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**A Th1/Th2-associated chemokine imbalance during infancy in children  
developing eczema, wheeze and sensitization**

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**Abstract**

*Background:* Analyses of circulating chemokines offer novel tools to investigate the Th1/Th2 imbalance in allergic disease *in vivo*.

*Objective:* To relate circulating Th1- and Th2-associated chemokines in infancy to allergic disease, sensitization and probiotic supplementation.

*Methods:* Circulating levels of Th1-associated CXC-chemokine ligand (CXCL)9, CXCL10 and CXCL11 and Th2-associated CC-chemokine ligand (CCL)17 and CCL22 were assessed with Luminex and CCL18 with ELISA at birth (n=109), 6 (n=104), 12 (n=116) and 24 months (n=123) in 161 infants completing a double-blind placebo-controlled allergy prevention trial with *Lactobacillus reuteri* during the last month of gestation and through the first year of life. The infants were followed regarding development of allergic disease and sensitization until two years of age.

*Results:* The Th2-associated chemokines CCL17 and CCL22 were as highest at birth and then decreased, whereas CCL18 and the Th1-associated chemokines increased with age. High Th2-associated chemokine levels were observed in children developing allergic disease. Sensitization was preceded by elevated levels of the Th2-associated CCL22 and reduced levels of the Th1-associated CXCL11 already at birth. The Th2-associated CCL17 was also elevated at birth in infants developing recurrent wheeze. A high Th2/Th1-ratio (CCL22/CXCL10) at birth associated with both sensitization and eczema development. Presence of *L. reuteri* in stool the first week of life was associated with low CCL17 and CCL22 and high CXCL11 levels at six months of age. High Th1-associated chemokine levels were associated with day-care.

*Conclusion and Clinical Relevance:* Allergic disease and sensitization in infancy was associated with low circulating Th1- and high Th2-associated chemokine levels already from birth. Circulating chemokines are useful for investigating the Th1/Th2 imbalance in allergic

disease *in vivo*. Elucidation of the role of chemokines in allergic diseases may lead to future treatments. (ClinicalTrials.gov NCT01285830)

**Key words**

Wheeze; eczema; chemokine; day-care; infant; *Lactobacillus reuteri*; pregnancy; probiotics; sensitization; skin prick test

## Introduction

Allergy development has been attributed to a delayed maturation of the immune system with a decreased allergen induced interferon- $\gamma$  (IFN- $\gamma$ ) production at birth [1] and a prolonged postnatal Th2-deviation in childhood [2, 3]. Yet, it is still somewhat controversial whether Th2-deviation increases the risk for allergic disease or not. For example, children in developing countries with a strong Th2-deviation, due to chronic parasite infection, do not run an increased risk for allergic disease [4]. Also, both Th1-associated autoimmune diseases, such as Crohn and diabetes mellitus, and allergic disease have increased in affluent countries [5]. Although the increase of allergic disease obviously depends on environmental factors, it has been difficult to link laboratory markers of Th2-deviation to such factors in humans *in vivo*. This might be a methodology issue. Appropriately powered prospective studies from birth are needed, as well as reliable markers in peripheral blood.

Circulating Th1 and Th2 cytokine levels are low and close to detection limit [6]. Chemokines, on the other hand, are easily detected in peripheral blood [6]. They comprise a large protein family responsible for the trafficking of leukocytes to the site of inflammation and the regulation of leukocyte maturation [7]. Their receptors are expressed on the surface of several cell types involved in the allergic inflammation: *e.g.* CCR4 on eosinophils and Th2 lymphocytes and CXCR3 on Th1 lymphocytes and natural killer cells [7]. Atopic dermatitis is associated with high circulating levels of the Th2-cytokine induced CCR4 ligands CCL17 and CCL22 [8-10], as well as CCL18 (unknown receptor) [11], in children and adults. Furthermore, increased levels of CCL17, CCL18 and CCL22 in bronchoalveolar lavage (BAL) fluid have been reported in asthmatics [12, 13] and after allergen challenge [14]. In contrast, the IFN- $\gamma$  induced CXCR3 ligands CXCL10 and CXCL11 are associated with Th1-like diseases, such as sarcoidosis, tuberculosis [15] and

Crohn's disease [16]. It is not known, however, whether any of these chemokines are primarily involved in the pathogenesis of allergic diseases or merely are secondary to a general immune deviation.

