Infant sex differences in human milk intake and composition from 1- to 3-month post-delivery in a healthy United States cohort

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ABSTRACT
Background: Macronutrient composition of human milk differs by infant sex, but few studies have examined sex differences in other milk components, or their potential modification by maternal body mass index (BMI).
Aim: We compared milk intake and human milk hormone and cytokine concentrations at 1- and 3-month post-delivery and tested infant sex by maternal BMI (OW/OB vs. NW) interactions.
Subjects and method: Data were analysed for 346 mother–infant dyads in the Mothers and Infants Linked for Healthy Growth (MILk) Study at 1- and 3-month post-delivery. Infant milk intake was estimated by the change in infant weight after test feedings. Concentrations of glucose, insulin, leptin, adiponectin, interleukin-6 (IL-6), and C-reactive protein (CRP) were measured using ELISA. Multivariable linear regression and linear mixed models were used to estimate sex main effects and their interaction with maternal BMI.
Results: Mean glucose concentration at 1 month was 2.62 mg/dl higher for male infants, but no difference at 3 months was observed. Milk intake and concentrations for the other milk components were similar for males and females at both time points. Associations with infant sex did not differ significantly by maternal BMI.
Conclusions: Among healthy United States mother–infant dyads, appetite, and growth-regulating factors in human milk did not differ significantly by infant sex.

Introduction

Human milk is a complex fluid that provides essential energy and nutrients to infants as well as growth factors, hormones, microbes, immune factors, whole cells, miRNAs, and other constituents believed to be bioactive for the infant during a critical period of development (Savino et al. 2013; Andreas et al. 2015; Lee and Kelleher 2016). Human milk composition is known to vary considerably between individual women and across populations, and by specific maternal characteristics, including diet, obesity status, metabolic state (i.e. obesity and diabetes), as well as behavioural factors, such as feeding patterns (Fields et al. 2016; Lee and Kelleher 2016). In contrast, infant characteristics that influence milk composition, including infant sex, are greatly understudied. Sexual dimorphism in mammalian morphology is apparent early in development onwards, arises initially from differing genetic and hormonal effects, and is associated with numerous sex differences in subsequent health and development (Becker et al. 2005). The need to conduct studies that take into account sex as a biological variable in medicine was highlighted by the 2001 Institute of Medicine (IOM) Committee report, “Exploring the Biological Contributions to Human Health: Does Sex Matter” (IOM 2001).

Studies in animals support the general notion that there are sex differences in milk output (Hinde 2009) and macronutrient composition of milk produced by non-human primates (Hinde 2009; Hinde et al. 2013), cattle (Hinde et al. 2014), and marsupials (Quesnel et al. 2017). Generally, higher levels of energy, energy density, protein, and fat are found in milk made for males as compared to that produced for female offspring in these species (Galante et al. 2018). The physiological mechanisms underpinning sexual dimorphism in milk composition are speculative, and include sex differences in placental hormones that differentially affect breast function (Enninga et al. 2015), the exchange of metabolic signals via retrograde flow of milk and infant saliva back into the breast during suckling (Ramsey et al. 2004; Biox-Amoros 2019), and sex differences in feeding behaviours (e.g. feeding frequency or suckling patterns) that may influence composition (Fields et al. 2017).

Understanding of sex differences in human milk is partial at this time. Initial human studies focussed primarily on the macronutrient content of human milk, reporting total energy...
and fat content differences for male vs. female offspring, as recently reviewed by Galante et al. (2018). While males are known to consume approximately 6% higher volume of milk per day than do females (Da Costa et al. 2010), findings are inconsistent with regard to the presence and direction of human sex-based milk differences (Powe et al. 2010; Fujita et al. 2012; Quinn 2013; Hahn et al. 2017; Fischer Fumeaux et al. 2019; Hosseini et al. 2020). The additional putatively bioactive elements of milk that shape infant gut development, appetite and satiety, and metabolism have been little studied in terms of sex differences. This is an important gap, particularly given the growing evidence around milk as a carrier of signaling molecules that induce metabolic programming of the infant, altering future body composition and disease risk (the so-called “lactational programming” literature) (Quinn 2021).

Evolutionary life course theory predicts that the quality of the prevailing nutritional environment may dictate developmental trajectories in light of trade-offs between achieving a rapid growth rate, for instance, and mounting a robust immunological defence or maximising fertility (Roff 1992; Stearns 1992), and that these strategies may also differ by sex. By extension, optimal ranges for growth and appetite-regulating components of mamalian milk for male and female offspring could depend on the current deficit or abundance of nutritional resources, frequently indexed by maternal nutritional status, or BMI. In a prior study, our group reported higher milk insulin and leptin concentrations in milk for female infants, but only when maternal BMI was in the overweight or obese range (Fields et al. 2017). Similarly, a recent study showed sex interactions with a number of maternal factors in the concentration of milk growth-related hormones and protein in the large Finnish STEPS cohort (Galante et al. 2020). Additional studies are needed to examine sex differences in a broader variety of potentially bioactive constituents of human milk, using rigorous methods and with control for potential confounding factors, across the range of maternal weight and nutritional status.

