Neurodevelopment of Preterm Infants at 24 Months After Neonatal Supplementation of a Prebiotic Mix: A Randomized Trial

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ABSTRACT

Objectives: Fetal brain maturation is disrupted by preterm birth. Inflammation during the neonatal period may further harm neurodevelopmental outcomes. The present study aimed to determine the effect of short-chain galacto-oligosaccharides/long-chain fructo-oligosaccharides/pectin-derived acidic oligosaccharides (scGOS/lcFOS/pAOS) on neurodevelopmental outcomes measured by Bayley Scales of Infant and Toddler Development in preterm infants at 24 months.

Methods: In this randomized controlled trial, scGOS/lcFOS/pAOS or placebo was supplemented between days 3 and 30 of life. Serum samples at day 1, 7, and 14 were analyzed for cytokine levels. Stool samples at day 1, 7, 14, and 30 were measured for bacterial count and bifidobacteria percentage. At 24 months corrected age infants were followed up by a blinded pediatric psychologist for the Bayley Scales of Infant and Toddler Development II or III.

Results: Seventy-seven of one hundred one (76%) eligible infants participated in the follow-up study. Neurodevelopmental outcomes were not different in the scGOS/lcFOS/pAOS and placebo group. Infections during the neonatal period, lower percentages of bifidobacteria at day 7 ($F = 3.8$, $P = 0.05$) and day 14 ($F = 5.0$, $P = 0.02$) and higher levels of Interleukine (IL)-1β ($F = 4.0$, $P = 0.04$) and IL-8 ($F = 8.0$, $P = 0.01$) at day 7 are associated with lower mental developmental index. Lower psychomotor outcomes are associated with IL-2 ($F = 4.0$, $P = 0.05$), IL-4 ($F = 6.0$, $P = 0.02$) at birth, and interferon gamma at day 7 ($F = 4.4$, $P = 0.04$).

Conclusions: scGOS/lcFOS/pAOS showed no significant improvement of neurodevelopmental outcomes at 24 months in preterm infants. Infections, lower bifidobacteria counts, and higher serum cytokine levels during the neonatal period were associated with lower neurodevelopmental outcomes at 24 months of age indicating the relevance of microbiome and immune responses in neurodevelopmental processes.

Key Words: cytokines, microbiota infections, neurodevelopmental outcomes, preterm infants, scGOS/lcFOS/pAOS supplementation

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What Is Known

- Fetal brain maturation is disrupted by preterm birth and further damaged by infections and inflammation.
- The mixture of short-chain galacto-oligosaccharides, long-chain fructo-oligosaccharides, and pectin-derived acidic oligosaccharides is designed to balance immune and microbiota development.

What Is New

- Short-chain galacto-oligosaccharides/long-chain fructo-oligosaccharides/pectin-derived acidic oligosaccharides was supplemented during the neonatal period. Long-term effects on neurodevelopmental outcomes on Bayley Scales of Infant and Toddler Development at 24 months of age have been investigated.
- Short-chain galacto-oligosaccharides/long-chain fructo-oligosaccharides/pectin-derived acidic oligosaccharides supplementation did not improve the Bayley Scales of Infant and Toddler Development outcomes.
- Serious neonatal infection, higher cytokine levels, and lower bifidobacteria counts during the neonatal period correlated with impaired Bayley Scales of Infant and Toddler Development outcomes at 24 months.

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R.M.E. and J.G. are employees of Danone Research (Nutricia Research Utrecht). Previously, R.M.E. was a neonatologist on the Neonatal Intensive Care Unit in the VU University Medical Center and became an employee of Danone Research (Nutricia Research Utrecht) after the data collection for the present study. Both R.M.E. and J.G. were not directly involved in the analysis of the data. The remaining authors report no conflicts of interest.

