Acute Effects of Endurance Exercise with Moderate and High Intensity on Breast Milk Composition Among Women with Overweight/Obesity

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Background

The period from conception to 2 years of age, often referred to as the "first 1000 days", is considered the most critical for the pathophysiological disorders leading up to obesity in childhood and later life.[1] The idea that nutrition during specific windows in early life influences later health is part of a broader concept called programming, which reflects the impact of a stimulus or insult during a sensitive time-period in producing long-term changes in the structure or function of the organism.[2] Extensive evidence in animal models shows that nutrition during a brief critical window in early postnatal life programmes obesity, insulin resistance, dyslipidaemia, cardiovascular disease, and even lifespan.[3] Also, observational data from human cohorts confirm that faster weight gain in **early** infancy is associated with a greater risk of later obesity[4-9] Indeed, rapid weight gain in the first 3 months of life is associated with several determinants of cardiovascular disease and type 2 diabetes in early adulthood.[10] The key limitation, however, of such observational studies is the lack of experimental evidence for a causal role of early nutrition in programming human health.

Identification of the molecular mechanisms contributing to rapid weight gain during infancy can guide strategies for optimal nutrition to prevent the development of childhood obesity, and thereby reduce the risk for type 2 diabetes and cardiovascular diseases later in life. During early postnatal life, the role of breastfeeding for later-life obesity is the one of the most recognised factors at play when discussing the nutritional background of childhood obesity. It has become clear during the recent decade that breastmilk composition is influenced by genetic variations and environmental factors, including geographical location, parity and mode of delivery, as well as lifestyle factors such as maternal smoking, body mass index (BMI) and diet.[11-16] One behavioural factor which has received very little attention in this regard is physical activity/exercise.

Regular exercise training is a formidable regulator of overall systemic metabolism through both acute effects driven by each exercise bout and through chronic adaptations in multiple tissues. Emerging work shows that the beneficial effects of exercise are not limited to adaptations within each tissue but from integration of inter-tissue communication by a variety of signalling molecules, hormones and cytokines collectively named 'exerkines'.[17,18] (Fig. 1) There are, however, little knowledge about how exercise affects human breastmilk.

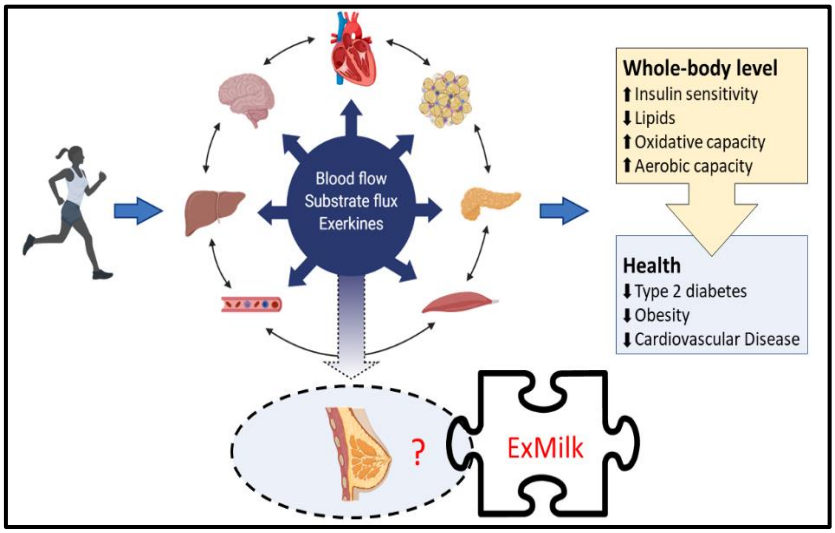

Fig. 1. Exercise induce multiple molecular adaptations in the heart, adipose tissue, the pancreas, skeletal muscle, circulation, the liver, and the brain, with inter-organ crosstalk. Together these molecular factors contribute to the exercise-induced health effects, e.g., improved insulin sensitivity and reduced hyperlipidemia, which improves cardiovascular and metabolic health. There is little evidence for exercise-induced adaptations in human breastmilk (beyond increased levels of single metabolites acutely after exercise).

