7

Neonatal modulation of serum cytokine profiles by a specific mixture of anti-inflammatory neutral and acidic oligosaccharides in preterm infants

Jolice P van den Berg, Ninke van Zwieteren, Elisabeth A.M. Westerbeek, Johan Garssen, Ruurd M. van Elburg

Cytokine 2013 Oct;64(1):188-95

Abstract

Background Infections are common in preterm infants and cause differences in cytokine levels. Aim of this study was to measure cytokine levels in preterm infants during the first year of life and to determine the effect of feeding a specific non-digestible carbohydrate mixture (scGOS/lcFOS/pAOS). Furthermore, other perinatal factors in relation to these cytokine levels were analyzed.

Methods In a randomized controlled trial, preterm infants (GA <32 weeks and/or birth weight <1500 g) received a scGOS/lcFOS/pAOS mixture or a placebo (maltodextrin) between days 3 and 30 of life. Cytokine levels (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-17, IFN-γ, and TNF-α) were analyzed at 5 time points during the study: before start of the study, at day 7, at day 14 and at 5 and 12 months after the start of the intervention.

Results In total, 55 preterm infants in the scGOS/lcFOS/pAOS group and 58 in the placebo group were included. During the neonatal period cytokine levels increased, followed by a decrease at 5 months and 12 months. Enteral supplementation of the non-digestible oligosaccharides decreased cytokine levels at day 7 but not at day 14, indicating a temporarily anti-inflammatory effect. In the neonatal period, serious infection before sampling increased all cytokine levels.

Conclusion Enteral supplementation of this specific non-digestible oligosaccharide mixture decreased cytokine levels in preterm infants at day 7 of life, although this effect disappeared thereafter.

Introduction

Preterm infants have an immature immune system which makes them vulnerable for infections.[166] Cytokines are a key part of the immune system, thereby playing a role in both the susceptibility and immune-defence of newborn infants towards infections. Cytokine levels in preterm infants differ from cytokine levels in term infants. In cord blood, pro-inflammatory cytokines such as Interleukin (IL)-2, IL-4, Il-5, IL-6, IL-8, IL-10 and tumor necrosis factor α (TNF-α) are higher in preterm infants than in term infants, while there are conflicting results regarding higher or even lower IL-1β levels in cord blood of preterm infants compared to term infants.[16,17] Levels of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, TNF-α and IFN-γ are higher in cord blood of preterm infants who were prenatally exposed to infection. IL-10, IL-18, IFN-γ, TGF-β and TNF-α might differentiate between infants with fungal and bacterial sepsis, later in the neonatal period.[18] Extremely low birth weight infants with blood stream infections have lower levels of IL-17 and higher levels of IL-6 and IL-8. The highest blood cytokines levels in preterm infants are found on the day of birth.[19] Levels of cytokines at birth or in the neonatal period in preterm infants are possible predictors of bronchopulmonary dysplasia, white matter brain damage and cerebral palsy.[20,21,23,24,185]

The infant's immune system matures by exposure to intestinal microbiota.[167,168] This interaction leads to metabolic/immunologic reactions by the epithelial cells and the underlying lymphoid cells: the bacterial-epithelial 'cross talk'.[186–188] Preterm infants have a delayed intestinal colonization and possibly as a consequence an inappropriate bacterial-epithelial 'crosstalk', resulting in an inadequate maturation of the host immune defence.[43,186,187,189]

In term infants, breastfeeding decreases the incidence of infections.[190] Human milk oligosaccharides (HMOS) are part of the factors in human milk thought to be responsible for this effect.[191–193] Non-human milk oligosaccharides such as short-chain galacto-oligosaccharides (scGOS) and long-chain fructo-oligosaccharides (lcFOS) are used to mimic the functions of HMOS.[75,181] Pectin derived acidic oligosaccharides (pAOS) are able to act as receptors analogs and are known to inhibit the adhesion of pathogens on the epithelial surface.[48] pAOS may also directly affect the immune cells via interaction with selectins, dendritic cell specific C-type lectin, integrins and other target receptors such as Toll-like receptors.[75] We recently described that, if supplemented in sufficient amounts, the combination of neutral oligosaccharides and acidic oligosaccharides decreases the incidence of infections in preterm infants.[61] It was hypothesized that enteral supplementation of a scGOS/lcFOS/pAOS mixture consisting of neutral and acidic oligosaccharides may positively modulate the immune system.

