Clinical Study Protocol

Impact of Maternal Sugar Intake on Breast Milk Composition and Breast Milk Variation over 24 hours.

Short title: **MATSUB: The Maternal Sugar and Breast Milk Study**

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Background

Breast milk composition is believed to be highly variable due to an extensive array of factors which include maternal diet. Many previous studies have provided insights into the potential role of maternal dietary intake on the concentrations of key macronutrient (fat, protein and lactose) in human milk. These studies have suggested that concentrations of lactose, the most abundant carbohydrate (Smilowitz et al. 2013), are not affected by the maternal diet (Smilowitz et al. 2013 and Nommsen et al. 1991). Similarly, total protein concentrations in human milk are thought to be relatively stable, with some evidence even showing a compensatory ability for women to maintain consistent protein concentrations in their milk despite consuming diets low in protein (Lonnerdal 1986). Fats are known to be the most variable macronutrient in human milk, with many studies demonstrating that both the fat content and fatty acid composition of human milk shifts rapidly in response to changes in these factors in the maternal diet (Mohammed et al. 2009 and Henjum et al. 2018). However, there are significant weaknesses in the existing literature base, since the majority of these studies have measured effects on human milk composition in pooled samples collected over a 24-hour period before analysis. This means that they have no capacity to consider how milk composition varies over a day, or the effects of dietary changes on human milk composition in the short-term.

The design of the proposed research is based upon our recently published UK pilot study where 9 women were each given a control/higher sugar/higher fat diet over 3 separate days (Ward et al. 2021). On each of the 3 days breast milk was collected hourly for a period of 12 hours. The study showed a significant increase in breast milk triglyceride concentrations following a higher sugar diet, compared to a control diet ($p<0.001$). It was also identified that statistically significant variations in breast milk protein and lactose concentrations occurred across the 12-hour sampling period, indicative of a circadian rhythm that had not been previously reported. Given the novelty of the study protocol, and the study findings, it is important to confirm these in a larger group of women.

The aim of this study is to explore in greater detail the impact of sugar on breast milk triglycerides and the changes in milk protein and lactose across the course of a day. This research will provide a positive contribution to the current knowledge base as to the source of variations in breast milk composition and lead to better formed advice for breastfeeding mothers and health care professionals.

Aims

1. To determine the effect of increased sugar consumption on the composition of breast milk.
2. To assess variations in breast milk composition across the day
3. To evaluate variations in breast milk composition across the different stages of lactation in general.

Study Design

The study design follows that of a similar pilot study which was completed in 2019, (Ward et al., 2021). Exclusively breastfeeding mothers will be recruited using online platforms and will be provided with two separate diets across two full days (diets will either be control

(aligned with the Australian Guide to Healthy Eating) or contain a higher amount of sugar). Concurrently participants will be asked to collect hourly breast milk samples by manual expression. Breast milk samples will be analysed for compositional elements such as macronutrients to determine if increased sugar consumption influences breast milk composition.

Study Population

40 mother/baby dyads in total; 20 with infants between 6-12 weeks and 20 with infants between 13-20 weeks at time of enrolment.

Recruitment

Advertisements for the study will be posted on social media websites such as Facebook and will also be shared by associations for breastfeeding mothers such as Australian Breastfeeding Association. These advertisements will include contact details for the primary researcher to allow prospective participants to express interest. Following expression of interest potential participants will receive information on the study design and eligibility criteria. If they are still interested and deem themselves eligible then a screening telephone call will be arranged. Planned recruitment will commence in March 2022 and will continue until we have obtained the required number of participants.