Human Milk Lipids

Human milk is rich in lipids, and one class of lipids that will be especially interesting are so-called "lipokines" since these lipids act as signalling molecules and can regulate inflammation and influence systemic metabolism.[19-21] Some lipokines have been discovered in human milk. Yu and colleagues recently identified a breastmilk-specific lipid species (an alkylglycerol), which may protect the infant from obesity.[22] The role of this signalling molecule is to impede the transformation of beige adipose tissue to lipid-storing white adipose tissue in the infant, suggesting that breastfeeding may activate adipocyte thermogenesis. Whether the abundance of this lipokine is influenced by maternal exercise is unknown. Another identified lipokine, 12,13-dyhydroxy-9Z-octadecenoic acid (12,13-diHOME) was shown to regulate brown adipose tissue fuel uptake and thermogenesis in both mice and humans.[23] It was recently reported that this lipokine is present in human milk and that its abundance in breastmilk is inversely associated with infant adiposity[24], yet again suggesting that differences in milk lipid composition can be functionally related to early-life obesity risk. 12,13-diHOME increases fatty acid uptake in skeletal muscle and its abundance in plasma has been shown to increase in response to acute exercise.[25,26] In the work I was involved in during my research stay in Australia, however, we found no sustained increase in 12,13-diHOME after daily exercise training (Moholdt et al, *in review*.), which suggest that these effects are transient. Even if recent preliminary data suggest an increase in breastmilk 12,13-diHOME in most women after acute exercise[24], there is no evidence for a lasting effect or, if so, whether this effect plays a causal role in early childhood obesity development.

Furthermore, recent data indicate that the relative quantity of short-chain fatty acids in human breastmilk play a role in weight gain and adiposity during infancy, with evidence for negative associations between short-chain fatty acids (butyrate, formic acid, and acetate) with measures of infant adiposity between the ages of 3 and 12 months.[27] These findings are supported by experimental data from animal models that identified a role for short-chain fatty acids in regulating lipid metabolism, insulin sensitivity, low-grade inflammation and body weight gain.[28,29] Variability in fatty acid composition in breastmilk may partially explain inconsistent protective effects of breastfeeding against excessive infant adipose deposition. In line with this, exposures to different subgroups of short-chain fatty acids (the arachidonic acid/docosahexaenoic + eicosapentaenoic acid ratio) during the first 4 months of life may contribute to the way infants accumulate adipose tissue.[30] The relative abundance of several lipid metabolites changes acutely after exercise, in a direction opposite of what has been implicated in cardiometabolic diseases.[26] We discovered that exercise alters the relative abundance of metabolites involved in lipid metabolism, with a partial reversal of high-fat diet-induced increase in sphingolipid synthesis.[31] Consequently, exercise might modify the relative abundance of lipid components also in breastmilk, and such exercise-induced effects can have implications for infant growth.

Cytokines, Adipokines and Hormones

Breastmilk contains several non-nutritive bioactive components, including hormones and cytokines. There is evidence that human milk exhibits substantial individual variation in the concentration of cytokines, adipokines and appetite-regulating hormones. Breastmilk insulin, insulin growth factor-1 (IGF-1) and leptin levels have been reported to be higher in the milk of mothers with overweight and obesity[32-35] and different concentrations of these hormones in breastmilk has been suggested as one possible reason for high growth velocity in infants of women with poor metabolic health. Breastmilk from obese mothers has also been shown to have elevated inflammatory markers, and a pro-

inflammatory fatty acid profile that were found to be associated with infant growth and adiposity at one month of age.[36,37] It is well established that chronic low-grade inflammation, indicated by increased pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), contributes to the development and progression of metabolic disorders and non-communicable diseases such as insulin resistance, obesity and type 2 diabetes.[38,39] Regular exercise induces suppression of TNF-α-induced insulin resistance, and this is one of the pathways explaining the protective effects of exercise against chronic diseases associated with low-grade inflammation.[40] To our knowledge, only one previous study exists on the associations between exercise and cytokines in breastmilk.[41] The data from this observational study of 58 women showed that exercise was associated with elevated proinflammatory cytokines in the milk. These correlational data do, however, not provide any information about a possible causal relationship.

Human Milk Oligosaccharides (HMOs)

Human breastmilk contains a host of substances that may have mechanistic roles for metabolic health in early childhood, such as macronutrients, micronutrients, metabolic hormones, adipokines, and proinflammatory markers.[33,42,43] In particular, human milk oligosaccharides (HMOs) have recently been linked to early infancy growth and body composition.[14,44,45] Specifically, 2'-fucosyllactose, which is the most abundant HMO, was positively associated with infant weight gain between birth and 5 months[44], whereas 6'sialyllactose and lacto-N-neotetraose have been reported to be inversely associated with measures of infant adiposity.[44,46] These findings indicate that certain HMOs can play a role in infant growth.