We have previously reported that *L. reuteri* treatment was associated with a lower incidence of sensitization and IgE-associated disease [17]. This study provided an opportunity to relate circulating Th1- and Th2-associated chemokines to the development of allergic disease, probiotic supplementation and environmental factors in infancy. We hypothesized that high levels of circulating Th1- and low levels of Th2-associated chemokines would be associated with *L. reuteri* supplementation and reduced incidence of sensitization and allergic disease during infancy, and that factors possibly decreasing the risk of allergic disease, *e.g.* day-care, would relate to elevated Th1- and reduced Th2-associated chemokines, while the chemokine pattern was opposite for factors increasing the risk.

## Methods

### *Study design*

This was a part of a prospective, double-blind, placebo-controlled, multi-centre trial in South Eastern Sweden comprising 232 families with at least one family member with allergic disease, assessing the potential allergy preventing effect of probiotics [17]. The mothers started taking *Lactobacillus reuteri* ATCC 55730 ( $1 \times 10^8$  CFU, BioGaia AB, Stockholm, Sweden) or placebo four weeks before term and continued to do so daily until delivery. After birth, the baby commenced with the same study product as the mother at 1-3 days of age and continued daily for one year. The infant was followed up another year regarding the development of allergic disease with or without positive skin prick test (SPT) or circulating IgE to food allergens.

As previously described in detail [17], clinical follow-up was done at 1, 3, 6, 12 and 24 and telephone interviews at 2, 4, 5, 8, 10 and 18 months by a research nurse and the final diagnosis by a paediatrician at 24 months of age. A questionnaire was completed on each occasion. In all, 188 infants completed the original study, and 161 of these, where blood samples were available, were included in the present study (81 in the treated and 80 in the placebo group). The clinical outcome in these 161 infants is described in Table 1. The active and placebo group did not differ regarding potential confounders, such as sex, birth order, duration of pregnancy, caesarean delivery, parental smoking, family history of allergic disease, breastfeeding, day-care and infections, except for antibiotics, that were more often prescribed in the active than the placebo group (Table 1). An informed consent was obtained from both parents before inclusion. The Regional Ethics Committee for Human Research at Linköping University approved the study. The study is registered at ClinicalTrials.gov (NCT01285830).

**Table 1.** Descriptive data of children completing the study.

	<i>Lactobacillus reuteri</i> % (n/N)	Placebo % (n/N)	p-value*
Boys	57 (46/81)	46 (37/80)	0.18
First born	51 (41/81)	55 (44/80)	0.58
Caesarean delivery	11 (9/81)	11 (9/80)	0.98
Parental smoking	7 (6/81)	13 (10/80)	0.28
Furred pets	16 (13/81)	10 (8/80)	0.25
Maternal atopy	74 (60/81)	75 (60/80)	0.89
Paternal atopy	67 (54/81)	66 (53/80)	0.95
Breastfeeding			
3 months, exclusive	68 (55/81)	59 (74/80)	0.41
6 months, partial	83 (67/81)	83 (66/80)	0.97
Antibiotics			
0-12 months	38 (31/81)	23 (18/80)	0.03
12-24 months	43 (35/81)	46 (37/80)	0.70
Day-care			
0-12 months	5 (4/81)	5 (4/80)	0.99
12-24 months	80 (65/81)	86 (69/80)	0.31
Allergic manifestations**			
Recurrent wheeze	6 (5/81)	10 (8/80)	0.37
Eczema	36 (29/81)	34 (27/80)	0.79
Allergic disease	44 (36/81)	44 (35/80)	0.93
Sensitisation	35 (22/63)	43 (27/63)	0.44
IgE-assoc. disease	14 (9/63)	32 (20/63)	0.02

