Breast Milk Protects Against Gastrointestinal Symptoms in Infants at High Risk for Autism During Early Development

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ABSTRACT

Objectives: Parents of children with autism spectrum disorders (ASDs) often report gastrointestinal (GI) dysfunction in their children. The objectives of the present study were to determine whether infants at high risk for developing ASD (ie, siblings of children diagnosed as having ASD) show greater prevalence of GI problems and whether this prevalence is associated with diet and age at weaning from breast milk.

Methods: Using questionnaires, diet history and GI problems were tracked prospectively and retrospectively in 57 high-risk infants and for comparison in 114 low-risk infants (infants from families without ASD history).

Results: In low-risk infants, prevalence of GI symptoms, in aggregate, did not vary with diet or age of weaning. By contrast, high-risk infants with GI symptoms were weaned earlier than those without symptoms ($P < 0.04$), and high-risk infants showed greater prevalence of GI symptoms, in aggregate, on a no breast milk diet than on an exclusive breast milk diet ($P < 0.017$). Constipation, in particular, was more prevalent in high-risk infants compared with low-risk infants ($P = 0.01$), especially on a no breast milk diet ($P = 0.002$). High-risk infants who completed weaning earlier than 6 months showed greater prevalence of constipation ($P = 0.001$) and abdominal distress ($P = 0.004$) than those fully weaned after 6 months.

Conclusions: The greater prevalence of GI symptoms in high-risk infants suggests that GI dysfunction during early infant development may be a part of the ASD endophenotype. Late weaning and exclusive breast milk were associated with protection against GI symptoms in high-risk infants.

Key Words: abdominal discomfort/pain, breast milk, breast-feeding, constipation, pervasive developmental disorder

What Is Known

- Gastrointestinal dysfunction is common in autism spectrum disorder.
- Weaning age, which has been suggested to affect the risk for autism spectrum disorder, may also play a role in gastrointestinal dysfunction.
- Infants with older siblings with autism spectrum disorder are at high risk themselves and may be used to determine early endophenotypes.

What Is New

- We show that infant gastrointestinal symptoms, particularly constipation, are an endophenotype for autism spectrum disorder.
- Prevalence of constipation in high-risk infants is associated with infant diet and weaning age.
- These findings may guide dietary recommendations for at-risk families and open new lines of investigation by establishing a new endophenotype for autism spectrum disorder that precedes diagnosis.

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In addition to the main hallmarks of autism spectrum disorders (ASDs), gastrointestinal (GI) dysfunction has been shown to be comorbid with ASD (1–4), although estimates for its prevalence vary considerably, with values ranging from 9% to 90% depending on the severity and type of dysfunction being quantified and the age studied (see (5) for a thorough review). Commonly listed GI symptoms are chronic constipation, abdominal pain with or without diarrhea, and encopresis (2,3,5). GI problems may also be underreported because GI conditions can present as non-GI manifestations such as behavioral changes and/or problem behaviors (5). As evidence that GI problems are associated with behavioral problems, it has been reported that individuals with both ASD and GI dysfunction are more likely to have a worsening of sensory overresponsiveness, and increased anxiety, compared with ASD individuals without GI dysfunction (6,7).

Retrospective data (based on parent interviews after children have been diagnosed as having ASD at 2 to 3 years of age or later) suggest that GI problems can occur quite early in development, that is, in the first year of life (1,8,9). To date, however, there are no

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prospective data on the early development of GI dysfunction in ASD (ie, data collected before diagnosis at 2 to 3 years). One way to obtain prospective data is to track GI development in infant siblings of children already diagnosed as having ASD. These infants are referred to as ''high-risk'' (see (10–12) for reviews) because their risk for developing ASD is ~10- to 20-fold higher than that seen in the general population (13–15). Because ASD has been shown to have a genetic component, (based on results from twin studies (16) and genetic linkage and association studies [see (17,18) for reviews]), much of the increased risk of developing ASD in high-risk infants has been attributed to them carrying some of the genes associated with ASD. Shared genetics may not be the full explanation, however, because high-risk infants also share environmental factors with their older siblings with ASD. Regardless of the extent to which the risk for developing ASD is because of genes or environment, many studies have now shown that even those high-risk infants who do not develop ASD show abnormalities compared with low-risk control children (from families without ASD history) (see (19) for review and (20) for evidence of GI problems in first-degree relatives of individuals with ASD). The advantage of the ''high-risk'' approach is that such abnormalities may elucidate the risk factors associated with developing ASD despite only a minority of these infants actually developing ASD. The present study investigated GI dysfunction in high-risk compared with low-risk infants to determine whether early GI dysfunction may also be an abnormality associated with ASD. These abnormalities that run in individuals with ASD and their first-degree relatives are referred to as ''endophenotypes'' of ASD (18,21).

As mentioned above, although there is strong evidence for a genetic component in ASD, there is also a contribution from the environment (16), even though there are relatively few studies directly testing this possibility. One possible environmental factor is infant diet. Breast-feeding for <2 months, compared with breast-feeding for ≥6 months, is associated with significantly increased chances of an infant in the general population developing ASD (22). One possible mechanism for the protective effect of breast milk is through its effects on the developing GI tract. Motivated by these findings implicating breast milk as a potential protective factor, the present study, using a mixture of prospective and retrospective data, tracked diet history and GI dysfunction in high- and low-risk infants. The aim is to determine whether GI dysfunction is related to group status (high- vs low-risk), dietary history, or an interaction between the two.

METHODS

Subjects

High-risk infants with an older sibling diagnosed as having ASD were recruited through advertisements in the San Diego area and through referrals from other laboratories studying ASD at the University of California, San Diego. The older siblings of the high-risk infants were diagnosed as having ASD (including autistic spectrum disorder, Asperger syndrome, or pervasive developmental disorder not otherwise specified [PDD-NOS]) by a licensed clinical psychologist or medical doctor not associated with this research, based on *DSM-IV-TR* criteria (the *DSM-V* no longer recognizes these separate categories of ASD; however, these subjects were recruited when the *DSM-IV* was still in effect.). They had no known specific neurological or genetic conditions (eg, Fragile X, Rett syndrome) that could account for their diagnosis of ASD. We confirmed the ASD diagnosis of each older sibling using the *Autism Diagnostic Observation Schedule* (*ADOS*) (23) and the *Autism Diagnostic Interview-Revised* (24). Detailed information for the older sibling of each high-risk infant is presented in supplemental Table 1 (*http://links.lww.com/MPG/A531*).

TABLE 1. Demographics of the low- and high-risk infants

	Low risk, N (range)	High risk, N (range)	*P*
	49 ± 39	47 ± 25	0.46
IPI, mo	114 (12–214)	57 (11–153)	
	35 ± 4	33 ± 4	0.045
Mothers' age, y*	114 (25–45)	55 (24–47)	
	37 ± 6	35 ± 5	0.053
Fathers' age, y*	111 (22–58)	53 (24–51)	
	16 ± 13	15 ± 14	0.20
Age First Q, mo†	114 (3–39)	57 (3–37)	
	27 ± 11	24 ± 11	0.052
Age Last Q, mo‡	114 (6–39)	57 (6–38)	
	−6.1 ± 8.0	−8.4 ± 10.6	0.17
GD	113 (−41–16)	57 (−54–10)	
Females, %	42.5	40.4	0.87
Race (white), %§	70.2	63.2	0.39
Ethnicity (Hispanic), %‖	14.0	12.3	0.82

GD = gestational duration based on number of days that birth date was pre-/postdue date; IPI = inter pregnancy interval.

