Relationship Between Milk Fat Globule-Epidermal Growth Factor 8 and Intestinal Cytokines in Infants Born Preterm

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Objectives To investigate the relationships between dietary intake and fecal concentrations of milk fat globule-epidermal growth factor 8 (MFG-E8), and between fecal concentrations of MFG-E8 and markers of intestinal inflammation in infants born preterm.

Study design Fecal samples were collected daily and enteral feedings were sampled weekly. MFG-E8 in enteral feedings and feces, and cytokine concentrations in feces were quantified by enzyme-linked immunosorbent assay.

Results Milk MFG-E8 concentrations were significantly greater in unfortified mother's own milk (MOM) and MOM with human milk fortifier than either donor human milk or preterm formula. MFG-E8 concentrations in fecal samples were positively correlated with MFG-E8 concentrations in respective milks. High MFG-E8 exposure (≥60 mL/kg/day of feedings that include MOM or MOM with human milk fortifier) was associated with lower concentrations of proinflammatory cytokines (interleukin-8, tumor necrosis factor-α, and monocyte chemoattractant protein-1) and higher concentrations of the anti-inflammatory cytokine interleukin-4 in feces, compared with low MFG-E8 exposure.

Conclusions Infants born preterm who were fed MOM had greater concentrations of MFG-E8 and lower concentrations of proinflammatory cytokines in fecal samples than other diets or no feedings. These data further support the protective role of MOM, possibly because of MFG-E8, against intestinal inflammation. *(J Pediatr 2021;230:71-5)*.

Milk fat globule-epidermal growth factor 8 (MFG-E8) is a secretory glycoprotein expressed in macrophages and intestinal epithelial cells. The anti-inflammatory role of MFG-E8 has been demonstrated in adult conditions such as sepsis, ischemia-reperfusion, and inflammatory bowel disease.[1-4] MFG-E8 expression is inversely correlated with mucosal inflammation and disease severity in patients with ulcerative colitis.[5] MFG-E8 also has been associated with decreased inflammation and evidence of end-organ injury in a murine model for neonatal sepsis.[6] MFG-E8 binds to macrophages, down-regulating the lipopolysaccharide-induced production and secretion of proinflammatory cytokines that play a role in sepsis and necrotizing enterocolitis (NEC).[7] As MFG-E8 is expressed in human milk, there is speculation that MFG-E8 may play a role in the prevention of intestinal inflammation in infants born preterm. We hypothesized that there is a positive relationship between dietary intake and fecal concentrations of MFG-E8 in infants born preterm, and that there is an inverse relationship between markers of intestinal inflammation and fecal concentrations of MFG-E8.

Methods

The study was approved by the Northwell Health Institutional Review Board, and informed consent was obtained from parents. A convenience sample of 40 mother-infant pairs was studied. Infants were enrolled in the first 7 days after birth if their gestational age was <33 weeks and birth weight <1501 g. Infants with major congenital anomalies were excluded. Samples of enteral feedings were collected weekly as available up to 4 times during the study. Diet samples included mother's own milk (MOM), mother's own milk fortified with a liquid bovine-based human milk fortifier (HMF) (Similac Human Milk Fortifier; Abbott Nutrition) (MOM+HMF), donor human milk (DHM) obtained from the New York Milk Bank, fortified DHM (Prolacta

DHM	Donor human milk
ELISA	Enzyme-linked immunosorbent assay
HMF	Human milk fortifier
IL-4	Cytokine interleukin-4
IL-8	Interleukin-8
MCP-1	Monocyte chemoattractant protein-1
MFG-E8	Milk fat globule-epidermal growth factor 8
MOM	Mother's own milk
NEC	Necrotizing enterocolitis
TNF-α	Tumor necrosis factor-α

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Ready to Feed), and preterm formula (Similac Special Care 24; Abbott Nutrition). Samples of feces were collected daily as available for 30 days after birth or until discharge, whichever came first. All samples were stored at −80°C until analyzed.

Analytical Methods

Fresh MOM samples were separated in aliquots from the total volume of milk expressed from one breast and stored at −80°C. At the time of analysis milk samples were centrifuged at 4000 rpm for 15 minutes at 4°C, and the fat layer was removed. Supernatants were aliquoted and stored at −80°C until analyzed further. Fecal samples were separated into 100 mg aliquots and stored at −80°C. Fecal samples were added to 800 mL of radioimmunoprecipitation assay buffer, centrifuged at 10 000 rpm for 30 minutes at 4°C, and the resulting supernatant was stored at −80°C until analyzed. MFG-E8 concentration in milk and fecal extracts were measured using Human MFG-E8 Quantikine enzyme-linked immunosorbent assay (ELISA) (R&D Systems). Cytokine analyses were performed by ELISA using Human Cytokine Array Proinflammatory Focused 13-plex (Eve Technologies). Fecal calprotectin was analyzed by ELISA (Eagle Biosciences).