To help address these gaps, we examined sex differences in infant milk intake and human milk concentrations of factors known to vary by maternal BMI: glucose, insulin, leptin, adiponectin, C-reactive protein (CRP), and interleukin-6 (IL-6) in a longitudinal cohort of healthy and predominantly exclusively breastfeeding women. We hypothesised that male infants would have higher milk intake than females, and that milk from mothers of male infants would have higher levels of growth and/or appetite promoting factors (glucose, insulin, and adiponectin) (Fields and Demerath 2012; Kratzsch et al. 2018), and lower levels of theoretically appetite and/or growth-inhibiting and pro-inflammatory factors (leptin, IL-6, and CRP) (Fields and Demerath 2012; Abd El-Maksoud et al. 2017; Fields et al. 2017; Saso et al. 2018; Nuss et al. 2019). Finally, we tested the modifying effect of the mother’s pre-pregnancy BMI on observed sex differences in milk composition.

Subjects and methods

Study population

Our study included participants in the Mothers and Infants Linked for Healthy Growth (MILk) study, a longitudinal cohort study of mother–infant dyads in Minneapolis/Saint Paul, MN, and Oklahoma City, OK, who intended to exclusively breastfeed for at least 3 months as described previously (Sadr Dadres et al. 2019; Tahir et al. 2019). Participants with singleton pregnancies in the second trimester were eligible for enrolment if they were between 21 and 45 years of age at the time of delivery, had a pre-pregnancy BMI between 18.5 and 40.0 kg/m², and reported an intention to exclusively breastfeed for at least 3 months. Exclusion criteria consisted of 1) any tobacco use during pregnancy and lactation, 2) alcohol use defined as >1 drink per week during pregnancy or lactation, 3) history of type 1 or type 2 diabetes or current diagnosis of gestational diabetes, or 4) presumed or known congenital birth defect. Participants who had a full-term delivery (37–42 weeks gestation) with a birthweight of 2500–4500 g and who were exclusively breastfeeding at 1-month postpartum were eligible to continue in the postpartum component of the study and provide milk samples at the 1- and 3-month postpartum follow-up visits. All participants provided written informed consent and the study protocols were approved by institutional review boards at the University of Minnesota, HealthPartners Institute for Education and Research, and the University of Oklahoma Health Sciences Centre. The MILk study is registered with clinicaltrials.gov (NCT03301753).

Of the 368 mother–infant dyads who enrolled during pregnancy and remained eligible in the post-partum period, 22 did not have breast milk samples available from either of the 1- or 3-month postpartum visits (due to a variety of reasons). Thus, a total of 346 mother–infant dyads who had breast milk samples available at either 1 or 3 months were included in the analytic sample.

Human milk collection

Mothers and infants visited the centres for follow-up at 1-month [median (25th and 75th) = 31.0 (28.0, 34.0) d] and 3-month postpartum [median (25th and 75th) = 92.0 (88.0 and 95.0) d]. Mothers were instructed to come to the test centre for a morning feeding, followed by completion of questionnaires and a collection of a milk sample. Upon arrival between 8:00 and 10:00 am, infants were weighed without clothing but including a fresh diaper on a high sensitivity weighing scale integrated into the Pea Pod (accuracy ± 2 g) (Ma et al. 2004), and then mothers were asked to feed their infant ad libitum from one or both breasts until their infant was satisfied, followed by a second weighing of the infant after the feeding, again without clothing but with the same diaper to insure that any expelled urine or faeces were included in the weights. The difference in post-feeding weight and pre-feeding weight (in grams) was determined for each infant at the 1- and 3-month visits. The test weighing method of assessing infant milk intake has been shown to be highly accurate and precise (Meier et al. 1990). The milk sample was collected at a standardised time, 2 h after the test feeding. Mothers were asked to express the entire contents of the right breast using a hospital-grade electric breast pump (Medela Symphony™, Medela Inc., McHenry,
IL). Within 20 min of collection, the milk sample was mixed, aliquoted, and stored at −80 °C.

Measurement of human milk composition

Analyses of human milk samples were conducted by the University of Oklahoma Health Sciences Centre Metabolic Research Program Laboratory. Milk fat was separated from the aqueous portion by centrifugation in preparation to assay samples of skimmed milk. Glucose was measured using the glucose oxidase method (2300 STAT Plus, Yellow Springs Instruments, Yellow Springs, OH). Insulin, leptin, adiponectin, CRP, and IL-6 were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer’s protocol, as previously reported (Fields and Demerath 2012; Whitaker et al. 2017; Sadr Dadres et al. 2019). Interassay variability was 5.6% for glucose, 6.2% for insulin, 5.1% for leptin, 5.9% for adiponectin, 5.5% for CRP, and 12.8% for IL-6. Intra-assay variability was 10.2% for glucose, 5.1% for insulin, 5.6% for leptin, 2.0% for adiponectin, 4.7% for CRP, and 9.1% for IL-6. Breast milk analyte concentrations of glucose (mg/dl), insulin (pg/ml), leptin (pg/ml), adiponectin (ng/ml), CRP (ng/ml), and IL-6 (pg/ml) were recorded at 1- and 3-month lactation. Adiponectin measurements in the 3-month samples were limited to a subset of 125 participants due to cost constraints.