Inclusion criteria

Inclusion

- Healthy mothers of singleton infants born at full term (>38 weeks gestation)
- Mothers between the ages of 21-35 years
- Mother with a BMI between 20 and 30 kg/m^2
- Infants between 6 and 20 weeks of age at the time of recruitment
- Mother and infants have no self-reported underlying medical conditions/special dietary requirements which result in restriction of any major food groups (e.g. vegan/dairy allergy)
- Mother able to give informed consent for themselves and their infants
- Mothers have been exclusively breastfeeding their infant since birth (with the exception of additional water or medication such as paracetamol suspensions)

Exclusion

- Infants who were born by caesarean section
- Infants who are fed formula milk or fed a mixture of breast milk and formula
- Mothers who are struggling with breast feeding and/or are unable to manually express milk.
- Mother and/or baby who has an allergy/intolerance/aversion to the study diet (e.g. lactose intolerance).

Informed Consent

Participants will provide a freely given verbal consent to undergo trial-related phone screening. The Principal Investigator or designee will ensure that all participants must provide written informed consent before undergoing any in-person screening activity or study procedure. Participants will have the opportunity to discuss their involvement with a friend or family member, and to ask study staff any questions they have about the study and have these answered to their satisfaction prior to providing informed consent. Participants

will be given a copy of the signed informed consent forms, whilst original copies will be kept by the researcher.

Withdrawals

Consent is entirely optional and can be withdrawn at any time throughout the study without the need to provide reasons. Participants will be notified of their ability to withdraw at any time within the participant information sheet and will be reminded verbally during the screening visit.

Any data collected before the point of expressing desire to withdrawal will be retained along with the rest of the study data.

The PI or designee may also withdraw a participant from the study at any time. In this event, the clinic will ensure the reasons have been clearly explained to the individual.

Study Visits and Procedures

The study design follows that of a similar pilot study which was completed in 2019, (Ward et al., 2021).

Screening telephone call

- A chance to ask questions and discuss any concerns about the study before signing informed consent form. A copy of the diets will be provided (appendix 3).
- A questionnaire regarding mother's health including height and weight information, infant's health and infant feeding patterns will be completed.
- Information regarding dietary data collection along with instructions on how to fill in the diet diaries will be provided (see appendix 2).
- Following confirmation of suitability mothers will be contacted and asked to complete the diet diary for 3 days prior to the home visit.

Visit 2&3/Intervention (within participants' home)

- Three main meals as well as snacks will be provided following specific diets (standardised/higher sugar). The meals may be tailored to personal preferences to a certain degree (allowing for all major food groups to remain included). Consumption at each meal will be measured by weighing anything not consumed at the end of the study day.
- Breakfast will be eaten when participants wake up, this will start the sample collection period. Lunch and dinner will be consumed at 5 and 10 hours from breakfast respectively. Snacks can be consumed at any time between meals and will be logged on a written document alongside fluid intake over the day.
- A 5-10mL sample of fore milk will be collected into a 10 mL polyprop vial and will be obtained by the mother by manual expression at hourly intervals in privacy within participants' homes.
- Women will be asked to record infant feeding times and duration.
- Record any adverse events, concomitant medication changes
- Babies will be weighed by the student investigator (or in the case of the Covid-19 safe protocol, scales will be provided for mothers to weigh their babies).

Covid-19 Safe Protocols

In the case of increased requirements for Covid-19 safety the below protocols will be adhered to. These are designed to limit contact and prevent entrance of the student investigator into participants' homes in order to protect both parties.

- Face masks and gloves will be worn at all times during food preparation.
- Each participants' meals and sample collection materials (including relevant paperwork and instructions) will be placed in separate boxes/carrier bags for transportation. Meals will be transported from CSIRO to participant homes in a crate which has been cleaned with alcohol wipes.
- During deliveries/collections a mask will be worn and hands will be sanitised before any handling of study materials. Unless exempt, the research participant will be asked in advance to wear a face mask during drop-off.
- Deliveries will be placed at the doorstep/agreed location, researcher will step back and wait until it has been collected by the participant. A minimum distance of 2 metres will be kept from one another at all times. The researcher will not enter the residence of research participants and drop offs/collections will always take place outside of the building or property.
- Collection of study materials and samples will occur in the same way; participants will place the collection on the doorstep/agreed location and step back until it has been collected. The student investigator will transport collected items in a crate to CSIRO and will clean sample tubes with alcohol wipes before proceeding. Any other items such as transportation crates/boxes will be cleaned.