* Mothers' age and Fathers' age refer to their age at the birth of infant.

† Age First Q represents the infant age when parents filled out the gastrointestinal questionnaire for the first time (effective enrollment age for this study).

‡ Age Last Q represents the oldest age point for which we have data on the infants.

§ The choices for race were American Indian/Alaska Native, Asian, Native Hawaiian or Other Pacific Islander, Black or African America, Other (not listed), or >1 race. Here, we present the percentage of whites.

‖ For ethnicity, the choices were Hispanic/Latino versus not. Here, we present the percentage of Hispanic/Latino.

Low-risk infants (infants from families without any history of ASD, that is, no biological siblings, parents, aunts/uncles, or cousins diagnosed as having ASD or any other developmental disorder) were recruited in San Diego via letters sent to parents. Like high-risk infants, all of the low-risk infants had ≥1 older sibling. For each high-risk infant, we recruited 2 low-risk infants, trying to match on sex, gestational duration (within 7 days), race, and ethnicity, so that the final samples of high- and low-risk infants would not differ significantly in these variables, which they did not (Table 1).

All of the subjects were screened to confirm they had an uncomplicated birth and no major medical problems. In accordance with the guidelines of our approved protocols from the internal review committee at the University of California, San Diego, the parent of each subject in our study signed a consent form to participate. The subjects in this study were part of a larger longitudinal study that tracked visual, cognitive, social, and language development, and GI symptoms, from 3 to 36 months of age (21,25,26).

GI and Diet Questionnaires

Parents completed questionnaires on their infant's GI health and diet history at 3, 6, 7, 8, 10, 14, 18, 24, and 36 months of age (chosen because these were time points in a larger longitudinal study (21,25,26)). To increase enrollment in the present study, parents of infants at any of the above ages in the longitudinal study were invited to join the present study. Therefore, our data regarding diet and GI dysfunction is a mix of both prospective and retrospective data. Relative to the age of diagnosis for ASD at 3 years, however, all data in the present study can be considered prospective or concurrent. When parents completed the questionnaires for the first time, they were asked to report about events between birth and

the present time. At subsequent visits, the questionnaire focused on events that occurred between the previous questionnaire and the present time. The questionnaires for the first and subsequent visits are provided in the Appendix (*http://links.lww.com/MPG/A531*).

The age at which parents began and completed the weaning of their child was used to determine when the infant was in each of 3 possible diet categories: exclusive breast milk (EBM), which is the diet before start of weaning, partial breast milk (PBM), which is the diet between the start and the completion of weaning, and no breast milk (NBM), which is the diet after complete weaning. The questionnaires also included a table of GI symptoms (eg, diarrhea, reflux, constipation; Appendix [*http://links.lww.com/MPG/A531*]). Parents were asked to report which, if any, of these symptoms their infant experienced and which diet category the infant was in at that time. (Of the symptoms on the questionnaires, ''trouble nursing'' was dropped from the analysis because its prevalence was below 6% in both groups regardless of diet category, and it is not typically associated with ASD. Also, recognizing that ''gassiness and/or bloating,'' ''abdominal discomfort/pain,'' and ''colic'' are likely indistinguishable to parents of preverbal infants; these symptoms have been combined into 1 category called ''abdominal distress/irritability.'') To reduce subjectivity, parents were instructed to mark only those symptoms severe enough that they sought medical advice or made a change to their infant's care. Rather than ask parents to exactly remember the age of their infant when a GI symptom occurred, we assumed the symptom could have occurred at any time in that diet category (hypothetical example provided in supplemental Fig. 1 [*http://links.lww.com/MPG/A53*]).

Data Analyses

In the first analysis, we investigated the aggregate prevalence of GI symptoms, without regard for a specific GI symptom, which we refer to as ''any GI symptom.'' For each month after birth, we calculated the number of infants in each of the diet categories and the number of infants within that category for whom any GI symptom was reported (supplemental Fig. 1 [*http://links.lww.com/MPG/A531*] for hypothetical examples), to determine the percentage of infants with GI problems as a function of diet category. We refer to this as the ''point prevalence,'' that is, the proportion of the population that has a GI problem at a specific point in time and/or in a specific diet category. Except for 1 infant, no infants were in the EBM category in the last 12 months. We, therefore, restricted this analysis to the period up to 12 months so that we would have enough infants in the EBM category to investigate the associations of diet with GI symptoms. At each time point, we determined whether there were significant differences in the point prevalence of any GI symptoms: across diet categories and between low- and high-risk infants for each of the 3 diet categories. As infants were transitioning between diet categories at different ages, it was not appropriate to compare point prevalence at one time point to point prevalence at another (ie, subject populations overlapped but were not identical across time points).

In our second analysis, we incorporated the effects of age by asking whether there were differences in the ages that weaning was started or completed between infants who had any GI symptoms versus those who did not.

In the third analysis, we investigated individual GI symptoms, calculating the prevalence of each. We performed this analysis for each of the 3 diet categories and without regard for the particular diet category, which we refer to as ''any diet.''

In the fourth analysis, we investigated the associations between individual GI symptoms and diet category while simultaneously adjusting for other factors using multivariate models. For the multivariate analyses, generalized estimating equations were used to conduct repeated measure logistic regression. This analysis takes into account the fact that different infants enter into each diet category at different ages and may enter into a different number of diet categories during the observation period of the study, thus contributing unequally to the data. Performing this type of multivariate analysis enabled us to distinguish independent associations for diet category, subject group, age, and other covariates.

Bonferroni-corrected t tests or Fisher tests were used for statistical tests in which we made multiple pairwise comparisons. The number of comparisons used to determine alphas (significance cutoffs) are described in figure legends (for comparisons between only 2 groups and for the multivariate analysis, $\alpha = 0.05$). We refer to findings as ''marginally significant'' if their P value is $>\alpha$ but <0.05 (or <0.1 for the multivariate analyses). Because of the paucity of previous research in this area of GI dysfunction in infant siblings and the wide variation in estimates (9%–90%) of GI dysfunction in adults and older children with ASD (5), we could not perform a power analysis in advance of the study.

Outcome Assessments

In our laboratory, at 24 and 36 months of age, the outcome of infants in our study was assessed with the *ADOS* and the *Mullen Scales of Early Learning* (27). The *Autism Diagnostic Interview-Revised* was conducted for any child who scored above the ASD cutoff on the *ADOS*. Of the 57 high-risk infants included in our analyses, 23 were not assessed by study completion because of their families being unavailable or because of being too young for the assessments. The remaining 34 were assessed for ASD (22 at 36 months and 12 at 24 months because these infants had not yet reached 36 months at study completion). Of these 34, eleven were diagnosed as having ASD (7 with autism and 4 with PDD-NOS). (We also tested low-risk infants at 24 and 36 months. Of the 114 low-risk infants, 57 were assessed for ASD at 36 months and an additional 75 were assessed for ASD at 24 months. Two additional infants were found to have PDD-NOS, and their data are not included in our analyses. Of the 39 untested infants, 9 were unavailable for testing because the family moved or was unreachable, and 30 were too young [ie,<24 months] to be tested. Given the frequency of ASD in the general population [$\sim$1%], there is an extremely small chance that 1 of our 39 untested low-risk infants will develop ASD.) Analyses were conducted with these 11 infants both included and excluded, to determine the degree to which they drove our findings. In addition, the prevalences of select outcomes were compared between low-risk infants and the 11 high-risk infants who developed ASD and between low-risk infants and the remaining 23 high-risk infants who were assessed and did not develop ASD.

