

**Response on pneumococcal  
vaccine in preterm infants  
after neutral and acidic  
oligosaccharides supplementation**

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## Abstract

**Background:** Supplementation of oligosaccharides in premature infants was shown to influence the immune system. We determined the effect of combined short-chain galacto-oligosaccharides (scGOS), long-chain fructo-oligosaccharides (lcFOS), and pectin-derived acidic oligosaccharides (pAOS) on antibody concentrations after pneumococcal conjugate vaccination in very preterm infants.

**Methods:** Very preterm infants with GA<32 weeks and/or BW<1500 g were randomized to receive enteral supplementation with scGOS/lcFOS/pAOS or placebo between days 3 and 30 of life. Blood samples were collected at birth, 5 and 12 months of age and compared with term samples from a Dutch cross-sectional population-based serosurveillance study. IgG antibody levels to pneumococcal capsular polysaccharides were determined by multiplex immuno assay.

**Results:** In total, 113 preterm infants were included with similar baseline and nutritional characteristics in both groups. After 3 primary pneumococcal vaccinations, the scGOS/lcFOS/pAOS-group had lower GMC antibody concentrations (  $\mu\text{g/ml}$ ) (serotype 4: 1.53, 6B :0.25, 9V: 1.19, 14: 2.39, 18C: 1.88, 19F: 7.42, 23F: 0.72) than the placebo group (serotype 4: 3.29, 6B: 0.79, 9V:2.64, 14: 4.52, 18C: 3.13, 19F: 14.64, 23F: 1.88; all  $p<0.05$ ), but comparable to those in the term control group (serotype 4: 0.97, 6B: 0.32, 9V: 1.67, 14: 3.24, 18C: 2.03, 19F: 5.06, 23F: 0.59, all  $p>0.05$ ). After the booster vaccination at 11 months, antibody levels were no longer different between the two preterm groups.

**Conclusion:** Enteral supplementation of scGOS/lcFOS/pAOS has a regulatory effect on the response to conjugated polysaccharide pneumococcal vaccine with normalization of the enhanced responses in preterm infants towards levels similar to healthy term infants.

## Introduction

Preterm infants are born with a still immature immune system. Prior to infant vaccinations, infants are presumed to be partly protected by maternal IgG obtained during pregnancy, mainly transported from mother to child after the gestational age (GA) of 32 weeks.<sup>33,34,125</sup>

Next to lower maternally derived IgG antibody levels, preterm infants are often observed to have decreased immunological responses and lower serological effectiveness after immunizations, supposedly due to immaturity of both humoral and cellular immune mechanisms.<sup>15</sup> With respect to polysaccharide-protein conjugate vaccines, lower Hib antibody responses upon vaccination have been found in preterm infants with a GA < 32 weeks compared with term infants.<sup>205-207</sup> Similarly, lower post-vaccination levels for pneumococcal conjugate vaccines (PCV) have been described in preterm infants.<sup>229-231</sup> Results are however conflicting since also higher antibody levels for PCV in preterm infants compared to term infants have been reported for serotypes 19F, 9V and 4 after completion of a 2-4-6 months of age primary immunization schedule.<sup>232</sup> In 2006, the heptavalent pneumococcal vaccine (PCV7) was implemented in the national immunization program of the Netherlands at the age of 2, 3 and 4 months followed by a booster dose at 11 months irrespective of GA at birth.<sup>204</sup>

Human milk influences microbial colonization in the gut and immune maturation. Among the many factors in human milk contributing to these influences are over 200 types of oligosaccharides. These human oligosaccharides are 80% neutral and up to 20% acidic.<sup>48</sup> Non-human milk oligosaccharides, such as short-chain galacto-oligosaccharides (scGOS), long-chain fructo-oligosaccharides (lcFOS) and pectin-derived acidic oligosaccharides (pAOS) have been developed to substitute the beneficial immunomodulatory, anti-adhesive, and antimicrobial effects<sup>169,211</sup> effects of these human milk oligosaccharides.<sup>215-217</sup> Impact on immune responses of these oligosaccharides has been described in mice, where fructo-oligosaccharides improve the immune response to live attenuated vaccination against *Salmonella*<sup>218</sup> and the combination of scGOS/lcFOS/pAOS enhances inactivated influenza virus vaccine-specific delayed type hypersensitivity responses in a dose-dependent manner.<sup>233</sup> This was accompanied by a reduction of the production of Th-2 cytokines suggesting a Th-1 skewed systemic immune response to the vaccine. We hypothesize that the combination of neutral and acidic oligosaccharides as 80% scGOS/lcFOS and 20% pAOS in very preterm infants supplemented early in life may affect the innate and adaptive priming of the immune system, and as a consequence modulate the immune response upon vaccinations.