Infant sex and covariates

Infant sex, maternal age, race/ethnicity, parity, infant birth-weight, and delivery mode (vaginal and caesarean section), along with pre-pregnancy height and weight were collected from the mother’s electronic medical record (EMR). Other characteristics were self-reported by mothers including annual household income. The first prenatal visit height and weight were used, limited to the range of dates recorded within 8 weeks of the estimated date of conception. Pre-pregnancy maternal BMI (kg/m²) was then calculated and categorised as normal weight (BMI 18.5 − 24.9 kg/m²), overweight (BMI 25.0−29.9 kg/m²), or obese (BMI ≥ 30 kg/m²). All infants were exclusively breastfed at 1 month. Breastfeeding status reported at the 3-month postpartum visit was defined as fully breastfeeding, mixed-feeding, or fully formula feeding. We defined full breastfeeding as maintaining breastfeeding with <24 oz of formula total since birth or the prior visit, and feeding only breast milk for the 2 weeks prior to the visit. Mixed feeding (only relevant at the 3-month visit) was defined as providing infants with >24 oz of formula since the last visit but also some breast milk. Fully formula feeding (only relevant at the 3-month visit) was defined as providing infants only formula (no breast milk) since the last visit. Observed infant weights and lengths were obtained during 1- and 3-month study visits as previously described by our group. Infant age at the time of visit was calculated as the difference between the date of delivery and the date of visit (in days).

Statistical analyses

Descriptive statistics were calculated to summarise characteristics of the study population, estimates of milk components, and estimates of infant milk intake at 1- and 3-month of lactation. Continuous variable distributions were examined by histograms, box plots, and Shapiro–Wilk normality tests. Natural log transformations were applied to the skewed distributions of the milk concentrations of insulin, leptin, adiponectin, CRP and IL-6, and geometric means and geometric standard deviations were reported. Arithmetic means and standard deviations or medians and 25th and 75th percentiles were calculated for other continuous variables as appropriate for the variable distribution. Unadjusted comparisons of milk intake and milk composition by infant sex were assessed using T-tests or Wilcoxon rank-sum tests. Frequencies and proportions were reported for categorical variables and bivariate comparisons were conducted using Chi-square or Fishers Exact tests.

Linear regression models were fit to estimate the effect of infant sex on infant milk intake at the 1-month (n = 325) and 3-month (n = 318) time points. Breast milk intake models at 3-months omitted one participant who had infant milk intake recorded but reported exclusive formula feeding and did not provide a milk sample. Modelling assumptions were assessed by examining plots of the residual vs. predicted values to confirm linearity and equal error variances. Linear mixed-effect models were used to estimate associations between infant sex and individual milk hormones and inflammatory markers at each time point (1-month: n = 342, 3-month: n = 325) accounting for assay batch as a random effect. Of the 346 participants providing milk samples at either time point, 4 did not have 1-month measures available and an additional 3 did not have 1-month adiponectin measures for analysis. Models for outcomes measured at 3-months omitted participants who did not provide milk samples at 3-months (n = 21). The interpretation of the model coefficients for the log-transformed milk analytes was facilitated by back-transforming the estimates to provide the ratio of the geometric means for males compared to females (i.e. geometric mean ratios and 95% confidence intervals [CIs]). We explored interactions with maternal pre-pregnancy weight by collapsing BMI into two categories of normal weight (NW, BMI 18.5−24.9) or overweight/obese (OW/OB, BMI ≥ 25.0) and adding interaction terms to the model. The GM ratios and 95% CIs are reported stratified by maternal weight status.

Confounding was assessed using the manual backwards selection approach, where covariates were retained in the adjusted model if removal changed the beta coefficient for infant sex by 10% or more. Covariates evaluated for confounding included maternal age (years), maternal race/ethnicity (white, other), parity (0, 1, ≥2), household income (<$30,000, $30,000−$60,000, $60,001−$90,000, >$90,000), pre-pregnancy maternal BMI (normal, overweight, and obese), mode of delivery (vaginal and caesarean section), gestational age at delivery (days), infant age at time of visit (days), study centre (OK, MN) and breastfeeding status (evaluated for 3-month measures only: fully breastfeeding, mixed feeding, and fully formula feeding). All evaluated covariates were accounted for in the models for outcomes.
met the 10% criterion for confounding and were controlled in the milk intake models. When confounding was evaluated for the association between infant sex and breast milk analyte concentrations, each covariate met the confounding criteria for at least one of the milk component models. Thus, for consistency with the intake analyses, adjusted results controlling for all evaluated covariates were also reported for the milk analytes. Finally, to check if any of the demonstrated sex differences were independent of infant size, we added birthweight (grams), infant weight (grams), and length (centimetres) at study visit as additional covariates to the models.

Sensitivity analyses were also conducted to consider the impact of breastfeeding status and outlier hormone concentrations on results. Analyses of 3-month measures were repeated after excluding mothers who did not report full breastfeeding at the 3-month visit (n = 20). Additionally, when the highest value of a milk hormone or inflammatory marker concentration was at least 2-fold greater than the preceding rank-ordered value, we defined this as an outlier and conducted sensitivity analyses after removing these extreme values (n = 4). This examined the impact of high maximum values for, adiponectin at three months (172.11 ng/ml), IL-6 at one month (1084.24 pg/ml), IL-6 at three months (514.87 pg/ml), and leptin at three months (6575.70 pg/ml). All analyses were conducted using SAS version 9.4 software (SAS Institute Inc., Cary, NC).

