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Impact of maternal overweight and obesity on human milk composition and infant growth

Overweight and obesity impact half of the pregnancies in the United States and can have negative consequences for offspring health. Studies are limited on human milk alterations in the context of maternal obesity. Alterations in milk are hypothesized to impact offspring development during the critical period of lactation. We aimed to evaluate the relationships between mothers with overweight and obesity (OW/OB, BMI ≥25 kg/m^2), infant growth, and selected milk nutrients. We recruited mother-infant dyads with pre-pregnancy OW/OB and normal weight status. The primary study included 52 dyads with infant growth measures through six months. 32 dyads provided milk at 2 weeks which was analyzed for macronutrients, long chain fatty acids (LCFAs), and insulin. We used multivariable linear regression to examine the association of: maternal weight status with infant growth, maternal weight status with milk components, and milk components with infant growth. Mothers with OW/OB had infants with higher weight-for-length (WFL) and body mass index Z-score at birth. Mothers with OW/OB had higher milk insulin, dihomo-gamma-linolenic, adrenic, and palmitic acid, and reduced conjugated linoleic and oleic acid. N6 long chain polyunsaturated fatty acid (LC-PUFA) driven factor 1 was associated with higher WFL, lower length-for-age (LFA) and lower head circumference-for-age (HCA) Z-score change from 2 weeks to 2 months in human milk fed infants; while N6 LC-PUFA driven factor 5 was associated with lower LFA Z-score change. Human milk composition is associated with maternal pre-pregnancy weight status and composition may be a contributing factor to early infant growth trajectory.

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Human milk, breast feeding, polyunsaturated fatty acids, macronutrients, infant growth, obesity

INTRODUCTION

In the United States, over 50% of pregnancies are complicated by overweight or obesity ("ACOG Committee opinion no. 549: obesity in pregnancy," 2013). Obesity has adverse effects on maternal health, impacts fetal development, and programs lifelong offspring risk for obesity (Leddy, Power, & Schulkin, 2008; Poston, Harthoorn, & Van Der Beek, 2011). In addition to the *in utero* environment, rapid weight gain in infancy has been linked to childhood obesity risk, pointing to the neonatal period as a critical window for adipose tissue development (Monteiro & Victora, 2005; Weng, Redsell, Swift, Yang, & Glazebrook, 2012).

The lactation period is a window during which alterations in milk composition impact infant metabolic outcomes (Prentice et al., 2016). In the setting of maternal disease, there are differences in milk nutrients, oxidative stress and inflammatory markers, and bioactive factors, many of which have been implicated in programming offspring growth and appetite (Ellsworth, Harman, Padmanabhan, & Gregg, 2018; Young, Johnson, & Krebs, 2012). Animal models demonstrate that maternal obesity and high fat diet influence milk composition leading to increased insulin and altered fatty acids in milk of obese dams and obesity in adult offspring (Gorski, Dunn-Meynell, Hartman, & Levin, 2006; Wahlig et al., 2012). Limited human research on maternal weight status during lactation has shown differences in milk composition of nutrients, hormones, bioactives and microbiota with suggested links to infant growth (Eriksen,

Christensen, Lind, & Michaelsen, 2018; Fields et al., 2017; Makela, Linderborg, Niinikoski, Yang, & Lagstrom, 2013; Panagos et al., 2016).

Milk composition has a high degree of variability despite well conserved macronutrients, with maternal weight status contributing to this variation (Ballard & Morrow, 2013). Maternal weight status has been associated with increased factors promoting adipogenesis including N6 long chain polyunsaturated fatty acids (LC-PUFAs) and metabolic regulating factors such as insulin (Ahuja et al., 2011; Amaral, Marano, Oliveira, & Moreira, 2019; Chan et al., 2018; Ley, Hanley, Sermer, Zinman, & O'Connor, 2012; Makela et al., 2013; Young et al., 2017). The impact of maternal weight status on milk composition may be an important aspect of developmental programming; however, the degree to which milk composition influences infant growth remains an area for exploration. Recent studies have suggested roles for mature milk growth factors, inflammatory factors, and fatty acids in the development of infant body composition (Fields & Demerath, 2012; Much et al., 2013). The aims of this study were twofold: (1) evaluate the influence of pre-pregnancy overweight and obesity on infant growth and early milk composition in the first weeks of life, and (2) investigate the potential for milk composition to impact infant growth trajectories during the first six months of life.

METHODS

Study Overview:

Mother-infant dyads were recruited after written informed consent was obtained from mothers for their own participation and assent on behalf of their infant. Mothers were given detailed instructions for milk collection. Maternal demographics were obtained through paper

survey with health history obtained through medical record review. Infant characteristics, growth measurements, and nutrition source were obtained through medical record review at birth and well child appointments at two weeks, two months and six months. Participants were given a stipend for their involvement. This study protocol was approved by the institutional review boards at participating hospitals.

Participants:

Fifty-five dyads were enrolled peripartum while admitted for birth at two participating hospitals in Ann Arbor, Michigan, United States. Three dyads opted out of the study. Dyads were categorized based on maternal pre-pregnancy body mass index (BMI) into normal weight (NW; BMI <25.0 kg/m^2) and overweight or obese (OW/OB; BMI ≥25.0 kg/m^2). Pre-pregnancy BMI was obtained through the medical record by obstetrics recorded pre-pregnancy weight or early first trimester weight with BMI then calculated using recorded height. Inclusion criteria included: maternal age >18, gestational age ≥35 weeks and healthy singleton birth with no maternal diabetes. Dyads were enrolled if the mother intended to breastfeed; however, not excluded if formula was supplemented. Infant growth trends and milk composition associations with growth were excluded for those receiving exclusive formula feedings.

Milk collection:

The research team verbally instructed mothers on milk sample collection with a written protocol provided. Samples were collected by mother at home on the morning of their infant's physician visit near two weeks of life, on average at postpartum day 16. This time point was

selected given expected improvement in milk supply to allow for milk in transition to mature milk to be provided without limiting the infant supply. Mothers collected non-fasting milk between 8:00 and 10:00 am at least 2 hours after feeding their infant. Mothers provided milk by hand expression or pumping emptying an entire single breast. The method of expression and which breast to empty were based on maternal preference. Milk was expressed into a container then inverted to mix prior to transfer of 25 mL into glass vials in 5 mL aliquots. Samples were stored in mother's home freezer then transported to clinic on ice for storage at -20 °C for less than one week before transport on ice for final freezing at -80 °C. Prior to analysis, samples were thawed on ice. Milk collection protocol was based on published milk collection with modifications as described for our study design and population (Fields & Demerath, 2012).

Milk analysis:

Macronutrients

Whole milk samples were analyzed using a mid-IR spectroscopy macronutrient analyzer (MIRIS HMA™, Uppsala, Sweden). For macronutrient analysis of carbohydrate, protein, fat, and total energy, 5 mL milk sample was warmed to 40 °C in a bead bath then underwent ultrasonic homogenization prior to immediate analysis per manufacturer protocol. Analysis was performed in duplicate for reproducibility and average value was used in analysis. As control prior to analysis, internal calibration using standards provided by MIRIS HMA™ was completed and verified with the range of targeted values established by the manufacturer. MIRIS HMA™ reported repeatability for fat, crude protein, true protein ≤0.05g/100 mL, carbohydrate

≤0.08g/100 mL and accuracy for fat ±12%, crude protein, true protein, carbohydrate ±15%. Milk macronutrient analysis was not completed for samples with under 5 mL volume available.