The aim of this study was to investigate the specific antibody levels against the 7 pneumococcal vaccine serotypes after vaccination with PCV7 in preterm infants and to compare those with term infants vaccinated during the same time period. Furthermore, we measured the effect of enteral supplementation of neutral and acidic oligosaccharides during day 3 till day 30 of life on vaccine responses in preterm infants in the first year of life.

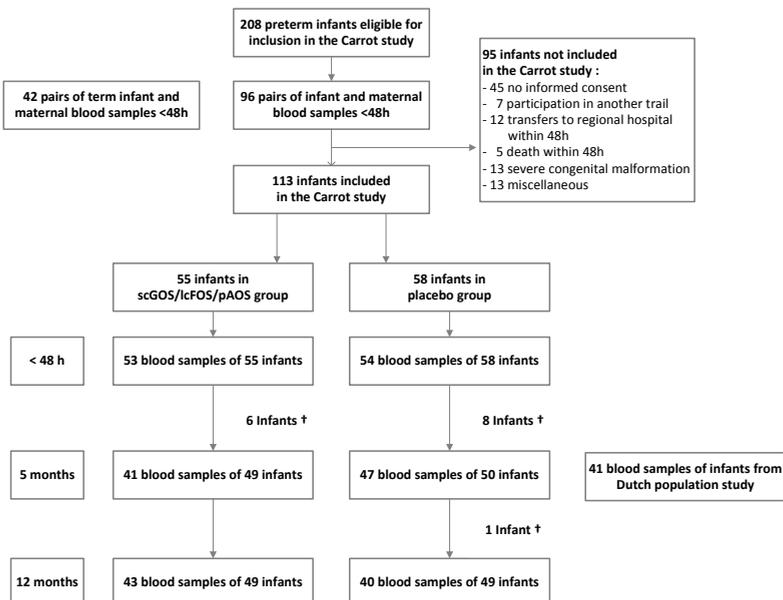
## Methods

### Study population

Infants born between May 2007 and October 2008 with a GA <32 weeks and/or birth weight (BW) <1500 gram who were admitted to the level III neonatal intensive care unit (NICU) of the VU University Medical Center (VUmc) of Amsterdam and their mothers were eligible for participation in the study. This study was conducted according to the guidelines laid down in the Declaration of Helsinki and the medical ethical review board of VUmc approved all procedures involving human patients. Written informed consent was obtained from all parents. This study was part of the CARROT-study, registered as ISRCTN16211826.

### Vaccinations and blood samples

All infants were scheduled to receive 4 doses of heptavalent conjugate pneumococcal vaccine (Prevenar-7<sup>®</sup>, Pfizer) at 2, 3, 4 and 11 months of age, simultaneously administered with DTaP-IPV-Hib vaccine (Infanrix<sup>®</sup>, GlaxoSmithKline or Pediacel<sup>®</sup>, Sanofi Pasteur MSD) in another limb according to the Dutch national immunization programme. Infants received the hexavalent DTaP-IPV-Hib-HepB vaccine (Infanrix hexa<sup>®</sup>, GlaxoSmithKline) if one of their parents was born in a hepatitis B endemic area. Immunizations were administered in the hospital or at a well-baby clinic. The timing of the immunizations of every infant was re-



**Figure 9.1.** Trial profile

† died

corded. Serum samples of preterm infants were collected within 48 hours after birth, 4-6 weeks after the third pneumococcal immunization and 4-8 weeks after the fourth (booster) immunization. Serum samples of the infants aged between 5-7 months from the national serum bank of a cross-sectional population-based serosurveillance study in the Netherlands conducted in the same years (ISRCTN20164309),<sup>234</sup> were used as term controls (Dutch population study) (Figure 9.1).

### **Transplacental transport**

Serum samples collected within 48h after birth of preterm infants in the scGOS/lcFOS/pAOS study, complemented with cord blood samples of preterm infants whose parents gave informed consent only for the cord blood sample, and serum samples of their mothers were collected. Cord blood of term infants (GA >37 weeks) born at the VUmc and their mothers served as controls only for serum samples at birth (Figure 9.1).