Results

Descriptive characteristics

Characteristics of the study population are presented in Table 1. The majority of participants were white (84.8%). When examining pre-pregnancy BMI, 45.8% of mothers were normal weight, 31.6% were classified as overweight, and 22.6% were obese. There were no statistically significant differences in maternal, sociodemographic, or delivery characteristics in male and female infants. The expected sexual dimorphism in size was observed, however. For example, female infants weighed, on average, less than male infants at birth (p = .01) and at 1 and 3 months (p < .0001). Similarly, female infants were shorter at both 1 and 3 months than were male infants (p < .0001).

| Table 1. Baseline characteristics of the study population (n = 346). |
|---------------------------------------------------------------|
| Male (n = 175) | Female (n = 171) | Total (n = 346) | p Value |
| Maternal race/ethnicity: | | | |
| Non-Hispanic white | 147 | 85.0 | 138 | 84.7 | .94<sup>b</sup> | 285 | 84.8 |
| Missing | 2 | 8 | 10 |
| Delivery mode | | | | |
| Vaginal delivery | 136 | 78.2 | 136 | 81.9 | .38<sup>b</sup> | 272 | 80.0 |
| Caesarean section | 38 | 21.8 | 30 | 18.1 | 68 | 20.0 |
| Missing | 1 | 5 | 6 |
| Pre-pregnancy body mass index | | | | |
| Normal (< 24.9 kg/m<sup>2</sup>) | 78 | 44.8 | 80 | 44.8 | .10<sup>b</sup> | 158 | 45.8 |
| Overweight (25.0 – 29.9 kg/m<sup>2</sup>) | 63 | 36.2 | 46 | 26.9 | 109 | 31.6 |
| Obese (≥30 kg/m<sup>2</sup>) | 33 | 19.0 | 45 | 26.3 | 78 | 22.6 |
| Missing | 1 | 0 | 1 |
| Household income | | | | |
| <$30,000 | 18 | 10.8 | 12 | 7.2 | .07<sup>b</sup> | 30 | 9.0 |
| $30,000–$60,000 | 40 | 23.9 | 37 | 22.3 | 77 | 23.1 |
| $60,000–$90,000 | 49 | 29.3 | 29 | 17.6 | 84 | 25.2 |
| > $90,000 | 60 | 35.9 | 82 | 49.4 | 142 | 42.6 |
| Missing | 8 | 5 | 13 |
| Parity | | | | |
| 0 | 48 | 27.4 | 51 | 30.2 | .85<sup>b</sup> | 99 | 28.8 |
| 1 | 72 | 41.1 | 67 | 39.6 | 139 | 40.4 |
| 2 or more | 55 | 31.4 | 51 | 30.2 | 106 | 30.8 |
| Missing | 0 | 2 | 2 |
| Centre | | | | |
| Minnesota | 108 | 61.7 | 123 | 71.9 | .04<sup>b</sup> | 231 | 66.8 |
| Oklahoma | 67 | 38.3 | 48 | 28.1 | 115 | 33.2 |
| Breastfeeding status at 3 months | | | | |
| Fully breastfeeding | 159 | 93.5 | 150 | 91.5 | .15<sup>c</sup> | 309 | 92.5 |
| Mixed feeding | 10 | 6.1 | 11 | 6.5 | 21 | 6.3 |
| Formula feeding | 0 | 0 | 0 | 0 | 4 | 1.2 |
| Missing | 5 | 7 | 12 | | | |
| Mean | | | | |
| Gestational age (weeks) | 39.7 | 1.1 | 39.8 | 1.0 | .25<sup>d</sup> | 39.7 | 1.1 |
| Maternal age at delivery (years) | 30.5 | 4.2 | 30.9 | 4.1 | .37<sup>d</sup> | 30.7 | 4.2 |
| Birth weight (g) | 3572.3 | 427.3 | 3461.5 | 405.7 | .01<sup>d</sup> | 3517.9 | 419.9 |
| Weight (g) 1 month | 4617.1 | 595.4 | 4317.5 | 500.2 | <.0001<sup>a</sup> | 4470.0 | 570.0 |
| Length (cm) at 1 month | 54.9 | 2.2 | 54.0 | 2.1 | .001<sup>d</sup> | 54.4 | 2.2 |
| Weight (g) 3 months | 6371.1 | 779.1 | 5854.3 | 551.2 | <.0001<sup>a</sup> | 6119.0 | 724.3 |
| Length (cm) at 3 months | 61.4 | 2.3 | 59.8 | 2.1 | <.0001<sup>d</sup> | 60.7 | 2.4 |

<sup>a</sup>Percentages are reported for non-missing values; <sup>b</sup>Chi-square test; <sup>c</sup>Fishers exact test; <sup>d</sup>T-test for independent means (equal variances); <sup>a</sup>T-test for independent means (unequal variances). Bold values represent p < .05.
Table 2. Mean human milk intake and human milk components by month of visit and infant sex.

|                        | 1-Month visit | 3-Month visit |
|------------------------|---------------|---------------|
|                        | Male infants  | Female infants|
| Infant breast milk intake (g) | 84.8 (34.5)  | 80.4 (32.5)  |
| Glucose (mg/dl)        | 31.1 (10.5)   | 28.8 (9.9)    |
| GM (GSD)               | GM (GSD)      | p             |
| Insulin (pg/ml)        | 510.23 (1.9)  | 982.4 (2.0)   | .60 |
| Leptin (pg/ml)         | 473.4 (2.2)   | 512.9 (2.3)   | .35 |
| Adiponectin (ng/ml)    | 18.2 (1.4)    | 19.1 (1.5)    | .27 |
| C-reactive protein (mg/ml) | 94.6 (3.1) | 97.5 (3.2)   | .84 |
| Interleukin-6 (pg/ml)  | 5.5 (4.7)     | 6.2 (4.1)     | .39 |
| GM (GSD)               | GM (GSD)      | p             |

