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ORIGINAL ARTICLE

The relationship between bioactive components in breast milk and bone mass in infants

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Human breast milk (HBM) contains numerous bioactive components, recently shown to be associated with growth and body composition in breastfed offspring. Reciprocity in adipogenic and osteogenic pathways suggests bone mass may also be influenced by these components. The association between bioactive components found in HBM and bone mineral content (BMC), to our knowledge, is unknown. The purpose of this proof-of-principle study was to evaluate the association between specific bioactive components in HBM in exclusively breastfed infants and skeletal health in the first 6 months of life and examine potential gender differences in these associations. Thirty-five mother–infant dyads were followed from 1 to 6 months. The contents of a single breast expression were used for analyses of bioactive components (insulin, glucose, leptin, interleukin-6 and tumor necrosis factor-α (TNFα), whereas BMC was evaluated by dual-energy X-ray absorptiometry. In the total sample, there was a positive association between TNFα and BMC at 1 ($P = 0.004$) and 6 months ($P = 0.007$). When stratified by sex, females exhibited a positive association between BMC and glucose and an inverse relationship between BMC and TNF-α at 1 month with TNF-α strengthening ($P = 0.006$) at 6 months. In males, at 6 months a positive relationship between BMC and HBM glucose and an inverse relationship with HBM leptin were observed with no associations observed at 1 month. Although preliminary, the associations between bioactive components in HBM highlight the importance HBM has on bone accretion. It is critically important to identify factors in HBM that contribute to optimal bone health.

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INTRODUCTION

Beyond the specific and nonspecific immunological defenses, functional interactions between bioactive factors in human breast milk (HBM) and growth and development in the first months of life[1–5] have been observed. Studies have largely centered on the association of growth, satiation and inflammatory factors and hormones in HBM with fat mass as a potential link between maternal factors and risk childhood obesity. These studies suggest that through transfer of some bioactive components in HBM, adipogenic pathways in the offspring may be activated or suppressed. The strongest evidence for a role of bioactive components in HBM and adipogenesis is for maternal glucose regulation and leptin.[4,6] Further, cytokines (for example, interleukin-6 (IL-6), tumor necrosis factor-alpha (TNFα)) have been associated with increased adiposity in infants,[7–9] children[10,11] and adults.[12] Given the purported antagonistic pathways linking adipogenesis and osteogenesis,[13–15] in the context of the rapid increases in linear growth and fat mass gain during the first 6 months of life, we hypothesize that bioactive factors (glucose, insulin, leptin, IL-6 and TNFα) in HBM may be have a role in bone health.

The relationship between bioactive components in HBM and bone health, to our knowledge, has not been examined. During the first 6 months of life, there is approximately a 50% increase in periosteal apposition (that is, external bone size); yet, little is known about factors that affect mineral accretion during this particularly active time of bone modeling. In general, the evaluation of dynamic changes in bone parameters in infancy is limited to outcome differences (that is, bone mineral content (BMC) and bone mineral density (BMD)) between breast- and formula-fed infants. Although the causal factor(s) remain unknown, breastfed infants have been reported to have higher BMC at 3 months,[16] whereas slower mineralization and

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maturation of bone at 2–4 months in formula-fed infants relative to breastfed infants has been observed.[17–20] Investigation of the non-nutritive factors could provide long-term bone health, which can be influenced in early life, particularly during the proximate postnatal period (that is, the first 6 months of life).

Thus, the purpose of this study was to evaluate the contribution of specific bioactive components in HBM (glucose, insulin, leptin, IL-6 and TNF-α) in breastfed infants that may influence skeletal health in the first 6 months of life. We have chosen these factors on the basis of the a priori association with fat mass gain in infants. Because linear growth patterns and body tissue partitioning may differ by sex, a secondary objective was to determine whether there were sex-specific associations between bioactive components in HBM and bone parameters.

Results

The pre-pregnancy characteristics of the mothers, concentration of bioactive components in HBM expressed at 1 and 6 months and infant anthropometrics and body composition variables at 1 and 6 months in the total sample as well as stratified according to offspring sex are presented in **Table 1**. Maternal characteristics did not differ in those having male or female offspring. Although boys were longer, no significant differences in BMC were observed at 1 month between males and females. Length remained greater in boys, and at 6 months BMC was higher in males ($P<0.01$). At 1 month, HBM from mothers of female offspring had greater insulin ($P<0.05$) and TNF-α ($P<0.05$) concentration than that of mothers of males. At 6 months, HBM from mothers of male offspring had greater glucose ($P<0.05$) concentration than that of female offspring.