As part of our randomized controlled trial, the aim of this study was to measure cytokine profiles during the neonatal period and during the first year of life of preterm infants. A unique analysis, which has never been done before in preterm infants, both during the neonatal period and the following year. In addition, the effect of enteral supplementation

with a mixture of non-digestible neutral and acidic oligosaccharides but also the effect of other perinatal factors on serum cytokine profiles were analyzed.

Methods

Subjects

Infants with a gestational age (GA) <32 weeks and/or birth weight (BW) <1500 g, admitted to the level III NICU of the VU University Medical Center, Amsterdam, were eligible for participation in the study. Exclusion criteria were: infants with a GA >34 weeks, major congenital or chromosomal anomalies, death <48 h after birth and transfer to another hospital <48 h 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.

Randomization, blinding and treatment

The infants were randomly allocated to treatment <48 h after birth to receive either enteral 80% scGOS/lcFOS and 20% pAOS or placebo powder (maltodextrin) as previously described.[61,116] The randomisation code was broken after data analysis was performed. Supplementation of the mixture or placebo was administered in increasing doses between days 3 and 30 of life to 1.5 g/kg/day to breast milk or preterm formula. Per 100 mL, the preterm formula provided 80 kcal, 2.4 g protein (casein-whey protein ratio 40:60), 4.4 g fat and 7.8 g carbohydrate. The preterm formula did not contain oligosaccharides. When infants were transferred to another hospital before the end of the study, the protocol was continued under supervision of the principal investigator.[61]

Nutritional support

Nutritional support was administered as previously described.[61] For each infant in the study a feeding schedule was proposed based on BW and the guidelines as mentioned previously.[61,75] The medical staff of the NICU and the responsible paediatricians in the regional hospitals had final responsibility for the administration of parenteral nutrition and advancement of enteral nutrition.

Cytokine analysis

Blood samples were collected before the start of the intervention, within 48 h after birth (birth), at postnatal day 7 (day 7) and day 14 (day 14), at 4-6 weeks after the last vaccination of the primary series of DTaP-IPV-Hib and pneumococcal vaccinations at 2, 3 and 4 months (5 months) and at 4-8 weeks after the DTaP-IPV-Hib and pneumococcal booster vaccination at 11 months (12 months). Collected blood was allowed to clot at room temperature for

1 h and centrifuged at 3000 rpm for 10 min. 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 (MIA) (Luminex xMAP technology) at the National Institute for Public Health and the Environment, Bilthoven, the Netherlands. Analysis was performed with a Bio-Plex 200 (Bio-rad laboratories, Hercules, CA, USA). Cytokine assay kits were purchased from Bio-rad (Hercules, CA, USA). Cytokine measurements were performed according to the instructions of the manufacturer with slight modifications. Samples were diluted with human serum diluent in a 1:1 ratio (normal 1:3). The standard curve was calibrated on a high Photo-Multiplier Tubes (PMT) setting. Paired samples from a single subject were always analyzed on the same plate.

Statistical analysis

The sample size of 113 infants was based on the sample size calculation for the primary outcome of the main trial (serious infectious morbidity). Normally distributed and nonparametric data are presented as means ± SD and medians (ranges) respectively. Patient and nutritional characteristics were compared between groups with *t* test, Mann-Whitney *U* test, chi-square test, or Fisher's exact test for continuous normally distributed, nonparametric continuous and dichotomous data, respectively. In the primary analysis, generalised estimating equations (GEE) were used to compare changes in cytokine levels over time between the groups. This method takes into account the dependency of the observations within a patient and the fact that samples may not be available at each measuring time. Cytokine levels below the lower limit of quantitation were assigned as half the mean lower limit of quantitation of all plates (IL-1β 0.14, IL-2 0.06, IL-4 0.03, IL-6 0.10, IL-8 0.10, IL-10 0.10, IL-17 0.32, IFN-γ 2.41 and TNF-α 0.23). All cytokine levels were expressed as geometric mean concentrations (GMC) with 95% confidence intervals (CI).

