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Early Elevation in Interleukin-6 is Associated with Reduced Growth in Extremely Low Birth Weight Infants

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Abstract

Objective—To determine whether reduced growth velocity (GV) in extremely low birth weight (ELBW) infants is preceded by elevated inflammatory cytokines.

Study Design—GV was determined at 36 weeks post-menstrual age (PMA) in 768 infants 401-1000 g birth weight (BW). Association between blood cytokines measured through day of life

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21 and GV was explored using linear regression models that adjusted for late-onset sepsis (LOS), BW, small-for-gestational age (SGA), gender, race, energy intake, and center.

Results—Serum interleukin-6 (IL-6) was increased at days 14 and 21 in LOS infants. LOS was associated with reduced energy intake and GV for weight (weight-GV) at 36 weeks PMA. Linear regression analysis controlling for LOS and energy intake showed significant relationships between increased IL-6 at days 14 and 21 with reduced weight-GV at 36 weeks PMA ($p<0.0001$). The relationship between day 21 IL-6 and weight-GV was not associated with LOS ($p=0.12$) when controlling for BW and energy intake. Both BW ($p=0.02$) and energy intake ($p=0.003$) influenced the relationship between day 14 IL-6 and weight-GV.

Conclusions—IL-6 elevation during the first month of life is associated with lower weight-GV at 36 weeks PMA and may have a direct effect upon energy balance and postnatal growth.

Keywords

cytokines; growth retardation; growth velocity; preterm infant; sepsis

INTRODUCTION

Very low birth weight (VLBW, <1500 gm BW) infants with major morbidities experience reduced growth velocity relative to comparable infants without these morbidities (1). Late-onset sepsis (LOS) comprises the largest proportion of associated morbidities in these infants. LOS increases total energy expenditure (TEE) (2) and is associated with reduced growth velocity (GV) at 36 weeks post-menstrual age (PMA) (3). Pro-inflammatory cytokines including TNF α and IL-6 have been associated with intrauterine and postnatal growth restriction in a number of different clinical settings (4, 5). Neonates have recently been shown to have immature anti-inflammatory responses, resulting in reduced production of the anti-inflammatory cytokine IL-10 relative to production of the pro-inflammatory cytokines IL-6 and TNF α (6, 7). Whether neonates who have experienced major morbidities may be especially prone to persistent up regulation of inflammatory cytokines and thereby reduced GV is not known.

Older children with chronic relapsing inflammatory conditions including inflammatory bowel disease (IBD) and juvenile rheumatoid arthritis (JRA) commonly experience increases in circulating cytokines and growth failure (8, 9). Either targeted anti-TNF α therapy or supplemental nutrition aimed at increasing protein and energy intake can return them to pre-morbid growth velocities (10–12). LOS is associated with a substantial increase in circulating cytokines including IL-6 (13), and increased circulating IL-6 has been associated with reduced growth velocity in preterm infants without major morbidities (4). We hypothesized that reduced GV in ELBW infants with major morbidities including LOS might therefore also be preceded by elevated inflammatory cytokines. We examined the relationship between early elevation in pro-inflammatory cytokines and postnatal growth outcomes in a large cohort of ELBW infants enrolled in the Neonatal Research Network (NRN) Cytokine Study.

PATIENTS and METHODS

Patient Cohort and Study Design

Data were collected prospectively for ELBW neonates (weighing 401–1000 g at birth) who were born between 1999–2001 and were cared for at one of the 17 Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) Neonatal Research Network sites. The Network maintains a registry of infants with birth weights of 401 to 1500 g who are admitted at participating centers within 14 days after birth. Trained research personnel collected maternal socio-demographic, pregnancy, and delivery data soon after birth and infant data from birth until 120 postnatal days, discharge, or death, using standardized registry forms. The institutional review board at each center approved participation in the registry, and written informed consent was obtained from the parents.