Fatty acid profiles

Lipids were extracted from milk samples (30 µL) following the method of Bligh and Dyer (Bligh & Dyer, 1959) with modifications. The volume of the milk was adjusted to 600 µL with water followed by addition of 2.25 mL of a mixture of chloroform-methanol (1:2) containing 0.01% of butylated hydroxytoluene (BHT) and 10 µL of 4 mM heptadecanoic (C17:0) acid as an internal standard. The mixture was homogenized on a vortex mixer. The homogenized sample was further treated with 0.75 mL each of chloroform and aqueous sodium chloride (NaCl 0.9%) solution followed by mixing and centrifugation at 3000 rpm for 5-6 min at 4 °C on a table top centrifuge. The upper aqueous layer was discarded by aspiration under vacuum and the lower organic (chloroform) layer containing the lipids were separated out, transferred into a screw caped glass tube and saved at -20 °C unless used as follows.

The fatty acid portion of the lipids were converted into their methyl esters with BF_3-methanol as described before with slight modification (Morrison & Smith, 1964). The solvents of the lipid extracts were removed under nitrogen. To the dry residues left in the screw caped glass tube, 2 mL of Boron trifluoride-methanol (BF_3-methanol, 14% solution from Sigma, Saint Louis, MO, US) was added; the mixtures were incubated at 60 °C for 2-2.5 hours. The fatty acid methyl esters (FAMEs) were extracted in hexane by adding 2 mL of hexane and 1 mL of water, mixing and centrifuging as above followed by transferring the upper hexane layer into a separate glass tube. The volume of the solvent was reduced under nitrogen and the crude FAMEs were purified by applying on thin layer chromatographic (TLC) plate (20 x 20 cm, silica gel 60, Merck

KGaA, Darmstadt, Germany, now operates as Millipore Sigma, Burlington, Massachusetts, US). The plate was developed with a solvent mixture of hexane-diethyl ether-acetic acid (80 mL-20 mL-1.5 mL respectively). Methyl ester bands were identified by comparing the retention flow (rf) of a commercial standard FAME (methyl heptadecanoate) applied on the same TLC plate on a side. The powder of the identified methyl ester was scrapped out into a test tube and extracted with 2 mL of chloroform by mild sonication on a bath sonicator followed by centrifugation at room temperature (22 °C) and then transferring the clear chloroform layer into a separate tube. The solvents were removed completely under nitrogen, purified methyl esters re-dissolved in 100 µL of hexane and the fatty acid compositions of the lipids analyzed by gas chromatography (GC).

GC analyses were performed on an Agilent GC (Santa Clara, CA, US), model 6890N equipped with flame ionization detector (FID), an auto sampler, model 7693 and Chemstation software (version C.01.04) for data analysis. The GC column used was Agilent HP 88, 30 meter, 0.25 mm I.D. and 0.20 µm film thickness. Hydrogen was used as a carrier gas and for the FID detector and nitrogen was used as a makeup gas. Analyses were carried out injecting 1 µL of sample via autoinjector mode. A temperature program of 125 °C for 0 minute, 125-145 °C at 39.52 °C/min, holding 145 °C for 5.3 minutes, 145-220 °C at 9 °C /min, hold 220 °C for 5 minutes was used. Each sample run was completed in 19.14 minutes. A calibration curve was prepared using known amounts of methyl heptadecanoate and other commercially available standard methyl ester mixtures containing saturated and unsaturated carbon chain length from 12 through 22 carbon on GC, and using the peak area ratio response of each methyl ester with respect to methyl heptadecanoate. The fatty acid methyl ester components in the sample were

identified comparing the retention time of the commercial standards and quantified with respect to the amount of methyl heptadecanoate internal standard added in each sample during liquid extraction and the peak area ratio response as in the calibration curve prepared. The quality control (QC) was done by analyzing a pooled sample (indistinguishable from the study samples) periodically and also before analysis of any study samples to ensure GC performances with respect to separation, retention times and peak response. The coefficient of variation (CV) was determined after multiple runs of the sample injection volume of the same pooled sample and calculating area percentages of methyl esters, their mean (average) values and standard deviations and then evaluating the ratio of the standard deviation to the mean. The CV for GC analysis was found to be within 2.3 to 3.7%. Individual long chain fatty acids (LCFAs) concentrations were calculated as a percentage of total LCFAs for 33 saturated, monounsaturated, and polyunsaturated LCFAs. N6:N3 LC-PUFA ratio was calculated based on total N6 and total N3 concentrations.

Insulin

Whole milk samples were delipidated to isolate the skim milk layer by centrifugation at 720 relative centrifugal force for 20 minutes at 4 °C, followed by removal of the bottom skim milk layer using a 21-guage needle with 1 mL syringe then repeated. Using 25 µL of undiluted skim milk, insulin was analyzed using Mercodia Insulin ELISA 10-1113-01 according to the manufacturer protocol with Mercodia Diabetes Antigen Control Human Low and High 10-1134-01 (Mercodia Ab, Uppsala, Sweden) (Whitmore, Trengove, Graham, & Hartmann, 2012). Analysis was performed in duplicate and average value was used in analysis. Milk insulin

analysis was not completed for all samples; if only minimal volume available then milk was prioritized for lipid analysis.

Infant growth measurements:

Infant anthropometrics were obtained by medical record review using birth and pediatric well-child routine visits at birth, two weeks, two months and six months. Age- and sex-specific Z-scores for weight-for-age (WFA), length-for-age (LFA), head circumference-for-age (HCA), weight-for-length (WFL), and BMI using the World Health Organization (WHO) growth standard for infants 0 to 2 years of age were extracted ("WHO Child Growth Standards based on length/height, weight and age," 2006).

Statistical analysis:

We described participant demographics including 52 dyads for growth and 32 dyads for which milk analysis was completed using descriptive statistics (Table 1). In all multivariable models, we included covariates based on prior knowledge of variables that are associated with the independent variable of interest and could be a potential determinant of the dependent variable assessed in each model (Hernan, Hernandez-Diaz, Werler, & Mitchell, 2002; Schriger, 2008).

Maternal weight status and infant growth

We examined associations of maternal weight status with infant growth at birth, two weeks, two months, and six months of age using multivariable linear regression based on 52 mother-infant dyads. In these models, OW/OB versus NW (referent) was the independent

variable of interest, and infant WFA Z-score, WFL Z-score, BMI-Z-score, LFA Z-score, and HCA Z-score at each of the time-points were the outcomes of interest. In these models, we adjusted for the following covariates: infant sex and feeding type (milk only or milk + formula) at the time of anthropometric assessment. For time-points beyond birth, we also adjusted for the anthropometric indicator of interest at birth; by doing so, the estimate of association can be interpreted as the average change in anthropometry from birth, while accounting for size at birth. In addition to evaluating maternal weight status as OW/OB versus NW, we also examined it as a continuous variable multiplied by 1 standard deviation (SD) of pre-pregnancy BMI for the study sample to enhance biological relevance, as we have previously done in other studies ("About Adult BMI | Healthy Weight | CDC," 2019; Perng, Gillman, Mantzoros, & Oken, 2014).