Furthermore, the effect of host and treatment related factors on cytokine levels (prenatal corticosteroids, chorioamnionitis, gender, mode of delivery, GA, serious infection <48 h before blood sample, antibiotics <48 h before blood sample, type of feeding and mild bronchopulmonary dysplasia (BPD) [61,75]) was determined by GEE analysis.[176] All statistical analysis was performed on an intention-to-treat basis. For all statistical analyses, a two-tailed *p* value <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 of 208 eligible preterm infants were included in the study (Figure 7.1). Baseline patient and nutritional characteristics were not different

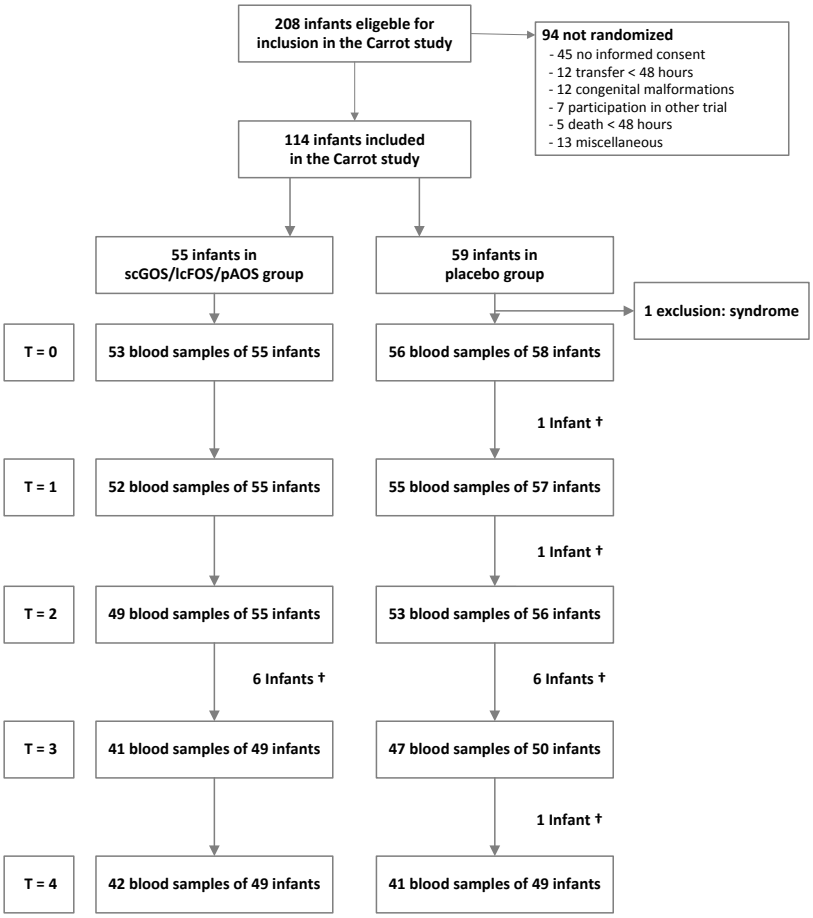

Figure 7.1. Trial profile
Time points T0 = <48h after birth, T1 = day 7, T2=day 14, T3= 5 months T4=12 months.
† died

in scGOS/lcFOS/pAOS (*n* = 55) and placebo group (*n* = 58) (Table 7.1). Nutritional and clinical characteristics at the time of cytokine measurements were not different in both groups (Table 7.2). In total, blood samples of 89% of the eligible infants at 5 months of age and 85% of the eligible infants at 12 months of age were collected (Figure 7.1, Table 7.2).