This was a secondary study using the patient data and analytical results from the NICHD Neonatal Research Network Cytokine Study. We studied 768 of 1067 infants in the NRN Cytokine Study who had GV data available at 36 weeks PMA. Inclusion criterion was BW 1000 g, (ELBW) and exclusion criterion was death < 7 days of age. Eleven infants with syndromes/major malformations (including femoral hypoplasia, ambiguous genitalia, atrial septal defect, sick euthyroid syndrome, hypoplastic pulmonary arteries, hydroureter, absent septum pellucidum, hepatic hemangioendotheliomatosis involving the entire liver, congenital schizencephaly, duodenal atresia, and skeletal dysplasia) were included in the analysis. Patient data included measurements of body weight, length and HC, nutritional intake, and the occurrence of major morbidities (bronchopulmonary dysplasia (BPD), necrotizing enterocolitis (NEC), severe intra-ventricular hemorrhage (IVH), and LOS). Total energy intake was calculated as the appropriately-weighted sum of the energy contained in administered parenteral components: dextrose, amino acids, and lipids; plus the caloric total for the consumed volume of enteral intake, expressed in kcal/kg/day. The outcomes are network-standard definitions. LOS was defined as culture positive septicemia/bacteremia diagnosed beyond 72 hours of age. Infants were considered to have NEC if they met or exceeded the modified Bell's staging criteria IIA. IVH was based on the cranial sonogram within 28 days showing the most severe hemorrhage. Severe IVH (grade 3–4) was defined as blood/echodensity in the parenchyma (grade 4), or blood/echodensity in the ventricle with ventricular size enlarged (grade 3). BPD was defined as supplemental oxygen at 36 weeks PMA (including discharge or transfer on oxygen if prior to 36 weeks).

Whole blood spots on filter paper (about 0.2 ml per day) were collected on days 0 (day of birth), 3±1, 7±1, 14±3, and 21±3 and frozen to –70°C. The stored blood spots were analyzed in a batch for 25 cytokines including IL-6, IL-1β, TNF-α, and IL-10 using a multiplex Luminex assay (Luminex Corp., Austin, TX) (14). The working range, defined as the range of concentrations for which the coefficient of variation (CV) is < 20%, for each of these was 7.8–4000 pg/mL. The intra- and inter-assay CV for each cytokine were: IL-1β: 6.7% and 11%, IL-6: 5.3% and 16%, IL-10: 5.4% and 13%, and TNFα: 5.5% and 16%, respectively. Cytokine levels below or above the detection limit were assigned the value of the detection limit. For example, “<3” was assigned a value of 3, and “>6400” was assigned a value of 6400.

Analysis

All statistical analyses were performed at the Neonatal Research Network Data Coordinating Center (RTI International). As previously described by Patel et al (15), growth velocity for weight was calculated in daily increments based on daily weights using the following formula: $\{ [\text{wgt}(n+1) - \text{wgt}(n)] * 1000 \} / \{ [\text{wgt}(n) + \text{wgt}(n+1)] / 2 \}$. When daily weights were missing, the values were divided by the number of days between the two growth measurements. The starting point for calculating GV was the day when birth weight was regained (or day 1 for infants who never lost birth weight). The overall GV for each infant was calculated by averaging all the daily GV values for that infant. The cohort was then separated by quartiles according to their growth velocity for weight (g/kg/day) through 36 weeks PMA or discharge. Since we did not have daily measurements of length and head circumference, GV-length and GV-HC were calculated as the differences between final and initial measurements divided by the number of weeks between measurements, and were expressed in cm/wk. Within each time point, median TNF α , IL-6, IL-1, and IL-10 levels across the four growth velocity quartiles were then compared using the non-parametric Median test. Given concerns about the need to consider the influence of NEC or LOS on cytokine levels, we also compared cytokine levels at each of the time points and GV for infants who experienced LOS between days 4–20, after day 20, or not at all. Non parametric median tests were used for all unadjusted comparisons of cytokine levels because of the skewed nature of these data.

Adjusted analyses / regression modeling was then performed for those cytokines that showed associations with LOS group or GV quartile in the unadjusted / bivariate analyses. Linear regression modeling was performed for the effect of IL-6 or IL-10 levels at each time point on GV, while adjusting for timing of LOS/NEC (days 4–13, 14–21, 21+, or no LOS/NEC), BW, SGA, gender, race, center and energy intake. The linear regression models were set up to predict overall growth velocity (starting at date regaining BW through 36 weeks PMA). All data were analyzed using SAS 9.1 (SAS Institute, Cary, NC), at RTI International (Research Triangle Park, NC).