To adjust for differences in concentration and intake in analyses of fecal samples, high MFG-E8 exposure was defined by an enteral intake of MOM and/or MOM+HMF ≥60 mL/kg/day whereas low MFG-E8 exposure was characterized as all other (or no) enteral feedings. As diet and intake varied during the study, we evaluated exposure only for the 48 hours prior to fecal collection. Fecal cytokine and calprotectin concentrations were measured weekly as available. Mean fecal MFG-E8 and cytokine concentrations were analyzed in relation to MFG-E8 exposure. Cytokine data were log-transformed for analysis. Data were analyzed using Minitab 14 (Minitab). The Pearson correlation coefficient was used to compare continuous variables, and Kruskal-Wallis test was used for comparisons of independent variables.

Results

From July 2018 to August 2019, 425 stool samples and 209 milk samples were collected from 40 infants and 36 mothers (**Table**; available at www.jpeds.com). Mean MFG-E8 concentrations in MOM and MOM+HMF samples were significantly greater than those in DHM, fortified DHM, and preterm formula (**Figure 1**). The mean concentration of MFG-E8 in unfortified MOM was significantly greater than that in fortified MOM (**Figure 1**). Significant differences persisted between MOM and other enteral samples, and between MOM and MOM+HMF, after adjustment for differences over time and between subjects.

Mean fecal MFG-E8 concentrations were significantly greater in samples from infants with a diet of MOM and MOM+HMF than those with of a diet of DHM, preterm formula, or no feeding in the 48 hours preceding fecal sample collection (**Figure 2**). Fecal MFG-E8 concentrations were

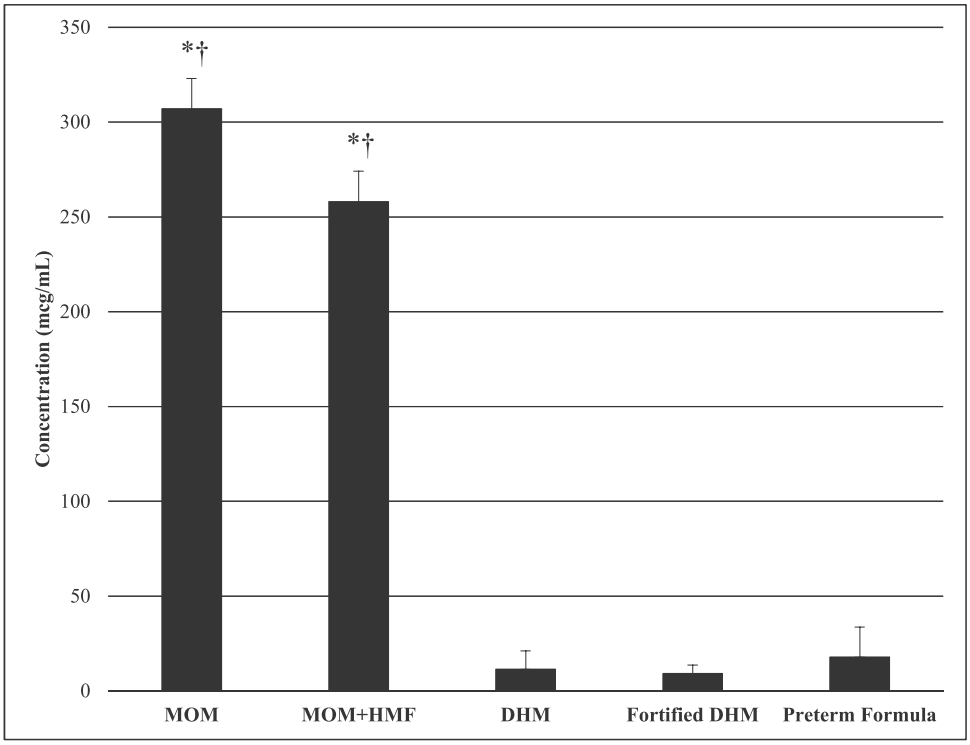

Figure 1. MFG-E8 concentrations in diets of infants born preterm. The MFG-E8 concentrations in samples of MOM (n = 112) and MOM+HMF (n = 75) were significantly greater than DHM (n = 5), fortified DHM (n = 13), and preterm formula (n = 4), $^{*}P < .001$. The concentration of MFG-E8 in MOM was significantly greater than that in MOM+HMF, $^{\dagger}P = .036$.