RESULTS

Demographics

In total, 114 low-risk and 57 high-risk infants contributed to the data in this study. The demographics of the 2 groups are presented in Table 1. The 2 groups did not differ significantly in basic demographics: sex, gestational duration, race, or ethnicity. The mean age at which the 2 groups were enrolled in this study, that is, age of the first questionnaire (Age First Q), did not differ ($P = 0.2$); however, the last data collection point (Age Last Q) was at an older age in low-risk infants than high-risk infants ($P = 0.052$), that is, 27 vs 24 months. The only demographic difference between the groups, which we did not attempt to match at the time of enrollment, was maternal and paternal age at the infant's birth. Both maternal and paternal ages were slightly greater (by $\sim$2 years) in the low-risk group (both P values near 0.05).

Insufficient numbers of parents responded with income data to analyze socioeconomic differences between groups.

Distribution of Diet as a Function of Age and Point Prevalence of Any GI Symptoms Within Each Diet Category

In our first analysis, we asked whether there were significant differences in the point prevalence of the aggregated GI symptoms: across diet categories and between low- and high-risk infants for each of the 3 diet categories. Results are shown in Figure 1. For low-risk infants (Fig. 1, upper panel), point prevalence of any GI dysfunction did not vary with diet category, at any age (all P values >0.15, Fisher test). There was a trend for greater point prevalence of GI symptoms for low-risk infants on an NBM diet at month 2, but this was nonsignificant with a low number of infants (ie, only 6) on NBM.

In contrast to low-risk infants, GI symptoms in high-risk infants (Fig. 1, lower panel) varied with diet category; high-risk infants exhibited greater point prevalence of GI dysfunction in the NBM category at the younger ages. Specifically, point prevalence of GI dysfunction in the NBM category, as compared with the EBM category, was significantly ($P < \alpha = 0.017$) or marginally significantly ($0.017 < P < 0.05$) greater through month 5 (and then marginally significant again at month 8). In addition, point prevalence of GI dysfunction in the NBM category, as compared with the PBM category, was greater by a marginal significance at 4 and 5 months of age ($P < 0.03$).

In addition to analyzing whether GI dysfunction varied with diet within each subject group, we also investigated whether there were differences across subject groups within each diet category. At most times in the first year of life, the high-risk group had a higher point prevalence of GI symptoms than the low-risk group when on NBM (although this result did not reach significance) but not when on EBM. In sum, these results suggest greater prevalence of GI symptoms in high-risk infants who were not receiving breast milk.

Time of Weaning

As a second analysis, we investigated group differences in the ages for starting and completing the weaning process. We additionally examined the effect of weaning age on ''any GI symptom'' data by asking whether there were differences between infants who had any GI symptoms versus those who did not in the ages at which weaning was started or completed. For the analysis of ''start of weaning age,'' the data from all of the infants were used because all the infants started to wean before their final questionnaire. The ''completion of weaning age,'' however, could not be obtained for all the infants because of the fact that some infants remained on partial breast milk even at the time of their final questionnaire. To address this latter limitation, we only included infants who were enrolled through $\geq$2 years of age. We chose the 2-year mark because it provided a good balance between overall inclusion (it captured 86% of all infants in our study) and completion of weaning (96% of the infants in this sample had completed weaning by 2 years). For the remaining 4% who had not completed weaning by 2 years, we set the upper limit of age of completion of weaning to 2 years, rather than using the last available data point (Age Last Q), to avoid the potential confound from the slight difference in Age Last Q (Table 1 and see above) between groups.

The results from these analyses showed that, regardless of whether an infant had any GI symptoms, high- and low-risk

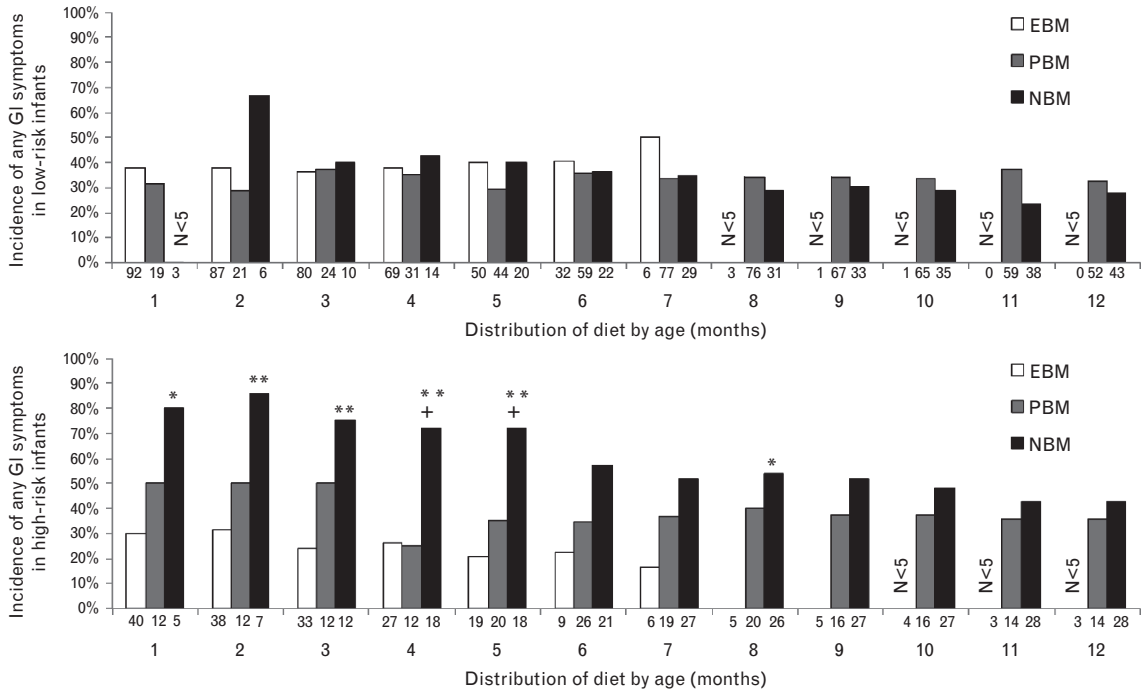

FIGURE 1. Any GI symptom. Point prevalence of infants who experienced any GI symptom, in each diet category, separately for low-risk (upper panel) and high-risk (lower panel) infants. The numbers below each bar show the number of infants in each diet category (EBM, PBM, and NBM) at each month in the first year of life. Groups with <5 infants were not included in the analysis because of low power. $^{**}P \leq 0.012$ NBM versus EBM, $^{++}P \leq \alpha$ NBM versus PBM. Note: $\alpha = 0.017$ (Fisher test, Bonferroni-correction factor of 3) because each diet category is used in 3 comparisons, such as the high-risk NBM group is compared with the high-risk EBM group, the high-risk PBM group, and the low-risk NBM groups. Single symbols indicate marginal significance ($\alpha < P < 0.05$). EBM = exclusive breast milk; GI = gastrointestinal; NBM = no breast milk; PBM = partial breast milk.

infants started the weaning process at the same age (low-risk 3.8 ± 2.4 months, high-risk 3.6 ± 4.0 months). By contrast, high-risk infants completed the weaning process significantly earlier (7.6 ± 6.5 months) than low-risk infants (11.1 ± 6.8 months) (Fig. 2A, $P = 0.003$). Interestingly, the amount of time each group spent on an NBM diet during our study (determined as Age Final Q minus age weaning was completed; infants still on PBM diet had a 0 value entered for this) was similar (15.2 ± 11.7 months for high-risk infants and 15.7 ± 11.7 months for low-risk infants, $P = 0.83$, including all infants in the study). This indicates that the greater prevalence of certain symptoms on an NBM diet in high-risk infants compared with low-risk infants (see below) is not because of increased time on that diet.