It was recently discovered that exercise training increases the abundance of the HMO 3'sialyllactose in mouse milk, and that this metabolite is a critical mediator to improve metabolic health and cardiac function in mouse offspring.[47] In the same publication, the researchers reported that 3'sialyllactose levels in human breastmilk two months postpartum were *correlated* with average steps taken per day. One main shortcoming in human breastmilk research is that almost all available data are from observational studies. As such, little is known about cause and consequences, and the associations reported may be solely due to confounding factors (such as dietary intake).

Exercise and Human Breastmilk

Exercise represents a major challenge to whole-body homeostasis and affects multiple cells, tissues, and organs in response to the increased metabolic activity of contracting skeletal muscles.[17] It was recently shown that there is a dramatic shift in > 80% of annotated metabolites in the circulating metabolome in response to a single endurance-type exercise session of just 12 minutes, with beneficial alterations in metabolites representing key metabolic pathways central to obesity, insulin resistance, and inflammation.[26] Such alterations can help explain the broad exercise-induced benefits on cardiovascular and metabolic health.

Apart from the scarce evidence on exercise-induced adaptations in breastmilk, there is very little knowledge about how maternal exercise training affects milk composition. Women are recommended to be physically active after childbirth, but the emphasis is on maternal weight control and fitness. Current guidelines for exercise in lactating women are sparse. The most recent recommendations from the American College of Obstetricians and Gynecologists[48] merely states that "*regular aerobic exercise in lactating women has been shown to improve maternal cardiovascular fitness without affecting milk production, composition, or infant growth*." These recommendations are based on observational studies[49,50] and one small RCT[51] from the 1990s, before today's

advancements in various 'omics' technologies and the application of computational and systems biology approaches. Furthermore, no prior studies have investigated the role of exercise intensity for adaptations in breastmilk composition. There is now substantial evidence for superior effects of high-intensity exercise, as compared to moderate-intensity exercise, in a range of clinically relevant health outcomes.[52,53]

Aim and hypotheses

The overall aim of this project is to determine the acute effect of maternal exercise on breastmilk composition. We will also compare the effects of moderate versus high intensity exercise on breastmilk composition. Our hypothesis is that: Exercise will induce substantial changes in breastmilk composition after a single endurance exercise session, with greater effects of high-intensity exercise than of moderate intensity exercise.

Methods

Participants

We will include 20-25 women with BMI > 25 kg/m^2 who are exclusively breastfeeding a singleton term infant. Participants will provide a written informed consent. The assessments will be completed between 5 and 12 weeks postpartum, with the first assessment 5-9 weeks postpartum. No formal power calculation is possible for the acute study due to the exploratory nature of the research. We will continue including participants until we have complete data for all three sets of conditions for at least 20 participants. The crossover design of this study will decrease the interindividual variation in the statistical analysis. The women will serve as their own controls, which will remove any inter-individual biological variance in breastmilk.

Experimental design

This will be a randomised cross-over study, in which all participants undergo three different conditions, in random order (Fig. 2):

1) Moderate intensity exercise; 48 min at ~ 70% of heart rate maximum
2) High-intensity exercise; 4 x 4 min at 90-95% of heart rate maximum
3) No exercise. Resting in the lab.

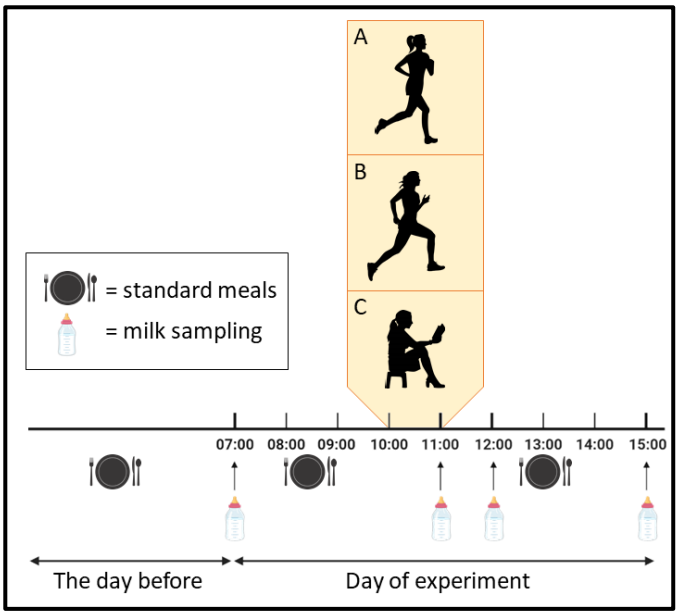

Fig. 2. Exercise will take place at 1000h, and breastmilk will be sampled at 0 h (immediately after exercise), + 1 h and + 4 h after exercise/no exercise. Dietary intake will be standardised on the day before and on the day of the experiment. Moderate intensity exercise (A), High intensity exercise (B) and No exercise (C) will be separated by one week and will be undertaken in random order.