Multiple linear regression revealed an inverse association between BMC and TNF-α in at 1 month ($P=0.05$) and a positive association ($P=0.007$) at 6 months in the total sample. When stratified by sex, there were marginal relationships observed between BMC and HBM glucose and BMC and TNF-α only in female offspring at 1 month. There were no associations detected between BMC and HBM at 1 month in male offspring. At 6 months, the inverse relationship between BMC and TNF-α in females strengthened ($P=0.007$), whereas a marginal association between BMC and HBM glucose and BMC and HBM leptin ($P=0.07$) was observed in males (**Table 2**). **Figure 1** illustrates the change (1 to 6 months) in BMC (**Figure 1a**) and the change in bioactive components in HBM (**Figures 1b and c**; expressed at 1 month vs concentration at 6 months) after adjusting for baseline BMC. Boys gained more BMC relative to girls ($P<0.05$). Circulating concentrations of insulin, leptin and IL-6 differed in HBM of male and female offspring ($P<0.05$, all). **Table 3** reports the linear regression analysis evaluating the association between the change in BMC from 1 to 6 months and the change in concentration of bioactive components of HBM from 1 to 6 months after adjusting for infant sex (in the total sample), infant weight change from 1 to 6 months and mother's

Table 1 Descriptive characteristics in the total sample and stratified by offspring sex (mean + s.e.)

Maternal characteristics	*Total sample (n = 35)*	*Males (n = 20)*	*Females (n = 15)*
Mother's age	29.1 ± 0.9	28.9 ± 1.2	29.7 ± 1.4
Mother's pre-pregnancy weight (kg)	72.9 ± 3.5	74.2 ± 5.2	72.0 ± 4.7
Pre-pregnancy BMI	26.8 ± 1.2	26.9 ± 1.7	27.0 ± 1.7
Infant characteristics		1 month	
Infant age (day)	39.3 ± 0.6	39.1 ± 0.9	39.4 ± 1.0
Infant weight (kg)	4.7 ± 0.1	4.9 ± 0.2*	4.4 ± 0.1
Infant weight change from birth (mean g^{-1} day^{-1})	1.1 ± 0.1	1.2 ± 0.1	1.0 ± 0.1
Infant length (cm)	55.5 ± 0.4	56.3 ± 0.6*	54.6 ± 0.4
Infant total fat (kg)	1.2 ± 0.1	1.2 ± 0.1	1.2 ± 0.1
Infant percent body fat	24.2 ± 0.5	23.6 ± 0.6	24.9 ± 0.7
Infant trunk fat (kg)	0.4 ± 0.02	0.4 ± 0.03	0.4 ± 0.03
Infant lean body mass (kg)	3.6 ± 0.1	3.8 ± 0.1*	3.4 ± 0.1
Infant BMC (g)	88.8 ± 3.4	91.5 ± 5.1	85.4 ± 4.0
HBM glucose (mg dl^{-1})	25.4 ± 1.52	27.4 ± 1.9	22.92 ± 2.37
HBM insulin (pg ml^{-1})	802.4 ± 132.4	527.5 ± 66.04*	1146.1 ± 266.2
HBM leptin (pg ml^{-1})	906.2 ± 119.9	836.7 ± 128.8	993.2 ± 219.8
HBM IL-6 (pg ml^{-1})	7.42 ± 1.7	5.07 ± 1.3	10.21 ± 3.3
HBM TNFα (pg ml^{-1})	5.8 ± 0.9	4.12 ± 1.04*	7.81 ± 1.4
		6 Months	
Infant age (day)	167.7 ± 1.0	166.8 ± 1.5	169.2 ± 0.6
Infant weight (kg)	7.1 ± 0.23	7.4 ± 0.3	6.6 ± 0.4
Infant weight change from birth (mean g^{-1} day^{-1})	3.5 ± 0.2	3.7 ± 0.2	3.2 ± 0.4
Infant length (cm)	65.1 ± 0.5	66.1 ± 0.6**	63.1 ± 0.7
Infant total fat (kg)	2.4 ± 0.1	2.5 ± 0.14	2.3 ± 0.2
Infant percent body fat	32.4 ± 0.7	32.4 ± 0.8	32.4 ± 1.5
Infant trunk fat (kg)	0.8 ± 0.6	0.8 ± 0.1	0.7 ± 0.1
Infant lean body mass (kg)	4.8 ± 0.1	5.0 ± 0.2*	4.5 ± 0.2
Infant BMC (g)	134.1 ± 3.8	141.8 ± 4.9**	121.5 ± 4.0
HBM glucose (mg dl^{-1})	25.7 ± 1.4	27.3 ± 1.3	23.08 ± 3.1
HBM insulin (pg ml^{-1})	786.2 ± 184.3	584.2 ± 111.4	1116.7 ± 445.7
HBM leptin (pg ml^{-1})	700.9 ± 708.8	678.93 ± 178.0	736.95 ± 199.0
HBM IL-6 (pg ml^{-1})	6.4 ± 1.8	5.8 ± 2.4	7.4 ± 2.8
HBM TNFα (pg ml^{-1})	6.2 ± 1.2	5.6 ± 1.32	7.2 ± 2.2