Mean (SD) Mean (SD) Mean (SD) Mean (SD) p

|                        | Male infants  | Female infants|
|------------------------|---------------|---------------|
| 1-Month visit          | 91.2 (46.8)   | 92.5 (44.7)   | .80 |
| 3-Month visit          | 29.9 (10.4)   | 29.8 (9.2)    | .88 |

SD: standard deviation; GM: geometric mean; GSD: geometric standard deviation

Infant sex and infant human milk intake

Infant milk intake was assessed at a standard morning test feed at both 1 and 3 months. Crude (Table 2) and multivariate-adjusted mean values (Table 3) show that there were no sex differences in infant milk intake between male and female infants at either time point. When controlling for maternal and infant characteristics, human milk intake at 1-month post-delivery was, on average, 5.14 g higher for male infants compared to female infants (95% CI: 0.42, 9.92), but the CI crossed zero. At 3-months milk consumption for males was 0.07 g higher than females (95% CI: -0.18, 0.22) when controlling for the same covariates but the CI crossed zero. As there were no significant sex differences after covariate adjustment, infant size metrics were not included in models for milk intake.

Infant sex and human milk composition

Mean glucose concentrations at 1-month were 2.3 mg/dl higher for mothers feeding male infants compared to females (p = 0.04, Table 2). This difference persisted after adjusting for maternal and infant characteristics (mean difference 2.62 mg/dl (95% CI 0.42, 4.83)) and the CI excluded zero (Table 3). Unadjusted and adjusted differences in mean glucose concentrations were less than one gram at 3-month lactation, and no longer statistically significant. For the other milk components, crude geometric mean concentrations of insulin, leptin, adiponectin, CRP, and IL-6 were similar for males and females at both 1 and 3 months (Table 2). This similarity remained after covariate adjustment, as shown by geometric mean ratios for male compared to female infants that were near 1.0 for all components (Table 3), indicating no sex difference. The 1-month estimates indicated concentrations of these components were, on average, 1–14% lower among males compared to females, with the strongest sex effect seen for IL-6, while the geometric mean concentrations of insulin at 1-month lactation were on average 4% higher among males compared to females. However, none of the CIs for these associations excluded 1.0, indicative of no statistically significant difference by sex. Additionally, at 3-months the GM ratios remained near 1.0 for all milk components (insulin, leptin, adiponectin, CRP, and IL-6). In sensitivity analyses excluding outliers for leptin, adiponectin, and IL-6 and when restricting the sample to those reporting only fully breast milk feeding at 3-months, the findings remained essentially unchanged (Supplemental Tables S1 and S2).

Sex differences in milk intake and composition by maternal weight status

When evaluating interactions between infant sex and maternal pre-pregnancy weight status on the outcomes, we found limited evidence that conclusions regarding sex differences differed by maternal weight (Table 4). Although the observed association between male sex and glucose concentrations appeared to be strengthened when mothers were OW/OB (mean difference: 3.58 mg/dl; 95% CI 0.72, 6.43), the observed association within NW mothers was in the same direction (mean difference: 1.46 mg/dl; 95% CI -1.88, 4.79) with largely overlapping CIs for the stratum-specific estimates (p for interaction = .30). Box plots displaying the distribution of breast milk glucose concentrations by infant sex and maternal BMI are shown in Figure 1. Infant sex differences in adiponectin concentrations at 3-months appeared to vary by maternal weight in our main analysis (p for interaction = .04), with male infants having 12% lower adiponectin concentrations than females among NW mothers (GM ratio 0.88, 95% CI 0.75–1.02) but 14% higher concentrations among OW/OB mothers (GM ratio 1.14, 95% CI 0.92–1.42).
higher in males than females; GM Ratio (95% CI) = 1.0 indicates levels lower in males than females. However, this apparent relationship within this smaller subgroup with available adiponectin measures did not hold up in sensitivity analyses that excluded the single outlying value for adiponectin or that excluded dyads who did not exclude the null value and largely overlapped. Sensitivity analyses excluding the few outlying concentrations and restricting analyses to fully breastfeeding infants at 3-months resulted in consistent conclusions of no interaction between infant sex and maternal weight on milk intake or composition (Supplemental Tables S3 and S4).

### Discussion

In this large prospective study of 346 mother–infant dyads, human milk intake, human milk appetite/growth-regulating hormones, and human milk pro-inflammatory cytokines did not differ meaningfully for male and female infants, contrary to our hypotheses, nor were the main effects of infant sex modified by our marker of maternal nutritional condition (pre-pregnancy BMI status). Mean glucose concentration in

### Table 3. Comparisons of human milk intake and bioactive components by infant sex.

| Outcome | 1-Month Visit | 3-Months Visit |
|---------|---------------|----------------|
| Breast milk intake (g) | 4.32 (3.0, 11.64) | 5.14 (2.41, 12.69) |
| Glucose (mg/dl) | 2.22 (0.66, 4.38) | 2.62 (0.42, 4.83) |
| Linear mixed models GM ratio (95% CI) | Unadjusted | Adjusted |
| Insulin (pg/ml) | 1.04 (0.90, 1.20) | 1.04 (0.90, 1.20) |
| Leptin (pg/ml) | 0.92 (0.77, 1.09) | 0.92 (0.78, 1.01) |
| Adiponectin (ng/ml) | 0.99 (0.80, 1.29) | 0.94 (0.76, 1.17) |
| Interleukin-6 (pg/ml) | 0.85 (0.62, 1.15) | 0.86 (0.63, 1.19) |