Participants will walk or run on a treadmill during both exercise conditions. *Inclusion criteria: Besteen* 5+6 and 8+6 weeks postpartum at the first condition in the study, exclusively breastfeeding a term infant. *Exclusion criteria:* Known cardiovascular disease, type 1 or 2 diabetes.

Breastmilk samples (~ 25 mL) will be collected at 4 timepoints in each condition. Samples obtained after exercise and in the control condition (no exercise) will be compared to a 'baseline' sample obtained at the first breastfeeding in the morning (06-0700 h) on the same day (Fig 2). Prior to the experimental protocol, all participants will undergo a maximal effort exercise test to establish their cardiorespiratory fitness (maximum oxygen uptake) and heart rate maximum. This test will be undertaken at a separate day, at least two days prior to the first experimental condition, and takes about 30 minutes. On the same day, we will also estimate the participants' body composition using a bioimpedance scale (InBody 720), and infant weight and length. We will estimate infant body composition using biompedance (BioScan tough i8-nano).

Standardised meals

Participants will be asked to register their last meal the day before the first day of experiment, as well as the breakfast and lunch on the first day of experiment using a food diary. They will be asked to repeat the exact same meals at standardised time-points in the two following days of experiment (Fig. 2).

Questionnaires

Participants will complete the International Physical Activity Questionnaire (IPAQ) at baseline. They will also provide information on their age, ethnicity, date of delivery, the infant's birth weight, length and head circumference, and whether they are smoking or taking medications.

Breastmilk sampling

Breastmilk will be sampled by the women themselves, using an electric breast pump provided to them (Medela Symphony, Switzerland). Participants will be given a detailed instruction sheet and a link to a video for milk collection procedures. Breastmilk will be collected at the described time-points in the experimental protocol. For each mother, breastmilk will be collected from one breast (of her own

choice) for the entire study. The collected single full breast milk samples will be mixed and aliquoted into 10 x 1.8 mL sterile cryotubes and 1 x ~20 mL sterile vial, with the remaining milk for the mother to use. Samples will be frozen immediately after collection. Samples obtained after exercise training will be immediately transferred (on ice) to -80°C. The remaining samples (morning samples) will be frozen by the participants themselves at -20°C for up to 4 days before it will be transferred (cold chain) to the laboratory where it will be frozen at -80°C until analysis. The 10 x 1.8 mL aliquots will be used in the planned analyses, described below, whereas the additional 20 mL sample will be stored in a biobank for future analyses when additional funding and/or collaborations become available.

Breastmilk composition

Targeted NMR Metabolomics

Metabolic profiling of breastmilk will be analysed using nuclear magnetic resonance (NMR) spectroscopy using a Bruker Avance III Ultrashielded Plus 600 MHz spectrometer (Bruker Biospin GmbH, Germany) at NTNU MR Core Facility. After removal of residual lipids and proteins as previously described[54], each filtered sample (350 µL) will be mixed with 350 µL of 0.1 M phosphate buffer solution (pH 7.4) containing sodium 3-trimethylsilyl-(2,2,3,3-2-H3)-1-propionate (TSP) (final concentration 2 mM9, and then 650 µL will be transferred into a 5 mm wide NMR tube. Metabolites will be assigned using Bruker AMIX software v.2.5 and Chenomx v.7.11 (Chenomx Inc., Canada), matching spectra to reference databases of metabolites. A mother's secretor status reflects her blood group antigens and determines her ability to synthesise certain HMOs, thus the secretor status for each individual will be determined by the high abundance (secretor) or near absence (non-secretor) of the HMO 2′-fucosyllactose in the respective milk samples with a cutoff of 100 nmol 2′FL/mL, as previously described.[55] Secretor status will be accounted for in the analysis by doing sub-group analyses of secretors vs. non-secretors.