Blood samples were taken at 14.4 ± 20.7 h (T0), 7.1 ± 0.8 days (T1), 14.5 ± 1.6 days (T2), 177 ± 21 days (T3) and 390 ± 23 days (T4) (Table 7.2). In the crude GEE analysis at day 7, serum levels of IL-1β, IFN-γ and TNF-α (p = 0.003, p = 0.02, p = 0.026) were lower after enteral supplementation of scGOS/lcFOS/pAOS compared to the control group. In addition, there was a trend to lower levels for IL-4, IL-6, IL-8 and IL-17 (p = 0.06, p = 0.09, p = 0.07, p = 0.06). At day 14, there was a trend towards lower levels of IL-1β (p = 0.06) in the scGOS/lcFOS/pAOS group compared with the placebo group (Figure 7.2). At 5 months, IL-1β was lower (p =

Table 7.1. Baseline and nutritional characteristics*†

	scGOS/lcFOS/pAOS (N=55)	Placebo (N=58)
Baseline characteristics		
Maternal age (y)	32.1± 4.6‡	31.1± 6.4
Maternal race, % Caucasian [*n (%)*]	43/55 (78%)	40/58 (69%)
Obstetric diagnosis [*n (%)*]		
Chorioamnionitis	11/55 (20%)	13/58 (22%)
PE,E or HELLP	16/55 (31%)	18/58 (31%)
Placental insufficiency	4/55 (7%)	3/58 (5%)
Antenatal antibiotics [*n (%)*]	11/55 (20%)	16/58 (28%)
Antenatal corticosteroids [*n (%)*]	30/55 (56%)	32/58 (56%)
Multiple birth [*n (%)*]	9/55 (16%)	13/58 (22%)
Vaginal delivery [*n (%)*]	31/55 (56%)	32/58 (55%)
Gestational age (wks)	29.9 ± 1.9	29.3 ± 2.1
Birth weight (kg)	1.32 ± 0.4	1.23 ± 0.3
Birth weight <10th percentile [*n (%)*]	12/55 (22%)	8/58 (14%)
Sex , % male [*n (%)*]	31/55 (56%)	36/58 (62%)
Apgar at 5 min <6 [*n (%)*]	9/55 (16%)	5/58 (9%)
pH umbilical artery <7.10 [*n (%)*]	2/55 (4%)	0/58 (0%)
Surfactant medication [*n (%)*]	22/55 (40%)	26/58 (45%)
PIVH [*n (%)*]		
None	45/55 (82%)	46/58 (80%)
Grades I or II	8/55 (15%)	10/58 (17%)
Grades III or IV	2/55 (4%)	2/58 (3%)
Antibiotics postpartum [*n (%)*]	41/55 (75%)	44/58 (76%)
Nutritional characteristics		
Age at start of study supplementation (d)	2.1 (1.54-5.25)§	2.1 (1.54-3.25)
Time to full supplementation dose (d)	11 (4-28)	11 (5-27)
Mean supplementation dose during study period (g/kg/d)	1.30 (0.10-1.60)	1.27 (0.17-1.79)
Age at advancement of enteral nutrition (d)	2.8 (0.58-27.50)	2.5 (0.33-18.04)
Exclusive breast milk during 30 day study period	38/55 (69%)	33/58 (57%)

*PE=preeclampsia; E=eclampsia; HELLP=syndrome of hemolysis, elevated liver enzymes and low platelets; PIVH=periventricular-intraventricular hemorrhage. † Student's *t* test, Mann-Whitney *U* test and chi-square test or Fisher's exact test are used to analyse continuous normally distributed, nonparametric continuous data, respectively

‡Mean±SD (all such valued) §Median: range in parentheses (all such values) ||There were no statistically differences ($p<0.05$) between both groups.

Table 7.2. Nutritional and clinical characteristics[1]