RESULTS

Clinical and demographic characteristics

Patient characteristics by GV quartile for weight are given in Table I. Both BW and gestational age (GA) were lower in the groups with lower GV. The groups did not vary based upon frequency of SGA, male gender, African-American race, or NEC. However, the frequency of BPD, grade III-IV IVH, and LOS were higher in the groups with lower GV. Length of stay was also longer in the group with the lower GV.

Energy intake

Differences in energy intake could contribute to differences in GV, particularly if elevated cytokines increased protein and energy requirements. Energy intake was therefore examined between days 1 and 60 as a function of GV quartile for weight. As shown in Supplemental Table I, median (IQ range) energy intake was reduced in GV quartile I compared to the other groups ($p < 0.0001$ by Median test). Energy intake was also examined between days 1 and 60

as a function of timing of LOS. As shown in Table II, median (IQ range) energy intake was also significantly lower for the groups with LOS compared to those without LOS both between DOL 4–20 and those with LOS after DOL 20 ($p<0.0001$ by Median test).

Blood cytokine levels stratified by GV quartiles

We then examined serum cytokine levels at each of the time points as a function of GV quartile. There were no significant relationships between any of the four cytokines (IL-6, IL-10, IL-1 β , TNF α) with GV quartile (see Supplemental Table II).

Blood cytokine levels and GV stratified by occurrence and timing of LOS

It would be expected that episodes of LOS could be associated with both elevation in circulating cytokines and alterations in GV. Therefore, we performed an analysis to examine the overall cytokine profiles and GV at 36 weeks PMA for these three groups: the 185 infants with LOS between DOL 4–20, the 154 infants who only experienced LOS on or after 21 days, and the 429 infants who never experienced LOS (see Supplemental Tables III and IV). LOS was associated with day 14 serum IL-6 ($p=0.03$), day 21 serum IL-6 ($p=0.03$), and day 14 serum IL-10 ($p=0.007$). By comparison, LOS was not associated with day 21 IL-10, TNF- α , or IL-1 β . Day 14 and day 21 serum IL-6 was significantly higher in infants who experienced LOS between DOL 4–20 and DOL 21+ compared to those who did not experience LOS (Figure 1). Median day 14 IL-10 was significantly higher in infants who experienced LOS between DOL 4–20 compared to those who did not experience LOS (see Figure 1). Median (IQR) GV for weight through 36 week PMA was lower in the groups with LOS [15.3 (12.9–17.5) gm·kg/d in the group without LOS compared to 14.7 (12.1–17.2) gm·kg/d in the group with LOS between DOL 4–20 and 13.9 (12.3–15.5) gm·kg/d in the group with LOS after DOL 20 (see Supplemental Table V, $p=0.002$ by Median test)].

Adjusted analysis using linear regression models

To further explore the relationship between those cytokines significantly associated with LOS in unadjusted analysis (day 14 or 21 blood IL-6, day 14 blood IL-10; see Supplemental Table III), timing of LOS, and GV, linear regression models that included BW, SGA, gender, race, center, total energy intake (day 1–60), IL-6 or IL-10 levels, and age at occurrence of LOS/NEC as covariates were examined. Blood IL-10 at day 14 was not associated ($p=0.11$) with weight-GV after adjustment and was not investigated further. The results from linear regressions for GV are shown in Table III for day 21 IL-6 and in Supplemental Table VI for day 14 IL-6. Data are presented as the parameter estimate (95% CI) and p-value. This parameter estimate can be interpreted as the average adjusted change in GV for each pg/mL increase in the level of serum IL-6. The LOS/NEC variable compared timing of infection (4–13 days, 14–20 days, 21+3 days) to infants without infection. The overall p-value is also given (this is the only categorical variable with more than two levels).

Significant ($p<0.0001$) relationships between day 14 or 21 serum IL-6 levels and weight-GV at 36 weeks PMA were identified. Thus, an increase of 100 pg/mL in the level of day 21 blood IL-6 was associated with a lower weight gain of about 0.5 g/kg/day in weight GV, and a similar increase in the level of day 14 blood IL-6 was associated with a lower weight gain of about 0.7 g/kg/day in weight GV. Energy intake ($p=0.003$) and birthweight ($p=0.02$)

significantly influenced the relationship between day 14 IL-6 and weight-GV. LOS/NEC ($p=0.12$), energy intake between day 1–60, and other clinical and demographic factors considered did not influence the relationship between day 21 IL-6 and weight-GV. There was no association between day 14 IL-6 and length-GV or HC-GV. The association between day 21 blood IL-6 and length-GV approached significance ($p=0.06$) though the size of the effect was relatively small. Length-GV was significantly associated with energy intake. There was no association between day 21 blood IL-6 and HC-GV. However, HC-GV was significantly associated with energy intake, LOS/NEC occurring after 21 days of age, BW, SGA, and race. Significant center variations were present in both length-GV and HC-GV.