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Fetal brain maturation is disrupted by preterm birth. During the neonatal period, the brain of a preterm infant faces multiple factors that can influence short- and long-term developmental outcomes. These factors include cerebral hypoxia, ischemia, infection, and inflammation, also underlying lesions such as periventricular leukomalacia and intraventricular hemorrhage. (1) During follow-up in preterm infants, major handicaps as cerebral palsy (incidence around 2%–7%), neurodevelopmental impairment (4%–5%), deafness (1%–2%), and blindness (1%–2%) are found. (2) Later in life, more subtle disabilities such as lapses of attention and poor visuospatial working memory are recognized in very preterm infants as well. (3)

Serious postnatal infections are known to have detrimental effects on mental and psychomotor development in preterm infants. (4,5) Preterm infants are more vulnerable for infections, because of their immature immune system. The influence of the gut on the brain, known as the gut to brain connection, has been discussed by several authors. (6–10) In case of gastrointestinal inflammation, intestinal epithelial cells become more permeable and enterochromaffin cells, lymphocytes, mast cells, and dendritic cells secrete neuroimmune factors that can stimulate enteric nerves. (11) Preterm infants are known to have both higher levels of gastrointestinal inflammation, as shown amongst others by higher calprotectin levels, and an impaired gut barrier function, resulting in increased intestinal permeability. (12,13) Therefore, lymphocytes and cytokines will increase in the circulation. Lymphocytes and several serum cytokines (interleukine [IL]-1β, IL-6, interferon gamma [IFN-γ], tumor necrosis factor alpha [TNF-α]) can pass the blood-brain barrier, and some cytokines (IL-1β, TNF-α) are even known to bind to brain endothelial cells, inducing an immune response in the brain. (14) The immune response in the brain may result in changed neuronal homeostasis. (8) Influences from the gut may influence indirectly memory formation, emotional arousal, and affective behavior. (8,15) Previously, we have shown a decrease in serum cytokine levels of IL-1β, IFN-γ, and TNF-α in preterm infants after supplementation of the mixture of short-chain galacto-oligosaccharides/long-chain fructo-oligosaccharides/pectin-derived acidic oligosaccharides (scGOS/lcFOS/pAOS) in the neonatal period (16) and a decrease in endogenous infections when the scGOS/lcFOS/pAOS was given in sufficient amounts. (17) Lower cytokine levels and fewer infections during the neonatal period may positively influence neurodevelopmental outcomes. Previously, Carlo et al (18) showed that there is a potential association between neurodevelopment and cytokines in infants, as cytokine levels differed between infants with and without cerebral palsy. Keunen et al (6) reviewed the specific types and mechanisms of brain damage in very preterm infants, with the potential effects of nutritional components on these detrimental mechanisms.

So far, long-term effects of neonatal scGOS/lcFOS/pAOS supplementation have not been reported for preterm infants. The aims of the present study were to evaluate the neurodevelopmental outcome at the corrected age of 2 years of very preterm infants after supplementation with scGOS/lcFOS/pAOS and possible associations with cytokine levels and bacterial counts in the feces during the neonatal period.

METHODS

Very preterm infants (gestational age <32 weeks and/or birth weight <1500 g), admitted to the level-III neonatal intensive care unit (NICU) of the VU University medical center, Amsterdam, were eligible for this randomized double-blind placebo-controlled trial of enteral supplementation of scGOS/lcFOS/pAOS. (19) Exclusion criteria were as follows: infants with gestational age (GA) >34 weeks, major congenital or chromosomal anomalies, death <48 hours after birth, and transfer to another hospital <48 hours after birth. The medical ethical review board of the hospital approved the study protocol. Written informed consent was obtained from all parents. This trial was registered at isrctn.org as ISRCTN16211826.

Effect of Enteral Supplementation of scGOS/lcFOS/pAOS

Protocol guidelines for the introduction of parenteral and enteral nutrition followed present practices at the NICU. Nutritional support was administered as previously described. (17,19) In short, the medical staff of our NICU had final responsibility for the administration of parenteral nutrition and advancement of enteral nutrition. During the study period, infants received from day 3 to 30 after birth daily scGOS/lcFOS/pAOS or placebo supplemented to breast milk or to preterm formula (Neonatal Start, Nutricia, Zoetermeer). Supplementation of the mixture or placebo was administered in increasing doses between days 3 and 30 of life to 1.5 $g \cdot kg^{-1} \cdot day^{-1}$ to breast milk or preterm formula. Per 100 mL, the preterm formula provided 80 kcal, 2.4 g protein, 4.4 g fat, and 7.8 g carbohydrates. After discharge, all infants received breast milk or preterm formula (Nenatal Start) followed by postdischarge formula (Nenatal 1, Nutricia, Zoetermeer) until the corrected age of 6 months. None of the formulae contained oligosaccharides. (17)