With regard to the effects of weaning age on symptoms, in low-risk infants there were no differences between infants with versus without GI symptoms in the ages at which they started or completed weaning (Fig. 2B). In contrast, high-risk infants with GI symptoms both started ($P = 0.02$) and completed ($P = 0.03$) their weaning at younger ages than high-risk infants without GI symptoms (Fig. 2C). The results from these analyses are consistent with the findings from the first analysis of GI symptom prevalence (above) showing that the prevalence of GI symptoms is higher in high-risk infants on NBM diet at younger ages.

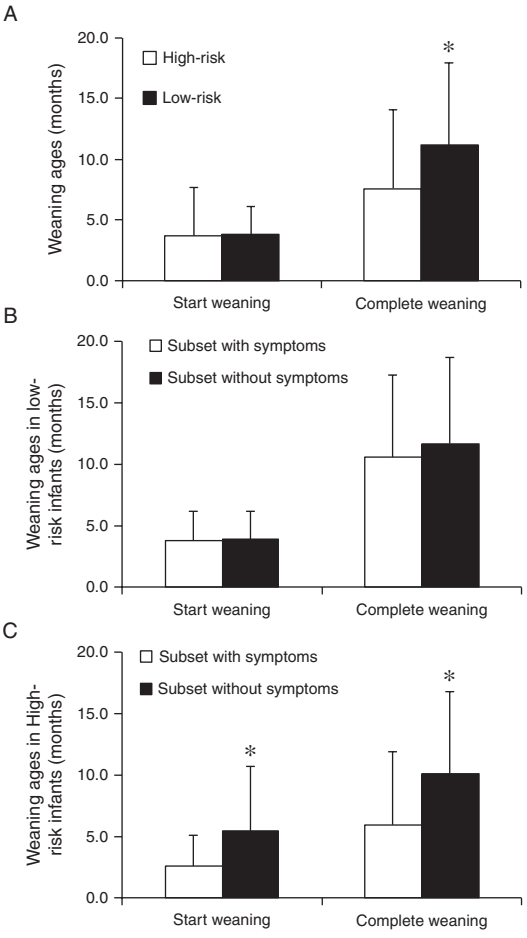

FIGURE 2. A, Ages at which high-risk and low-risk infants started and completed weaning. $^{*}P = 0.003$ low-risk versus high-risk. B and C, Ages at which low-risk (B) and high-risk (C) infants, with versus without symptoms, started and completed weaning. $^{*}P < 0.04$ without versus with symptoms. N (start weaning) = 57 (35 with symptoms) high-risk and 114 (60 with symptoms) low-risk infants. N (finish weaning) = 47 (28 with symptoms) high-risk and 100 (50 with symptoms) low-risk infants. Note, $\alpha = 0.05$ (*t* tests, no Bonferroni correction).

Prevalence of Individual GI Symptoms

In the third analysis, we investigated individual symptoms, calculating the prevalence of each symptom in each individual diet category and without regard for the particular diet category, which we refer to as ''any diet.'' Results are shown in Figure 3. In low-risk infants (upper panel), the most commonly reported GI symptom was ''Spitting Up or Reflux'' (hereafter referred to as reflux), and this symptom was significantly more likely to occur while on an EBM diet than on an NBM diet ($P < 0.0001$). No other symptoms in the low-risk group appeared to be associated with diet. High-risk infants (lower panel) had a similar prevalence of reflux in ''any diet,'' but showed less specificity to the EBM phase for this symptom. In comparison with low-risk infants, high-risk infants had a significantly greater prevalence of constipation ($P = 0.01$). Constipation was significantly more likely to occur while these high-risk infants were on an NBM ($P < 0.0001$) or PBM ($P = 0.006$) diet than on an EBM diet.

To incorporate the start and completion of weaning age information into the symptom analysis, we subdivided the infant populations into 2 groups, one that completed weaning before, and one that completed weaning after, 6 months, the median age at which high-risk infants completed weaning. In low-risk infants, reflux was dependent on diet (being most common on an EBM versus an NBM or PBM diet, $P < 0.0001$ and $P = 0.004$, respectively; Fig. 4, third row, spitting up and reflux group) in those weaned after 6 months but not for those weaned before 6 months (ie, no significant differences; Fig. 4, top row). This suggests, indirectly, that reflux may be more dependent on age than diet category (see Discussion). In high-risk infants, prevalence of constipation was significantly greater in infants on NBM diet compared with EBM diet in those who completed weaning before 6 months (Fig. 4, second row) but not in those who completed weaning after 6 months (Fig. 4, fourth row).

With respect to comparisons between groups, the prevalence of constipation in high-risk infants was not different from low-risk infants in infants who completed weaning after 6 months but was significantly higher in infants who completed weaning before 6 months (Fig. 4). This difference was driven primarily by constipation in high-risk infants on an NBM diet. Abdominal distress was also more likely in high-risk than low-risk infants who completed weaning before 6 months.

We also repeated this analysis using 3 months as the cutoff point, and here the effects of age of weaning were even more pronounced than when we used 6 months as the cutoff. Results are shown in supplemental Figure 2 (*http://links.lww.com/MPG/A531*). Prevalence of constipation was significantly greater in high-risk infants weaned before versus after 3 months (56% vs 21%, $P = 0.013$). Abdominal distress was also significantly greater in high-risk infants who completed weaning before (61%) versus after 3 months (23%, $P = 0.008$). Of note, abdominal discomfort in low-risk infants on a PBM diet was also greater (marginal significance, $P = 0.05$) in those completely weaned before 3 months compared with those weaned after, suggesting that the transition from breast milk may be difficult even for the general population when it occurs before 3 months of age. With respect to comparisons between groups, the prevalence of constipation in high-risk infants was not different from low-risk infants in infants who completed weaning after 3 months but was significantly higher in infants who completed weaning before 3 months (56% vs 0%, $P = 0.001$).