Maternal weight status and human milk composition

We examined the relationship between maternal weight status and milk composition, including macronutrients, LCFAs, N6:N3 LC-PUFA ratio, and insulin for the 32 mother-infant dyads from which milk samples were provided. Here, we explored the ratio of N6:N3 as well as the percentage of fatty acid compared to the total LCFAs using student's *t*-tests and Mann-Whitney U-test as appropriate based on normality of data distribution.

To process the LCFA data for regression analysis, we used principal components analysis (PCA) to consolidate the 33 lipids into five factors based on the Scree plot and Eigenvalues >1 similar our previously published work (Perng, Gillman, Fleisch, et al., 2014; Perng et al., 2017). Use of PCA to derive factors results in clusters of lipid metabolites that are correlated with one another and have been shown to reflect true biological processes (e.g., the clustering of branched chain amino acids and acylcarnitines in a metabolomics analysis of

childhood obesity reflects the fact that acylcarnitines are downstream catabolites of branched chain amino acid catabolism (Perng, Gillman, Fleisch, et al., 2014). These factors each have a continuous and normally distributed score that represents distinct lipid metabolite patterns. A higher and positive score for a given factor means that a woman's milk lipid composition more closely represents that particular factor. Conversely, a lower and negative score for a pattern suggests that a woman's milk lipid composition does not reflect the pattern captured by a given factor. We examined associations of maternal weight status with milk composition, we used linear regression models where OW/OB versus NW was the dichotomous independent variable, and each of the milk components were assessed as separate continuous outcomes.

Human milk composition and infant growth

We explored associations of milk composition with infant growth from two weeks to two months, and two months to six months of age for the 32 mother-infant dyads from which milk samples were provided. Here, we used multivariable linear regression models where each milk component was entered as a continuous independent variable, change in the anthropometric indicators were the outcomes. In these models, we also accounted for infant sex. Given the variation in infant fluid status at birth, the two week time point was used to assess change in growth. We evaluated associations with infant growth for infants receiving any type of nutrition (which included any degree of human milk feedings at 2 weeks, 2 month, or 6 month) with additional analysis including only those infants receiving exclusive human milk (EHM) feedings at 2 weeks, 2 months and 6 months.

In the multivariable analyses, we focused our interpretation on the direction and magnitude of beta estimates and the precision of the 95% confidence intervals (CIs), while also

considering statistical significance as $p<0.05$. All models met standard assumptions of multivariate normality.

All analyses were carried out in SAS version 9.4 (Cary, NC, USA) with the exception of demographics and individual LCFA analysis which was carried out using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Characteristics of the 52 dyads enrolled from which the 32 milk samples were collected are presented in Table 1. Birth was at term gestational age with an equal percent of male and female infants of primarily Caucasian race/ethnicity. Of the 52 infants, 17.3% were large for gestational age, defined as birth weight percentile greater than the 90th percentile, and no infants were small for gestational age. For 32 dyads with milk available for analysis, 6 mothers with OW/OB provided supplemental formula plus human milk at 2 weeks. At 2 months, 3 mothers with OW/OB provided supplemental formula plus human milk. By 6 months of age in our cohort of 52 dyads, 1 infant of NW and 7 infants of OW/OB had transitioned to exclusive formula feedings while 2 infants of NW and 8 infants of OW/OB received supplemental formula plus human milk.

Maternal weight status and infant growth

The associations of maternal pre-pregnancy BMI category (NW versus OW/OB) with infant WHO Z-score anthropometry from birth to six months are shown in Table 2. Maternal OW/OB was associated with higher infant WFL Z-score and BMI Z-score only at birth. There were no associations at the later time points.

Maternal weight status and human milk composition

Milk samples were analyzed for LCFA profiles as presented in Table 3. The distributions of milk components are presented in supplementary Figure S1. We observed no significant difference in N6:N3 LC-PUFA ratio by maternal weight status (NW 12.82 vs OW/OB 12.98). However, individual LCFAs were statistically higher in OW/OB including palmitic acid, dihomo-gamma-linolenic acid (DGLA) and adrenic acid. Oleic acid and conjugated linoleic acid were lower in OW/OB. Linear regression analysis of maternal pre-pregnancy weight status and LCFA profiles demonstrated positive association with palmitic acid, adrenic acid, DGLA and negative association with oleic acid as shown in supplemental Figure S2. Milk LCFAs were further assessed using PCA to create latent variables representing metabolite patterns ("factors"). We retained five factors from the PCA, which together accounted for 66.8% of variance in the original LCFA data set. This is compared to other analyses using PCA to reduce high-dimensional data into factor scores (Perng, Gillman, Fleisch, et al., 2014; Perng et al., 2017). LCFA composition and factor loadings for key metabolites within each factor (factor loading >|0.4|) are shown in Table S1. Lipid factor 1 is primarily composed of N6 LC-PUFAs including DGLA, bosseopentaenoic acid, and arachidonic acid (AA) in addition to gadoleic acid. Saturated long chain fatty acids comprise lipid factor 2, including stearic acid, arachidic acid, pentadecyclic acid, and palmitic acid. Lipid factor 3 is derived from mainly N3 LC-PUFAs including docosahexaenoic acid (DHA), docosapenaenoic acid (DPA), eicosapentaenoic acid (EPA), and well as a N6 LC-PUFA docosadienoic acid which is an elongation product of DGLA. Lipid factor 4 contains very long chain fatty acids with 22-22 carbons including lignoceric acid, nervonic acid, brassidic acid, and behenic acid. The top lipid factor 5 metabolites include N6 LC-PUFAs,

eicosadienoic acid, linoleic acid, adrenic acid, and arachidonic acid. Lipid factor 2 score was higher in OW/OB as shown in Table 4.

Milk macronutrient analysis revealed no significant difference between maternal groups as shown in Table 4. Milk from mothers with OW/OB had higher insulin levels.

Human milk composition and infant growth

We then evaluated milk macronutrient, LCFA factors, N6:N3 LC-PUFA ratio and insulin associations with infant growth by WHO Z-score change from two weeks to two months and two weeks to six months as presented in Table 5 for infants receiving any type of nutrition (which included any degree of human milk feedings at 2 weeks, 2 month, or 6 month) and infants receiving EHM at 2 weeks, 2 months, and 6 months. Milk fat was positively associated with WFA Z-score change from 2 weeks to 2 months in EHM fed infants only with no other associations between milk macronutrients and infant growth. N6 LC-PUFA driven lipid factor 1 and 5 were associated with infant growth. Lipid factor 1 was associated with higher infant WFL, lower LFA and lower HCA Z-score change from 2 weeks to 2 months with lower HCA Z-score also noted from 2 weeks to 6 months. N6 LC-PUFA driven factor 5 was also associated with lower LFA Z-score change from 2 weeks to 2 months in EHM fed infants. Milk N6:N3 LC-PUFA ratio was positively associated with infant WFA, WFL, and BMI Z-score change from 2 weeks to 6 months in infants receiving all types of nutrition; however, this trend persisted but was no longer significant when evaluating infants receiving EHM feedings. Milk insulin was positively associated with HCA and WFA Z-score changes in infants receiving any type of nutrition; however, this association was small and not significant when evaluating EHM fed infants.