Cytokine Measurements

Cytokine measurements during the neonatal period have previously been described by van den Berg et al. (16) In short, blood samples were collected before the start of the intervention, within 48 hours after birth (birth), at postnatal day 7 (day 7) and day 14 (day 14). Serum was collected and stored at −80°C until analysis. Serum samples were analyzed for levels of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, IFN-γ, and TNF-α. Cytokine levels were measured using a fluorescent bead-based multiplex immunoassay (Luminex xMAP technology), and cytokine assay kits were purchased from Bio-rad (Hercules, California).

Measurement of Fecal Microbiome

Fecal sample preparation and fluorescent in situ hybridization analysis were performed as previously described. (20) The 7 used probes represent the major groups of microorganisms in term infants and were commercially synthesized and 5′-labelled with Cy3 (Biolegio BV, Nijmegen, the Netherlands). For total cell counts, slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and counted by an automated Olympus AX70 epifluorescence microscope. The percentage of labeled bacteria per sample was determined by counting all cells and all labeled bacteria in the same field with a DAPI filter set (SP100) and CY3 filter set (41007), respectively (Chroma Technology Corp, Brattleboro, VT).

Neurodevelopmental Outcome

As part of our routine follow-up program, a pediatric psychologist assessed all of the participating infants at the corrected age of 2 years. To assess cognitive and motor development, Mental Development Index (MDI) and Psychomotor Development Index (PDI) of the Bayley Scales of Infant and Toddler Development (BSID) were used. (21) During the period of the present study, the present available BSID-III was administered in the Netherlands, but Dutch norms were not yet available. Therefore, the American norm was used for the infants tested with BSID III. (22) Infants in the present study could therefore be tested by the BSID II or BSID III.