\*  $X^2$  test. \*\* Cumulative incidence until 2 years of age.*Diagnostic criteria of allergic disease*

Allergic disease included eczema, recurrent wheeze, allergic rhinoconjunctivitis, allergic urticaria and gastrointestinal allergy. Eczema was defined as a pruritic, chronic or chronically relapsing non-infectious dermatitis with typical features and distribution. Wheeze was defined as an episode with obstructive airway symptoms, and recurrent wheeze as  $\geq 3$  wheezing episodes, at least once verified by a physician. A diagnosis of allergic rhinoconjunctivitis required watery discharge at least twice in contact with the same allergen and no signs of infection. Urticaria was defined as allergic when appearing at least twice in conjunction with

a certain food. A diagnosis of gastrointestinal allergy required vomiting, diarrhoea or systemic reaction after ingestion of a potentially allergenic food and a confirmation by challenge, unless there was a clear history of a severe systemic reaction. Allergic urticaria, ARC and gastrointestinal allergy were not analyzed separately since the incidence of these manifestations was low (2.1%, 2.7% and 2.1%, respectively). Infants were regarded as sensitized if they had at least one positive SPT and/or detectable circulating allergen specific IgE antibodies. Allergic disease was classified as IgE-associated if the symptomatic infant also was sensitized.

### *Sensitization*

Skin prick tests were done on the volar aspects of the forearm with egg white, fresh skimmed cow milk (lipid concentration 0.5%) and standardised cat, birch and timothy extracts (Soluprick®, ALK, Hørsholm, Denmark) at 6, 12 and 24 months of age. Histamine hydrochloride (10 mg/ml) was used as positive and albumin diluents as negative control. The test was regarded as positive if the mean diameter of the wheal was  $\geq 3$ mm.

Circulating IgE antibodies to egg white and cow's milk were analysed at 6, 12, and 24 months of age in venous blood (UniCap® Pharmacia CAP System™, Pharmacia Diagnostics, Uppsala, Sweden). The cut off level was 0.35 kU/L, according to the protocol of the manufacturer. In addition, circulating IgE to a mixture of food allergens, including egg white, cow's milk, cod, wheat, peanut and soy bean, was analyzed at 6, 12 and 24 months of age (UniCap® Pharmacia CAP System™, fx5, Pharmacia Diagnostics).

### *Bacteriological analyses*

The bacterial analyses in faecal samples are described in detail elsewhere [18]. Briefly, stool samples were collected from the baby at 5 or 6 days of age, weighted, thawed and diluted in

saline buffer and plated on modified de Man-Rogosa-Sharpe (MRS) agar with 50 mg/L Vancomycin and 2% sodium acetate (MRS-3, Acumedia, Ljusne, Sweden). The plates were incubated anaerobically in anaerobic jars with GasPack Plus (Becton Dickinson, Stockholm, Sweden) at 37°C for 48 hours. *Lactobacillus reuteri* colonies were confirmed by detecting reuterin production after addition of glycerol.

#### *Chemokine analyses in venous blood*

Venous blood were collected from the umbilical cord (n=109) and at 6 (n=104), 12 (n=116) and 24 months (n=123) and stored in -20°C as heparinized plasma or serum until assessment. The chemokines CXCL9, CXCL10, CXCL11, CCL17 and CCL22 were analyzed with a Luminex assay, modified from [19].