### **Effect of supplementation of scGOS/lcFOS/pAOS**

After assignment to 1 of 3 birth weight groups (<799, 800–1199, and 1200 g), the infants were randomly allocated by an independent researcher using a computer-generated randomization table (provided by Danone Research, Friedrichsdorf, Germany) for treatment within 48 h after birth to receive either enteral scGOS/lcFOS/pAOS or placebo powder (maltodextrin). Investigators, parents, medical and nursing staff were unaware of the treatment allocation.<sup>61</sup> Protocol guidelines for the introduction of parenteral and enteral nutrition followed current practices at the NICU. Nutritional support was administered as previously described.<sup>61</sup> 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 3-30 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 1.5g/kg/day to breast milk or preterm formula. Per 100 mL, the preterm formula provided 80 kcal, 2.4 g protein, 4.4g fat and 7.8g carbohydrate. After discharge, all infants received breast milk or preterm formula (Neonatal Start, Nutricia, Zoetermeer) followed by post-discharge formula (Neonatal 1, Nutricia, Zoetermeer) until the corrected age of 6 months. None of the formulae contained oligosaccharides.<sup>61</sup>

### **Laboratory analyses**

Blood samples collected within 48 h after birth of both term and preterm infants, and of their mothers, and follow up serum of preterm infants collected 4-6 weeks after the third pneumococcal immunization (at 5 months) and 4-8 weeks after the fourth (booster) immunization at 11 months were collected and transported to the lab at room temperature. All samples were centrifuged and serum was stored at –80°C until analysis. Serum samples were

analyzed as previously described by Elberse et al.<sup>235</sup> In short, serum samples were tested for antibodies to the 7 pneumococcal vaccine (PCV7) serotypes (4/6B/9V/14/18C/19F/23F) with a multiplex immuno assay (Bio-Rad Laboratories, Hercules, CA) using Luminex technology. Geometric mean concentrations of the serotypes determined by Luminex might be slightly higher than determined by ELISA. Correlations between the assays are high ( $R^2$ : 0.84 to 0.91 for all serotypes except serotype 19F, for which  $R^2$  was 0.70).<sup>235</sup> Additionally, serum samples were tested for 6 other vaccine serotypes (1/3/5/6A/7F and 19A, not part of PCV7 but included in Prevenar-13<sup>®</sup>, Pfizer) using the same Luminex technology.

### Data analyses

Normally distributed and nonparametric data were presented as means ( $\pm$  SD) and median (range), respectively. For statistical analyses, antibody levels below the lower limit of quantitation were assigned as half the lower limit of quantitation (0.001 microgram/ml). All IgG antibody levels were expressed as geometric mean concentrations (GMCs) in  $\mu\text{g/ml}$  and with 95% confidence intervals (CIs). Internationally assigned protective concentrations were used to determine the percentage of infants with presumed protective IgG levels ( $\% > 0.35 \mu\text{g/ml}$  at 5 months of age and  $\% > 1.0 \mu\text{g/ml}$  at 12 months of age).<sup>236</sup> Patient and nutritional characteristics were analyzed with Student's t-test, chi-square test or Fisher's exact test for continuous normally distributed and dichotomous data, respectively. If parameters had a skewed distribution, a natural logarithmic transformation was performed before analysis. ANOVA was performed to analyse differences between more than two groups. A linear regression analysis was performed to determine the influence of GA, BW, cord blood antibody concentrations, breastfeeding and infections during the first 80 days of life. All analyses were performed on an intention to treat basis. For all statistical analyses, a two-sided p value of  $< 0.05$  was considered significant. SPSS 20.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis.

### Results

Between May 2007 and November 2008, 113 preterm infants were included in the CARROT study (Figure 9.1). Baseline patient and nutritional characteristics were similar in the scGOS/lcFOS/pAOS ( $n=55$ ) and placebo group ( $n=58$ ) (Table 9.1). Serum samples around birth were collected of 96 preterm and 42 term infants and their mothers. Serum samples of 88 preterm infants at 5 months (89% of the eligible infants, mean age 177 days) and of 83 infants at 12 months (85% of the eligible infants, mean age 390 days) were available (Figure 9.1). From the Dutch population-based serosurveillance study, 41 term infants (mean age 183 days, 49% male) were eligible for comparison with the preterm infants of 5 months. No serum