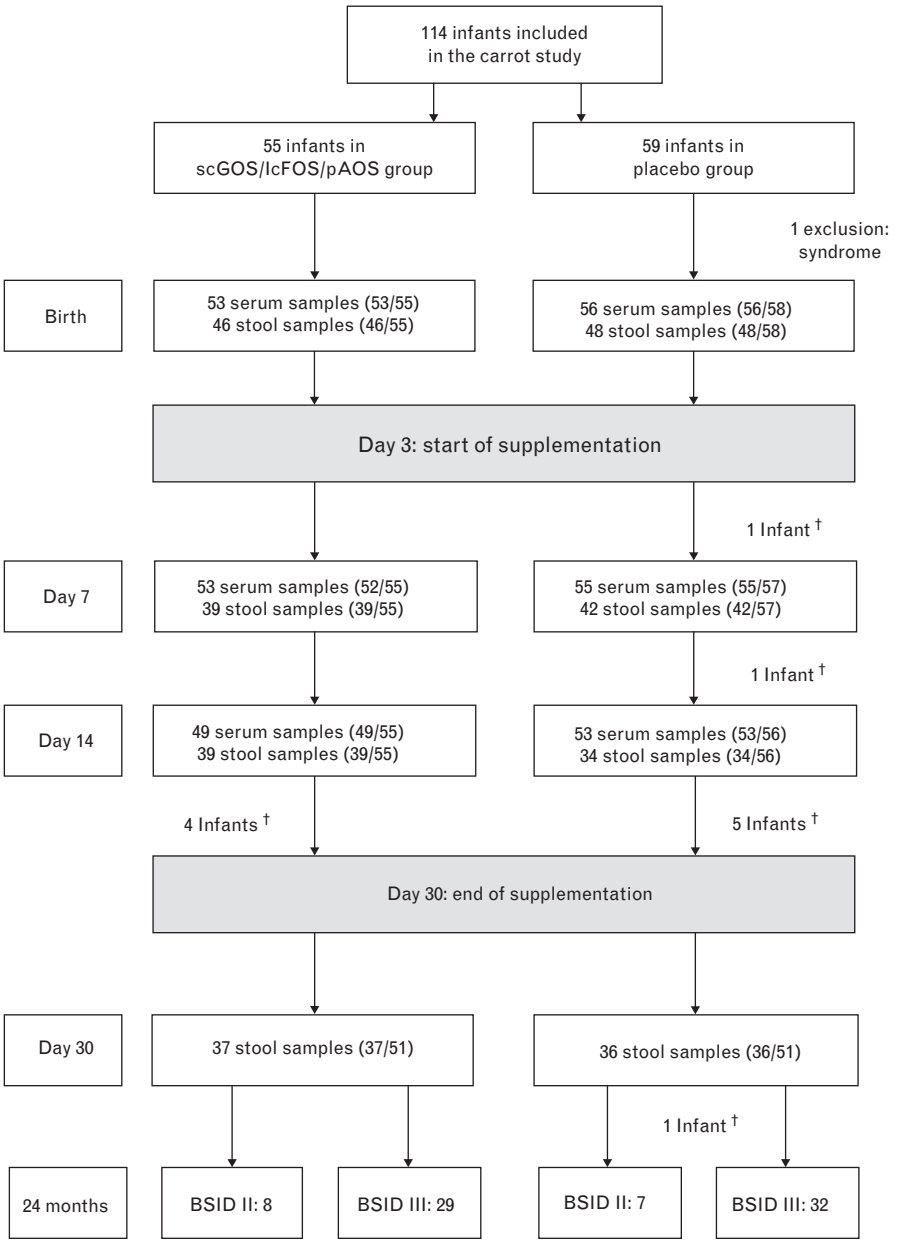

FIGURE 1. Trial profile. BSID = Bayley Scales of Infant and Toddler Development; scGOS/lcFOS/pAOS = short-chain galacto-oligosaccharides/long-chain fructo-oligosaccharides/pectin-derived acidic oligosaccharides.

The BSID III consists of 5 scales but the results of the gross motor function, fine motor function were combined for the PDI and the mental development for the MDI to compare the results with the BSID II. Infants were screened during the routine follow-up for cerebral palsy by a pediatrician and a physiotherapist. Both parents and investigators were unaware of treatment allocation in the neonatal period during the follow-up.

Statistical Analysis

The power analysis of this follow-up study is based on the primary study outcome (serious neonatal infectious morbidity). Normally distributed and nonparametric data are presented as mean ± standard deviation (SD) and median (ranges). Patient and nutritional characteristics were analyzed with Student t test, χ^2 test, or Fisher exact test for continuous normally distributed and dichotomous data, respectively. To adjust for potential confounding variables, data were analyzed by multiple linear regression analysis (for continuous data) or logistic regression analysis (for dichotomous data). Adjustments were made for gestational age, birth weight, sex, and 1 or more serious neonatal infection. Influence of neonatal cytokine levels and bacteria measured in the feces on BSID outcomes at 24 months was analyzed by ANOVA with covariates. A $P < 0.05$ (2 tailed) was considered significant. SPSS 20.0 (SPSS Inc, Chicago, IL) was used for data analysis.

RESULTS

At the corrected age of 2 years, 101 of 113 (89%) infants were eligible to participate in the follow-up study. Twelve infants had died before the age of 2 years (4 in the scGOS/lcFOS/pAOS and 8 in the placebo group). Of these 101 infants, 77 infants (76%) finally participated in the study of which 38 infants received scGOS/lcFOS/pAOS supplementation and 39 infants received placebo supplementation during the neonatal period (Fig. 1). Reasons for not participating were as follows: family could not be reached (n = 9), family did not show up (n = 7), exclusion (n = 3), difficulties with Dutch language (n = 3), and migration abroad (n = 2) Figure 2.