Abbreviations: BMC, bone mineral content; HBM, human breast milk; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha.
Asterisks denote statistically significant differences between male and female offspring; *$P<0.05$, **$P<0.01$.

Table 2 The relationship between BMC and bioactive components in HBM at 1 month and 6 months after controlling for infant sex (in total sample), weight change and mother's pre-pregnancy

	Total sample (n = *35*)		*Males* (n = *20*)		*Females* (n = *15*)	
	β	P-*value*	β	P-*value*	β	P-*value*
1 Month						
HBM glucose (mg dl^{-1})	0.10	0.44	0.02	0.93	0.45	*0.09*
HBM insulin (pg ml^{-1})	−0.11	0.49	−0.23	0.19	−0.42	0.21
HBM leptin (pg ml^{-1})	−0.05	0.78	−0.22	0.31	−0.01	0.98
HBM IL-6 (pg ml^{-1})	0.05	0.74	0.23	0.25	−0.19	0.50
HBM TNFα (pg ml^{-1})	−0.29	**0.05**	0.07	0.71	−0.52	*0.07*
6 Months						
HBM glucose (mg dl^{-1})	0.11	0.42	0.33	*0.10*	0.15	0.57
HBM insulin (pg ml^{-1})	0.08	0.53	−0.08	0.69	0.15	0.66
HBM leptin (pg ml^{-1})	−0.29	*0.07*	−0.45	*0.07*	0.07	0.83
HBM IL-6 (pg ml^{-1})	0.14	0.21	0.14	0.39	−0.08	0.76
HBM TNFα (pg ml^{-1})	0.31	**0.007**	0.03	0.86	−0.76	**0.006**

Abbreviations: β, standardized parameter estimate; BMC, bone mineral content; HBM, human breast milk; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha. A significant *P*-value ($P < 0.05$, bold) indicates an independent association of bioactive component in HBM on BMC. Italicized *P*-values indicate a marginal relationship $0.10 \leqslant P > 0.05$.

pre-pregnancy body mass index (BMI). In the total sample, increased BMC was associated with lower HBM leptin concentration ($P = 0.003$) and greater TNF-α concentration ($P = 0.009$), which after stratifying by sex, significance remained only in males ($P = 0.001$, 0.02, respectively).

Discussion

The association between the bioactive components of HBM (glucose, insulin, leptin, IL-6 and TNF-α) and body composition in the infant suggests functional relevance of concentrations in HBM in infant growth and development. In particular, maternal glucose/insulin homeostasis, the leptin profile and adipocytokines could be considered as a potential programing factor regulating adipogenic pathways during rapid postnatal growth. To our knowledge, this represents the first report of an association of bioactive components in HBM with BMC in infants. Although the associations are modest, the data presented highlight the importance in identifying factors during early infancy, which may contribute to optimal (and suboptimal) bone health.

It is generally accepted that sexual dimorphism in BMC emerges in puberty.[21,22] However, our findings provide evidence of an earlier sex-dependent difference and offer the possibility for postnatal initiation with exposures in HBM as a potential provocateur. Sex differences in the relationship between HBM insulin, glucose, leptin, TNF-α and IL-6 with infant BMC were readily apparent beginning at 1 month.