Study Intervention

The intervention within this study includes two different diets; one control and one higher in sugar. A cross-over design will be used where control and higher sugar diets will be provided in a random order to each woman across visit 2 and 3. Participants will be encouraged to try and avoid consuming foods other than those provided but can resume their normal eating habits once the last sample has been collected. If women consume anything other than the foods provided, they will be asked to record this information, similarly, if they do not consume all food provided they will be asked to retain the food for the student investigator to weigh and estimate the percentage of consumption at each meal.

There will be 7 days between these visits to allow for a washout period between diets. There are no dietary restrictions during the washout period.

Participants will be discouraged from consuming caffeine or alcohol during the study period (i.e. days when intervention diets are being consumed).

The paired design means that in the analysis of the data each woman will act as her own control.

Considering dyads at two different infant ages will enable the project to address the third aim and determine whether milk composition varies across lactation and whether sensitivity to maternal diet may change over time.

Diets

Both diets will consist of ~2500 kilocalories over the course of the day and have been designed with the help of a registered dietitian at University of Nottingham. We aim to

provide a healthy balanced diet meeting as many of the recommended daily intakes for lactating women as possible. The designed diets for this study are the same (allowing for differences in brands/products available) as those provided during the UK pilot study. The higher sugar diet will be similar to the control, however, sugar content will be higher across the day amounting to an extra ~65g. E.g. high sugar cereals instead of low sugar cereals, jam added to toast. An itemised breakdown of both diets is included in the supplementary material.
Meals may be variable between participants as there will be the opportunity for some personalisation to accommodate preferences or any dietary constraints (e.g. vegetarian). Women will be asked to record fluid intake during the study.

Preparation/Storage
Food preparation will take place in the CSIRO Nutrition and Health Research Clinic in line with the Food Risk Assessment Team (FRAT) guidelines. An application for FRAT approval will be submitted alongside this application for HREC approval. Meals will be delivered to participants homes by the student investigator. Food will be stored in cool bags with ice packs to ensure temperature control between preparation and delivery.

Data Collection and Analysis

Dietary data
Dietary data will be collected using an online diet diary application (Appendix 2). Details of this and instructions for use will be supplied at screening visits and participants will be instructed to record their dietary and fluid intake. Participants will be requested to complete the diary for 3 full days including one weekend day. Dietary data will be recorded between the screening visit and visit 2 and is designed to collect data representative of each woman's habitual diet.

Sample collection
A 5-10mL sample of fore breast milk will be collected into a 10 mL polyprop vial and will be obtained by the mother by manual expression before breakfast in the morning. Further milk samples will be collected at hourly intervals in the same way. Samples will be collected by participants in privacy within their own homes. Mothers will be encouraged to express for a short time to collect the sample prior if timings coincide with baby feeding and will be discouraged from collecting milk from the opposite breast to the one baby is feeding on ('drip milk').

Sample storage
Following collection in 10 mL polyprop vials breast milk samples will be immediately frozen and stored at -20ºC in participants' homes. Samples will be collected within 24 hours by the student investigator and transferred on ice to the laboratory where they will be stored at -20 ºC until time of processing/analysis and thereafter. All laboratory assays will be completed in a batch once all samples have been collected. We anticipate samples will be stored for a maximum of 6 months prior to analysis.

Sample analyses
Total breast milk triglycerides will be quantified using colorimetric assay kits as previously reported (Ward et al 2021). Briefly samples will be loaded onto a 96 well plate alongside standards. Colorimetric reagent is added (previously Thermofisher Infinity kits were used)

and absorption recorded. Protein concentrations of breast milk samples will be determined from liquid samples using the Bradford (1976) assay. Milk lactose concentrations will be determined using liquid chromatography-mass spectroscopy (Ward et al 2021). Fatty acid profiles of milk will be determined following collection onto dried spot cards as reported by Liu et al. (2014). Hormone assays may be completed depending on time constraints, the methods for this have not yet been decided.