DISCUSSION

The period between regaining birth weight and hospital discharge is a critical window for preterm infants to establish an optimal growth velocity (16). VLBW infants with major morbidity (defined as LOS, BPD, NEC, or severe IVH) experience reduced growth velocity relative to comparable infants without these morbidities (1). This is reflected in a longer time to regain birth weight and persistently lower weight over time. For each 100-g birth weight (BW) interval examined between 501–1500 g, LOS comprised the largest proportion of associated morbidities (1). Therefore, LOS may be a particularly important factor associated with subsequent reduced growth velocity in VLBW infants. Despite nutritional intake which should be sufficient to meet estimated protein and energy requirements, these infants do not exhibit catch-up growth prior to hospital discharge (1). This suggests that factors such as circulating cytokines associated with morbidities including LOS may cause a resistance to what would otherwise be adequate nutrition.

In the current study, we found that ELBW infants who experience LOS have elevated IL-6 levels during the first month of life and reduced GV through 36 weeks PMA when compared to ELBW infants without LOS and elevated levels of IL-6. It should be noted that the magnitude of the IL-6 effect upon weight gain was relatively modest over the range of cytokine levels measured (approximately a 0.5 g/kg/day decrease in weight gain for every 100 pg/mL increase in day 21 IL-6). Nevertheless, our results suggest that elevation in IL-6 may in fact cause a resistance to what would otherwise be adequate nutrition.

Our study is in agreement with the report by Ahmad et al in which the relationship between serum IL-6, IGF-I, and GV was explored in a prospective cohort of 51 preterm infants without co-morbidities. In contrast to the current study, this group was enrolled following initial NICU stabilization, after removal of all indwelling catheters, once they were receiving enteral nutrition of at least 100 kcal/kg/d (4). During the observation period, weight and IGF-I increased while IL-6 decreased. Importantly, IGF-I and IL-6 were inversely correlated suggesting an influence of circulating IL-6 upon anabolic metabolism even in a cohort of otherwise healthy preterm infants. Regression analysis confirmed an inverse association between serum IL-6 levels and growth parameters.

Inflammatory cytokines which are up regulated during an APR, including TNF α and IL-6, have been associated with intrauterine and postnatal growth restriction in a number of different clinical settings. IL-6 was higher in neonates with sepsis and NEC than in neonates

without infection (17). In some studies, neonates have been shown to have immature anti-inflammatory responses characterized by reduced production of the anti-inflammatory cytokines IL-10 and TGF β relative to the pro-inflammatory cytokines TNF α and IL-6 (6, 7). Median IL-10 was elevated at day 14 for infants with LOS but neither IL-10 nor LOS/NEC was significantly associated with GV in adjusted linear regression. Therefore, our data do not support a defect in anti-inflammatory IL-10 production contributing to persistent IL-6 elevation in preterm infants with poor growth.

Even a brief infection can induce a significant nutritional deficit. Inflammation may induce a catabolic state characterized by increased metabolic rate (18). If these extra nutritional requirements, in particular increased protein relative to energy, are not met following the infection, catch-up growth will not occur. Protein catabolism may increase during neonatal sepsis and may explain growth failure (19, 20). Protein intake must increase from 6% of calories to 15% in order for IGF-I to increase and catch-up growth to occur following shigellosis in undernourished children (21). This could translate into a requirement for an additional 1 gram/kg/day of protein for catch-up growth to occur in VLBW infants following infection. Moreover, if a chronic inflammatory process persists, re-feeding therapy will not succeed. In a study examining re-feeding of malnourished elderly patients, failure of re-feeding was associated with elevated circulating IL-6 and a persistent catabolic state (22). Increased circulating levels of pro-inflammatory cytokines may persist for several years following a neonatal infection. For example, children who have had RSV infection as infants exhibit increased circulating IFN- levels at 6 to 10 years of age (23). Unless chronic inflammation is identified and addressed, growth velocity which declines during an acute infection may remain sub-optimal. In this regard, the energy intake of the infants in GV quartile I or with LOS may not have been sufficient to overcome the additional demands induced by the increase in circulating IL-6. Similar to fetal HC and length sparing observed in asymmetric intrauterine growth restriction, our results suggest that postnatal energy intake may support HC and length growth before weight gain.