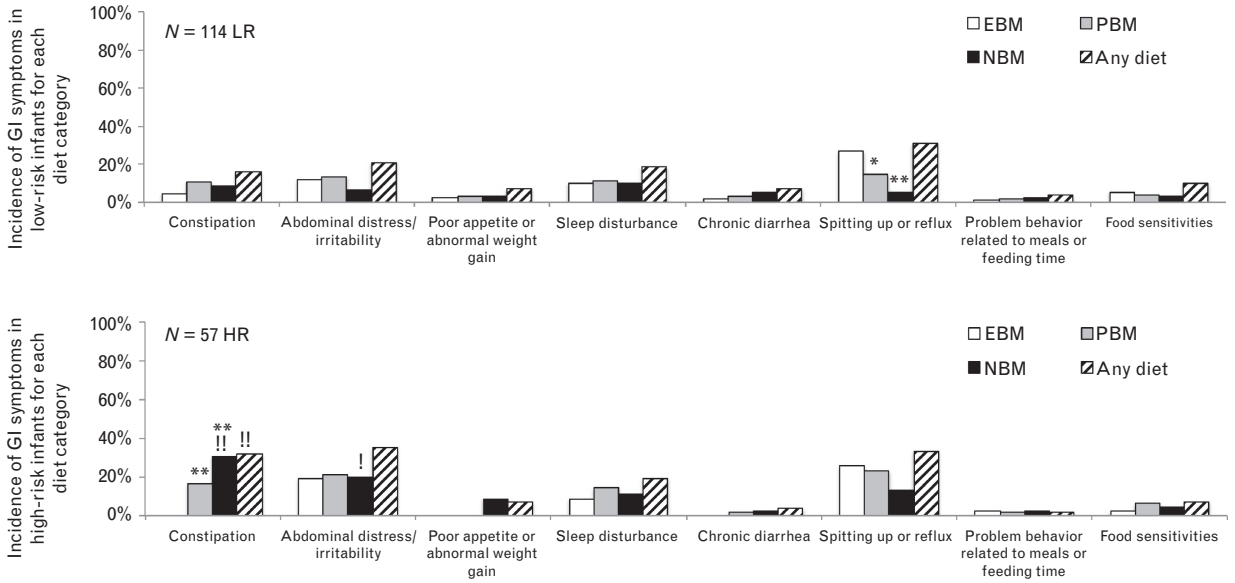

FIGURE 3. Prevalence of GI symptoms within each diet category for low-risk (top) and high-risk (bottom) infants. $^{**}P < 0.006$ NBM or PBM versus EBM, !!$P < 0.011$ high-risk versus low-risk group. Note, $\alpha = 0.017$ (Fisher test, Bonferroni-correction factor of 3) for differences between EBM, PBM, and NBM categories (each is compared against the other 2 diet categories and the same diet category in the other risk group) but $\alpha = 0.05$ for the ''any diet'' group because it is only compared with the ''any diet'' category of the other risk group. Single symbols indicate marginal significance ($\alpha < P < 0.05$). EBM = exclusive breast milk; GI = gastrointestinal; NBM = no breast milk; PBM = partial breast milk.

To investigate even further, we looked at the effects in infants who had started to wean by 3 months, with the data presented in supplemental Figure 3 (*http://links.lww.com/MPG/A531*). The effects in this group were similar to those shown in Figure 4 (with completion of weaning before 6 months). Though EBM diet is recommended through 6 months of age (28), it was not feasible to compare infants who started weaning before 6 months with those who started weaning after 6 months because of the low number of either high-risk (7 of 57) or low-risk (9 of 114) infants who met the 6-month recommended duration of EBM diet.

Multivariate Analysis

To more thoroughly investigate the predictors of GI symptoms, we performed multivariate logistic regression analyses for the 3 GI symptoms that appeared to be elevated by diet or risk category (based on the above analysis). The predictor variables were diet (EBM, PBM, and NBM) and subject group (low-risk and high-risk), and the covariates (included to account for variance in the data unrelated to the main predictor variables) were infant weaning ages and sex, and the age of mother and father at birth. Results of these analyses, reported in Table 2, suggest that diet type is independently associated with higher likelihood of constipation symptoms but not abdominal distress or reflux. More specifically, the odds of constipation were increased by 6.0-fold for PBM diet ($P = 0.001$) and 9.2-fold for NBM diet ($P = 0.004$), compared with the EBM diet. Subject group category appears to magnify this effect of diet, as shown by a marginally significant interaction between NBM diet and subject group (adjusted odds ratio [AOR] = 15.73, $P = 0.091$, N = 46 NBM + high-risk, and 93 NBM + low-risk infants). In other words, in line with our analyses of the prevalence of individual GI symptoms (Fig. 3), constipation appears particularly elevated in high-risk infants who are not on an EBM diet.

The results of these analyses also revealed the effects of the covariates, as follows. Increased infant's age at diet transition was independently associated with lower odds of both constipation (AOR = 0.93, $P = 0.037$) and reflux (AOR = 0.81, $P = 0.001$) but not abdominal distress. Mother's and father's age at the time of birth were associated with abdominal distress but in opposite directions. Older ages at birth for mothers were associated with increased odds of abdominal distress (AOR = 1.17, $P = 0.009$), whereas older ages for fathers were associated with lower odds of abdominal distress (AOR = 0.91, $P = 0.015$).

ASD Versus Low-Risk Group

In our data set, 11 high-risk individuals developed ASD (assessed at 24 and 36 months), allowing us to ask whether, as suggested previously (1,8,9), ASD is associated with the appearance of GI symptoms before ASD diagnosis (as put forward in the introduction, it is already well established as a comorbidity after diagnosis). Even with this small sample size, a significant percentage of the ASD infants were reported to have had constipation as a GI symptom before diagnosis as compared with low-risk infants (45% vs 16%, N = 11 and 114, respectively, $P = 0.03$).

In addition, we repeated the weaning age analysis (Fig. 2) and symptom analysis (Fig. 3), excluding the 11 high-risk individuals diagnosed as having ASD. This restricted analysis yielded similar results to that in the full population, showing higher prevalence of GI symptoms in high-risk than low-risk infants (supplemental Figs. 4 and 5 [*http://links.lww.com/MPG/A531*]). Even restricting our population to only those high-risk infants who were tested for, and found not to have, ASD (N = 23), constipation on a NBM diet was still more prevalent in that group (27%) than in low-risk infants (9%, N = 93, $P = 0.027$). These additional analyses suggest that our finding of elevated GI symptoms is not driven by the infants who went on to develop ASD. In fact, the prevalence of constipation, regardless of diet, was not significantly different between assessed high-risk infants who developed ASD (45%) and those who did not (26%, $P = 0.43$). Taken together, the finding of higher prevalence of early GI symptoms in both high-risk infants who do and do not go on to