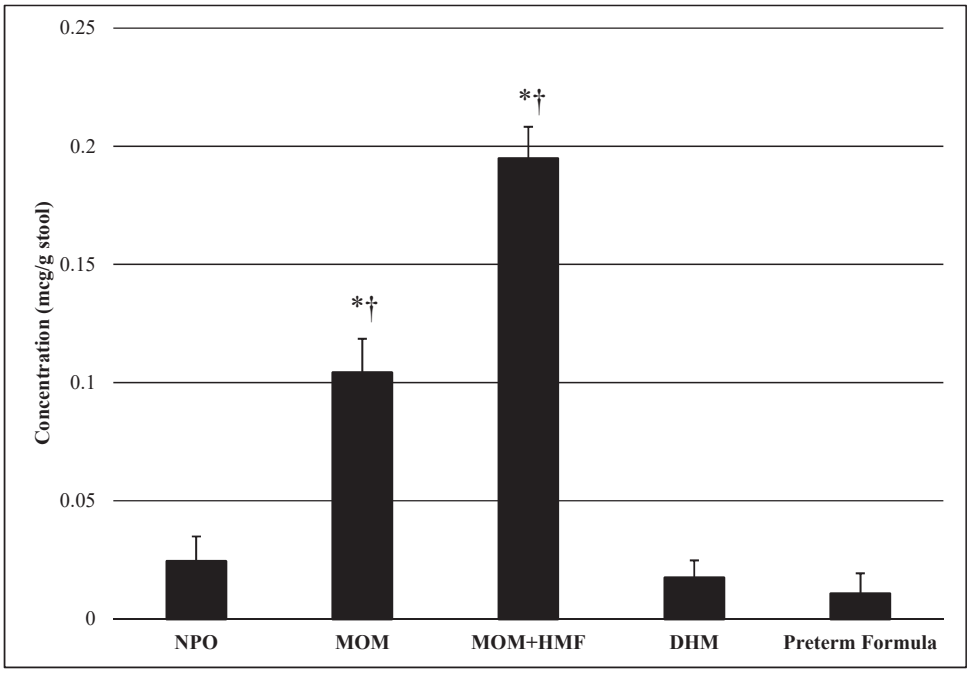

Figure 2. Fecal MFG-E8 concentration vs infant diet. The mean fecal MFG-E8 concentration was significantly greater in fecal samples from infants with a diet of MOM (n = 111) and MOM+HMF (n = 230) than DHM (n = 37), preterm formula (n = 13), or no feeding (n = 34) in the 48 hours preceding fecal sample collection, $^{*}P < .001$. The concentration of MFG-E8 in MOM was significantly greater than that in MOM+HMF, $^{\dagger}P < .001$.

positively correlated with MFG-E8 concentration in diet samples (r = 0.42, $P < .001$) (**Figure 3**) and with enteral intake (r = 0.23, $P < .001$). MFG-E8 was significantly higher in fecal samples after high dietary MFG-E8 exposure compared with samples after of lower dietary MFG-E8 exposure (0.099 mcg/g stool vs 0.003 mcg/g stool, $P < .001$).

We next examined the relationship between dietary MFG-E8 exposure and mean fecal concentrations of pro- and anti-