### Table 4. Adjusted associations between infant sex and outcomes of human milk intake and milk composition stratified by maternal pre-pregnancy body mass index.

| 1-Month visit | Normal weight mothers | Overweight/obese mothers | p for interaction |
|---------------|------------------------|--------------------------|------------------|
| Breast milk intake (g) | 3.29 (2.13, 4.45) | 3.43 (2.28, 4.58) | .59 |
| Glucose (mg/dl) | 1.46 (1.22, 1.70) | 1.48 (1.24, 1.72) | .30 |
| Linear mixed models GM ratio (95% CI) | Unadjusted | Adjusted |
| Insulin (pg/ml) | 0.96 (0.78, 1.18) | 1.00 (0.82, 1.21) |
| Leptin (pg/ml) | 0.84 (0.69, 1.02) | 0.85 (0.69, 1.04) |
| Adiponectin (ng/ml) | 0.94 (0.84, 1.05) | 0.96 (0.85, 1.08) |
| Interleukin-6 (pg/ml) | 0.91 (0.65, 1.26) | 0.95 (0.70, 1.29) |

| 3-Month visit | Coefficient (95% CI) | p for interaction |
|---------------|----------------------|------------------|
| Breast milk intake (g) | 0.45 (−0.18, 1.78) | −0.16 (−1.37, 1.14) | .91 |
| Glucose (mg/dl) | 1.17 (1.39, 3.74) | 0.27 (−2.88, 3.43) | .59 |
| Linear mixed models GM ratio (95% CI) | Unadjusted | Adjusted |
| Insulin (pg/ml) | 1.06 (0.85, 1.33) | 0.97 (0.78, 1.20) |
| Leptin (pg/ml) | 0.82 (0.65, 1.03) | 0.84 (0.67, 1.05) |
| Adiponectin (ng/ml) | 0.88 (0.75, 1.02) | 1.14 (0.92, 1.42) |
| Interleukin-6 (pg/ml) | 1.11 (0.79, 1.55) | 0.73 (0.53, 1.02) |

*Female are defined as the reference group.

Linear regression model coefficients represent mean differences in infant breast milk intake for males compared to females.

Linear mixed model coefficients for breast milk components were estimated with random effects for laboratory batch.

Geometric mean ratios are reported for log-transformed breast milk components by back-transforming the model coefficient; GM Ratio > 1.0 indicates levels higher in males than females; GM Ratio < 1.0 indicates levels lower in males than females.

Adjusted for centre, maternal race/ethnicity, delivery mode, parity, BMI categories, income, maternal age, infant age at 1-month, and gestational age (weeks).

Adjusted for centre, maternal race/ethnicity, delivery mode, parity, BMI categories, income, maternal age, infant age at 3 months, gestational age (weeks), and breastfeeding status at 3 months.
Human milk intake

Hypotheses about sex-based biologic investment through early life nutrition have prompted animal and human investigations of sex differences in lactation. Lactation is an energetically costly function, and under this hypothesis, milk composition may differ by sex of the offspring depending on maternal nutritional and environmental status (Hinde et al. 2014). Studies examining sex-based lactation among mammals have assessed milk yield and milk composition in rhesus macaques and dairy cows. Hinde (2009) reported that milk yield in rhesus macaques was higher in daughters than sons, but was offset by the production of higher gross energy (“richer milk”) for male offspring (Hinde 2009). Among dairy cows, Hinde et al. (2014) also reported a 1.3% increased milk yield following birth of a female vs. a male offspring (Hinde et al. 2014). Human studies of sex differences in human milk intake are limited. Da Costa et al. (2010) examined sex differences in human milk consumption among infants 0.4–24 months old (with an average age of 5.2 months) by pooling 1106 measurements from 737 mother–infant dyads across 16 international studies that used stable isotope tracer methodology to measure milk intake over a 14-d time period (Da Costa et al. 2010). Their study found that the median daily milk intake for male infants was approximately 5% higher than female infants; however, the comparisons by infant sex did not adjust for infant age at measurement, mode of delivery, indicators of infant size, or other maternal or infant characteristics including exclusive vs. partial breastfeeding status (Da Costa et al. 2010). The authors attribute the observed difference to the possible influence of both biological and behavioural factors, where male infants have more lean mass than females across infancy, which may influence intake levels, and mothers may feed males differently if assumed to have greater energy requirements (Da Costa et al. 2010). In contrast, mean human milk intake in our study did not differ significantly by infant sex. While the estimated average intake at one month was 4 g greater for males, and 5 g greater for males after adjusting for maternal and infant characteristics, the CIs did not exclude the null value. Furthermore, when examining human milk intake at 3 months, unadjusted mean intake estimates were more similar between male and female infants, and indicated a less than 1 g higher intake for male infants after covariate adjustment. Differences in milk intake assessment likely contributed to these inconsistencies; Da Costa et al. (2010) utilised deuterium-labelled water method, which is considered the gold standard, and measured consumption over a 14-d time period whereas our study utilised infant test-weighing during only a single feeding at each time point. The theoretically greater measurement error around true total milk intake in our study therefore may have resulted in misclassification and thereby a bias towards the null hypothesis of no sex difference.