DISCUSSION

The purpose of this study was to evaluate for associations between maternal weight status and infant growth, the impact of maternal weight status on the composition of human milk at two weeks post-partum, and the role of specific milk components in early infant growth. We found that maternal pre-pregnancy weight status was positively associated with infant birth WFL and BMI Z-scores. Mothers with OW/OB were found to have higher LCFAs that may be obesity promoting, but no overall difference in the N6:N3 LC-PUFA ratio. Milk composition of N6 LC-PUFAs were associated with infant growth changes over the first months of life in exclusively human milk fed infants. Because maternal pre-pregnancy weight status was not associated with infant growth beyond birth, these associations highlight the contribution of milk composition in the early postnatal period to infant growth.

Our study supports prior associations between increased fetal growth based on birth anthropometrics and maternal pre-pregnancy BMI (Sewell, Huston-Presley, Super, & Catalano, 2006; Starling et al., 2015). In this cohort, we did not find an association between maternal weight status and infant growth beyond birth. Our ability to detect this difference may be limited by a comparatively small amount of data. With this lack of association, our study identifies the importance of alterations in milk as a significant factor in programming of infant growth outcomes. Studies have shown that rapid early infant growth by increased WFL Z-score over the first six months of life, independent of maternal BMI, predicted obesity of children at three years old (Taveras et al., 2009). Additional evaluation with a larger cohort and longer follow-up is necessary to broaden conclusions on the role of milk composition differences in infant programming.

Based on animal models of maternal obesity, altered mammary gland lipogenesis and decreased fatty acid synthesis has been shown (Saben et al., 2014). Limited human studies on the impact of maternal obesity on milk lipid composition in mature milk have shown higher total saturated fatty acids with increased N6:N3 LC-PUFA ratio, while others have shown increased PUFAs. These outcomes highlight the need for additional research examining specific maternal populations and periods of lactation (Makela et al., 2013; Marin, Sanjurjo, Rodrigo, & de Alaniz, 2005). Our study did not find a difference in the overall milk N6:N3 LC-PUFA ratio. Many human milk LC-PUFAs have been shown to be influenced by maternal dietary intake; however, maternal diet was not assessed in this study.

Fatty acid profile analysis in this study was novel for increased percentages of potentially obesity promoting and inflammation-associated LCFAs, particularly palmitic acid, DGLA, and adrenic acid in mothers with OW/OB when assessed individually. While the mechanisms of action of these fatty acids ingested in milk need further elucidation, studies on serum fatty acid level and dietary intervention models give insight. Pro-inflammatory palmitic acid, as seen in lipid factor 2, has been shown to stimulate inflammatory and metabolic responses through the toll like receptor signaling pathways, while diets high in palmitic acid have been associated with lowered fatty acid oxidation, impaired insulin signaling and increased fat mass (Kien, Bunn, & Ugrasbul, 2005; Rogero & Calder, 2018). Studies have shown that increased insulin results in increased conversion of gamma linolenic acid to DGLA and decreased conversion to arachidonic acid (metabolites in lipid factor 1), which may alter metabolic regulation (Vessby, Gustafsson, Tengblad, Boberg, & Andersson, 2002). This unique association with insulin may contribute to the findings in our cohort. DGLA has been identified as a biomarker for obesity with

maternal plasma levels during pregnancy associated with increased childhood BMI at seven years (de Vries et al., 2014). Elevated adrenic acid, as seen in lipid factor 5, has been found in the colostrum of mothers with OW/OB and plays a protective role in infant brain development (Hadley, Ryan, Forsyth, Gautier, & Salem, 2016; Sinanoglou et al., 2017). Oleic acid and conjugated linoleic acid were lower in mothers with OW/OB in our study. Oleic acid has been shown to reduce obesity and improve metabolic and circulating lipid profiles in adults on high oleic acid diets (Liu et al., 2016). While conjugated linoleic acid found in milk in our diet protects against obesity and diabetes in animal models, adult human studies have not shown consistent benefits (Fuke & Nornberg, 2017).

Grouped lipid metabolites identified through PCA provides insight into potential biological processes impacting infant growth trends. Lipid factors 1 and factor 5 highlight distinct lipid metabolite patterns of primarily N6 LC-PUFAs, many of which have been linked to obesity and adiposity in both children and adults. These factor 5 LC-PUFAs include linoleic acid-derived eicosadienoic acid, DGLA and downstream byproduct AA which has pro-inflammatory properties, as does gadoleic acid (de Vries et al., 2014; Kaska et al., 2020; Vidakovic et al., 2016). In our cohort, lipid factors 1 and 5 were negatively associated with early infant LFA Z-score change and factor 1 positively associated with WFL Z-score change which may be driven by reduced length growth. These may be related to the biological impacts of N6 LC-PUFAs on obesity risk. Additional potentially protective LC-PUFAs are highlighted in lipid factor 3 including the N3 LC-PUFAs docosahexaenoic acid, docosapenataenoic acid, and eicosapenataenoic acid, which have been shown to be associated with lower body fat in children when plasma levels are high in their mothers (Vidakovic et al., 2016). However, our cohort found no significant

impact of this factor on growth in EHM infants and no overall differences in these top metabolites between NW and OW/OB mothers. Lipid factor 2 was comprised of saturated fatty acids including palmitic acid, which alone was higher in OW/OB mothers. The higher palmitic acid may reflect an overall higher concentration of saturated fatty acids related to dietary intake in mothers with increased weight status and leads to mixed infant outcomes (Jenkins, West, & Koulman, 2015; Panagos et al., 2016; Petersen et al., 2019; Pfeuffer & Jaudszus, 2016; Vidakovic et al., 2015). This is consistent with a lack of relationship of lipid factor 2 with infant weight and length growth in our cohort. Very long chain fatty acids including lignoceric acid and nervonic acid, identified as top metabolites contributing to factor 4, have also been linked to adolescent obesity and metabolic syndrome. There was, however, no significant impact of factor 4 on growth in our cohort (Gunes et al., 2014; Karlsson et al., 2006). Studies specifically evaluating the impact of the lactation period and human milk lipid composition on infant growth are limited and an area of need for future research.

With the rising levels of N6:N3 LC-PUFA ratio in the western diet and epidemic levels of childhood obesity, adipogenic promoting effects of N6 LC-PUFAs have been hypothesized (Ailhaud & Guesnet, 2004). Our study showed a positive association between milk N6:N3 LC-PUFA ratio and infant growth from two weeks to six months with higher infant WFA, WFL and BMI Z-score change in infants receiving any degree of human milk with continued positive trends in EHM feed infants. Panagos showed that increased N6:N3 LC-PUFA ratio was associated with increased WFL Z-score at two months (Panagos et al., 2016). On the other hand, a human supplementation study showed that milk N6 LC-PUFAs were negatively associated with infant BMI and lean body mass at four months and increased N3 LC-PUFAs in

milk stimulated early fat accumulation (Much et al., 2013). Given these conflicting results, research is necessary to arrive at a consensus on the impact of milk fatty acid profiles on infant growth with studies differentiating early versus late time frames of lactation.