**Table 9.1.** Baseline and nutritional characteristics of preterm infants

	scGOS/lcFOS/pAOS n = 55		Placebo n = 58	
<b>Baseline characteristics</b>				
Chorioamnionitis	11	(20%)	13	(22 %)
PE, E or HELLP	17	(31%)	18	(31%)
Placental insufficiency	4	(7%)	3	(5%)
Antenatal antibiotics	11	(20%)	16	(28%)
Antenatal corticosteroids	31	(56%)	32	(56%)
Multiple birth	9	(16%)	13	(22%)
Vaginal delivery	31	(56%)	328	(55%)
Gestational age (wks)	29.9	(1.9)	29.3	(2.1)
Gestational age ≤ 28 wks	11	(20%)	16	(28%)
Birth weight (kg)	1.3	(0.4)	1.2	(0.3)
Birth weight <10th percentile	12	(22%)	8	(14%)
Sex, male	31	(56%)	36	(62%)
Apgar at 5 min <6	9	(16%)	5	(9%)
Antibiotics postpartum	41	(75%)	44	(76%)
<b>Nutritional characteristics</b>				
Age at start of study supplementation (d)	2.1	(1.5-5.3)	2.1	(1.5-3.3)
Time to full supplementation dose (d)	11	(4-28)	11	(5-27)
Supplementation during study period (g/kg/d)	1.30	(0.1-1.6)	1.27	(0.2-1.8)
Age at advancement of enteral nutrition (d)	2.8	(0.6-27.5)	2.5	(0.3-18.0)
Exclusive breast milk during day 3-30	38	(69%)	33	(57%)

PE, preeclampsia; E, eclampsia; HELLP, syndrome of hemolysis, elevated liver enzymes and low platelets; There were no statistically differences ( $p < 0.05$ ) between both groups.

samples were available from term infants 12 months of age from that study for comparison with preterm infants.

At birth GMCs in  $\mu\text{g/ml}$  were not different between the scGOS/lcFOS/pAOS and placebo group for all pneumococcal serotypes, except for 18C (table 9.2).

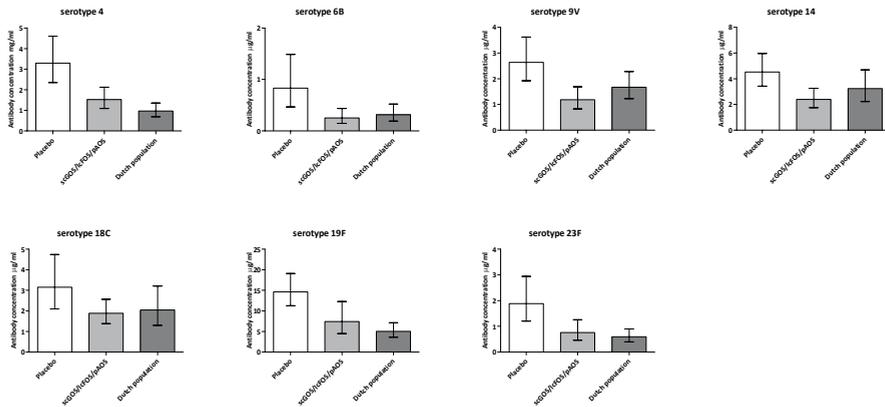
### Transplacental transport of pneumococci specific IgG

GMCs with 95% CIs and ranges of pneumococci antibody levels in maternal and cord blood serum samples are summarized in Supplemental digital content 2. The median placental transfer ratio of the pneumococcal antibodies was 0.35 in preterm infants and 0.86 in term infants ( $p < 0.01$ ).

**Table 9.2.** Number of samples tested, geometric mean concentration (GMCs) and 95% confidence intervals (CIs) of pneumococcal serum IgG levels of antibodies to pneumococci 4, 6B, 9V, 14, 18C, 19F and 23F in preterm and term infants at birth, age 5 and 12 months.