			scGOS/lcFOS/pAOS group (n=55)	Placebo group (n=58)
Day 1 (T0)	Blood samples taken at (hours)		15.35 ± 19.79[2]	13.54 ± 21.27
n = 110	Bloodtype	- venous	3/54 (6%)	3/56 (5%)
		- capillair	4/54 (7%)	6/56 (11%)
		- arterial	11/54 (20%)	17/56 (30%)
		- umbilical cord	36/54 (67%)	29/56 (52%)
		- missing	0/54 (0%)	1/56 (2%)
Day 7 (T1)	Blood samples taken at (days)		6.98 ± 0.63	7.19 ± 0.89
n=107	Infection ≤ 48 h for sample		10/52 (19%)	13/55 (24%)
	Antibiotics ≤ 48 h for sample		17/52 (33%)	24/55 (44%)
	Exclusive breast milk feeding		32/52 (62%)	34/55 (62%)
	Bloodtype	- venous	2/52 (4%)	0/55 (0%)
		- capillair	26/52 (50%)	32/55 (58%)
		- arterial	21/52 (40%)	22/55 (40%)
		- umbilical cord	1/52 (2%)	0/55 (0%)
		- missing	2/52 (4%)	1/55 (2%)
Day 14 (T2)	Blood samples taken at (days)		14.54 ± 2.03	14.44 ± 1.21
n=102	Infection ≤ 48 h for sample		1/49 (2%)	6/53 (12%)
	Antibiotics ≤ 48 h for sample		21/49 (43%)	22/53 (42%)
	Exclusive breast milk feeding		32/49 (66)	37/53 (70%)
	Bloodtype	- venous	0/49 (0%)	1/53 (2%)
		- capillair	43/49 (88%)	42/53 (79%)
		- arterial	4/49 (8%)	9/53 (17%)
		- missing	2/49 (4%)	8/53 (15%)
5 months (T3)	Blood samples taken at (days)		176 ± 21	179 ± 21
n=88	Bloodtype	- venous	38/41 (93%)	42/47 (89%)
		- capillair	3/41 (7%)	5/47 (11%)
12 months (T4)	Blood samples taken at (days)		389± 23	391 ± 23
n=82	Bloodtype	- venous	31/41 (76%)	35/41 (85%)
		- capillair	9/41 (22%)	6/41 (15%)
		- arterial	1/41 (2%)	0/41 (0%)

[1] Student's *t* test, Mann-Whitney *U* test and chi-square test or Fisher's exact test are used to analyse continuous normally distributed, nonparametric continuous data, respectively [2] Mean±SD (all such valued) ||There were no statistically differences ($p<0.05$) between both groups.