In summary, the evidence in humans for differential maternal investment in male vs. female infants in terms of amount of milk provided indicates differences are small, and inconsistent across studies. This conclusion is based on few studies to have examined the question to date, and in a wide range of populations which may or may not reflect the
types of environmental forces that would reveal the hypothesised phenomenon. Also, human studies suffer from the difficulty in accurately disarticulating differences in maternal supply for male and female infants from infant milk intake differences, which are not only determined by supply but also by infant-specific appetite, sleep, growth, and feeding behaviours.

**Human milk glucose**

The one milk component that was observed to have a small sex difference in our study was glucose, which was observed to be approximately 8% higher at 1 month in milk made for male infants than for female infants. This finding withstood covariate adjustment and was also more evident in women with overweight and obesity than in women with normal body weight status prior to pregnancy. The specific source of glucose concentrations in human milk is unclear, but Neville found milk glucose to be proportional to the rate of milk secretion (Neville et al. 1990), with greater glucose thereby indicative of higher milk volume. For that reason, we would expect, according to the differential maternal investment hypothesis, higher milk glucose in milk made for male infants in settings of nutritional sufficiency/abundance, as in this study. Further, higher concentrations of glucose have been observed in the colostrum (Fujimori et al. 2015) and mature human milk (Ahuja et al. 2011) of overweight and obese mothers compared to normal-weight mothers. It is notable that in our study the magnitude of the male-related increase in glucose concentrations was greatest among OW/OB mothers than among NW mothers. This finding is compatible with the observation that maternal pre-pregnancy obesity or overweight is associated with a 3 times greater risk of obesity among sons, but not daughters (Bridgman et al. 1998; Das 2001; Wang X et al. 2013). The measurement of human milk carbohydrates in general is characterised by variability (Neville et al., 2013), little is known about factors affecting insulin levels in human milk. Insulin is a hormone involved in blood glucose homeostasis and is also characterised as having appetite-suppressing effects (Pliquett et al. 2006). While insulin concentrations in human milk are well-documented (Kulski and Hartmann 1983), and insulin is known to play a critical role in lactation at the level of the lactocyte (Neville et al, 2013), little is known about factors affecting insulin levels in human milk. CRP and IL-6 are markers of inflammation, circulating levels of which are elevated in chronic states of inflammation and are associated with loss of lean body mass (Papanicolaou et al. 1998; Das 2001; Wang X et al. 2013).

Although studies specifically designed to address sex-difference in these bioactive human milk compounds were not identified, we located three studies that reported on sex differences while addressing other aims related to human milk...

**Human milk hormones and cytokines**

Research is emerging evaluating the significance of non-nutritive bioactive components of human milk, with key appetite-regulating hormones and inflammatory markers gaining attention for their potential role in infant growth and adiposity. The most frequently studied non-nutritive components in human milk are adiponectin and leptin, which are hormones controlling food intake. Early exposure to adiponectin and leptin through breast milk is believed to influence infant appetite regulation and metabolic programming (Hassiotou and Geddes 2014; Badillo-Suárez et al. 2017; Larson-Meyer et al. 2021). This is supported by epidemiologic evidence indicating that measures of infant body weight and adiposity are inversely related to human milk leptin concentrations and positively associated with adiponectin, although conflicting findings exist (Schuster et al. 2011; Fields et al. 2017; Kratzsch et al. 2018; Palou et al. 2018; Larson-Meyer et al. 2021).

We found no observed sex differences in milk leptin or adiponectin. Initially, we found an apparent interaction of maternal weight status with infant sex on 3-month adiponectin concentrations, such that adiponectin concentrations among NW mothers were 12% lower when the offspring were male compared to female, while concentrations were 14% higher for male infants when the mothers were OW/OB. Nonetheless, neither stratum-specific CI excluded the null value, and our sensitivity analyses revealed that the observed effect was likely spurious. Similarly, a Finnish study evaluating human milk concentrations of leptin and adiponectin reported no differences by infant sex when examining milk samples collected approximately 3 months after birth from 501 mothers (Galante et al. 2020). In additional analyses evaluating conditional effects of infant sex on milk composition, Galante et al. observed evidence of a potential interaction between infant sex and maternal factors (Galante et al. 2020). As in this analysis, they found no significant interactions between maternal pre-pregnancy BMI and infant sex on milk leptin or adiponectin. Thus, in the two human studies that specifically aimed to address the question of sex-related differences in breast milk leptin and adiponectin concentrations, no significant differences have been found.