Untargeted Global Metabolomics and Complex Lipid Panel Analysis

In parallel, untargeted metabolomics profiling panels will be applied as an unbiased screening for the relative concentrations of metabolites. These analyses will be subcontracted to Metabolon, an internationally renowned metabolomics service company who provides high sensitivity analyses of more than 1000 metabolites. The principal investigator has previous experience with Metabolon data from a diet-exercise study.[31] Samples will be analysed using ultra-high-performance liquid chromatography-tandem mass-spectrometry (UPLC-MS/MS; positive mode), UPLC-MS/MS (negative mode) and gas chromatography-MS (GC-MS).[56] The Metabolon platform combines multiple MS methods and a laboratory information managements system with the industry's largest reference library of authenticated metabolite standards and has a suite of patented informatics and quality-control software. These features collectively allow us to overcome the 'signal-to-noise' challenges of other metabolomics platforms. In addition to the global metabolomics profiling, we will undertake Complex Lipid Panel profiling at Metabolon. Complex lipids are a diverse class of metabolites that play important roles in the development of metabolic diseases and inflammation. Because these metabolites have a diverse array of chemical structures and a high degree of isomeric overlap, lipids are exceptionally challenging to accurately identify and quantify. The Complex Lipid Panel, developed by Metabolon, is currently the best (and only) lipidomic platform to provide complete speciation data. The Complex Lipid Penal identifies up to 1,100 individual lipid species, offering unparalleled insight into the lipidome.

Cytokine and hormone profiling

We will measure a network of 27 cytokines of relevance for inflammatory status[57] (Human Cytokine Group I multiplex panel) using Luminex xMAP Technology on a Bio-Plex 200 system (Bio-Rad) at the Cellular and Molecular Imaging Core Facility (CMIC), NTNU. Since subclinical mastitis, which is a common condition, induces substantial changes in inflammatory breastmilk components[58], information of mastitis prevalence will be recorded and adjusted for in the statistical analyses. Breastmilk insulin, leptin, adiponectin will be measured by enzyme-linked immunosorbent assays (ELISA) using DS2 ELISA processing system (Dynex Technologies) at the Dept. of Circulation and Medical Imaging.

Micro-RNA sequencing

We will profile miRNA after sample preparation for extracellular vesicle enrichment of cell- and debris-free, defatted breastmilk, by precipitation (ExoQuick Exosome Precipitation Solution). Isolated RNA will be sequenced at NTNU's internal genomics facility with their standard small RNA protocol and Illumina's TruSeq SmallRNA Library Preparation Kit as per manufacturer's instructions and sequencing with a NextSeq HighOutput flow cell on a NextSeq 500 instrument (Illumina Inc.).

Biobanking and future directions

We will obtain additional milk samples for later (when funding and/or new collaborations become available; from the Research Council Norway, Horizon 2020/Horizon Europe, or other funding bodies). The samples will be stored in a research biobank specific to this project, with Trine Moholdt as responsible for the biobank.

Ethics and privacy protection

Participation is voluntary and the participants can withdraw their written consent at any time. They can also request that their samples are destroyed, if they have not already been included in analyses. The project involves very little risk to the participants, except for a theoretical risk of an adverse event during exercise. Women are recommended to exercise postpartum. The knowledge gained through this project is highly needed and therefore outweighs the potential risk of exercise.

Samples may be transferred to other countries. In such instances, only the biological material (breastmilk) and ID numbers linking the participant to the sample will be transferred, and no other information about the participant.

Team, environment, and facilities

This project will be undertaken at the Dept. of Circulation and Medical Imaging, NTNU. We will use facilities provided by NTNU, at St.Olav's Hospital, including: the exercise physiology laboratory at the NextMove Core Facility, the Dept. of Circulation and Medical Imaging's laboratories at the 3rd floor at AHL, laboratories at the Dept. of Clinical and Molecular Medicine, NTNU, and the MR Core Facility. Principal investigator is Researcher Trine Moholdt, PhD, Dept. of Circulation and Medical Imaging (ISB), NTNU. Collaborators: PhD student Maëliss Lemoine, ISB, NTNU, PhD student Emily Rose Ashby, ISB, NTNU, Postdoc Ankit Tanwar, ISB, NTNU, Reesarcher Marta Riise Moksnes, ISB, NTNU, Medical student Rebecca Lyng Holm, ISB, NTNU, Prof. Ann-Charlotte Iversen, Dept. of Clinical and Molecular Medicine (IKOM), NTNU, Staff Engineer Guro Rosvold, Msc, ISB, NTNU, Senior Engineer Trygve Andreassen, ISB, NTNU, Senior Engineer Liv Ryan, IKOM, NTNU, Senior Engineer Ragnhild Røsbjørgen, ISB, NTNU, Ass. Professor Guro

Giskeødegård, Dept. of Public Health and Nursing (ISM), NTNU, Ass. Professor Melanie Rae Simpson, ISM, NTNU, Professor Pål Sætrom, IKOM, NTNU.

Budget

The costs for the project are covered by an ERC Starting Grant to Trine Moholdt.

Dissemination

The results from the project will be published in a peer-reviewed journal, with open access. We will also communicate our results to the public and to health care workers seeing women in reproductive age (such as midwives).

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