Baseline infant and maternal characteristics were not different (all $P > 0.05$) in the participating (n = 77) and nonparticipating (n = 24) groups (data not shown). Baseline infant and maternal characteristics were not different in the scGOS/lcFOS/pAOS-supplemented and the placebo group (Table 1).

Supplementation of scGOS/lcFOS/pAOS

MDI and PDI were not different in the scGOS/lcFOS/pAOS (95 [80–115] and 100 [71–130]) and placebo group (100 [65–115] and 97 [69–145]). Adjustment for serious infection and other possible confounders (administration of antenatal corticosteroids, mode of delivery, GA, and sex) did not change the results of the primary analysis (Table 2). The supplementation of scGOS/lcFOS/pAOS during the neonatal period did not influence the incidence of cerebral palsy or neurodevelopmental impairment. None of the infants was blind or had hearing disability.

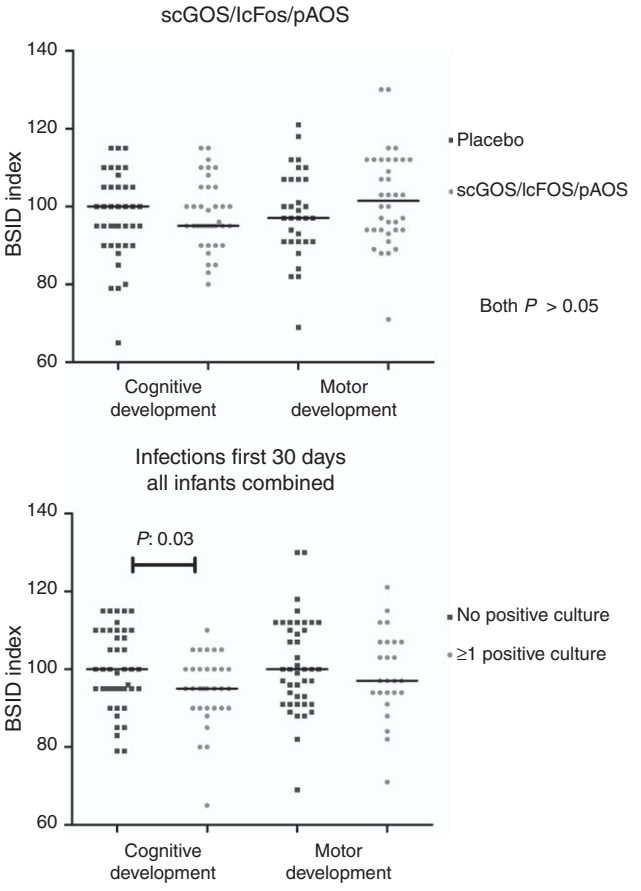

FIGURE 2. Influences of scGOS/lcFOS/pAOS and infections on neurodevelopmental outcomes. Medians of the groups are shown. BSID = Bayley Scales of Infant and Toddler Development; scGOS/lcFOS/pAOS = short-chain galacto-oligosaccharides/long-chain fructo-oligosaccharides/pectin-derived acidic oligosaccharides.

Influence of Infections, Microbiota, Cytokines, and Infections During Neonatal Period and BSID

Serious neonatal infection was associated with lower levels of MDI scores ($P = 0.03$), but not PDI scores ($P > 0.05$). As evaluated by ANCOVA, higher percentages of bifidobacteria in the microbiome analyses at day 7 ($F = 3.8$, $P = 0.05$) and day 14 ($F = 5.0$, $P = 0.02$) of life were associated with higher MDI scores. Total bacterial count in the feces did not influence MDI or PDI scores (all $P > 0.05$).

Higher cytokine levels at day 7 for IL-1β ($F = 4.0$, $P = 0.04$) and IL-8 ($F = 8.0$, $P = 0.01$) were associated with lower MDI scores at 24 months of age, and trends were found for TNF-α at birth ($F = 3.5$, $P = 0.06$) and IL-10 ($F = 1.1$ $P = 0.06$). Higher cytokine levels at birth for IL-2 ($F = 4.0$, $P = 0.05$), IL-4 ($F = 6.0$, $P = 0.02$), and IFN-γ at day 7 ($F = 4.4$, $P = 0.04$) were associated with lower PDI scores.