Although not significant, glucose was higher in HBM of mothers of male offspring compared with those of females (27.4 ± 1.9 vs 22.9 ± 2.4 mg dl^{-1}). The greater increases in lean mass and in turn BMC in males may have been at least in part related to increased glucose uptake of the musculoskeletal system in boys.[1] Conversely, HBM insulin was significantly higher in mothers of female offspring relative to those of male offspring at 1 month. Insulin exerts direct effects on mesenchymal stem cells, promoting adipocyte differentiation,[13–15] at the expense of fat-free mass (lean and bone mass) accrual.[2] Further, in an insulinemic environment, females in general have been shown in numerous studies to display a lower capacity to alter fuel utilization pathways.[3,4,23]

In aging, bone resorption is viewed as anti-osteogenic. However, during the modeling/remodeling process in growth, resorption is essential for establishing the strength-structural properties of the skeleton; thus, the positive association between TNFα and BMC likely represents a beneficial effect on bone remodeling. TNFα, although associated with bone resorption during periods of stasis in terms of bone morphology and mineral accrual (that is, adulthood), is integral in modeling geometric properties of bone and resorptive aspects of bone, which are essential for improving strength and structure during growth and development.[24,25] Whereas an inverse association was observed at 1 month in males, at 6 months a positive relationship was observed, concomitant with a significant increase in BMC and linear growth.[26–28] This is in line with reports indicating the first 6 months as a period of rapid bone modeling, in which bone is mineralized at a slower rate than the bone is growing.[24–26] During growth, exposure to inflammatory factors may have a dual role as 'kines' released as anabolic factors, and may elicit differential effects on metabolic programing and tissue partitioning.[29,30]

It has been hypothesized that the coordination among neonatal calcium accrual and maternal bone metabolism is established by altering parathyroid hormone–related peptide (PTHrP) content.[31] PTHrP becomes the primary calcium balance regulator during lactation, and it is then that it is found in its greatest concentration in systemic circulation. Moreover, concentration in HBM is about 1000 to 10 000 times greater than in maternal circulation; yet, a function in neonatal growth and development is not clear.[32] Investigation in animals suggested that mammary production of PTHrP might help avoid hypocalcemia in the neonates when milk calcium content drops.[33] PTHrP has been shown to modulate several transcription factors essential for embryonic growth, including chondrocyte proliferation and differentiation.[34] Despite high concentration in HBM, a limited (if any) systemic absorption of PTHrP by the intestinal tract with no effect on bone mass in human neonates was recently reported.[32] We were unable to

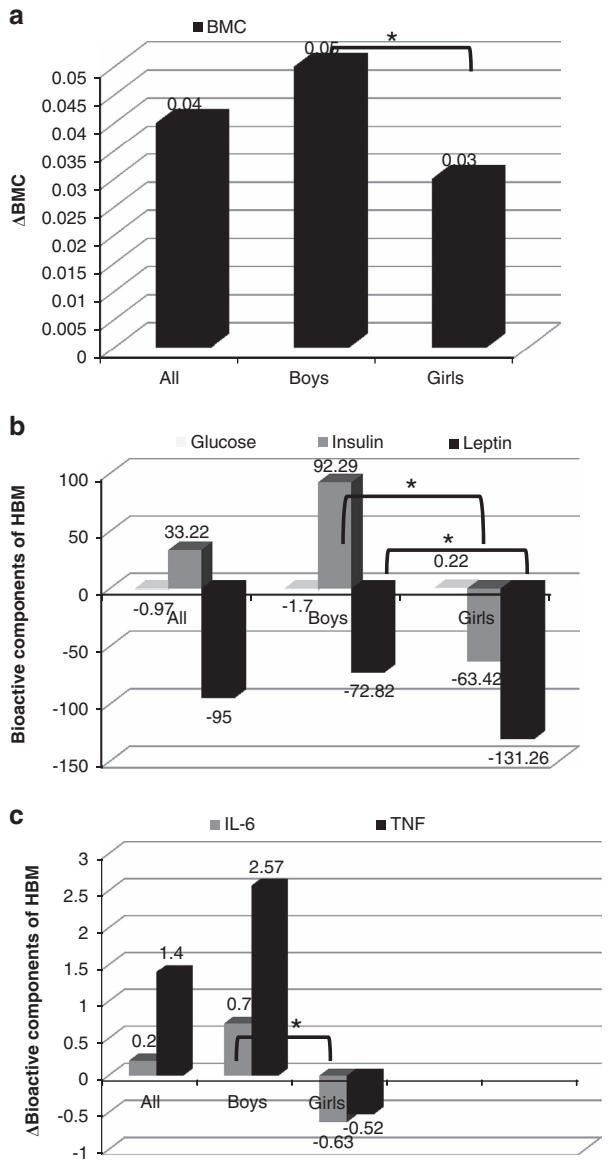

Figure 1 Change in BMC and bioactive components of HBM from 1 to 6 months. Panel **a** shows the change (1 to 6 months) in BMC (**a**) and the change in bioactive components in HBM (panel **b**: light gray = glucose; dark gray = insulin; black = leptin and panel **c**: dark gray = interleukin-6; black = tumor necrosis factor-alpha, expressed at 1 month vs concentration at 6 months) after adjusting for baseline BMC in the total sample, boys and girls. *Represents a significant difference ($P<0.05$) in change values between boys and girls.

evaluate maternal or neonatal PTHrP levels in this study sample, but we recognize that the highly regulated process of bone mineralization is representative of a distinct metabolic milieu that may likely differs from any other life stage, and PTHrP is a central regulator.