Ours was the first study to our knowledge designed to assess sex differences in insulin, IL-6, and CRP in human milk. Insulin is a hormone involved in blood glucose homeostasis and is also characterised as having appetite-suppressing effects (Pliquett et al. 2006). While insulin concentrations in human milk are well-documented (Kulski and Hartmann 1983), and insulin is known to play a critical role in lactation at the level of the lactocyte (Neville et al, 2013), little is known about factors affecting insulin levels in human milk. CRP and IL-6 are markers of inflammation, circulating levels of which are elevated in chronic states of inflammation and are associated with loss of lean body mass (Papanicolaou et al. 1998; Das 2001; Wang X et al. 2013).
composition. Consistent with our aggregate conclusions, none of these studies reported evidence of sex dimorphisms for the human milk hormones (insulin, leptin, and adiponectin) or inflammatory markers (CRP and IL-6) examined here. For example, Sims et al. (2020) reported that infant sex was not associated with leptin, insulin, and CRP concentrations or macronutrient content (Sims et al. 2020). In their cohort of 174 healthy, full-term infants and their mothers, morning breast milk samples were collected at 8-time points beginning at 0.5 months, providing an extensive longitudinal assessment of this relationship. The estimates for infant sex, however, were not shown. Similarly, a small study examining maternal adiposity in 25 exclusively breastfeeding, non-diabetic mothers in Alabama reported that 1-month concentrations of adiponectin, leptin, and insulin did not differ by infant sex (Schneider-Worthington et al. 2020). In an additional study evaluating immune markers in human milk samples from 115 mother–infant dyads in South Carolina (Burch et al. 2013), infant sex was among several perinatal characteristics examined that also included maternal asthma, maternal infections, maternal smoking, maternal demographics, and season of birth. Human milk samples were collected an average of 3 weeks after delivery (range of 1–8 weeks). Consistent with our findings, Burch et al. (2013) reported no statistical differences in IL-6 concentrations by sex of the infant.

In summary, we found no consistent or biologically meaningful differences between male and female infants in the concentrations of milk leptin, insulin, adiponectin, IL-6, or CRP and these findings were in line with a small number of other reports.

Strengths and limitations

In addition to our large cohort of mother-infant dyads, a major strength of this study is the use of rigorous, standardised methods for human milk collection, which controlled for diurnal variation and lactation stage (Ballard and Morrow 2013) by timing a morning collection of one entire breast using a standardised hospital grade pump precisely 2 h after the previous breastfeeding. Additionally, our analyses controlled for numerous confounding factors and accounted for clustering by assay batch. A limitation of our study is the relatively homogeneous racial and ethnic composition of our study population, which included predominantly non-Hispanic white women in the United States. Thus, the generalisability of our findings to minority populations may be limited. The infant test weighing method for assessing milk intake, while a valid estimate of the infant milk intake during the test feed itself, may not accurately capture 24-h routine intake. Maternal prandial state was not controlled by fasting prior to milk collection; thus, it is possible that glucose variability could have been introduced if feeding patterns for males and female infants systematically influenced maternal food intake patterns based on related energy needs. Additionally, the smaller number of available adiponectin measurements at 3 months reduced statistical power to detect associations of small magnitude for this component. Despite these limitations, the study does provide one of the more rigorous assessments of sex differences in human milk appetite and growth-regulating hormones and cytokines given its relatively large sample size, repeated milk sampling, and controlled study design. We would argue therefore that the study does assist in understanding the “how much” question regarding the magnitude of sex differences in some specific human milk hormones and adipokines in a healthy United States population sample. What is less clear is whether this study can address the “how and why” question regarding these sex differences. Evolutionarily shaped phenomena, including the putative differential signalling via human milk for optimising growth for male and female infants of interest here, may not be detectable within a generally healthy population of United States women with prenatal care, high dietary quality, and of predominantly moderate to high socioeconomic status. It is likely that the inherited human-environmental signal detection system, geared to promote optimal reproductive outcomes, like the hypothesised differential shaping of milk for boys and girls, may be obscured when the environmental-quality signals that we are adapted to detect are relatively subtle or mismatched.

Conclusion

This study adds to the limited research evaluating sex differences in non-nutritive human milk composition and milk intake. Our findings indicate that in a well-nourished population, the maternal–infant dyad in the first month following birth may exhibit greater milk glucose concentrations for male infants. This sex differential in milk glucose may be more prominent when mothers are overweight or obese, but these differences did not remain evident at 3-month post-delivery. This observation of early sex-related milk glucose concentrations is consistent with the hypothesis of increased biological investment in male infants in an environment with plentiful nutritional resources. However, the relatively small magnitude and transience of the sex-related glucose pattern, and the absence of notable sex differences in human milk intake and concentrations of appetite-regulating hormones and inflammatory factors, do not provide broad support for the idea that these particular aspects of milk composition and lactation are strongly shaped by infant sex. Additional work is needed on sex differences in other aspects of milk, including milk exosomal miRNAs and the myriad of growth-regulating factors now being discovered in human milk.

Acknowledgements

The authors acknowledge and thank all the women and health care providers who contributed to the MILK study and the MILK study teams, including Neely Miller and Kristin Sandness from the Center for Neurodevelopmental Behavior and Rebecca Hollister from the Center for Pediatric Obesity at the University of Minnesota, the University of Oklahoma Health Sciences Center, and the HealthPartners Institute. A special thank you to Laurie Foster and the Clinical and Translational Research Services support team at the Clinical and Translational Science Institute at the University of Minnesota.
Disclosure statement
The authors disclose research funding from the National Institutes of Health (R01HD080444, U54GM104938, and T32DK083250).

Funding
This work was supported by the National Institute of Child Health and Human Development [R01HD080444] and the National Institute of General Medical Sciences [U54GM104938] of the National Institutes of Health. EMN was supported by the National Institute of Diabetes and Digestive and Kidney Diseases [T32DK083250] and supported by grant number UL1TR002494 from the National Institutes of Health’s National Center for Advancing Translational Sciences. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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