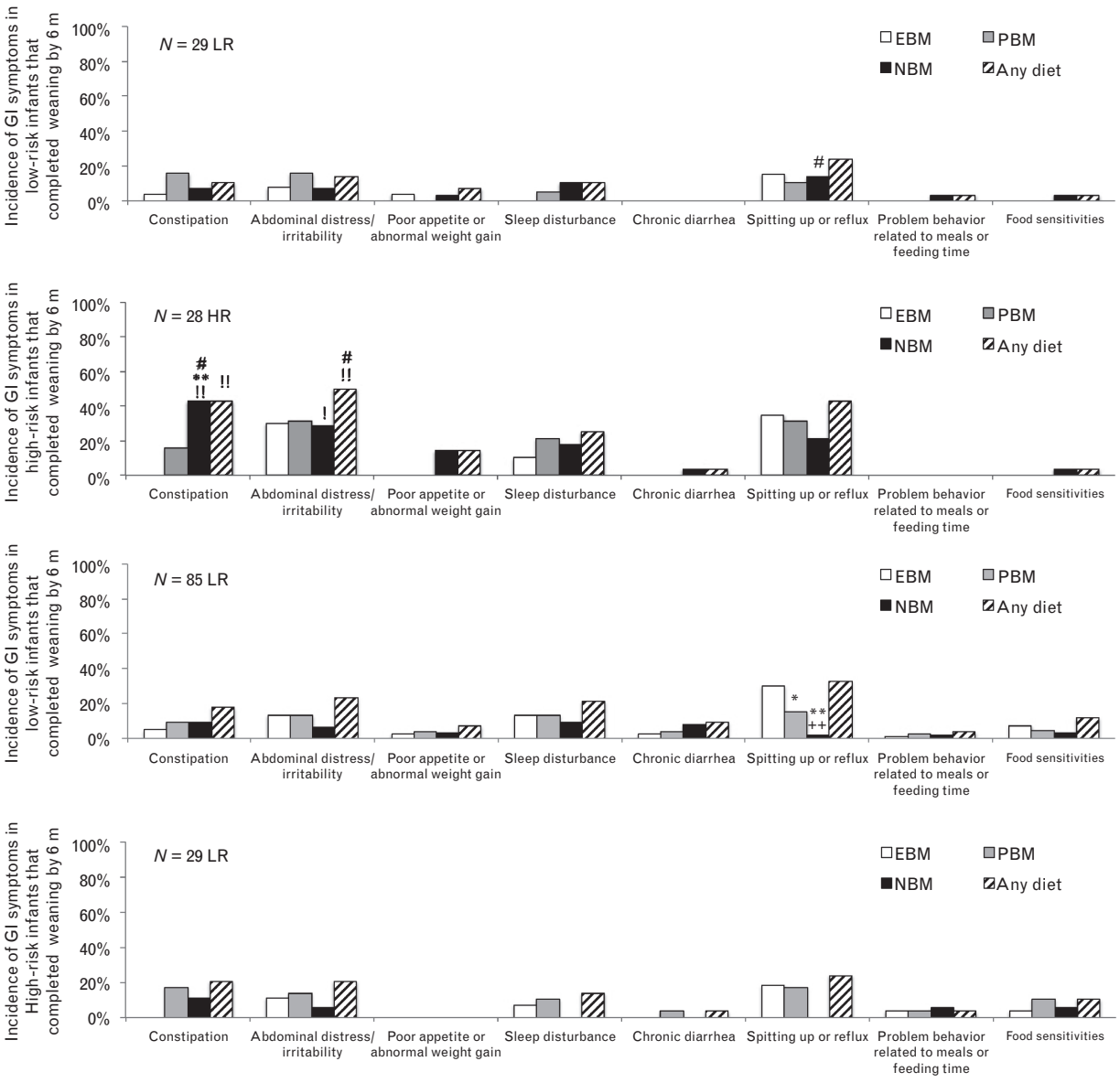

FIGURE 4. Infants divided into those who completed weaning before versus after 6 months. Prevalence of GI symptoms within each diet category for the subset of (first and third rows) low-risk and (second and fourth rows) high-risk infants who completed weaning (top 2 rows) before 6 months of age or (bottom 2 rows) after 6 months of age. $**P < 0.0006$ NBM or PBM versus EBM, $^{++}P = 0.004$ NBM versus PBM, $^{!!}P < 0.008$ high-risk versus low-risk group, and $^{\#\#}P < \alpha$ infants who completed weaning before versus after 6 months. Note, $\alpha = 0.013$ (Fisher test, Bonferroni-correction factor of 4) for differences involving EBM, PBM, and NBM categories (each is compared against the other 2 diet categories and against the same diet category in the other risk group and the other weaning age subset), but $\alpha = 0.025$ for the ''any diet'' groups because they are only compared with the ''any diet'' category of the other risk group and other weaning age subset. Single symbols indicate marginal significance ($\alpha < P < 0.05$). EBM = exclusive breast milk; GI = gastrointestinal; NBM = no breast milk; PBM = partial breast milk.

develop ASD supports the inclusion of early GI symptoms (specifically constipation while on an NBM diet) as an ASD endophenotype.

Analysis of Potential Recall Bias

As explained in the Methods section, the infants in the present study were part of a larger longitudinal study of high-risk infants. Some of the infants enrolled in the present GI study were enrolled at the same time as their enrollment in the larger longitudinal study, whereas others were enrolled in the present GI study much later. The parents of the older enrollees therefore needed to remember events farther back in time than the parents of new enrollees, potentially introducing a recall bias. More important, there was no difference between high- and low-risk groups in the mean age of enrollment in the present study (Table 1), so group differences in the report of GI symptoms should not be confounded with recall bias. Still, to evaluate the potential effect of recall bias on our findings (within each group), we repeated the main analyses excluding those infants enrolled at >1 year of age (ie, excluded 53 low-risk infants and 23 high-risk infants) (supplemental Figs. 6 and 7 [*http://links.lww.com/MPG/A531*]). As in the

TABLE 2. Results of multivariable logistic regression analysis (N = 171)

Variable	AOR (95% CI)	P
Model 1: constipation symptom		
Diet category		
EBM (ref)		
PBM	5.99 (2.10–17.11)	**0.001**
NBM	9.15 (2.92–28.61)	**0.004**
Risk category		
Low (ref)		
High	1.79 (0.77–4.18)	0.178
Age*	0.93 (0.86–0.99)	**0.037**
Female sex	1.11 (0.51–2.41)	0.792
Mother's age at birth	0.98 (0.87–1.09)	0.685
Father's age at birth	1.02 (0.94–1.12)	0.572
Model 2: abdominal distress symptom		
Diet category		
EBM (ref)		
PBM	1.37 (0.61–3.10)	0.445
No breast milk	1.73 (0.58–5.14)	0.327
Risk category		
Low (ref)		
High	1.39 (0.63–3.07)	0.414
Age*	0.90 (0.79–1.03)	0.128
Female sex	1.70 (0.76–3.79)	0.196
Mother's age at birth	1.17 (1.04–1.32)	**0.009**
Father's age at birth	0.91 (0.84–0.98)	**0.015**
Model 3: reflux symptom		
Diet category		
EBM (ref)		
PBM	1.31 (0.74–2.30)	0.354
No breast milk	1.04 (0.39–2.80)	0.939
Risk category		
Low (ref)		
High	1.50 (0.73–3.10)	0.274
Age*	0.81 (0.71–0.92)	**0.001**
Female sex	0.95 (0.47–1.92)	0.877
Mother's age at birth	1.06 (0.97–1.16)	0.222
Father's age at birth	1.03 (0.96–1.11)	0.423

Boldface type indicates statistical significance. AOR = adjusted odds ratio; CI = confidence interval; EBM = exclusive breast milk; NBM = no breast milk; PBM = partial breast milk.

* For this repeated measures model, each diet category an infant participates in is a "measure." "Age" refers to the age associated with each measure (ie, the age that diet category began) and thus incorporates both start and completion of weaning ages.

full analysis (Fig. 2), high-risk infants completed weaning earlier than low-risk infants, but in the restricted population they also started weaning significantly earlier than low-risk infants (supplemental Fig. 6A, *http://links.lww.com/MPG/A531*). High-risk infants with any GI symptoms were still significantly more likely to have started and completed weaning earlier than high-risk infants without GI symptoms, and there remained no difference in weaning ages in low-risk infants (supplemental Figs. 6B and C, *http://links.lww.com/MPG/A531*). In the individual symptom analysis (shown in supplemental Fig. 7, *http://links.lww.com/MPG/A531*), as in the full analysis (Fig. 3), the prevalence of constipation in high-risk NBM infants remained significantly greater than that in both high-risk EBM and low-risk NBM infants, and the prevalence of reflux in low-risk EBM infants remained significantly greater than that in low-risk NBM infants. These findings suggest parental recall did not introduce a bias into our study.