The strengths of our study include the large prospective cohort design and the rigorous clinical data collection methods. However, we were not able to control for all factors which may affect both the incidence and severity of major morbidities and associated growth in the analysis. We did not analyze data on fortified human milk versus preterm formula. Feeding with greater than 50% of enteral calories as fortified human milk has been shown to reduce the incidence of late-onset sepsis and NEC, presumably because of the presence of beneficial immune factors including IgA and TGF- β (24). However, this was also associated with reduced fat absorption and growth velocity relative to preterm formula (25). Moreover, the exploratory nature of the unadjusted/bivariate analysis, which did not control for multiple comparisons, may have over-estimated differences observed. Most importantly, we did not measure circulating IL-6 beyond day 21, and so were not able to determine whether this remained chronically elevated in infants with reduced weight gain. Severity of pulmonary disease and the use of steroids for management of BPD (26) have also been shown to influence growth velocity, but our models did not include details on severity of lung disease. Dexamethasone treatment causes GH resistance and has been shown to reduce circulating IGF-I and growth velocity in preterm infants (27).

LOS is associated with a substantial increase in circulating cytokines including IL-6. However, IL-6 levels typically decrease precipitously in most infants with LOS within 24 hours (13). Additional host factors may influence persistent elevation of serum IL-6 following the initial inciting event. Genetic variation (-174 G/C) in the IL-6 gene promoter regulates IL-6 production and has been associated with variation in growth in children with Crohn's disease (28). The IL-6 gene promoter -174 G/C genotype has also recently been associated with chorioamnionitis and sepsis in preterm infants (24). It will be important in future work to consider the IL-6 promoter genotype in interpreting differences in circulating IL-6 and growth in preterm infants.

Our data suggest that early elevation in blood IL-6 is associated with reduced weight-GV at 36 weeks PMA in ELBW infants. Persistent elevations of IL-6 could directly affect energy balance and postnatal growth. Assessment of this will be required to provide adequate protein and energy and potentially targeted anti-inflammatory therapy to preterm infants with reduced GV associated with chronic inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data collected at participating NRN sites were transmitted to RTI International, the data coordinating center (DCC) for the NRN, which stored, managed, and analyzed the data for this study. On behalf of the network, Dr. Abhik Das (DCC PI) and Mr. Scott A. McDonald (DCC Statistician) had full access to all the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis.

Appendix

The following investigators participated in this study:

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Abbreviations

APR	acute phase response
BPD	bronchopulmonary dysplasia
BW	birth weight
CI	confidence interval
CV	coefficient of variation
DOL	day of life
ELBW	extremely low birth weight
EOS	early-onset sepsis
GA	gestational age

GH	growth hormone
GV	growth velocity
HC	head circumference
IBD	inflammatory bowel disease
IGF-I	insulin-like growth factor 1
IL	interleukin
IQ	interquartile
IVH	intra-ventricular hemorrhage
JRA	juvenile rheumatoid arthritis
LOS	late-onset sepsis
NICHD	Eunice Kennedy Shriver National Institute of Child Health and Human Development
NEC	necrotizing enterocolitis
NRN	Neonatal Research Network
OR	odds ratio
PMA	post-menstrual age
SGA	small-for-gestational age
TEE	total energy expenditure
TNF	tumor necrosis factor
VLBW	very low birth weight

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Figure 1. Serum Cytokines and Timing of Late-Onset Sepsis

Serum IL-6, IL-10, IL-1 β , and TNF α concentration was determined on day of life 1, 3, 7, 14, and 21. The median value for the groups with no LOS, LOS between DOL 4–20, or LOS after DOL 20 is shown. *p<0.05 by median test.

Table 1

Patient Characteristics by Growth Velocity (GV) Quartile.