Concentrations of leptin, a potential mediator of PTHrP in HBM, have been positively correlated with maternal BMI and adiposity[6,35,36] and negatively correlated with childhood BMI in offspring of lean mothers.[37] One study has also reported that the change in leptin concentration underlies infant BMI; however, the association was reported in infant leptin concentration.[35] Although the contribution of mammary-derived leptin to growth and development is unknown and may differ according to growth tempo, the observed inverse relationship of HBM leptin with BMC suggests that lower exposure benefits the infant skeleton. In fact, we previously reported that greater HBM leptin was associated with lower adiposity.[3]

Although this study encompasses longitudinal assessment including robust measures of body composition, it is not without limitations. First, because of the preliminary nature of the investigation, the sample size was modest. The 35 mother–child dyads produced 90% power to observe a gender difference in BMC of 6 g, assuming a Type I error rate of 5%, not accounting for multiple comparisons. In addition, infant and/or maternal circulating concentrations of leptin, glucose, IL-6, TNF-α and insulin were not obtained, and maternal dietary intake was not measured or analyzed, which could affect breast-milk composition. In addition, fatty acid profile in breast milk (essential and non-essential) would explain even stronger correlations between fat and BMC. Further, as there is a growing evidence showing that the supplement of probiotics found in breast milk can effect BMC, as well as BMD,[38] it would have added even greater significance of HBM and growth if such factors contributed to our findings. However, given the limited assessments of nutritive and bioactive factors in HBM in this sample, we were unable to perform this interrogation but will be considered in the design of future studies. Lack of experimental evidence makes any discussion speculative but highlights the need for long-term assessments of qualitative aspects of bone in infancy.

Conclusion

This study for the first time showed bioactive components in HBM (glucose, TNF-α and leptin) contributing to overall bone health in the first 6 months of life. Although preliminary, this study shows biological relevance and a possible link between substances in HBM and skeletal health with potential sexual dimorphic implications.

Materials and Methods

Study overview

Thirty-five exclusively breast-feeding mother/infant pairs (exclusivity defined as no formula supplementation) were tracked for the first 6 months postpartum. Maternal consent was obtained prior to all testing procedures. All testing took place at the University of Oklahoma Health Sciences Center. All procedures for human participation were approved by the Institutional Review Board at the University of Oklahoma. The following inclusion criteria were used: (1) maternal age between 18 and 45 years at the time of delivery, (2) gestation lasting $\geqslant$37 weeks, (3) singleton birth and (4) a postpartum hospital stay for mother and infant less than 3 days. The following exclusion criteria were used: (1) any tobacco use, (2) alcohol consumption ($>$1 drink per week), (3) pre and gestational diabetes and (4) presumed or known congenital birth defects. Electronic medical records were used to establish the mother's age, parity, pre-pregnancy weight and weight gained during gestation. Only in a few rare instances was self-report used.

HBM and infant body composition were assessed at 1 and 6 months, as described previously.[2–4] Briefly, at each time point, dyads were encouraged to report to the University of Oklahoma Health Sciences Oklahoma City campus between 8:00 and

Table 3 Linear regression analysis evaluating the change in BMC from 1 to 6 months and a change in the concentration of bioactive components in HBM at 1 month and 6 months after controlling for infant sex (in total sample), weight change and mother's pre-pregnancy

	Change from 1 to 6 months					
	Total sample (n = *35*)		*Males* (n = *20*)		*Females* (n = *15*)	
	β	P-*value*	β	P-*value*	β	P-*value*
HBM glucose (mg dl^{-1})	0.29	0.09	0.39	0.12	0.10	0.75
HBM insulin (pg ml^{-1})	0.04	0.84	−0.08	0.78	−0.19	0.68
HBM leptin (pg ml^{-1})	−0.58	**0.003**	−0.89	**0.001**	0.37	0.43
HBM IL-6 (pg ml^{-1})	0.10	0.56	−0.03	0.91	0.12	0.69
HBM TNFα (pg ml^{-1})	0.47	**0.009**	0.63	**0.02**	0.01	0.97

Abbreviations: β, standardized parameter estimate; BMC, bone mineral content; HBM, human breast milk; IL-6, interleukin-6; TNF-α, tumor necrosis factor- alpha. A significant *P*-value ($P<0.05$, bold) indicates an independent association of the change bioactive component in HBM on BMC.

