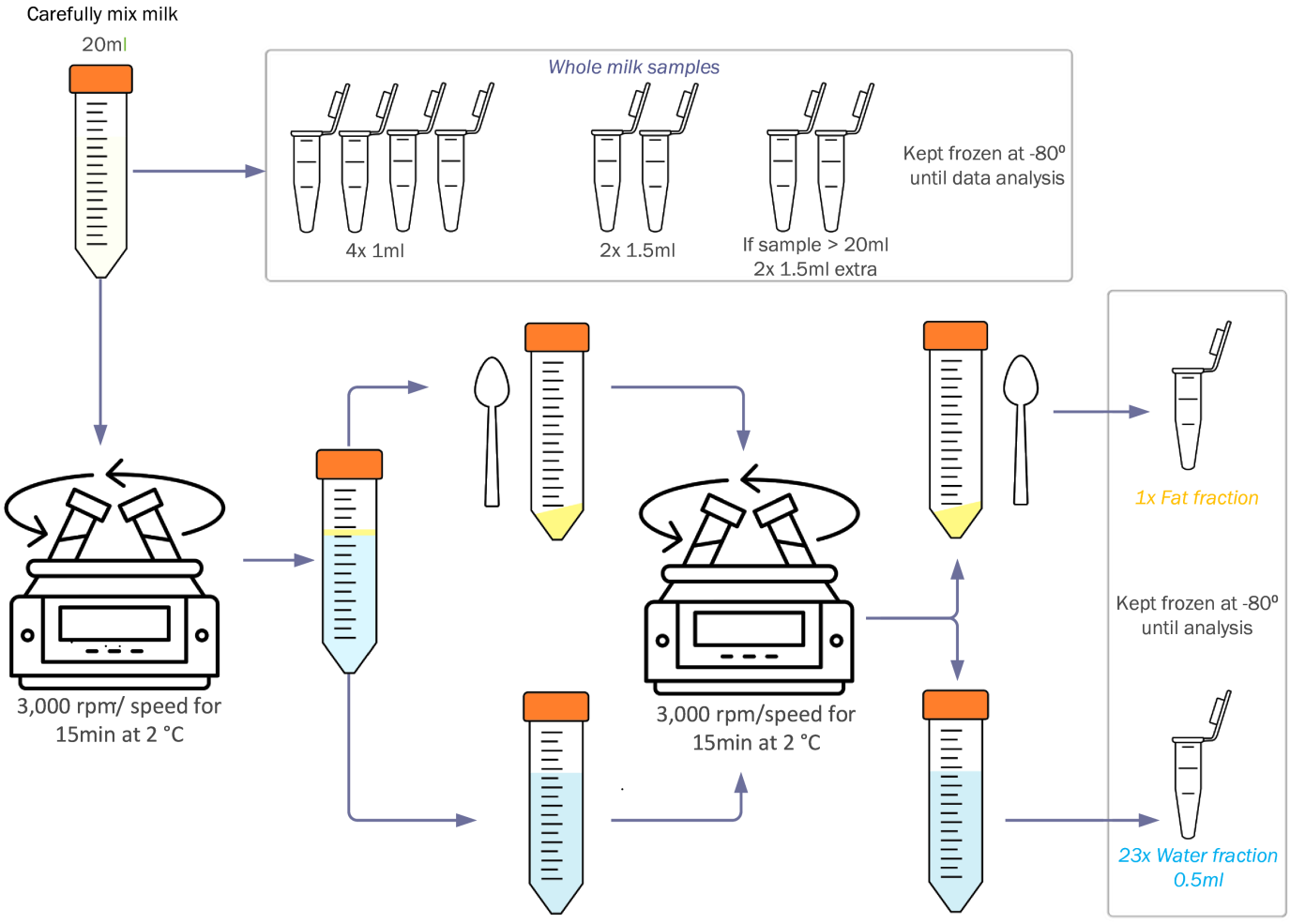

Figure 2 Human milk sample processing procedures.

composition and relative abundance in faeces, and the approach described in the paper of Henderickx *et al* will be followed for this analysis.[38] For microbiota analysis, bacterial DNA will be isolated from faeces. Setup, statistical analysis and interpretation of the infant gut microbiota analysis will be performed in cooperation with the Wageningen University microbiology group.