In this cohort, we found no significant differences in milk macronutrient composition based on maternal weight status. This conservation of macronutrients, particularly in the initial stages of lactation, is consistent with milk literature (Ballard & Morrow, 2013). Pre-pregnancy overweight and obesity may result in whole body insulin resistance, thus contributing to altered milk insulin (Badillo-Suarez, Rodriguez-Cruz, & Nieves-Morales, 2017). Our study showed higher milk insulin in mothers with OW/OB for milk obtained near two weeks postpartum. Published studies on mature milk insulin in relation to maternal BMI have shown a positive association with maternal pre-pregnancy BMI at later time points in lactation (Ahuja et al., 2011; Ley et al., 2012; Young et al., 2017).

We detected higher insulin levels in milk at two weeks in mothers with elevated BMI and found milk insulin to be positively associated with higher infant WFA from two weeks to six months and HCA Z-score change from two weeks to two months in infants receiving any type of nutrition. Given the minimal increases in growth and the lack of association when infants were receiving human milk exclusively, the clinical significance is uncertain. Studies examining associations between milk insulin and infant weight gain are limited with conflicting results. Fields reported lower infant weight gain with higher milk insulin at one month; however, further studies by Fields did not show significant associations at six months of age (Fields & Demerath, 2012; Fields et al., 2017). A Canadian cohort showed infant WFL Z-score with milk insulin having a U-shaped association with growth at age four (Chan et al., 2018). Whether ingested

milk insulin has biological effect in infants beyond a role in gut maturation remains controversial, with some evidence to support that ingested insulin in infants is resistant to degradation in the stomach and may have systemic effects (Shehadeh, Sukhotnik, & Shamir, 2006). Further research on the role of insulin in regulation of infant metabolic health is needed.

This study has several strengths including the analysis of milk from an early time point when milk is transitioning from colostrum to mature milk, which has not been included in many milk studies. This time point allows for unique assessment of lactational programming influences on growth outcomes at a time when mothers may be using less formula supplementation and when milk supply has been achieved. However, this early stage milk provides a limited period of nutrition for infants, and it is important to recognize that mature milk composition will also contribute to the pattern of ongoing organ development. This study included milk samples stored at -80 °C with analysis within 12 months after collection and minimization of repetitive freeze-thaw cycles, as these factors may impact milk composition to an unknown degree (George, Gay, Trengove, & Geddes, 2018; Nessel, Khashu, & Dyall, 2019).

Limitations of this study include a smaller sample size from a middle-class primarily Caucasian midwestern population, thus the results may not be applicable to a more diverse population. Other factors influencing milk composition including maternal diet, vitamin supplementation, and milk volume were not measured in this study. As the amount of formula supplementation was not quantified, the impact on infant growth trajectory may vary based on the degree of supplementation. When comparing infant growth associations in Table 5, loss of statistical significance between infants receiving all feeding types and EHM feeds as well as lack of growth associations beyond 2 months of age may be in part due to reduced sample size

or additional contributing factors to infant growth including genetics and environmental influences. Infant growth assessments were performed by non-research medical staff with weight, length, and head circumference documented in the medical record. The lack of trained research staff obtaining growth measurements may introduce inconsistency in infant measurements. Maternal pre-pregnancy weight similarly was obtained through medical record review. An assessment of infant body composition would be of great importance for future work and offer additional insight than WFL or BMI Z-score alone. Finally, while we used PCA to consolidate milk LCFAs into lipid metabolite patterns that likely reflect the clustering of lipids in biochemical pathways, we assessed other components of milk (macronutrients and insulin) individually given differences in the unit of measurement for each. This approach may not accurately represent the interactions among milk components. Future studies with more sophisticated analytical techniques to model joint effects of multiple milk components with health outcomes are warranted.

CONCLUSION

Our study has described early alterations in milk composition with increases in potentially obesity promoting fatty acids, reduction in protective fatty acids, and elevated insulin levels in milk from mothers with overweight and obesity. We found associations of higher milk N6 LC-PUFAs with altered infant length growth trajectory through two months of age. While we have shown implications of maternal increased BMI on milk composition and milk composition on infant growth, our study did not show an overall association between maternal weight status and infant growth after birth. The importance of human milk as the primary source of nutrition for

infants cannot be understated; however, as we gain more knowledge regarding the multi-faceted and complex changes in milk over the period of lactation, we must appreciate that factors in milk may impact early developmental programming. Future research to understand the complex relationships between maternal health, milk and early indicators of programmed infant metabolic disease risk is important to design interventions to optimize beneficial aspects of maternal milk for at risk infants.

KEY MESSAGES

- Animal models of maternal obesity have shown roles for milk nutrients, fatty acids, oxidative stress markers, inflammatory markers, hormones, and bioactive factors in programming of offspring growth. Human studies with longitudinal infant outcomes are limited.
- This study is novel in investigating the impact of pre-pregnancy overweight and obesity on the early macronutrient, fatty acid profile and insulin content of human milk during the period of transition from colostrum to mature milk and the association with infant growth through six months.
- This study found associations of higher milk N6 LC-PUFAs with infant growth trajectory.

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Table 1. Participant characteristics

	Participant Characteristics (n=52)		**Participant Characteristics with human milk (n=32)**	
	NW (n=22)	**OW/OB** (n=30)	**NW** (n=16)	**OW/OB** (n=16)
Maternal Age (years)	31.1 (3.9)	29.1 (4.7)	30.9 (4.1)	29.9 (3.8)
Maternal Pre-Pregnancy BMI (kg/m2)	21.1 (2.2)	**31.4 (4.8)**	21.3 (2.0)	**30.2 (4.5)**
Maternal Race or Ethnicity (%)				
Caucasian	12 (54)	22 (73)	9 (56)	14 (88)
African American	1 (5)	5 (17)	0 (0)	1 (6)
Hispanic	1 (5)	0 (0)	1 (6)	0 (0)
Asian	4 (18)	0 (0)	4 (25)	1 (6)
Other/Not available	4 (18)	3 (10)	2 (13)	0 (0)
Gestational Age	40 wk 0d (7 d)	39 wk 6 d (8 d)	40 wk 0d (7 d)	39 wk 5 d (10 d)
Mode of Birth (%)				
Vaginal	14 (64)	17 (57)	10 (62)	12 (75)
C-section	8 (36)	13 (43)	6 (38)	4 (25)
Infant Sex (%)				
Male	10 (45)	13 (43)	7 (44)	8 (50)
Female	12 (55)	17 (57)	9 (56)	8 (50)
Infant Birth Weight (kg)	3.5 (0.4)	3.6 (0.4)	3.5 (0.3)	3.5 (0.4)
Infant Age at 2 week collection (days)	16.1 (2.9)	16.2 (3.0)	16.1 (3.2)	16.7 (3.3)

Data presented as mean (standard deviation) or mean (percentage) as appropriate. Abbreviations: mothers with normal weight (NW), mothers with overweight or obesity (OW/OB); body mass index (BMI), week (wk), days (d). Bolded font indicates statistical significance at $p<0.05$ based on a *t*-test.