DISCUSSION

The present study investigated the effects of diet on GI symptoms in both typically developing, low-risk infants and those who are at high risk for developing ASD. In the low-risk infants, the prevalence of GI symptoms, in aggregate, did not vary with diet or age of weaning (Figs. 1 and 2). The analysis of individual symptoms showed an apparent greater prevalence of reflux (the most common GI symptom in low-risk infants) on an EBM diet (Fig. 3). This is likely an indication that reflux occurs at younger ages rather than an association with diet per se because this effect only appeared in subsets of low-risk infants who were weaned relatively later, increasing the odds of the symptom appearing while on the EBM diet (Fig. 4 and supplemental Figs. 2 and 3 [*http://links.lww.com/MPG/A531*]). The idea that reflux is more dependent on age at the time of the symptom than diet category is also in agreement with the finding of no difference in weaning age between low-risk infants with symptoms and those without (Fig. 2B). The possibility that reflux is linked to EBM diet is further opposed by the multivariate analysis findings of no significance of diet category and decreased risk of reflux with increased weaning age (Table 2, Model 3).

In contrast to low-risk infants, the prevalence of GI symptoms for high-risk infants varied with diet and age of weaning. Specifically, high-risk infants on a NBM diet in any of the first 5 months of life had a significantly greater prevalence of GI symptoms on that diet than those on an EBM diet (Fig. 1). In addition, high-risk infants with GI symptoms started weaning significantly earlier than those without GI symptoms (Fig. 2). The analysis of individual symptoms suggests that this effect may have been driven by constipation in particular, which was closely associated with both diet (occurring primarily when high-risk infants were on an NBM diet and not when on an EBM diet, regardless of weaning age) and age (prevalence dropping significantly in those completely weaned after 3 months). With regard to comparisons between groups, the prevalence of constipation was significantly greater in high-risk than low-risk infants both on "any diet" and on an NBM diet in particular (Fig. 3). This was most evident in the subsets of infants who started weaning before 3 months (supplemental Fig. 3 [*http://links.lww.com/MPG/A531*]) or completed weaning before 6 months (Fig. 4 and supplemental Fig. 2 [(*http://links.lww.com/MPG/A531*]). Abdominal distress showed no specificity for diet category. Nevertheless, it occurred at a greater prevalence in high-risk infants weaned at younger ages compared with either low-risk infants weaned at younger ages (Fig. 4 and supplemental Fig. 3 [*http://links.lww.com/MPG/A531*]) or with high-risk infants weaned at older ages (supplemental Fig. 2 [*http://links.lww.com/MPG/A531*]).

Interestingly, despite relatively low prevalence of constipation in the low-risk group, the multivariate regression analysis revealed that the greatest independent contributor to the risk of constipation was diet category. Although subject group was significantly associated with constipation in univariate analysis (Fig. 3), it was not significantly associated with any of the GI symptoms in multivariate analysis, which may reflect the low power for that type of analysis with regard to the number of high-risk infants enrolled, a common difficulty in the high-risk infant approach. Nevertheless, the large magnitude, marginally significant interaction between the subject group and the NBM diet suggests that the effect of diet may be driven more by the high-risk, than the low-risk, group, which is qualitatively in line with the results of our univariate analyses (Fig. 3).

Demographic differences between groups were limited to slightly greater maternal and paternal ages in the low-risk group. This age difference is unlikely to account for group differences, and would, if anything, predict greater prevalence of GI symptoms in the low-risk group (because, in general, developmental problems

are associated with advanced parental age), which was not the case. In addition, the last data collection point was at a slightly older age in low-risk infants than in high-risk infants. This allows more time for symptoms to appear, and be reported, in low-risk infants and could have led to a greater prevalence of reported GI symptoms in the low-risk group compared with the high-risk group, but this did not occur.

Limitations

Because the collection of GI history data was partially retrospective, it was not practical to ask parents to remember events from the past regarding the exact timing of their child's GI symptoms (unlike weaning ages, which parents typically remembered clearly). Therefore, it was not possible to include infant age at the time of the symptom in our analyses.

A second limitation is that report of symptoms in this study is not based on confirmed diagnoses but on parents perceiving a symptom severe enough that they sought medical attention or changed their infant's care. Although it is possible that group differences were driven by parents who already have 1 child with ASD (ie, in the high-risk group) having a lower threshold for seeking medical attention for GI issues and/or having a better memory for early GI problems, than parents who do not have a child with ASD (ie, in the low-risk group); we think these possibilities are unlikely because this would predict group differences across the board, which was not the case. Instead, group differences were restricted to mainly the NBM diet category and the symptoms of constipation and abdominal distress. We noted that diarrhea, one of the symptoms occurring at a higher prevalence in older children with ASD (2,3,5), was not prevalent in high-risk infants.

Early GI Dysfunction Is an ASD Endophenotype

The results of our study suggest that infants with an older sibling with ASD are at an increased risk for GI problems, most notably when they are on an NBM diet. Our results provide the first evidence that early GI dysfunction may be an ''endophenotype'' in ASD, defined as an abnormality that occurs more commonly in both individuals with ASD and their family members, and is thought to reflect a genetic predisposition for the disorder (18,21,29,30). With this in mind, we further suggest that the endophenotype (predisposition for GI problems) interacts with the environment, in this case, diet.

The rationale for the inclusion of early GI dysfunction as an ASD endophenotype is stronger if it can be shown that the phenotype occurs at greater prevalence in both the high-risk infants who develop ASD and those who do not compared with the low-risk population. Eleven of the 34 (32%) high-risk infants assessed were found to have ASD. This gave our study enough power to observe a significantly greater prevalence of early constipation in infants who develop ASD as compared with low-risk control infants. Likewise, in the subset of high-risk infants who were tested and found not to have ASD ($N = 23$), we observed a higher prevalence of constipation on an NBM diet compared with low-risk infants on an NBM diet. These findings provide additional support to the inclusion of early GI dysfunction as an ASD endophenotype. Note that, in theory, the better a trait fits the description of ''endophenotype,'' the more often it appears in first-degree relatives and the less useful it becomes as a predictor of whether a first-degree relative will also be diagnosed as having a disorder; therefore, it is not surprising that GI dysfunction was not dramatically different in high-risk infants who developed ASD versus those who did not. Power analysis suggests ~140 high-risk infants would need to be assessed to determine whether the difference in the prevalence of constipation between high-risk infants who do versus do not go on to develop ASD is significant. In addition to investigating prevalence, other studies would be required to determine whether existing GI dysfunction is more severe in high-risk infants who do versus do not develop ASD. Regardless, it will be important to consider the possibility that early GI dysfunction plays a causal role in the development of ASD.