#### Luminex assay:

Before commencing the multiplexed assay, monoclonal capture antibodies were covalently coupled to carboxylated microspheres, by the procedure recommended by the manufacturer (Luminex Corporation, Austin, TX, USA). 5 µg antibody/10<sup>6</sup> microspheres of monoclonal anti-human CXCL9 (clone no 49106, R&D Systems, Abingdon, UK), CXCL10 (clone 4D5, BD Biosciences, Stockholm, Sweden), CXCL11 (clone 87328, R&D Systems), CCL17 (clone 54026, R&D Systems) and CCL22 (clone 57226, R&D Systems) antibodies were used.

2000 coupled microspheres dissolved in 50µl PBS (Medicago AB) with 1% bovine serum albumin (BSA, Sigma-Aldrich, Stockholm, Sweden) were added to each well of a 1.2µm pore-size filter plate (Millipore multiscreen, Millipore Corporation, Bedford, USA).

Recombinant human CXCL9, CXCL10, CXCL11, CCL17 and CCL22 (R&D Systems) were used as standards. 50 µl blank and diluted samples (final dilution 1:2) were also added to the

microspheres and incubated over night at 4°C. After 2 washes, the microspheres were resuspended in 100 µl biotinylated anti-human CXCL10 (1000 ng/ml, clone BD Biosciences, Stockholm, Sweden), CXCL9 (500 ng/ml, BAF392), CXCL11 (500 ng/ml, BAF320), CCL17 (500 ng/ml, BAF364) and CCL22 (200 ng/ml, BAF336) antibody (R&D Systems) solution. After a 1-h incubation, the microspheres were washed twice, resuspended and incubated in 100 µl of 1 µg/ml Streptavidin R-phycoerythrin conjugate (Molecular Probes, Eugene, USA) for 30 minutes. After 2 washes, the samples were analysed on a Luminex<sup>100</sup> instrument (Biosource, Nivelles, Belgium) and the data were acquired using the StarStation 2.3 software (Applied cytometry systems, Sheffield, UK) with 5-parametric-curve fitting. Blank values were subtracted from all readings.

The possibility of cross-reactivity between the different microsphere sets was investigated by comparing the median fluorescence intensity (MFI) generated from a standard curve of the monoplex assay with the MFI generated from the corresponding standard curve of the pentaplex assay. This was performed for all combinations of microsphere sets and the MFI generated from the pentaplex assay was similar to those generated from the monoplex assays. The specificity of the assay was also evaluated by mixing all microsphere sets with a single chemokine standard in the middle part of the curve (2500 pg/ml for CXCL9, 2000 pg/ml for CXCL11, 500 pg/ml for CCL17 and 1000 pg/ml for CXCL10 and CCL22). A cocktail of all biotinylated detection antibodies were added to the wells. Positive readings were achieved for the microspheres coupled with the specific capture antibody for the current chemokine, whereas irrelevant chemokines did not show any readings above background for any microsphere sets. Taken all together, no cross-reactivity between the assay components was observed in the present setting.

The limit of detection was 14 pg/ml for CXCL9 , 6 pg/ml for CXCL10, 6 pg/ml for CXCL11, 2 pg/ml for CCL17 and 2 pg/ml for CCL22.

All samples were analysed in duplicates and the sample was re-analysed if the coefficient of variance (CV) was >15%. In total, 6.9 % of all samples were reanalyzed. The intra-assay variation was <10%, evaluated by testing one internal control sample in 12 wells at the same time point. Since samples from the study groups consisted of both plasma and serum samples, the correlation between plasma and serum samples was evaluated. Both serum and plasma samples were collected at the same time point from eleven children. Chemokine levels correlated mostly well in these samples: CXCL9 (p<0.001, rho=0.90), CXCL10 (p=0.12, rho=0.48), CXCL11 (p=0.03, rho=0.63) CCL17 (p=0.02, rho=0.68), CCL18 (p=0.01, rho=0.84), CCL22 (p=0.048, rho=0.58).