Table 2. Adjusted associations of maternal pre-pregnancy BMI with infant anthropometry at birth, 2 weeks, 2 months, and 6 months of age among mother-infant dyads

	Mean ± SE		β (95% CI)	*Per 1 SD (6.4 kg/m^2) BMI*[c]
	NW (*n* = 22)	**OW/OB (*n* = 30)**	**OW/OB vs. NW**	
Birth[a]				
WFA z-score	0.41 ± 0.17	0.52 ± 0.15	0.11 (-0.31, 0.54)	0.03 (-0.19, 0.24)
WFL z-score	-1.48 ± 0.24	-0.53 ± 0.21	**0.95 (0.34, 1.56)**	**0.40 (0.09, 0.71)**
BMI z-score	-0.79 ± 0.20	-0.23 ± 0.17	**0.56 (0.05, 1.06)**	0.24 (-0.02, 0.49)
LFA z-score	1.51 ± 0.23	1.02 ± 0.19	-0.50 (-1.06, 0.07)	-0.24 (-0.52, 0.05)
HCA z-score	0.49 ± 0.21	0.20 ± 0.18	-0.29 (-0.81, 0.24)	-0.08 (-0.34, 0.19)
Two weeks of age[b]				
WFA z-score	-0.01 ± 0.31	-0.27 ± 0.25	-0.26 (-0.64, 0.13)	-0.05 (-0.26, 0.14)
WFL z-score	-0.94 ± 0.43	-0.88 ± 0.35	0.07 (-0.49, 0.63)	-0.07 (-0.36, 0.22)
BMI z-score	-0.56 ± 0.39	-0.58 ± 0.32	-0.02 (-0.52, 0.47)	0.06 (-0.20, 0.32)
LFA z-score	0.74 ± 0.47	0.44 ± 0.38	-0.30 (-0.88, 0.29)	-0.05 (-0.37, 0.26)
HCA z-score	0.78 ± 0.47	0.38 ± 0.38	-0.40 (-0.99, 0.19)	-0.21 (-0.53, 0.11)
Two months of age[b]				
WFA z-score	-0.43 ± 0.23	-0.43 ± 0.17	0.00 (-0.35, 0.35)	0.08 (-0.10, 0.25)
WFL z-score	-0.59 ± 0.42	-0.77 ± 0.31	-0.18 (-0.86, 0.50)	0.03 (-0.30, 0.37)
BMI z-score	-0.60 ± 0.35	-0.79 ± 0.26	-0.19 (-0.74, 0.36)	0.03 (-0.25, 0.30)
LFA z-score	-0.05 ± 0.29	0.23 ± 0.22	0.28 (-0.17, 0.74)	0.12 (-0.11, 0.35)
HCA z-score	0.14 ± 0.38	0.20 ± 0.28	0.06 (-0.52, 0.65)	0.05 (-0.25, 0.35)
Six months of age[b]				
WFA z-score	0.45 ± 0.30	0.34 ± 0.23	-0.11 (-0.59, 0.37)	0.11 (-0.14, 0.36)
WFL z-score	-0.09 ± 0.33	-0.19 ± 0.25	-0.11 (-0.67, 0.45)	0.12 (-0.16, 0.41)
BMI z-score	-0.18 ± 0.33	-0.30 ± 0.24	-0.13 (-0.67, 0.42)	0.10 (-0.17, 0.38)
LFA z-score	0.79 ± 0.41	0.97 ± 0.31	0.17 (-0.50, 0.85)	0.17 (-0.18, 0.52)
HCA z-score	-1.01 ± 2.69	1.54 ± 2.01	2.55 (-1.80, 6.90)	2.04 (-0.17, 4.25)

[a] Estimates are adjusted for infant sex.
[b] Estimates are adjusted for infant sex, anthropometric indicator of interest at birth, and feeding preference at the time of anthropometric assessment.
[c] All estimates are multiplied by 1-standard deviation of pre-pregnancy BMI (6.4 kg/m^2) for population-level relevance.
Abbreviations: weight-for-age z-score (WFAZ), weight-for-length z-score (WFLZ), body mass index z-score (BMIZ), length-for-age z-score (LFAZ), head circumference-for-age z-score (HCAZ)
Bolded font indicates statistical significance at $P<0.05$ based on a Wald Chi-squared test.

Table 3. Human milk long chain fatty acid profiles

	NW (n=16)	**OW/OB** (n=16)	***P***
N6:N3 LC-PUFA ratio	12.82 (3.04)	12.98 (3.08)	0.78
	%nmol of total LCFA		
Saturated LCFA			
14:0, myristic acid	3.75 (0.99)	4.63 (1.76)	0.09
15:0, pentadecylic acid	0.26 (0.09)	0.28 (0.07)	0.37
16:0, palmitic acid	**21.96 (2.96)**	**24.34 (2.54)**	**0.02**
18:0, stearic acid	7.44 (1.86)	7.82 (1.27)	0.50
19:0, nonadecylic acid	0.14 (0.11)	0.16 (0.09)	0.29
20:0, arachidic acid	0.22 (0.05)	0.22 (0.03)	0.95
21:0, heneicosylic acid	0.08 (0.03)	0.10 (0.03)	0.09
22:0, behenic acid	0.09 (0.04)	0.08 (0.03)	0.93
24:0, lignoceric acid	0.08 (0.02)	0.09 (0.04)	0.62
Mono-unsaturated LCFA			
14:1 (n-5), myristoleic acid	0.18 (0.08)	0.22 (0.08)	0.19
16:1 (n-7)c, palmitoleic acid (cis)	2.03 (0.78)	2.40 (0.59)	0.14
16:1 (n-7)t, palmitoleic acid (trans)	0.24 (0.05)	0.24 (0.05)	0.87
18:1 (n-7), vaccenic acid	2.83 (0.62)	3.04 (0.63)	0.34
18:1 (n-9), oleic acid	**40.81 (5.16)**	**36.37 (3.37)**	**0.01**
20:1, gadoleic acid	0.10 (0.12)	0.2 (0.23)	0.60
22:1, brassidic acid	0.09 (0.02)	0.10 (0.03)	0.44
24:1, nervonic acid	0.09 (0.04)	0.10 (0.03)	0.72
Poly-unsaturated LCFA			
18:2 (n-6)cc, linoleic acid (cis)	16.08 (2.93)	15.82 (4.06)	0.83
18:2 (n-6)tt, linoleic acid (trans)	0.07 (0.04)	0.10 (0.04)	0.06
18:2 (n-7,9) Conjugate, conjugated linoleic acid	**0.45 (0.18)**	**0.34 (0.18)**	**0.02**
18:3 (n-3), alpha-linolenic acid	1.02 (0.31)	1.06 (0.59)	0.52
18:3 (n-6), gamma-linolenic	0.08 (0.03)	0.09 (0.03)	0.41
18:4 (n-3), stearidonic acid	0.05 (0.03)	0.04 (0.04)	0.78
20:2 (n-6), eicosadienoic acid	0.38 (0.07)	0.42 (0.10)	0.21
20:3 (n-6), dihomo-gamma-linolenic acid (DGLA)	**0.38 (0.09)**	**0.54 (0.20)**	**0.01**
20:4 (n-6), arachidonic acid	0.50 (0.14)	0.58 (0.24)	0.29
20:5 (n-3), eicosapentaenoic acid	0.07 (0.03)	0.09 (0.07)	0.62
20:5 (n-6), bosseopentaenoic acid	0.06 (0.01)	0.07 (0.04)	0.17
22:2 (n-6), docosadienoic acid	0.05 (0.07)	0.03 (0.02)	0.98
22:4 (n-6), adrenic acid	**0.10 (0.04)**	**0.14 (0.06)**	**0.02**
22:5 (n-3), docosapentaenoic acid	0.14 (0.08)	0.12 (0.04)	0.35
22:5 (n-6), osbond acid	0.006 (0.004)	0.006 (0.005)	0.45
22:6 (n-3), docosahexaenoic acid (DHA)	0.19 (0.16)	0.16 (0.09)	0.66