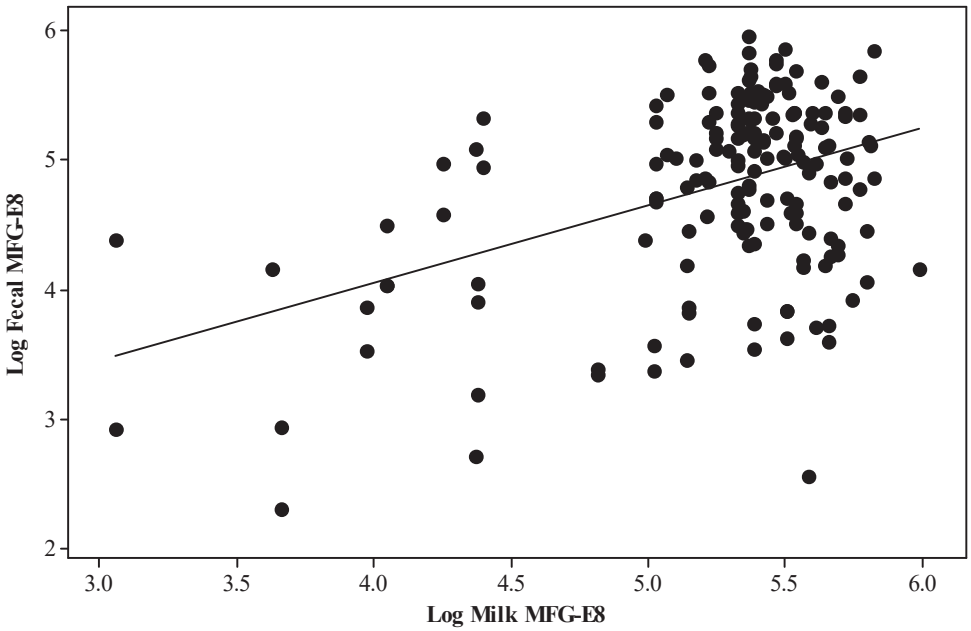

Figure 3. Diet and fecal MFG-E8 concentration. MFG-E8 concentrations in fecal samples (n = 196) were positively correlated with MFG-E8 concentration in respective diet samples, r = 0.42, $P < .001$.

inflammatory cytokines (Figure 4). High dietary MFG-E8 exposure was negatively correlated with proinflammatory cytokines interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1 (MCP-1), and positively correlated with the anti-inflammatory cytokine interleukin-4 (IL-4), when compared with low dietary MFG-E8 exposure. In addition, we found a significant negative relationship between milk MFG-E8 and fecal calprotectin (r = −0.18, P = .025) and positive relationship between calprotectin and IL-8 in fecal samples (r = 0.28, P = .001). Finally, we found no relationship between comorbidities and MFG-E8 in fecal samples. However, fecal MFG-E8 concentrations in infants with spontaneous intestinal perforation and NEC (0.026 mcg/g stool) trended lower than in infants without these conditions (0.062 mcg/g stool, P = .056).

Discussion

Concentrations of MFG-E8 were significantly greater in MOM and MOM+HMF samples compared with either DHM or preterm formula. In addition, we noted that infant diet correlated with fecal concentrations of MFG-E8; a diet including MOM was associated with significantly greater concentrations of fecal MFG-E8. We found that higher exposure to MFG-E8 was associated with lower fecal concentrations of the proinflammatory cytokines IL-8, TNF-α, and MCP-1, as well as fecal calprotectin, and with higher fecal concentrations of the anti-inflammatory cytokine IL-4.

Various cytokines have been associated with intestinal pathology in infants born preterm.[9,10] IL-8 is produced by intestinal lamina propria cells and has been correlated with both the degree of mucosal inflammation as well as clinical severity.[11-13] TNF-α also has been shown to be elevated in patients with NEC. This cytokine stimulates leukocyte migration and the resulting acute phase response, and plays a role in apoptosis and the onset of shock.[14,15] IL-4, meanwhile, downregulates the inflammatory cascade and leads to decreased tissue injury.[9] MCP-1 has not been described in relation to NEC, but may play a role in regulation of migration and infiltration of leukocytes.

Intestinal inflammation is a major concern in infants born prematurely.[8] Abnormal intestinal motility and treatment with antibiotics often causes dysbiosis in infants born preterm, with a predominance of pathogenic bacteria colonizing the intestine. This can trigger activation of inflammatory cascades via the interaction between lipopolysaccharides and toll-like receptor 4.[16-18] Consequently, activated leukocytes secrete proinflammatory cytokines, leading to cytotoxicity and epithelial injury.[19] Our finding that high MFG-E8 intake is associated with lower inflammatory cytokine and calprotectin content in feces suggests that MFG-E8 similarly dampens intestinal inflammation in infants born prematurely. We speculate that MFG-E8 may contribute to the protective effects of MOM in reducing the incidence of NEC.