#### ELISA:

CCL18 existed in substantially higher concentration in peripheral blood than the other chemokines. Therefore, CCL18 was assessed separately with sandwich ELISA as previously described [6] using monoclonal anti-human CCL18 (clone 64507, R&D Systems) for coating and biotinylated anti-human CCL18 antibody (BAF394, R&D Systems) for detection. The cord blood samples were diluted 1/200 and the 6, 12 and 24-month samples were diluted 1/800. The detection limit for CCL18 was 8 pg/ml. All samples were analysed in duplicates and the sample was re-analysed if the CV was >15%.

#### *Statistical analysis*

Non-parametric tests were used, since the chemokine levels were not normally distributed. The correlations were analyzed with Spearman's rank order correlation coefficient test, comparisons between paired groups with Wilcoxon's test and unpaired groups with Mann-

Whitney U-test. Friedman's test was employed in analyses of multiple longitudinal measures obtained from the same subject, and repeated-measures ANOVA in analyses of multiple longitudinal measures of a specific chemokine in subjects in two different groups. When a sample was missing from a subject at any age, the value corresponding to the median value for the specific chemokine at that age and group was given before repeated-measures ANOVA was performed. Because of the non-normal distribution, a log transformation was performed before ANOVA. Logistic regression and repeated-measures ANOVA were employed in adjustments for potential confounders affecting the chemokine analyses. Potential confounders were defined as factors that both significantly affected the outcome and were associated with the specific chemokine at that age. The  $\chi^2$  test was used to compare the prevalence of outcome variables and background factors between the groups. A probability level of  $<0.05$  was considered to be statistically significant. The calculations were made with the statistical package SPSS 16.0 for Windows (SPSS Inc, Chicago, IL, USA).

## Results

### *Kinetics of Th1 and Th2 chemokines*

The highest Th2-associated chemokine levels were detected at birth and then decreased, whereas the Th1-associated chemokines increased with age (Table 2). The exception from this pattern was the Th2-associated chemokine CCL18, which increased with age (Table 2). Friedman's test for the whole period was significant for all chemokines ( $p < 0.001$ ) except CXCL11. The CXCL11 levels were significantly higher at 24 months than in cord blood, however (Table 2). The Th2-associated chemokines CCL17 and CCL22 correlated at every time point and so did the Th1-associated chemokines CXCL10 and CXCL11 (Supplementary Table 1). Furthermore, the Th2-associated chemokines CCL17 and CCL22 were inversely correlated with the Th1-associated chemokines CXCL10 and CXCL11.

### *Probiotic treatment*

There was no significant difference in chemokine levels between the *L. reuteri* and placebo treated group. Presence of *L. reuteri* in stool the first week of life was associated with lower CCL22 and CCL17 and higher CXCL11 levels at six months of life, however [median, (interquartile range) pg/ml: 445 (332-754) vs. 830 (531-943)  $p=0.02$ , 194 (82-338) vs. 368 (235-574)  $p=0.006$  and 358 (129-728) vs. 115 (72-390)  $p=0.03$ ;  $p=0.016$  for CCL22 with repeated measures ANOVA for the samples from 6 to 24 months of age].

### *Relationship with eczema and recurrent wheeze*

Eczema development was associated with high levels of all Th2-associated chemokines the first year of life (Table 3,  $p=0.004$  for CCL17,  $p=0.01$  for CCL18 and  $p=0.02$  for CCL22 with repeated-measures ANOVA). In contrast, low Th1-associated chemokines associated with eczema, although only significantly so for CXCL10 at 12 months (Table 3,  $p=0.09$  for

**Table 2.** The kinetics of circulating Th1 and Th2-associated chemokines levels [median (25-75th percentiles)] from birth until 24 months of age in 161 children regardless of treatment and presence of allergic symptoms