DISCUSSION

In this follow-up study, preterm infants receiving enteral supplementation of a prebiotic mixture consisting of neutral and acidic oligosaccharides during day 3 to 30 of life showed no improvement in the neurodevelopmental outcomes at 24 months of corrected age, as reflected by MDI and PDI of BSID. Serious neonatal infection was associated with lower MDI scores, as is also true for lower percentages of bifidobacterial counts, whereas higher cytokine levels were associated with both lower MDI and PDI scores. Although previously scGOS/lcFOS/pAOS supplementation was found to decrease both serious infectious morbidity with noncoagulase negative streptococci bacteria and cytokine levels, this effect did not have any impact on the MDI and PDI scores at 24 months of age.

This absence of effect may be related to various factors between the neonatal period and the corrected age of 2 years. Firstly, although serious neonatal infections were less common in the scGOS/lcFOS/pAOS group than in the control group, these infections did occur in both the groups. Secondly, those infants who had early signs or symptoms of neurodevelopmental problems would receive treatment, which may have reduced the magnitude of the neurodevelopmental impairment as assessed by BSID. Previously in this group was shown that children with a history of serious neonatal infection more often had an abnormal neurodevelopmental assessment, significantly delayed motor ability and a higher incidence of neurodevelopmental impairment at 6 months, but no difference at 12 months of age. (23) Thirdly, the overall MDI and PDI were close to 100, which is close to the reference values for term born infants. Therefore, general neurodevelopmental outcome was quite good for these preterm infants, which may limit the potential effect of the intervention in the study population.

The associations between serious neonatal infections, microbiome, and cytokine-levels early in life with BSID outcomes later in life suggest that there may be an opportunity to influence neurodevelopment of these preterm infants via cytokine and microbiome modulation. The gut-brain-immune axis, a bidirectional pathway between the gut and the brain can influence the development and functionality of the brain. (7–10,24,25) Influence of the brain on intestinal diseases has been shown for, for example, inflammatory

TABLE 1. Baseline characteristics

	scGOS/lcFOS/pAOS (n = 38)	Placebo (n = 39)	*P*
Maternal characteristics			
Maternal age at birth (y, mo)	32.2 (3.7)	31.4 (7.0)	0.53
Maternal race (% Caucasian)	31/38 (82%)	29/39 (74%)	0.58
High maternal education	21/35 (60%)	16/38 (42%)	0.16
Infant characteristics			
≥1 course of antenatal corticosteroids	23/38 (60%)	21/39 (54%)	0.65
Vaginal delivery	20/38 (53%)	22/39 (56%)	0.82
Sex (% male)	21/38 (55%)	24/39 (62%)	0.65
Gestational age (wk)	29.9 (1.7)	29.6 (2.1)	0.45
Birth weight (kg)	1.32 (0.38)	1.28 (0.28)	0.59
Birth weight ≤10th percentile	8/38 (21%)	5/39 (13%)	0.38
Birth weight (*z* score)	0.07 (1.15)	−0.07 (0.84)	0.52
Head circumference (*z* score)	−0.11 (0.02)	0.11 (1.41)	0.34
5-min apgar ≤6	5/38 (13%)	2/39 (5%)	0.26
Surfactant	15/38 (40%)	15/39 (39%)	1.0
PIVH ≥ grade III	2/38 (5%)	1/39 (3%)	0.61
≥1 neonatal infection(s)	15/38 (40%)	17/39 (44%)	0.82
Human milk feeding (%)	27/38 (71%)	22/39 (56%)	0.24
Age at discharge NICU (days)	16 (1–90)	17 (3–105)	0.55
Weight a term (*z* score)	−0.01 (0.79)	0.01 (1.18)	0.95
Head circumference a term (*z* score)	−0.10 (1.11)	0.10 (0.89)	0.42
Age at discharge hospital (days)	54 (30–111)	54 (30–168)	0.80
Weight increase during hospital stay (kg)	1.23 (0.48)	1.20 (0.49)	0.77
Age at follow-up (mo)	26 (23–31)	26 (24–33)	0.27