	Birth			5 months			12 months		
	n	GMC (CI), µg/ml	n	GMC (CI), µg/ml	n	GMC (CI), µg/ml	n	GMC (CI), µg/ml	
4	Placebo	55	0.02(0.02-0.04)	47	3.29 (2.36-4.60)*	40	4.55 (3.28-6.30)		
	scGOS/lcFOS/pAOS	53	0.02(0.01-0.03)	41	1.53 (1.10-2.12)*	43	4.28 (3.15-5.82)		
	Dutch population	42	0.04 (0.02-0.06)	41	0.97 (0.69-1.36)*				
6B	Placebo	55	0.05 (0.03-0.10)	47	0.79 (0.43-1.47) *	40	5.39 (2.83-10.25)		
	scGOS/lcFOS/pAOS	53	0.06 (0.03-0.12)	41	0.25 (0.15-0.44)*	43	3.33 (2.01-5.51)		
	Dutch population	42	0.25 (0.13-0.50)*	41	0.32 (0.19-0.52)*				
9V	Placebo	55	0.06 (0.03-0.09)	47	2.64 (1.92-3.62) *	40	5.25 (3.85-7.16)		
	scGOS/lcFOS/pAOS	53	0.07 (0.04-0.12)	41	1.19 (0.83-1.69)*	43	4.00 (2.93-5.45)		
	Dutch population	42	0.12 (0.07-0.21)*	41	1.67 (1.23-2.28)*				
14	Placebo	55	0.14 (0.08-0.24)	47	4.52 (3.43-5.96) *	40	8.14 (6.01-11.02)		
	scGOS/lcFOS/pAOS	53	0.20 (0.11-0.34)	41	2.39 (1.76-3.26)*	43	5.76 (4.16-7.96)		
	Dutch population	42	1.65 (1.05-2.61)*	41	3.24 (2.24-4.69) *				
18C	Placebo	55	0.06 (0.04-0.10)	47	3.15 (2.09-4.73) *	40	5.40 (3.59-8.13)		
	scGOS/lcFOS/pAOS	53	0.15 (0.10-0.23)*	41	1.88 (1.38-2.57)*	43	4.73 (3.78-5.92)		
	Dutch population	42	0.32 (0.18-0.56)*	41	2.03 (1.29-3.21) *				
19F	Placebo	55	0.33 (0.23-0.50)	47	14.64 (11.25-19.06)	40	11.50 (8.43-15.68)		
	scGOS/lcFOS/pAOS	53	0.48 (0.29-0.81)	41	7.42 (4.48-12.28)*	43	10.69 (8.15-14.03)		
	Dutch population	42	1.47 (0.97-2.22)*	41	5.06 (3.59-7.13) *				
23F	Placebo	55	0.10 (0.06-0.15)	47	1.88 (1.21-2.94) *	40	7.36 (5.14-10.55)		
	scGOS/lcFOS/pAOS	53	0.08 (0.05-0.13)	41	0.72 (0.41-1.26)*	43	4.62 (3.35-6.36)		
	Dutch population	42	0.25 (0.15-0.41)*	41	0.59 (0.38-0.90)*				

\*Differed from placebo group: p<0.05



**Figure 9.2.** Geometric mean antibody concentrations with 95% CI for 7 pneumococcal serotypes at 5 months of age in the placebo group, scGOS/lcFOS/pAOS group and the Dutch population group in µg/ml.

### Effect of scGOS/lcFOS/pAOS supplementation

At 5 months of age, the IgG antibody responses to the 7 pneumococcal vaccine serotypes after the completed primary series of vaccinations were lower for 7 out of the 7 vaccine serotypes in the scGOS/lcFOS/pAOS group compared to placebo group (table 9.2). IgG antibody levels in preterm infants supplemented with scGOS/lcFOS/pAOS were similar to that of term infants at age 5 months, while IgG antibody levels in preterm infants supplemented with placebo were higher for 5 of the 7 vaccine serotypes (serotypes 4, 6B, 9V, 19F, 23F,  $p < 0.05$ ) than those in term infants (Figure 2 and Table 9.2).

At 12 months of age, there was a significant booster response in both preterm groups and the GMCs of pneumococcal antibody levels after the booster dose for all serotypes were not different in the scGOS/lcFOS/pAOS and placebo group (table 9.2).

### Proportion of preterm infants with protective IgG levels

Protective antibody concentrations  $> 0.35$  µg/ml post primary vaccinations at 5 months and  $> 1.0$  µg/ml post booster vaccination at 12 months in the scGOS/lcFOS/pAOS and placebo group are shown in table 9.3. At 5 months, more preterm infants in the placebo group were protected for serotype 6B compared with the scGOS/lcFOS/pAOS group ( $p < 0.01$ ) and more infants in the placebo group had a level above 0.35 µg/ml for all 7 serotypes ( $p < 0.01$ ). After the booster vaccination at 12 months, no differences in the protective antibody concentrations were found between the two groups.