	<u>cord blood</u>		<u>6 mo</u>		<u>12 mo</u>		<u>24 mo</u>	
	p-value*		p-value*		p-value*			
<b>n=</b>	109		104		116		123	
<b><u>Th1- associated chemokines</u></b>								
<b>CXCL9</b>	<b>102</b>	<0.001	<b>188</b>	0.03	<b>233</b>	ns	<b>222</b>	
<b>pg/ml</b>	(79-142)		(131-311)		(239-321)		(152-337)	
<b>CXCL10</b>	<b>21</b>	<0.001	<b>54</b>	0.02	<b>69</b>	ns	<b>78</b>	
<b>pg/ml</b>	(14-32)		(30-96)		(45-112)		(53-119)	
<b>CXCL11</b>	<b>195**</b>	ns	<b>258</b>	ns	<b>504**</b>	ns	<b>521**</b>	
<b>pg/ml</b>	(137-352)		(109-646)		(159-668)		(334-675)	
<b><u>Th2- associated chemokines</u></b>								
<b>CCL17</b>	<b>474</b>	<0.001	<b>275</b>	<0.001	<b>159</b>	<0.001	<b>103</b>	
<b>pg/ml</b>	(270-676)		(148-503)		(54-327)		(54-209)	
<b>CCL18</b>	<b>30</b>	<0.001	<b>123</b>	0.01	<b>151</b>	ns	<b>157</b>	
<b>ng/ml</b>	(22-41)		(83-179)		(103-206)		(83-179)	
<b>CCL22</b>	<b>622</b>	ns	<b>590</b>	<0.001	<b>315</b>	<0.001	<b>244</b>	
<b>pg/ml</b>	(406-828)		(353-895)		(241-477)		(151-348)	

\* Wilcoxon's test for paired samples. Friedman's test was significant for all chemokines ( $p < 0.001$ ) but CXCL11 ( $p = 0.66$ ). \*\*When comparing the levels of CXCL11 in cord blood with the levels at 12 and 24 months,  $p = 0.009$  and  $p = 0.02$ , respectively (Wilcoxon's test).

CXCL10 and  $p = 0.03$  for CXCL11 with repeated-measures ANOVA). A Th2/Th1 ratio indicates the Th2/Th1-balance, and the CCL22/CXCL10-ratio at birth has previously been associated with subsequent allergic disease [6]. Therefore this ratio was assessed also in our study. Indeed, eczema was preceded by an elevated CCL22/CXCL10 ratio already at birth (Fig. 1a,  $p = 0.02$  with repeated measures ANOVA).

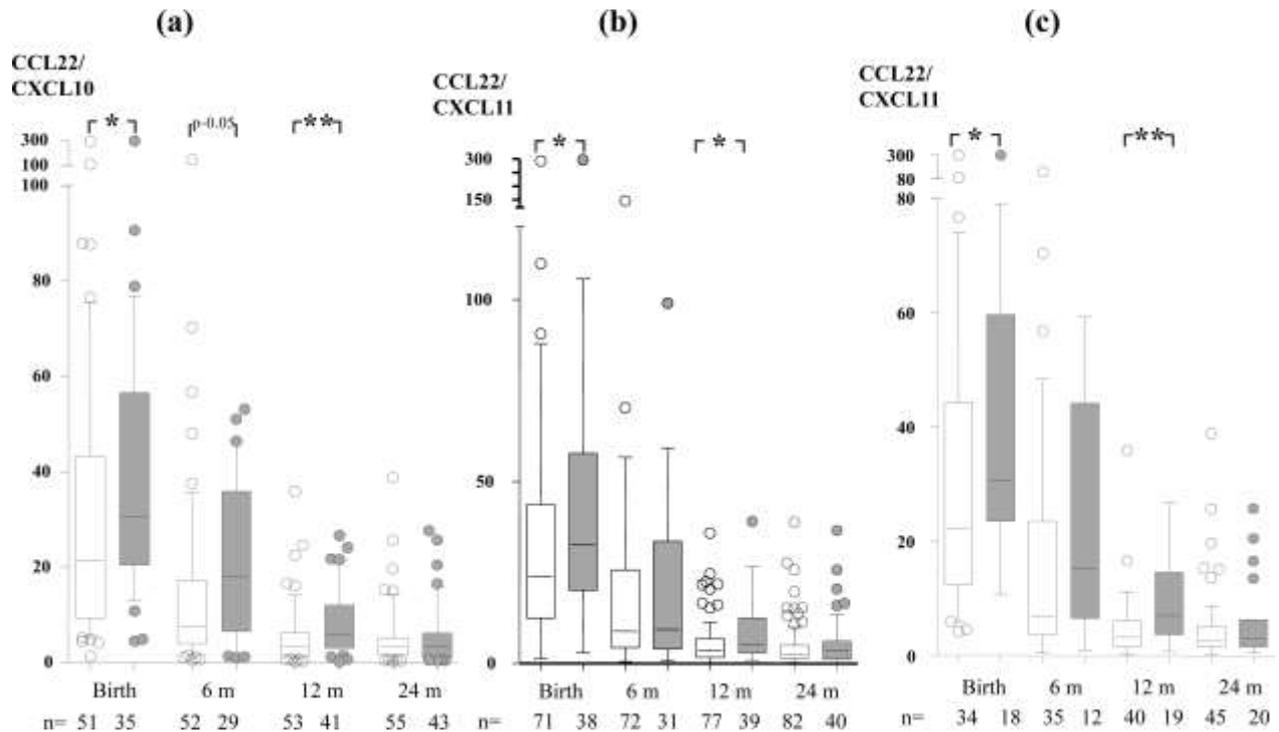
The relationship between recurrent wheeze and chemokine levels displayed a somewhat different pattern. Although recurrent wheeze was associated with elevated levels of all Th2-