Statistical Analysis Plan

Statistical analyses will be performed using SPSS software (version 21; IBM, Armonk, NY). Statistically significant differences in the variability of milk compositional concentrations will be determined using repeated and single measures ANOVA with Tukeys HSD post hoc tests where appropriate. A Bonferroni correction will be used to control for multiple comparisons. Statistical significance will be accepted with a *P* value of <0.05 and all results will be presented as mean with ± standard error of the mean.

Adverse Events

Participants will be asked at each visit to report any AEs they may have experienced since their last visit. All AEs will be recorded from Visit 1 and will be followed until either completely resolved or until expression of the desire to withdraw from the study.
As the intervention for this study is a moderate change to diet, severe AEs are not expected as a result.

Reporting of AEs

Any AEs will be reported on a source paper document provided to each participant and will capture the following information:

- Date of onset
- Description of the AE
- Duration
- Actions taken
- Outcome
- Medical Investigator's opinion on severity and causality.

AEs should be reported as diagnoses, if available, instead of individual signs and symptoms. All unresolved AEs will be followed up until the final study visit unless participants express the wish to withdraw or the event resolves itself. Details of AE resolution must be documented and stored with the rest of the study data.

Data Handling

All data collected from a Participant will be recorded in source documents (paper and/or direct to electronic). The student investigator will enter de-identified data from the participants' source notes into electronic databases which will be stored securely within password protected files. After completion of the study and publication of results, all study-related documents and data will be sent to archives and will be retained at least for 15 years. Upon request, the Investigator will permit access to source data and documents for trial-related monitoring, audits, HREC review and regulatory inspections. In the case of electronic sources, read-only access will be provided.

Ethical Considerations

The study has been designed and will be performed in accordance with the ethical principles stated within the Declaration of Helsinki.
Prior to beginning recruitment written approval must be received from the HREC for this protocol and all supplementary material including; ICF, other written information to be provided to the participants and advertisements to be used for recruitment. The Principal Investigator is responsible for informing the HREC of any amendment to the protocol and will report promptly any new information that may affect the safety or conduct of the study. Any necessary extensions or renewals of HREC approval must be obtained, in particular, for changes to the study such as modification of the protocol, the informed consent forms and any information provided to participants.

Participant Confidentiality

Study data will be stored in a computer database, maintaining confidentiality in accordance with national data legislation. Participants in this study will be identified by participant numbers assigned at the time of recruitment. All personal data collected and processed for the purposes of this study should be managed by site staff with adequate precautions to ensure confidentiality of those data and in accordance with the applicable privacy regulations per the investigators country/jurisdiction. The information collected will only be used for the purposes identified in this protocol. All raw data for processing, analysis and reporting will be handled within CSIRO. Any summaries of final study data, including tables, figures and listings will not contain any identifying data. The recipients of the final study data summaries will maintain confidentiality within limits of the local law.

Statistical Power

The sample size for this study was calculated based on the pooled data collected for milk triglycerides in our UK pilot study (Ward et al 2021) where we observed a 5.36 g/dL increase in triglycerides following the higher sugar diet compared to the control diet ($p<0.001$).
A power calculation was performed using the tool available at Statulator.com for a paired samples design and amended the calculation (90% power and 1% significance level) to allow for a smaller effect size than that observed in the pilot. On this basis 13 mother/baby dyads would be needed for each arm of the study (6-12 weeks and 13 to 20 weeks). The early months following the birth of a baby are challenging for new parents and so it is likely that we will see a number of drop-outs from the study due to personal circumstances, which might include fatigue, ill-health of mother or baby or cessation of exclusive breastfeeding. To allow for this we will aim to recruit 20 women for each infant age group amounting to 40 participants in total.

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