**Table 9.3.** Number of samples tested, geometric mean concentration of pneumococcal serum IgG levels (GMCs) and transplacental transport ratios of antibodies to pneumococci 4, 6B, 9V, 14, 18C, 19F and 23F in preterm and term infants.

Pneumococci		GMCs (95% CI) and transplacental transport ratio				
		N	Cord serum	N	Maternal serum	Ratio
4	Preterm	96	0.02 (0.02-0.03)	88	0.06 (0.04-0.09)	0.36 <sup>a</sup>
	Term	42	0.04 (0.02-0.06)	39	0.06 (0.04-0.10)	0.64
6B	Preterm	96	0.07 (0.05-0.11)	88	0.18 (0.12-0.28)	0.37 <sup>a</sup>
	Term	42	0.25 (0.13-0.50)	39	0.30 (0.14-0.63)	0.89
9V	Preterm	96	0.06 (0.04-0.09)	88	0.15 (0.10-0.22)	0.41 <sup>a</sup>
	Term	42	0.12 (0.07-0.21)	39	0.15 (0.08-0.26)	0.81
14	Preterm	96	0.12 (0.07-0.20)	88	0.39 (0.24-0.64)	0.30 <sup>a</sup>
	Term	42	1.65 (1.05-2.61)	39	1.64 (0.99-2.72)	1.00
18C	Preterm	96	0.09 (0.06-0.13)	88	0.26 (0.18-0.37)	0.35 <sup>a</sup>
	Term	42	0.32 (0.18-0.55)	39	0.38 (0.21-0.68)	0.82
19F	Preterm	96	0.33 (0.21-0.53)	88	1.22 (0.86-1.74)	0.29 <sup>a</sup>
	Term	42	1.47 (0.97-2.22)	39	1.73 (1.04-2.86)	0.88
23F	Preterm	96	0.08 (0.05-0.12)	88	0.21 (0.13-0.32)	0.36 <sup>a</sup>
	Term	42	0.25 (0.15-0.41)	39	0.28 (0.16-0.49)	0.94

**NOTE.** Maternal serum samples were obtained from mothers between 2 days before and after delivery and cord serum samples were obtained from umbilical cords. CI, confidence intervals; <sup>a</sup> p<0.01 preterm vs term

**Table 9.4.** Number of samples tested, geometric mean concentration (GMCs) and 95% confidence intervals (CIs) of pneumococcal serum IgG levels of antibodies to non PCV7 pneumococci 1, 3, 5, 6A, 7 and 19A in preterm infants at age 5 months.

		5 months	
		n	GMC (CI), µg/ml
1	Placebo	47	0.05 (0.04-0.06)
	scGOS/lcFOS/pAOS	41	0.05 (0.04-0.07)
3	Placebo	47	0.16 (0.12-0.22)
	scGOS/lcFOS/pAOS	41	0.12 (0.10-0.16)
5	Placebo	47	0.02 (0.01-0.02)
	scGOS/lcFOS/pAOS	41	0.01 (0.01-0.02)
6A	Placebo	47	0.03 (0.02-0.04)
	scGOS/lcFOS/pAOS	41	0.04 (0.02-0.07)
7	Placebo	47	0.02 (0.02-0.03)
	scGOS/lcFOS/pAOS	41	0.02 (0.02-0.03)
19A	Placebo	47	0.14 (0.10-0.20)
	scGOS/lcFOS/pAOS	41	0.09 (0.06-0.13)

### Additional vaccine serotypes

At 5 months, the 6 additional measured (vaccine) serotypes not included in PCV-7 showed low GMCs and were not different in the scGOS/lcFOS/pAOS and the placebo group; for all serotypes  $p > 0.05$  (table 9.4.).

### Influences of perinatal factors

At 5 months, preterm infants with higher GA showed higher pneumococcal antibody concentrations of serotype 6B ( $R^2=0.11$ ,  $p=0.01$ ), 18C ( $R^2=0.09$ ,  $p=0.02$ ) and 23F ( $R^2=0.10$ ,  $p=0.01$ ) and a trend towards higher antibody concentrations of serotype 4 ( $R^2=0.08$ ,  $p=0.07$ ) and 19F ( $R^2=0.01$ ,  $p=0.06$ ). At 12 months, preterm infants with higher GA showed higher pneumococcal antibody concentrations for serotype 6B ( $R^2=0.03$ ,  $p=0.02$ ) and 14 ( $R^2=0.10$ ,  $p=0.04$ ). Birth weight, breast feeding, infections during the first 30 days of life and cord blood pneumococcal antibody concentration had no influence on the antibody concentrations at 5 and 12 months (data not shown,  $p > 0.05$ ).