10:00 hours (to minimize diurnal variation in HBM composition). Anthropometrics and a whole-body dual-energy X-ray absorptiometry (DXA) scan were performed in the infant at each visit. The mother completed a full breast-milk expression from a single breast. At collection, the mother was strongly encouraged to empty the entire contents of one breast at 1 month (mean volume 2.4 ± 0.2 oz.). which were collected using an electric breast pump (Medela Inc., McHenry, IL, USA), ensuring the collection of fore-, mid-, and hind-milk within each sample. Approximately half of the mothers were of normal weight defined as a pre-pregnancy BMI $<25.0\,kg\,m^{-2}$ ($n=18$) and half were overweight/obese defined as pre-pregnancy BMI $\geqslant 25.0\,kg\,m^{-2}$ ($n=17$). Birth measurement variables were obtained from medical records where possible.

Infant growth, body composition and bone

Body weight and length were collected at each visit. Nude weight and length were obtained in duplicate using a Seca 728 electronic infant scale and Seca 416 infantometer (Seca, Hamburg, Germany; accuracy ± 0.1 g, 0.5 cm, respectively). Total body composition and total BMC and BMD were collected using a Lunar iDXA (GE, Fairfield, CT, USA) scanner as described previously[2–4] while the infant wore only a diaper. To improve compliance and minimize movement, the infant was swaddled using a light receiving blanket provided by the laboratory.

Breast-milk collection

Mothers were encouraged to pump the entire contents of a single breast expression for the analyses of HBM components (insulin, glucose, leptin, IL-6 and TNFα) as described previously.[3,4] Briefly, thoroughly mixed milk was divided into aliquots and stored at −80 °C. Prior to analyses, aliquots were thawed and milk fat was separated by centrifugation. The resulting skimmed milk was assayed by commercially available immunoassay kits for insulin, leptin, IL-6 and TNF-α. Glucose was measured by the glucose oxidase method (2300 STAT Plus, Yellow Springs Instruments, Yellow Springs, OH, USA).

Statistical analyses

Descriptive statistics were obtained for the pre-pregnancy characteristics of all the mothers as well as stratified according to offspring sex. Similarly, mean (+s.d.) concentration of bioactive HBM components collected at 1 and 6 months was generated for all mothers and stratified according to sex of the offspring. Infant characteristics including anthropometrics and body composition variables at 1 and 6 month (mean ± s.d.) were determined for all infants and by sex. The associations between BMC at 1–6 months and HBM (glucose, insulin, leptin, IL-6 and TNF-α) were also investigated using linear regression with infant BMC as the dependent variable, controlling for sex, weight change from birth and mother's pre-pregnancy BMI. Infant percent body fat was in the initial models but did not contribute to the relationship so was removed for parsimony. To further clarify these findings by studying the role sex may have in these observed relationships, male and female offspring were evaluated separately. Next change variables were calculated subtracting BMC and HBM bioactive components at 1 month from the value obtained at 6 months. The associations between the change in BMC from 1 to 6 months and the change in HBM were also investigated using linear regression controlling for sex (in the total sample), weight change from birth and mother's pre-pregnancy BMI. Although dramatic increases in BMD occur in the first 36 months of life, the capacity of DXA to detect changes is limited during this period.[39,40] Given that the accuracy and reliability of areal BMD in infants have been plagued by poor precision, only results for BMC as dependent variable are reported.

Conflict of Interest

DAF received funding for this study from Mead Johnson Nutrition, which did not have editorial control of the paper with the CMRI Metabolic Research Program at the University of Oklahoma Health Sciences Center providing additional support. The opinions expressed are those of the authors and not necessarily those of the NIH or any other organization with which the authors are affiliated. KC and LJH declare no conflict of interest.

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Author contributions: Krista Casazza conceptualized the analysis, drafted the initial manuscript, reviewed and revised the manuscript and approved the final manuscript as submitted. Lynae J Hanks contributed to the initial analysis, reviewed and revised the manuscript and approved the final manuscript as submitted. David A Fields conducted the study and played a part in the conceptual design, reviewed and revised the manuscript and approved the final manuscript as submitted.

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