Although the concentration of MFG-E8 was greater in MOM+HMF compared with donor milk or preterm formula, MOM+HMF had significantly lower MFG-E8

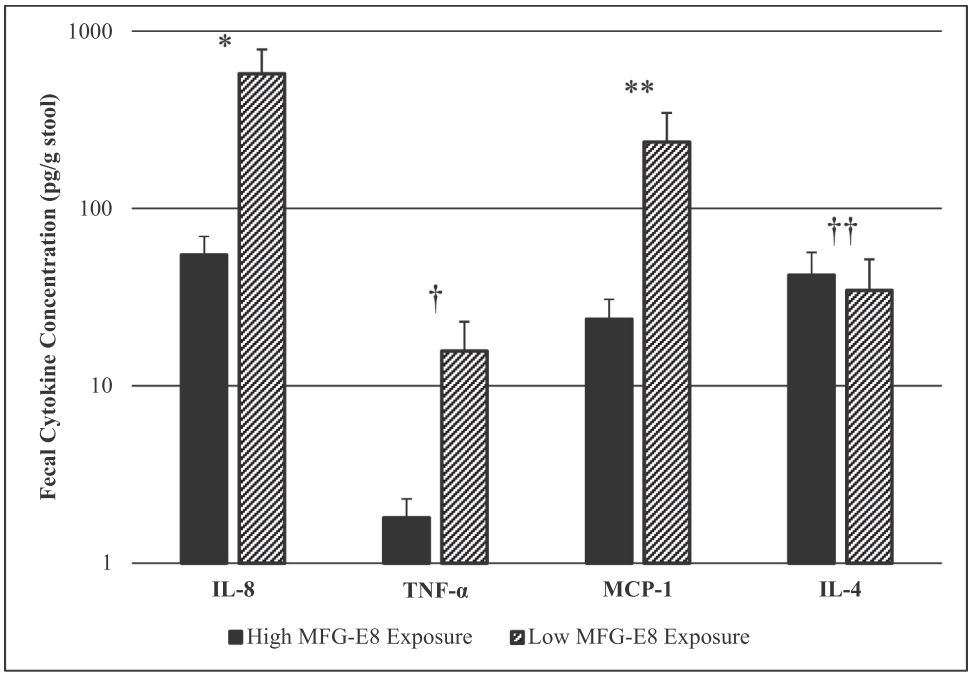

Figure 4. Relationship between fecal MFG-E8 and cytokine concentrations. High MFG-E8 exposure (n = 107) was correlated negatively with proinflammatory cytokines (IL-8, TNF-α, and MCP-1) in fecal samples, and positively correlated with the anti-inflammatory cytokine IL-4 compared with low exposure (n = 49), *P = .001, †P = .016, **P = .002, ††P = .026.

than MOM alone. We speculate that this was a dilutional effect of adding the liquid bovine-based human milk fortifier to MOM, displacing a portion of its volume. Although there was a difference between MFG-E8 concentrations in MOM and MOM-HMF, both diets were an order of magnitude higher than DHM, fortified DHM, or preterm formula. The heat lability of MFG-E8 may account for the limited quantities of this component in DHM, fortified DHM, and preterm formula.[20,21]

There were limitations to our study. Fecal samples were collected inconsistently due to the sporadic nature of infant fecal production. In addition, there was significant variation in the diet of each subject from week to week. For this reason, our analysis was performed by cohorting fecal samples and not study subjects. As this was a pilot study, the small sample size precluded broader conclusions regarding MFG-E8 intake and comorbid conditions.

In conclusion, fecal MFG-E8 concentrations correlated with a diet containing MOM, and were inversely associated with intestinal inflammation. It is unclear from this investigation whether MFG-E8 exerts a direct anti-inflammatory effect or is a surrogate marker for the protective effects of a human milk diet. ■

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Table. Maternal/neonatal characteristics

Mothers (n = 36)	
Age, y	32 ± 6*
Cesarean delivery, n (%)	30 (75%)
Steroid treatment, n (%)	40 (100%)
Antibiotic treatment, n (%)	21 (53%)
Infants (n = 40)	
Female/male, n (%)	21 (53%)/19 (49%)
Gestational age, wk	28 ± 2*
Birth weight, g	1071 ± 260*
Age feeding initiated, d	3 (2;4)†
Age human milk fortification, d	9 (7;11)†
Age complete enteral nutrition, d	14 (12;20)†
Discharge weight, g	2330 ± 644*
Age at discharge, d	61 ± 27*
Comorbid infant conditions	
Bronchopulmonary dysplasia	6 (15%)
Spontaneous intestinal perforation	3 (8%)
NEC (total)	6 (15%)
Medical	5 (13%)
Surgical	1 (2%)
Sepsis	8 (20%)
IVH (grade I-II)	11 (28%)
IVH (grade III-IV)	0
Retinopathy of prematurity (total)	11 (28%)

IVH, intraventricular hemorrhage.
*Mean ± SD.
†Median (IQR, 25th; 75th percentile).