## Discussion

This study shows that preterm infants without oligosaccharide supplementation to the enteral feeding have a higher pneumococcal antibody response to the PCV serotypes 4, 6B, 9V, 19F and 23F than term infants at 5 months of age. Enteral supplementation of scGOS/lcFOS/pAOS mixture during days 3-30 of life in preterm infants leads to a diminished antibody response to pneumococcal polysaccharides (PS) in preterm infants, but importantly they showed similar anti-PS IgG antibody levels as term infants after the primary series immunizations. After the PCV-7 booster vaccination at 12 months of age, differences between the 2 preterm groups no longer persisted.

The lower IgG levels to the 7 vaccine serotypes in the scGOS/lcFOS/pAOS supplemented infants at 5 months might reflect either a direct or indirect (through modulation of the microbiota) immunomodulatory effect of the neutral and acidic oligosaccharides in the first months of life. In the postnatal maturation of the immune system, the intestinal microbiota plays an important role.<sup>209,233</sup> In preterm infants, intestinal colonization with health promoting bacteria such as bifidobacteria is delayed compared to term infants, whereas colonization with potentially pathogenic bacteria is increased.<sup>43</sup> It is also known that breastfeeding modulates intestinal colonization for example by promoting colonisation with Bifidobacteria.<sup>209</sup> Singhal et al<sup>194</sup> emphasized that early nutrition provides long-term health effects and especially the first month of life is a critical window. Previously, our group showed that supplementation of scGOS/lcFOS/pAOS during day 3 -30 of life in preterm infants had both a direct immunomodulatory effect reflected by lower pro-inflammatory cytokine levels at day 7 of life<sup>61</sup> and by a reduced rate of serious neonatal infections.<sup>237</sup> There was also an

indirect effect by increasing the postnatal intestinal colonisation, reflected by a higher total bacterial count and a trend towards increased Bifidobacteria counts.<sup>62</sup> Prebiotics have been used in several studies in preterm infants with positive effects on fussing and crying,<sup>238</sup> stool viscosity and pH<sup>63</sup> and intestinal colonization<sup>62</sup> during the neonatal period and infections during the first year of life.<sup>61,239</sup> Probiotics have shown to induce a reduction in NEC in preterm infants,<sup>240</sup> and in term infants an increased vaccination response.<sup>241</sup> Therefore, future studies combining prebiotics and probiotics may even have a more pronounced effect on the immune system than prebiotics alone, which is supported by a study in healthy term infants supplemented with a probiotic during the first 6 months of life.<sup>242</sup> The exact mechanism behind the long term (5 months) effect of scGOS/lcFOS/pAOS supplementation on the antibody responses after pneumococcal vaccination in preterm infants however remains to be elucidated. Although we had expected that antibody responses after pneumococcal vaccination would be lower in preterm infants than term infants, the antibody responses of preterm infants in the placebo group were actually higher at 5 months than those in healthy term infants in a Dutch serosurveillance population-based study in the same time period. Preterm infants supplemented with scGOS/lcFOS/pAOS however, had similar antibody responses to the term infants of the Dutch population study. This seems to indicate that the preterm infants in general show a more abundant vaccination response to PCV7 at 5 months of age and that supplementation with scGOS/lcFOS/pAOS attenuates the increased vaccine response. Higher responses to pneumococcal conjugate vaccinations in preterm infants have been previously described by Shinefield et al, but the authors do not speculate on the aetiology of this increased response.<sup>232</sup> An excessive vaccine response might be explained by the immature immune system of preterm infants that might lead to a less balanced reaction on conjugate vaccinations. Van den Biggelaar et al<sup>243</sup> previously showed that in term neonates the reaction to a neonatal PCV-7 vaccine (given at birth) is more Th-2 skewed, but becomes more Th-1 skewed as the vaccination is administered later in life. The immature immune system of preterm infants is known to be more Th-2 skewed and might therefore react more abundantly to this Th-2 biased vaccine, even at 5 months of age.<sup>244</sup> PCV-7 might induce a more Th-2 skewed immune response because of the adjuvants Alum, which induces the release of IL-1 $\beta$ , which stimulates the production of IL-6 and IL-10 and has effect through circulation of inflammatory dendritic cells that induces Th-2 responses. Alum is also used as adjuvant in the DTaP-Hib vaccine, but this is aluminium hydroxide instead of aluminium phosphate which is used in PCV-7.<sup>245</sup> Maybe this leads to a different immune response, but this needs further investigation. In a murine model, van't Land et al. showed that enteral scGOS/lcFOS/pAOS induces immune modulation with a prominent role of CD25+ Tregs towards enhanced Th1 vaccine responsiveness, possibly by suppression of the Th2 vaccine responsiveness.<sup>200</sup> This would make the response to a Th-2 biased vaccine of the more Th2- skewed preterm infants receiving scGOS/lcFOS/pAOS more comparable to the more Th1 skewed term infants. Furthermore, a murine model showed that the serotype