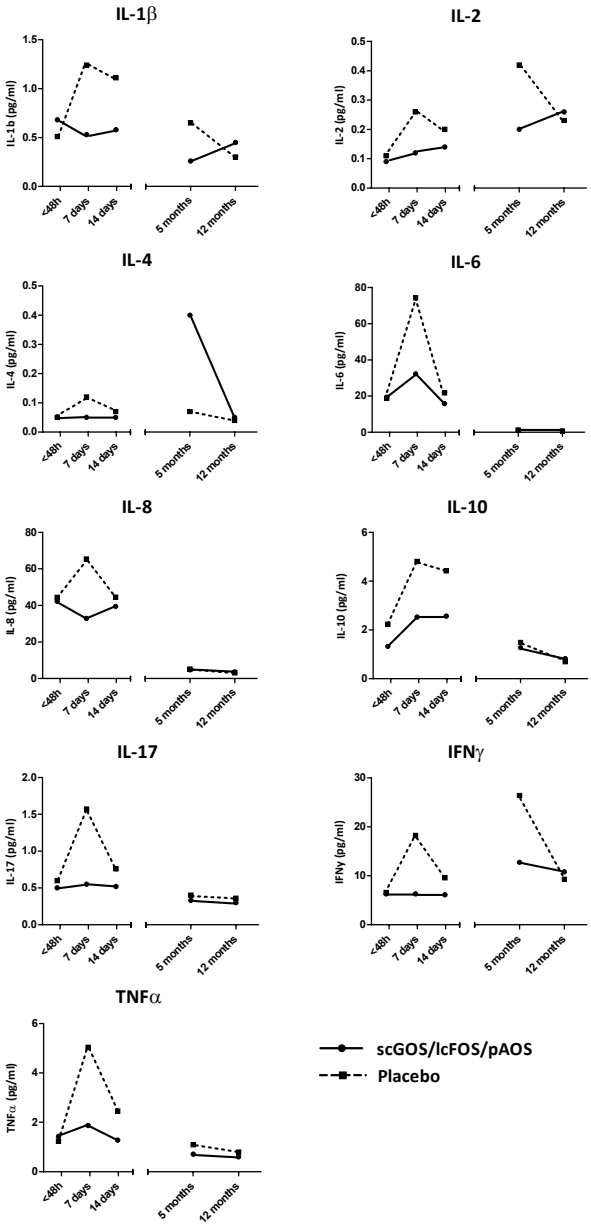

Figure 7.2. Cytokine profiles in preterm infants supplemented with scGOS/lcFOS/pAOS and placebo group. Time points: before start of the study (t=0), day 7 (t=1), day 14 (t=2), 5 months (t=3) and 12 months (t=4) after birth. Generalized estimating equations (GEE) were used to assess differences between the scGOS/lcFOS/pAOS mixture and placebo group at baseline and for changes over time (compared with baseline values)

Table 7.3. Influence of perinatal factors on cytokine levels[1]

Factor	IL-1β	IL-2	IL-4	IL-6	IL-8	IL-10	IL-17	IFN-γ	TNF-α
Prenatal corticosteroids	1.08 (0.73-1.57)[1]	1.32 (0.90-1.95)	1.34 (0.96-1.87)	1.34 (0.84-2.12)	1.17 (0.88-1.55)	1.37 (0.94-1.98)	1.10 (0.78-1.55)	1.42 (0.98-2.06)	1.34 (0.85-2.10)
Chorioamnionitis	1.29 (0.70-2.13)	1.03 (0.61-1.75)	0.96 (0.60-1.53)	1.18 (0.66-2.11)	1.14 (0.81-1.60)	1.11 (0.64-1.91)	0.80 (0.49-1.29)	1.00(0.59-1.68)	1.31 (0.73-2.36)
Gender	0.84 (0.56-1.26)	1.20 (0.82-1.77)	1.12 (0.80-1.58)	1.23 (0.77-1.96)	0.93 (0.70-1.24)	1.37 (0.92-2.04)	1.26 (0.87-1.81)	1.23 (0.83-1.81)	1.15(0.72-1.85)
Cesarean section	1.00 (0.65-1.52)	0.95 (0.63-1.42)	0.85 (0.59-1.22)	0.81 (0.49-1.34)	0.79 (0.59-1.07)	1.01 (0.67-1.51)	0.84 (0.57-1.24)	0.85 (0.56-1.29)	0.84 (0.51-1.37)
Gestational age	0.98 (0.85-1.13)	0.94 (0.83-1.06)	0.93 (0.82-1.04)	1.00 (0.84-1.19)	1.03 (0.94-1.14)	1.13 (0.99-1.29)	0.88 (0.77-1.02)	0.92 (0.81-1.05)	0.90 (0.76-1.07)
Infection ≤ 48 h for sample*	2.16 (1.14-4.09)‡	4.14 (1.83-9.37)†	5.56 (2.63-11.77)†	6.04 (2.54-14.37)†	2.11 (1.17-3.79) ‡	11.41(4.97-26.20)†	7.82 (3.25-18.86)†	5.34 (2.59-11.05)†	7.95 (3.39-18.67)†
Antibiotics ≤ 48 h for sample	1.22 (0.84-1.76)	1.05 (0.76-1.47)	1.13 (0.84-1.53)	1.57 (1.03-2.42) ‡	1.24 (0.92-1.69)	1.50 (1.07-2.11) ‡	1.26 (0.88-1.80)	1.31(0.93-1.85)	1.24 (0.82-1.86)
Exclusive breastfeeding	0.75 (0.51-1.10)	1.18 (0.81-1.70)	1.20 (0.86-1.67)	0.93 (0.59-1.48)	1.03 (0.78-1.37)	1.01 (0.67-1.51)	0.89 (0.61-1.29)	1.07(0.73-1.56)	0.97 (0.61-1.55)
Mild BPD	1.43 (0.59-3.51)	1.18 (0.39-3.62)	0.96 (0.37-2.49)	1.08 (0.40-2.95)	1.41 (0.76-2.62)	2.16 (0.75-6.23)	0.84 (0.33-2.11)	1.00 (0.32-3.11)	0.70 (0.23-2.16)