Data presented as mean (standard deviation). Values for fatty acids are given as percentage nmol of the fatty acid/total long chain fatty acid. Abbreviations: mothers with normal weight (NW), mothers with overweight or obesity (OW/OB), long chain fatty acid (LCFA), N6:N3 long chain polyunsaturated fatty acid (N6:N3 LC-PUFA). Bolded font

indicates statistical significance at $P<0.05$ based on a *t*-test or Mann-Whitney U-test as appropriate based on distribution.

Table 4. Associations of maternal pre-pregnancy weight status with human milk composition near 2 weeks postpartum

	Pre-pregnancy weight status		
	NW (mean±SD)	**OW/OB** (mean±SD)	***P***
Macronutrients	*n* = 15	*n* = 15	
Protein (g/100 mL)	1.2 ± 0.2	1.2 ± 0.4	0.89
Carbohydrate (g/100 mL)	7.0 ± 0.3	6.8 ± 0.5	0.24
Fat (g/100 mL)	3.7 ± 1.1	3.5 ± 0.9	0.43
Lipids	*n* = 16	*n* = 16	
Factor 1	-0.23 ± 1.00	0.23 ± 0.98	0.18
Factor 2	**-0.41 ± 0.87**	**0.41 ± 0.97**	**0.01**
Factor 3	-0.22 ± 0.72	0.22 ± 1.20	0.19
Factor 4	0.18 ± 1.34	-0.18 ± 0.46	0.29
Factor 5	0.14 ± 0.93	-0.14 ± 1.08	0.41
N6:N3 LC-PUFA ratio	12.8 ± 3.0	13.0 ± 3.1	0.88
Metabolic components	*n* = 16	*n* = 13	
Insulin (mU/L)	**14.5 ± 15.7**	**29.5 ± 21.3**	**0.02**

Data presented as mean ± standard deviation. Abbreviations: mothers with normal weight (NW), mothers with overweight or obesity (OW/OB), N6:N3 long chain polyunsaturated fatty acid (N6:N3 LC-PUFA). Bolded font indicates statistical significance at $P<0.05$ based on linear regression models.

Table 5. Associations of human milk composition near 2 weeks postpartum with change in offspring weight-for-age z-score, weight-for-length z-score, body mass index-for-age z-score, length-for-age Z-score, and head circumference-for-age Z-score between 2 weeks and 2 months, and 2 weeks and 6 months of age