	Quartile I	Quartile II	Quartile III	Quartile IV	p-value [‡]
GV-weight (gm/kg/day)	11.1 (9.1,11.9) *	13.7 (13.2,14.3)	16.0 (15.4,16.5)	18.7 (17.8,20.1)	<0.0001
GV-length (cm/wk)	0.61 (0.41,0.68) *	0.82 (0.78,0.86)	0.97 (0.94,1.00)	1.11 (1.07,1.20)	<0.0001
GV-HC (cm/wk)	0.59 (0.50,0.65) *	0.75 (0.71,0.78)	0.86 (0.83,0.88)	1.00 (0.94,1.08)	<0.0001
BW (g)	748 ± 144 **	748 ± 129	790 ± 132	785 ± 137	0.0010
GA (wk)	25.5 ± 1.9 **	25.8 ± 1.9	26.1 ± 1.7	26.3 ± 2.1	0.0012
SGA (%)	12.5	15.6	14.1	18.2	0.46
Male (%)	52.1	49.5	45.3	45.3	0.47
Black (%)	43.8	54.7	50.0	51.6	0.18
BPD (%)	55.8	54.8	47.6	34.4	0.0002
NEC (%)	9.4	7.3	11.5	12.0	0.39
IVH 3 (%)	18.9	9.9	11.0	15.1	0.047
LOS (%)	51.0	47.4	43.8	34.4	0.007
Length of stay (days)	107 ± 60 **	105 ± 42	88.6 ± 34	73.7 ± 33	<0.0001

* Median(Interquartile range)

** Mean ± SD

[‡] P-values from Median Test (GV-weight, GV-length, GV-HC), Kruskal-Wallis Test (BW, GA, Length of stay), or Fisher's Exact Test.

Table II

Total Energy Intake Per Day (kcal/kg), Days 1–60 by LOS group

	N	Mean (SD)	Median (IQR)
No LOS	429	91.8 (25.2)	97.5 (85.1 – 107.0)
LOS – DOL 4–20	185	78.5 (28.4)	86.4 (71.0 – 98.6)
LOS – DOL 21+	154	87.8 (15.6)	88.7 (81.3 – 97.3)

Median Test p-value < 0.0001

Table III

Adjusted Linear Regression Model for Predicting Growth at 36 weeks PMA.

Variables	GV-Weight	GV-Length	GV-HC
IL-6 @ d 21 (pg/mL)	−0.005 (−0.006, −0.003) <0.0001	−0.00006 (−0.0001, 0.000002) 0.06	−0.000005 (−0.00005, 0.00004) 0.85
LOS/NEC	<i>0.12*</i>	<i>0.997*</i>	<i>0.02*</i>
Days 4–3	−1.35 (−2.97, 0.27) 0.10	−0.0001 (−0.06, 0.06) 0.997	−0.03 (−0.07, 0.02) 0.20
Days 14–20	1.22 (−0.62, 3.06) 0.19	0.006 (−0.06, 0.07) 0.85	−0.03 (−0.08, 0.02) 0.23
Days 21+	−0.06 (−1.47, 1.34) 0.93	0.004 (−0.05, 0.06) 0.87	−0.06 (−0.10, −0.02) 0.002
Birth Weight	0.003 (−0.001, 0.008) 0.13	0.0001 (−0.000006, 0.0003) 0.06	0.0002 (0.00005, 0.0003) 0.005
SGA	−0.07 (−1.62, 1.48) 0.93	−0.008 (−0.06, 0.05) 0.78	0.06 (0.02, 0.10) 0.007
Male Gender	0.17 (−0.91, 1.24) 0.76	0.02 (−0.02, 0.06) 0.36	0.003 (−0.03, 0.03) 0.85
Black Race	0.64 (−0.53, 1.82) 0.28	0.02 (−0.02, 0.07) 0.26	0.04 (0.01, 0.08) 0.007
Total Energy Intake (d1–60)	0.03 (−0.004, 0.05) 0.09	0.005 (0.004, 0.006) <0.0001	0.002 (0.002, 0.003) <0.0001
Center	varies 0.62	varies 0.01	varies 0.0001

Data presented as parameter estimate (95% CI) and p-value. Models were adjusted for all variables shown in the table.

* Overall p-value for LOS/NEC effect.