[1]Data indicate the effect of a factor on cytokine levels (generalised estimated equations). The effect can be interpreted as follow: in case of prenatal corticosteroids, IL-1β is 1.15 (95% CI) times as high as without prenatal corticosteroids. Corrected for the influence of scGOS/lcFOS/pAOS

*Sepsis, meningitis, pyelonephritis, pneumonia or arthritis as diagnosed by a combination of clinical signs and a positive culture, †P≤0.001, ‡ p<0.05

0.002) in the scGOS/lcFOS/pAOS group and there was a trend towards lower IL-4 (p = 0.06) in the scGOS/lcFOS/pAOS group. At 12 months IL-10 was higher in the scGOS/lcFOS/pAOS group compared to the control group (p = 0.03).

At day 14, there was a trend toward fewer infections in infants in the scGOS/lcFOS/pAOS group compared with the placebo group (p = 0.06). Adjustment for serious infection and other possible confounders (chorioamnionitis, administration of prenatal corticosteroids, mode of delivery, GA, gender, type of feeding, administration of antibiotics, serious infection, mild BPD) did not change the results of the primary analysis.

Data of both groups were combined for analysis of the role of perinatal factors that may influence cytokine levels (Table 7.3). Serious infection before sampling increased all cytokines levels (IL-1β, p = 0.02; IL-8, p = 0.01; IL-2, IL-4, IL-6, IL-10, IL-17, IFN-γ and TNF-α $p \leq 0.001$).

There was a trend toward less serious infections in the scGOS/lcFOS/pAOS group compared to the placebo group, therefore we adjusted for serious infections to analyse the change of cytokine levels over time. In general, cytokine levels increased between birth and day 7, except for IL-8. Cytokine levels at day 14 did not show any significant difference with either day 1 or day 7, except for IL-1B and IL-10 which increased from birth to day 14 (p = 0.04 and p = 0.02), also after adjustment for serious neonatal infections.

Compared to the neonatal period (14 days) a decrease of IL-1β (12 months $p < 0.001$), IL-6 (5 months $p < 0.001$), IL-8 (5 months $p < 0.001$), IL-10 (5 months p = 0.002), IL-17 (5 months p = 0.02) and TNF-α (5 months p = 0.048) was seen during the follow-up. IFN-γ showed an increase at 5 months (p = 0.01) but showed a trend towards a decrease at 12 months (p = 0.10). IL-2 (p = 0.07 / p =0.64) showed no significant change (Figure 7.1).

Discussion

This study shows a general increase in the cytokine levels in the neonatal period compared to cord blood levels in preterm infants. The precise reason for this increase is not known but it might be a consequence of an immature immune system, which in turn leads to higher infectious pressure. After the neonatal period cytokine levels decrease as shown at 5 months and 12 months of age.

It is known that preterm infants have a more immature immune system compared to term infants at birth. Preterm birth may lead to an immunological cascade resulting in an unbalanced pro-inflammatory/anti-inflammatory cytokine profile with increased susceptibility to infections.[34] Especially the first months of life may represent a critical window in the maturation of the immune system.[194] It has been hypothesised that each type of immune response, either pro- or anti-inflammatory, develops its own regulatory population and a balanced immune response can be controlled by distinct regulatory T-cell populations.

To our knowledge this is the first study describing cytokine profile changes during the entire first year of life in a cohort of preterm infants. Overall, the analyses show that after the neonatal period serum cytokine levels decrease until the age of one year.

Differences between preterm infants and term infants might be explained by differences in perinatal, host and nutrition related factors. Causes for preterm delivery such as chorioamnionitis are known to cause changes in cytokine profiles.[18] In our study higher levels of TNF-α were measured at birth and day 7 after a pregnancy complicated by chorioamnionitis. After birth the cytokine profiles of all measured cytokines in preterm infants are increased during or shortly after infections. In our study the use of antibiotics increased cytokine levels of IL-6 and IL-10 during the neonatal period. This is in line with Sood et al. who found higher cytokine levels during sepsis, especially in bacterial sepsis.[195]