None of the infants received postnatal steroids. NICU = neonatal intensive care unit; PIVH = periventricular-intraventricular hemorrhage; scGOS/lcFOS/pAOS = short-chain galacto-oligosaccharides/long-chain fructo-oligosaccharides/pectin-derived acidic oligosaccharides.

bowel diseases, (26) and influence of the gut on the brain has been described in autism, although it remains to be elucidated what is the cause and what is the consequence. (7)

Very preterm infants (<32 weeks) are born with a brain with a predominance of preoligodendrocytes because they are exposed to extrauterine life in a period during which their brains are still developing. Therefore, very preterm infants are born with a brain that is very vulnerable for both ischemic and inflammatory processes. (27) In the present study, it was demonstrated again that preterm infants with infections during the first month have lower outcomes at the MDI scores of the BSID. This may be due at least in part to the activation of microglia, described as the pathway of cell-death of preoliogosaccharides by infectious processes. (28) In case of intestinal inflammation, intestinal epithelial cells become more

TABLE 2. Influence of scGOS/lcFOS/pAOS on neurodevelopmental outcomes at 24 months of age

		scGOS/lcFOS/pAOS	Placebo	Analysis	B	95% CI	*P*
BSID II	MDI	98 (83–115)	102 (79–115)	Crude	1.8	−14.0–17.7	0.81
				Adjusted	5.4	−13.2–24.1	0.52
	PDI	95 (71–109)	99 (69–121)	Crude	−2.8	−18.6–13.0	0.71
				Adjusted	−2.4	−19.7–13.4	0.68
BSID III	MDI	95 (80–115)	100 (65–115)	Crude	−0.4	−5.2–4.4	0.87
				Adjusted	−0.3	−5.0–4.4	0.91
	PDI	103 (88–130)	97 (82–145)	Crude	6.7	−3.1–16.4	0.18
				Adjusted	7.1	−3.0–17.2	0.17
Combined	MDI	95 (80–115)	100 (65–115)	Crude	0.3	−4.5–4.9	0.89
				Adjusted	0.1	−4.5–4.6	0.99
	PDI	100 (71–130)	97 (69–145)	Crude	4.5	−3.7–12.8	0.28
				Adjusted	5.5	−2.8–13.9	0.19
MDI ≤ 85		4/37 (11%)	5/39 (13%)	Crude	1.2	0.3–4.9	0.79
				Adjusted	1.2	0.3–4.9	0.83
PDI ≤ 85		1/35 (3%)	4/33 (12%)	Crude	5.4	0.5–54.4	0.16
				Adjusted	5.3	0.4–67.8	0.20
CP		0/37 (0%)	1/39 (3%)				

Data are median (range) or number (%). B = beta adjusted for sex, gestational age (weeks), birth weight <10th percentile and ≥1 neonatal infection. BSID = Bayley Scales of Infant and Toddler Development; CI = confidence interval; MDI = Mental Development Index; PDI = Psychomotor Development Index; scGOS/lcFOS/pAOS = short-chain galacto-oligosaccharides/long-chain fructo-oligosaccharides/pectin-derived acidic oligosaccharides.

permeable and enterochromaffin cells, lymphocytes, mast cells, and dendritic cells secrete neuroimmune factors that can stimulate enteric nerves. Preterm infants are known to have an impaired gut barrier function and therefore an increased intestinal permeability. (12,13) Preterm infants are not only more vulnerable for (gastrointestinal) infections but also show intestinal inflammation reflected by higher calprotectine levels. (29) In addition to the stimulation of enteric nerves, cytokines and lymphocytes will migrate to the circulation, among others, because of increased intestinal permeability. Subsequently, lymphocytes and serum cytokines (IL-1β, IL-6, IFN-γ, TNF-α) can pass the blood-brain barrier. IL-1β and TNF-α are known to bind to brain endothelial cells inducing an immune response in the brain. (8,14) A study in mice describes a possible role for gut microbiota in the regulation of the blood-brain barrier, showing a more permeable blood-brain barrier in the absence of certain gut microbiota. (30) We, therefore, hypothesized that preterm infants, with a delayed colonization of the gut, may have a more permeable blood-brain barrier as well, resulting in increased passage of cytokines across this barrier and combined the data about gut microbiome and cytokine profiles during early life to determine their long-term influence on neurodevelopment. In the present study, it was found that lower MDI scores in very preterm infants were associated with a lower percentage of bifidobacteria, although no association was found with overall bacterial count.