	WFAZ		WFLZ		BMIZ		LFAZ		HCAZ	
	Difference (β [95% CI]) in Δanthropometry in offspring between two weeks and two months of age									
	Any feeding preference	EHM feeding preference	Any feeding preference	EHM feeding preference	Any feeding preference	EHM feeding preference	Any feeding preference	EHM feeding preference	Any feeding preference	EHM feeding preference
Macronutrients (AFP n=29, EHM n=21)										
Fat	0.06 (-0.17, 0.30)	**0.27 (0.05, 0.50)**	-0.01 (-0.39, 0.37)	0.35 (-0.07, 0.77)	0.08 (-0.43, 0.28)	0.20 (-0.21, 0.61)	0.12 (-0.09, 0.33)	0.09 (-0.16, 0.35)	-0.13 (-0.41, 0.14)	-0.02 (-0.36, 0.33)
Protein	0.43 (-0.31, 1.17)	0.27 (-0.41, 0.95)	0.77 (-0.43, 1.97)	0.58 (-0.62, 1.77)	0.65 (-0.48, 1.78)	0.40 (-0.73, 1.53)	-0.05 (-0.74, 0.65)	-0.06 (-0.77, 0.64)	-0.05 (-0.97, 0.86)	-0.15 (-1.09, 0.80)
Carbohydrate	0.17 (-0.40, 0.74)	0.08 (-0.69, 0.86)	-0.26 (-1.19, 0.68)	0.14 (-1.23, 1.50)	-0.02 (-0.89, 0.86)	0.26 (-1.01, 1.53)	0.38 (-0.12, 0.90)	-0.11 (-0.90, 0.68)	0.00 (-0.71, 0.71)	-0.42 (-1.51, 0.67)
Lipids (AFP n=31, EHM n=23)										
Factor 1	-0.06 (-0.26, 0.13)	0.04 (-0.19, 0.26)	0.02 (-0.35, 0.39)	**0.38 (0.01, 0.75)**	-0.03 (-0.34, 0.29)	0.21 (-0.15, 0.57)	-0.12 (-0.33, 0.09)	**-0.30 (-0.52, -0.08)**	-0.21 (-0.47, 0.05)	**-0.32 (-0.60, -0.03)**
Factor 2	0.15 (-0.05, 0.34)	0.13 (-0.06, 0.33)	0.28 (-0.09, 0.64)	0.22 (-0.14, 0.58)	0.24 (-0.07, 0.56)	0.20 (-0.13, 0.53)	-0.05 (-0.27, 0.18)	-0.02 (-0.25, 0.22)	**0.28 (0.02, 0.54)**	0.22 (-0.05, 0.49)
Factor 3	-0.11 (-0.31, 0.09)	-0.03 (-0.26, 0.20)	-0.11 (-0.49, 0.27)	0.11 (-0.30, 0.53)	**-0.31 (-0.62, -0.00)**	-0.28 (-0.65, 0.08)	0.03 (-0.19, 0.26)	0.02 (-0.23, 0.29)	-0.10 (-0.37, 0.17)	0.07 (-0.24, 0.38)
Factor 4	-0.10 (-0.29, 0.10)	-0.05 (-0.24, 0.14)	-0.15 (-0.52, 0.21)	-0.07 (-0.42, 0.28)	-0.05 (-0.37, 0.26)	0.01 (-0.31, 0.33)	-0.02 (-0.24, 0.19)	-0.03 (-0.25, 0.18)	-0.13 (-0.40, 0.14)	-0.03 (-0.30, 0.25)
Factor 5	-0.03 (-0.23, 0.16)	-0.07 (-0.28, 0.14)	0.27 (-0.09, 0.63)	0.14 (-0.24, 0.51)	0.20 (-0.11, 0.51)	0.12 (-0.22, 0.47)	**-0.29 (-0.48, -0.10)**	**-0.24 (-0.45, -0.02)**	0.04 (-0.22, 0.31)	-0.06 (-0.35, 0.22)
N6:N3 PUFA	0.03 (-0.03, 0.09)	0.01 (-0.07, 0.09)	0.08 (-0.04, 0.20)	-0.01 (-0.15, 0.13)	0.06 (-0.05, 0.16)	0.00 (-0.13, 0.13)	-0.02 (-0.09, 0.05)	0.02 (-0.07, 0.10)	0.04 (-0.05, 0.13)	-0.04 (-0.15, 0.08)
Metabolic components (AFP n=28, EHM n=28)										
Insulin (mU/L)	0.01 (-0.01, 0.02)	0.01 (-0.01, 0.02)	0.00 (-0.02, 0.02)	-0.01 (-0.03, 0.02)	0.00 (-0.02, 0.02)	0.00 (-0.03, 0.02)	0.01 (-0.00, 0.02)	0.01 (0.00, 0.03)	**0.02 (0.00, 0.03)**	0.01 (-0.01, 0.03)
	Difference (β [95% CI]) in Δanthropometry in offspring between two weeks and six months of age									
Macronutrients (AFP n=26, EHM n=19)										
Fat	-0.03 (-0.37, 0.32)	0.28 (-0.13, 0.69)	-0.06 (-0.49, 0.38)	0.34 (-0.18, 0.86)	-0.11 (-0.50, 0.28)	0.22 (-0.27, 0.71)	-0.01 (-0.35, 0.33)	0.05 (-0.39, 0.49)	-2.18 (-5.26, 0.89)	0.06 (-0.38, 0.49)
Protein	0.42 (-0.67, 1.52)	0.17 (-0.92, 1.27)	1.29 (-0.01, 2.58)	1.16 (-0.12, 2.45)	0.91 (-0.29, 2.10)	0.69 (-0.56, 1.95)	-0.42 (-1.49, 0.65)	-0.66 (-1.76, 0.45)	2.80 (-7.34, 12.95)	-0.30 (-1.41, 0.83)
Carbohydrate	0.40 (-0.43, 1.23)	0.14 (-1.08, 1.36)	-0.12 (-1.18, 0.94)	-0.31 (-1.85, 1.22)	0.10 (-0.85, 1.06)	-0.07 (-1.51, 1.36)	0.66 (-0.13, 1.45)	0.46 (-0.80, 1.72)	2.00 (-5.97, 9.97)	-0.41 (-1.71, 0.90)
Lipids (AFP n=28, EHM n=21)										
Factor 1	-0.17 (-0.51, 0.17)	-0.18 (-0.55, 0.19)	-0.17 (-0.61, 0.27)	0.05 (-0.42, 0.52)	-0.13 (-0.53, 0.26)	-0.05 (-0.49, 0.39)	-0.17 (-0.51, 0.17)	-0.35 (-0.72, 0.01)	-0.24 (-0.59, 0.10)	**-0.47 (-0.81, -0.13)**
Factor 2	-0.06 (-0.43, 0.30)	-0.18 (-0.54, 0.17)	-0.03(-0.50, 0.44)	-0.26 (-0.70, 0.18)	0.02 (-0.40, 0.43)	-0.16 (-0.58, 0.26)	-0.09 (-0.46, 0.27)	-0.07 (-0.45, 0.31)	0.19 (-0.18, 0.55)	0.16 (-0.21, 0.53)
Factor 3	-0.07 (-0.39, 0.25)	0.08 (-0.28, 0.44)	-0.11 (-0.52, 0.31)	0.14 (-0.31, 0.58)	-0.28 (-0.63, 0.07)	-0.24 (-0.65, 0.17)	0.07 (-0.25, 0.39)	0.16 (-0.21, 0.53)	-0.14 (-0.46, 0.17)	0.00 (-0.36, 0.36)
Factor 4	**-0.60 (-1.08, -0.12)**	-0.47 (-0.96, 0.01)	-0.60 (-1.24, 0.03)	-0.36 (-0.99, 0.27)	-0.38 (-0.96, 0.21)	-0.22 (-0.82, 0.38)	-0.35 (-0.86, 0.16)	-0.34 (-0.87, 0.19)	-0.14 (-0.70, 0.42)	0.05 (-0.53, 0.63)
Factor 5	0.06 (-0.28, 0.40)	-0.02 (-0.39, 0.35)	0.40 (-0.01, 0.81)	0.27 (-0.18, 0.71)	0.33 (-0.04, 0.70)	0.24 (-0.18, 0.65)	-0.30 (-0.62, 0.02)	-0.31 (-0.67, 0.05)	-0.08 (-0.41, 0.26)	-0.12 (-0.49, 0.24)
N6:N3 PUFA	**0.13 (0.03, 0.22)**	0.10 (-0.02, 0.22)	**0.18 (0.06, 0.31)**	0.12 (-0.04, 0.27)	**0.16 (0.05, 0.27)**	0.12 (-0.02, 0.26)	0.01 (-0.10, 0.12)	0.02 (-0.12, 0.15)	0.03 (-0.08, 0.14)	-0.02 (-0.16, 0.12)
Metabolic components (AFP n=25, EHM n=18)										
Insulin (mU/L)	**0.02 (0.00, 0.04)**	0.01 (-0.02, 0.03)	0.02 (-0.01, 0.04)	0.00 (-0.03, 0.03)	0.02 (-0.00, 0.04)	0.00 (-0.02, 0.03)	0.01 (-0.01, 0.03)	0.01 (-0.02, 0.03)	0.02 (-0.00, 0.04)	0.01 (-0.02, 0.04)

Estimates are adjusted for infant sex.
Bolded font indicates statistical significance at $P<0.05$ based on a Wald Chi-square test.
Abbreviations: change (Δ), weight-for-age z-score (WFAZ), weight-for-length z-score (WFLZ), body mass index z-score (BMIZ), length-for-age z-score (LFAZ), head circumference-for-age z-score (HCAZ), N6:N3 long chain polyunsaturated fatty acid (N6:N3 LC-PUFA), confidence interval (CI). Any feeding preference (AFP) includes infants receiving a degree of human milk at 2 weeks, 2 months, and 6 months. Exclusive human milk (EHM) feeding preference includes infants receiving only human milk at 2 weeks, 2 months, and 6 months.

SUPPORTING FIGURE LEGENDS

Figure S1. Significant differences in human milk composition profiles comparing normal weight (NW) and overweight & obesity (OW/OB) for insulin, N6:N3 LC-PUFA ratio, palmitic acid [16:0], oleic acid [18-1 (n-9)], conjugated linoleic acid [18:2 (n-7,9)], dihomo-gamma-linolenic acid [20:3 (n-6)], adrenic acid [22:4 (n-6)]. LCFAs represented as percentage (%) of total LCFAs. *P<0.05

Figure S2. Linear regression of maternal pre-pregnancy BMI and human milk composition: A. N6:N3 LC-PUFA ratio, B. Palmitic acid [16:0], C. Oleic acid [18-1 (n-9)], D. Conjugated linoleic acid [18:2 (n-7,9)], E. Dihomo-gamma-linolenic acid [20:3 (n-6)], F. Adrenic acid [22:4 (n-6)], G. Insulin. LCFAs represented as percentage (%) of total LCFAs. *P<0.05