The influence of the type of feeding have been described by Field et al. [196] Exclusively breastfeeding decreased cytokine levels in term infants. Kainonen et al [197] found an anti-inflammatory cytokine milieu in breast-fed infants compared to formula-fed infants. Human milk oligosaccharides (HMOS) are thought to be responsible for at least a part of this effect. [191–193] HMOS interact with the local immune system of the gastro-intestinal tract.[48,56,169] The immunomodulatory effect of HMOS is thought to be related to the development of a bifidogenic intestinal microbiota.[167,168] Furthermore, neutral and acidic HMOS can be absorbed and cross the border membrane of the intestine, suggesting that HMOS may also act systemically and that their effect is not restricted to the intestine itself. For preterm infants breast milk is considered to be the optimal nutrition.[49,198,199] In the first 10 days of life of preterm infants, exclusive breast milk feeding is rare, caused by delayed onset of lactation after preterm birth.[49] Previous studies show that early exposure to microbes induces pro-inflammatory responses that shift presumed neonatal default anti-inflammatory responses towards a balanced pro-inflammatory/anti-inflammatory cytokine profile. In a vaccination model in mice, prebiotic oligosaccharides (scGOS/lcFOS/pAOS) shifted the immune response towards a more pronounced anti-inflammatory type of responsiveness. Consequences in terms of immune health and related disorders such as allergies, inflammation and infections are extensively described. Regulatory cells seem to play a key role in this effect.[75,168,200]

Our study shows that enteral supplementation of scGOS/lcFOS/pAOS decreased cytokine levels in preterm infants at day 7, indicating a temporarily anti-inflammatory effect. This effect mimics the effect of breast milk feeding as shown in term infants.[196] After day 7 this effect disappears and cytokine levels are comparable at day 14 in the scGOS/lcFOS/pAOS and the placebo group. However IL-1b and IL-4 are still affected, possibly indicating an anti-allergic and anti-inflammatory stimulus. An explanation for this finding might be that we supplemented the oligosaccharides both to breast milk feeding and preterm formula feeding. We hypothesise that since preterm infants are seldom exclusively breast fed during the first 10 days of life, the beneficial effects of human milk oligosaccharides might be exceeded by the oligosaccharides in the scGOS/lcFOS/pAOS mixture. Another possible explanation

for this might be that preterm infants have their peak incidence of sepsis around day 7. The influence of infections is larger than the influence of the scGOS/lcFOS/pAOS on the cytokine levels and therefore abolishes this effect at day 14. This might be explained by the direct influence of infections on cytokine levels but we hypothesise that the use of broad spectrum antibiotics during infections in preterm infants diminishes the anti-inflammatory effect by their opposite effect on the gut to neutral and acidic oligosaccharides.[43,201]

Some aspects of the study design need to be addressed. An optimal supplementation dose of 1.5 g/kg/day was reached at a median postnatal age of 11 days due to restriction of the maximal osmolarity of the enteral feeding. Therefore, infants may not have received an optimal dose of scGOS/lcFOS/pAOS-supplementation to reach the maximal effect at postnatal day 7 or 14. Furthermore, some of the cytokine levels were undetectable especially in IL-2 and IL-4, as we choose to measure unstimulated levels of cytokines to better reflect the natural cytokine response to oligosaccharide supplementation in preterm infants.

In summary, this study describes serum cytokine levels in preterm infants in the first year of life. During the first week after birth, cytokine levels were relatively high, followed by a significant decline later in the first year of life. External factors such as infections and antibiotics influence this profile. Additionally, dietary intervention with a unique mixture of non-digestible oligosaccharides (scGOS/lcFOS/pAOS) between days 3 and 30 of life decreases most cytokine levels at day 7, indicating a temporary anti-inflammatory effect. This effect disappears at day 14. Most cytokines decrease after the neonatal period (IL-1β, IL-6, IL-8, IL-10, IL-17 and TNF-α), while IFN-γ increases at 5 months, indicating skewing towards Th1 activity, which is highly desirable at this age. The increased IL-10 levels at 12 months are promising and might help in understanding the findings in other preclinical and clinical studies in which the oligosaccharide mixture lead to less severe allergy and/or lower incidence of allergy related disorders.[202,203]