Serious neonatal infections also attribute to critical illness and hemodynamic instability, which induces hypoxia-ischemic damage in preterm infants. The combination of both ischemia and inflammation activates 2 different pathways leading to microglia activation and ultimately brain injury. (27) Hanssen-Popp et al (31) previously found associations between cytokine levels for TNF-α and IL-8 in the first hours and neurodevelopmental outcomes at 2 years of age. In the present study, associations between possible brain-affecting cytokines, IL-1β, IL-8, and a trend for TNF-α on the MDI scores were found. In the study of Hansen-Popp, 20 of 69 (29%) preterm infants (GA < 32 weeks) had PDI scores <85, whereas in the present study with preterm infants with a comparable GA only 5 of 69 (7%) had PDI scores <85. This may partly be explained by the introduction of the BSID III, which is suggested to be overestimating the developmental outcomes in preterm infants. (32) It has previously been shown by Vohr et al (33) that infants receiving human milk during their NICU period have better outcomes on the BSID at 30 months of age. The scGOS/lcFOS/pAOS mimics human milk oligossacharides, and we therefore hypothesized that it would have a similar although potentially smaller effect. The distribution of human milk-fed infants in the 2 groups was comparable.

In addition to cognitive impairments, altered brain development in very preterm infants has been found to be associated with various attention problems at school age explained (mediated) by extremely slow responses and deficits in visuospatial working memory. (3) Brain development in preterm infants is, therefore, an important area for research. Future research focused on the consequences of disturbed immune and microbiota development in the neonatal period on brain development may reveal new opportunities to improve brain development in this vulnerable group of patients.

Some limitations of the study need to be addressed. The sample size was calculated for the primary outcome of the initial study: serious neonatal infections. Lost to follow-up decreased the sample size of the follow-up, which may induce a type-II error. Second, during the study period, the BSID III was introduced in our hospital. Because Dutch reference indices were not available, the US reference indices were used. This may give an overestimation of the neurodevelopmental outcome. The infants tested with the BSID III may show higher test scores, especially for scores in the lower regions. (32) Because the use of BSID II and BSID III is equally distributed in the scGOS/lcFOS/pAOS and placebo group, these differences did not influence the differences at group level, but this may explain why we had a lower percentage of preterm infants with PDI < 85 compared with the study of Hanssen-Popp. (31) The predictive value of the BSID for later development in preterm infants was limited as discussed by Luttikhuizen et al. (34) Presently, the BSID, however, remains the most used test for neurodevelopment at the age of 2 years. Third, the supplementation of the scGOS/lcFOS/pAOS was only during the first 30 days. Although interventions during the first month of life have shown to have long-term effects, these effects could possibly be more pronounced with longer supplementation. Furthermore, the method used to investigate the bacterial content of the stool was able to detect only major groups of microorganisms. This is a disadvantage compared with the presently more used DNA-sequencing methods that can separate different microorganisms. The imbalance of the gut microbiome may be more important than the increased number of bifidobacteria or other microorganisms, and therefore, more modern techniques could have shown a more specific effect of the microbiome in the first month on the outcomes at 24 months.

In conclusion, this is the first follow-up study on the long-term effects of the supplementation of scGOS/lcFOS/pAOS during the neonatal period on neurodevelopmental outcome at the corrected age of 24 months. Supplementation of scGOS/lcFOS/pAOS during the neonatal period did not change the neurodevelopmental outcome of preterm infants at the corrected age of 24 months, but associations between infections, microbiota, and several cytokine levels early in life and later neurodevelopment outcome assessed by BSID were found. These associations illustrate the potential role of nutritional modulation of the compromised development of the brain of very preterm infants.

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