of *Streptococcus pneumoniae* modified the Th-1/Th-2 cytokine profile in vivo; serotype 19F showed a more Th2 skewed profile compared to serotype 14.<sup>246</sup> In line with these findings, we found in our study no difference between preterm infants and term infants for serotype 14 (more Th-1 skewed profile) but higher levels for serotype 19F in preterm infants (more Th-2 skewed profile). In measurements of IgG subclasses, we found no difference in IgG subclasses for the PCV-7 serotypes between the scGOS/lcFOS/pAOS and placebo group (data not shown). Previously we found no differences between the scGOS/lcFOS/pAOS and placebo group for the combined Diphtheria, Tetanus and Pertussis vaccine responses at 5 months of age, although there was a trend towards lower levels in scGOS/lcFOS/pAOS group for Haemophilus influenzae type B vaccine responses, also a conjugate vaccine.<sup>247</sup> We also found no differences between the Dutch population group (n=41) and the preterm infants for these vaccines, except for the pertussis antigen Pertactin, which was higher in preterm infants (79.4; 95% CI 64.1-98.3) than in term infants (40.8; 95% CI 29.0-57.4 IU/ml, p=0.03).

To further elucidate the mechanism of this response and to exclude a specific effect not caused by vaccination, we performed additional measurements of antibodies to 6 pneumococcal serotypes which are not included in PCV-7 but are included in the follow-up vaccine PCV-13. The GMCs of these 6 serotypes were very low indicating no immune reaction to other serotypes than to the vaccinated serotypes induced by the previous PCV-7 vaccinations.

Transplacental transport of pneumococcal antibodies was lower in preterm infants compared to term infants. The ratios of this transport are consistent with the ratios found for antibodies specific for Hib and MenC as previously described.<sup>98</sup> Pneumococcal, Hib and MenC antibodies are mainly IgG2 subclass antibodies. IgG2 transplacental transport is less effective compared with other IgG subclasses like IgG1 and IgG3 which are the main antibodies of DTaP.<sup>77,78</sup> Maternal antibodies at birth did not influence the response to pneumococcal vaccinations at 5 months of age in preterm infants.

There are several limitations to our study. The primary endpoint of the CARROT study of neutral and acidic oligosaccharides was the number of infections during the first 80 days of life, and therefore the study was not powered on vaccination response. For the best comparison between the preterm and term infants, we should have included term infants with the same follow up as the preterm infants. Although no term born infants with a booster vaccination with PCV7 were available for comparison at the time of our study, similar antibody levels at 12 months after a booster vaccination at 11 months were found in term infants in a later study.<sup>248</sup> Prymula et al. found that prophylactic administration of paracetamol impaired the immunization response to pneumococcal vaccination.<sup>228</sup> In our study, we did not record information on the number of infants who received prophylactically paracetamol before the immunization but there is no indication that this might be different between the groups and prophylactically paracetamol use is usually low in the Netherlands.<sup>249</sup>

### Conclusion

Short-term supplementation of scGOS/lcFOS/pAOS during day 3-30 of life decreased the pneumococcal vaccine antibody response after the primary series of PCV7 at 5 months in preterm infants to levels, which are similar in term infants from a Dutch population study. However, after the booster vaccination at 12 months this effect of the scGOS/lcFOS/pAOS on the pneumococcal conjugate vaccine response had disappeared.