Dietary intake

Maternal dietary intake

Habitual diet and short-term dietary intake will be assessed by using four 24-hour food records using Traqq, a validated app for dietary intake assessment in the Dutch population.[39] The app allows users to log their consumed foods and drinks throughout the day, providing options for portion sizes (ie, household measures (cups, spoons, glasses), standard portion sizes (small, medium, large), or weight in grams) and mealtimes. It includes an extensive food list based on the Dutch Food Composition Database (NEVO) 2021 updated to cover food items commonly consumed by the three ethnicities of interest. If a food item is not listed, participants can note these food items on the provided paper lists. Intake of food groups, energy and nutrients will be calculated using the Compl-eat computation module,[40] which multiplies consumed amounts by energy or nutrient content from the NEVO 2016 database. Habitual maternal intake will be defined as the mean intake of all four food records combined. To capture less frequently consumed food products, participants will answer questions about the consumption frequency of foods known to impact human milk composition. These items include fish, shellfish, legumes and pulses and goat milk products. The FFQ-type questions, based on the validated FFQ used in the GLIMP2 study,[41–43] will be administered at the end of the study period.

Demographics, lifestyle and general health

Maternal characteristics

The general maternal questionnaire includes questions about demographics, including birth place, number of pregnancies, number of children and education; current work situation; general health and history of diseases such as diabetes mellitus, GI health, thyroid disorders, other chronic disorders and medication use; current bodyweight; use of supplements; current and previous smoking and drinking habits such as the amount smoked or alcohol consumed. These questions are based on well-established questionnaires of the NQ-plus study.[37]

Participants are classified into three educational levels based on their highest completed education. Those with no formal education, primary education or lower vocational education are categorised as having a low educational level. Participants who completed lower secondary education or intermediate vocational education are classified as having an intermediate educational level. Individuals who attained higher secondary education, higher vocational education or a university degree are categorised as having a high educational level. Never smokers did not smoke before, during or after the pregnancy, former smokers only smoked before pregnancy and current smokers were classified as smoking during or after the pregnancy. Data on participants' habitual physical activity over the preceding week will be collected using the validated Activity Questionnaire for Adults and Adolescents (AQuAA).[44] The primary outcomes measured are the total physical activity score and the duration of time spent in sedentary, light, moderate and vigorous intensity activities, expressed in minutes per week. Sleep and activity times are recorded using the Munich Chronotype Questionnaire.[45] Participants are asked specific questions regarding their sleeping habits, such as their typical bedtime, the duration needed to fall asleep, the number of awakenings during the night and their wake-up time. The primary parameters derived include estimates of sleep duration, sleep onset time, number of awakenings, rise time and mid-point of sleep. Stressful life events are measured by an adapted version of the List of Threatening Experiences,[46] which measures perceived stress in different aspects of life, including housing, work, relations with others, free time, finances and health. Weight and height are measured during home visit of the research staff. Weight is measured without shoes and heavy clothing (eg, sweaters) and with empty pockets to the nearest 0.1 kg on a calibrated scale. Height is measured to the nearest 0.1 cm without shoes using a stadiometer and the BMI (kg/m^2) will be calculated.

Infant characteristics

Most recent weight and length will be measured during the home visit of the researcher using a calibrated infant weighing scale. Infant length will be measured while lying on an infant length board, designed for the purpose of accurately measuring infant recumbent length, with the legs carefully stretched as much as possible. Additionally, mothers are asked for consent to access their children's growth records at the 'baby well centre' or to share the growth information of their child for up to 1 year themselves. A standardised online questionnaire will be used to assess the infant's sex; gestational age; mode of delivery; general health including recent infections of the lungs, eyes or ears; complications or use of medication after birth; weight, length and head circumference at birth; and nursing frequency. The infant's sleeping habits including the time spent sleeping during the night, the number and duration of awakenings and manner of putting the baby to sleep are also being assessed by the questionnaire. Stooling, crying and fussiness are assessed online by an adaptation of the Infant Gastrointestinal Symptom Questionnaire (IGSQ).[47] The questionnaire is a shortened version of the IGSQ used by the PRIMA cohort,[48] with 5 questions selected to reduce participant burden. Severity of complaints is assessed for each question, yielding a summary score ranging from 5=no complaints to 23=extreme complaints. Infant development is assessed online by the system Development First (Van Baar, A.L., Krijnen, L.J.G., Hessen, D. & Verhoeven, M. (2024). Development First! Creation of Dutch norms for a digital system to monitor child development from 0 to 6 years, in preparation), based on the questions of the Ages & Stages Questionnaires – extended.[49] The questions concern developmental milestones in five areas of infant development, communication, gross and fine motor functioning, problem solving and personal-social behaviour. Each question is answered by the parents and has three possible answer options (yes, sometimes and not yet). The system is tailored to the infant's age and abilities. The scores are automatically calculated and expressed in percentiles based on norms for the Dutch population. An overview of all exposure and outcome variables can be found in table 1.