Possible Explanations for Earlier Weaning in High-Risk Infants

With or without GI symptoms, high-risk infants completed weaning earlier than low-risk infants. There are a number of possible explanations for this result. First, there is evidence from Schultz et al (22) that children who develop ASD are more likely to have weaned earlier than those who do not develop ASD. Presuming that the older siblings with ASD in our families were, in fact, weaned early, it may be that the infant sibling (in the present study) was also weaned early. Unfortunately, we did not obtain weaning ages for the older siblings in our study to determine the degree to which early weaning is a familial trait. Second, parents with 1 older sibling with ASD may be more stressed or have more constrained schedules (eg, treatment, therapy, and so on for the older sibling) than those of infants with a typically developing older sibling, which may lead them to complete weaning earlier. Third, the low-risk families that enroll in our study may provide a skewed representation of typically developing infants. These parents, who are particularly devoted to medical research, may be the ones who keep their infants on breast milk longer than the general population. A fourth possibility is that high-risk infants may experience more feeding issues resulting in early weaning; however, this is unlikely because we found no greater prevalence of ''trouble nursing,'' ''problem behavior related to meals or feeding time,'' or ''food sensitivity'' symptoms in high-risk infants.

Although early weaning may cause GI dysfunction in the high-risk group, we also consider the ''reverse causality'' explanation; early weaning may be a result of early GI dysfunction. For example, if reflux is particularly bad or infants have trouble sleeping through the night, parents may switch to formula earlier than they would do normally. We think this is less likely the case, for 2 reasons: on an EBM diet, the 2 groups showed no hint of a difference in any type of GI symptom; constipation, the only symptom significantly more prevalent in high-risk infants as a whole compared with low-risk infants, was only significantly more prevalent compared with low-risk infants after complete weaning to the NBM diet (Fig. 3). We did not ask parents the reason for weaning and cannot address these possibilities in the present study.

Is Breast Milk Protective or Is Formula Detrimental?

One of the advantages of examining the partial breast milk (PBM) category is that it allows us to address whether differences in GI prevalence between the EBM and NBM categories are because of EBM protecting against GI dysfunction, in which case, PBM infants should resemble EBM infants, and/or the introduction of harmful non–breast milk (eg, formula), in which case, PBM infants should resemble NBM infants. The results addressing this issue are mixed. Figure 1 shows that PBM may grant high-risk infants some protection from GI symptoms (ie, PBM infants do not differ from EBM infants at any age, whereas PBM infants show marginally less GI dysfunction than NBM infants at 4 and 5 months). Nevertheless, the symptom-specific analysis in Figure 3 indicates that, at least for constipation, introduction of nonmilk in the high-risk infants may be detrimental (ie, PBM infants do not differ from NBM infants, whereas PBM infants show significantly more GI dysfunction than

EBM infants). The effects of breast milk or nonmilk are likely dose dependent. A more detailed prospective diet history will be necessary to resolve this issue.

Related Literature

As indicated, several previous studies report greater prevalence of GI symptoms in older individuals with ASD (reviewed in (5,31)). In many of these studies, similar to our high-risk group on a NBM diet, constipation is the most commonly reported symptom (2,32,33). To our knowledge, there is only 1 previous study by Black et al that investigated the prevalence of GI problems in individuals with ASD before ASD diagnosis (33a), although unlike the present study, the data from the study by Black et al were collected using medical records after ASD diagnosis. Specifically, the researchers evaluated early medical records of children who had developed ASD. They found that the prevalence of GI dysfunction was low (~9%) before the first diagnosis of ASD and did not differ from control children. Although these results appear to contradict those of the present study, the difference between results may be explained by the severity of the GI problems that Black et al included in their analysis. Black's team looked for the existence of serious GI disorders (eg, celiac disease, chronic gastroenteritis, ulcerative colitis) or severe GI symptoms, defined by 3 medical records (eg, doctor visits) of the same symptom within a 6-month period, whereas the present study used less severe GI dysfunction criterion. It is likely that the study by Black et al did not find constipation and abdominal distress, as in the present study, because constipation in infants can be easily treated with diet changes, addition of laxatives, and/or use of lubricants, and so parents are less likely to need 3 doctor visits for that issue. Indeed, the study by Black et al made no mention of constipation. Even though constipation is easily treated, our present hypothesis is that it is also a potential marker for an injurious mechanism in the intestine, which may be responsible for much of the abdominal distress/irritability in our high-risk infants, as described in the next section.

Constipation, Free Fatty Acids, Diet, and Intestinal Damage

We found the clearest associations with constipation, a condition that refers to the compactness of stool and difficulty passing it. It is often thought that stool hardness is determined by water content, and it is true that increasing water content in the diet (eg, increasing water consumption or adding fiber or stool softeners that draw water into the large intestine) reduces stool hardness. Direct measurements of stool hardness, however, indicate that the primary determination of hardness, at least in infants, is the level of calcium soaps in the stool (34,35). Calcium soaps are insoluble complexes that form when calcium or calcium phosphate binds to nonesterified (ie, ''free'') fatty acids (FFAs). Though FFAs can appear in stool unbound to calcium (eg, if the concentration of FFA exceeds the available calcium) (36,37), the presence of calcium soaps indicates that there are FFAs not getting absorbed by the small intestine. Thus, constipation in infants may be associated with hard stool, calcium soaps, and unabsorbed FFAs.

Diet affects calcium soap formation in the intestine. Stool from formula-fed infants was found to be harder than breast milk–fed infants throughout the first 20 weeks of life (38), and bolus obstruction of the infant intestine was associated with formula feeding and calcium soap formation in premature infants (35). This suggests that reduced calcium soap formation may be a mechanism by which breast milk protects from constipation. In support of this hypothesis, an in vitro study showed that lipase-digested formula releases 6 times as much FFA as lipase-digested breast milk (39). We found a strong association of early NBM diet with constipation in the high-risk group but no high prevalence of constipation in any diet category in the low-risk groups, suggesting that high-risk infants may have differences from low-risk infants in fat digestion and/or calcium or FFA absorption leading to accumulation in the intestine and calcium soap formation.

High concentrations of FFAs are able to damage the intestine (36,37,40–43), and infants are at particular risk because of the immaturity of their mucosal barriers (43–45). Because of high concentrations of FFAs, digested formula is cytotoxic whereas digested breast milk is not (39). There are several mechanisms whereby breast milk may reduce or prevent this damage that are lacking in infant formula (see review in (46)). Because unabsorbed cytotoxic levels of FFAs may be present even in the absence of sufficient calcium to form calcium soaps, damage may occur by this mechanism even in the absence of hard stool (36,37). FFA-induced intestinal damage may be the source of the abdominal distress/irritability in the high-risk infants, most apparent in those fully weaned before 3 or 6 months (11 of the 19 high-risk infants with constipation in our study also had abdominal distress/irritability). Alternatively, abdominal distress/irritability could indicate neural dysfunction in the GI tract (eg, a lower pain threshold).

Even in individuals without concomitant GI symptoms, ~40% of children and adults with ASD have hyperpermeable intestines compared with controls (20,47), suggesting that a large subpopulation of ASD individuals have either a genetic defect in their mucosal barrier and may have increased susceptibility to damaging mediators from the lumen (eg, FFAs, other partially digested food, digestive enzymes, and pathogens) or have ongoing intestinal damage causing impaired barrier function. We hypothesize that ASD may be associated with abnormalities in fat digestion or absorption leading to the accumulation of cytotoxic levels of FFAs in the intestine for which constipation may be a marker and/or delayed or deficient maturation of the mucosal barrier increasing susceptibility to damaging factors in the intestinal lumen. Additional studies are required to further test these hypotheses.

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