Statistics

Sample size

Sample size calculations were performed using G*Power particularly focusing on maternal dietary intake and human milk composition. Existing data[50] showed a correlation of 0.33 (0.23–0.42) between maternal DHA intake and DHA levels in human milk. With a probability of α=0.05, power (1-ß) of 95%, and approximately 10% drop-out rate, a total of 120 participants are needed to detect diet-related differences in human milk DHA. The sample size calculation by ethnicity was challenging due to limited existing data. Initial calculations, based on a study comparing DHA levels in human milk between Asian and New Zealand European mothers (ie, 0.016±SE0.004 vs 0.008±SE0.001, d=0.87),[51] required n=22 per group (80% power, α=0.05). Considering our primary aim of assessing dietary intake and human milk composition (requiring 120 participants), we initially aimed for 40 Dutch, 40 Turkish and 40 Chinese mothers. However, despite intensive recruitment efforts, the recruitment of Turkish and Chinese women remains challenging. Given larger effect sizes reported in other studies (eg, d=1.79, n=7 per group[52] and d=2.27; n=5 per group),[53] we adjusted our sample size during the recruitment period for the Turkish and Chinese women to 10 per group, making these analyses rather exploratory in nature.

Analysis

Descriptive statistics

For descriptive analyses of the study population, we will use all data, and apply basic summary statistics, such as mean and SD for normally distributed variables, median and IQR for skewed variables. If

Table 1 Overview of exposure and outcome measures of the MELK study

Exposure measures	Outcome measures
Maternal characteristics	**Human milk composition**
Demographics	Total fat
Occupation status	Total protein
Education level	Total lactose
Medication use	Ash
Smoking and drinking habits	Fatty acid composition
Sleep	Functional proteins
Stress	Amino-acid composition*
Number of children	HMO profile
BMI (kg/m^2)	Minerals*
Physical activity	Vitamins*
Maternal diet	**Human milk expression**
Food diaries	Total volume
Short FFQ	Time of expression
24-hour urine	Time of last feeding
Infant characteristics	Chosen breast side
Weight (g)	Last chosen breast side
(recumbent) length (cm)	**Infant health measures**
Head circumference at birth (cm)	Gut microbiota composition
Weight (g) and length (cm) at birth	Sleeping
Sex	Stooling
Gestational age (weeks)	Crying and fussiness
Mode of delivery	Cognitive development
Complications	
Use of medication after birth	
Nursing frequency	

*Future analysis.
BMI, body mass index; FFQ, food frequency questionnaire; HMO, human milk oligosaccharide.

necessary, dietary intakes will be log-transformed to better approximate normality. Human milk composition variables will be analysed as dependent variables in regression models, and it is expected that linear regression will suffice, but assumptions will be checked beforehand, to see whether another method needs to be employed, or additional transformations are needed.

Diet–human milk composition

The association of maternal diet of all 120 mothers with human milk composition will be assessed by means of linear mixed effects models, to account for the multiple sampling days.

Potential confounders or effect modifiers will be determined using Directed Acyclic Graphs (DAGs),[54] and may include maternal ethnicity, maternal characteristics (ie, age, BMI, lifestyle factors, medication and supplementation use) and infant characteristics (ie, sex, weight). Based on these results, the final models will be built.

Given the recruitment challenges of Turkish and Chinese participants and the resulting limited sample size, data analysis on maternal ethnicity and human milk composition will largely be exploratory. Data on ethnicity will be analysed similarly to the data obtained on dietary intake, by means of a (linear) regression.

Ethics and dissemination

The study has been approved by the Medical Ethical Committee Oost-Nederland (NL79447.091.21), and all participants provided written informed consent before entering the study. Findings will be published in international scientific, peer-reviewed journals and widely disseminated at international conferences and meetings including the annual Nutrition & Growth conference and ESPGHAN.

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Contributors EMB-B, LvL and EF obtained funding and wrote the initial proposal. IP, PW, EMB-B, LvL and EF designed the final study, wrote the protocol and obtained METC approval. PW, KAH, CB and IP developed laboratory protocols. IP and CvH performed data collection. IP, PW, CvH, LvL, KAH, CB, AvB, EF and EMB-B wrote and edited the manuscript. EMB-B is the guarantor of this work.

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Competing interests IP is currently, and LvL and CvH have previously been employed at Ausnutria B.V. EMB-B and EF received research support from Ausnutria B.V. and Regiodeal FoodValley. EMB-B received an EWUU grant. KAH is having an unpaid role in the scientific advisory board 'International Milk Genomics Consortium' and received travel budget to the Asian conference of nutrition 2023, Chengdu, China.

Patient and public involvement Patients and the public were not involved in the design, setup, or interpretation of this study. However, participants contributed to the research by providing biological samples and completing questionnaires, which were essential for data collection.

Patient consent for publication Not applicable.

Provenance and peer review Not commissioned; externally peer reviewed.

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