**Table 3.** The levels of Th1- and Th2-associated chemokines in cord blood and in peripheral blood at 6, 12 and 24 months in infants developing eczema and infants without any allergic manifestation until two years of age, including both probiotic and placebo treated children [median (25-75th percentiles)]

	<u>cord blood</u>		<u>6 months</u>		<u>12 months</u>		<u>24 months</u>	
	<u>eczema</u>		<u>eczema</u>		<u>eczema</u>		<u>eczema</u>	
	no	yes	no	yes	no	yes	no	yes
<b>n=</b>	51	35	53	30	53	42	55	43
<b><u>Th1-associated chemokines</u></b>								
<b>CXCL9</b>	104	101	169	168	238	246	225	172
<b>pg/ml</b>	(71-136)	(80-169)	(124-290)	(131-336)	(165-323)	(194-308)	(170-347)	(118-278)
<b>CXCL10</b>	21	19	54	46	81*	59*	82	67
<b>pg/ml</b>	(14-35)	(13-29)	(32-98)	(27-68)	(48-141)	(43-75)	(54-121)	(49-110)
<b>CXCL11</b>	214	192	445	186	527	405	490	545
<b>pg/ml</b>	(156-471)	(125-225)	(133-644)	(99-463)	(330-725)	(124-622)	(317-631)	(394-670)
<b><u>Th2-associated chemokines</u></b>								
<b>CCL17</b>	412	513	230*	359*	129*	239*	96	138
<b>pg/ml</b>	(227-660)	(344-618)	(129-376)	(223-571)	(47-232)	(90-430)	(31-143)	(57-234)
<b>CCL18</b>	28	30	105	144	131*	164*	153	161
<b>ng/ml</b>	(22-37)	(23-38)	(76-158)	(87-187)	(96-188)	(125-207)	(96-191)	(125-207)
<b>CCL22</b>	535	608	458*	781*	279**	368**	240	244
<b>pg/ml</b>	(338-789)	(410-836)	(328-732)	(433-910)	(195-350)	(270-624)	(146-304)	(138-400)

\*p<0.05, \*\* p<0.01 with Mann-Whitney U-test.

associated chemokines from birth until 2 years of age (Table 4, p=0.001 for CCL17, p<0.001 for CCL18 and p=0.02 for CCL22 with repeated-measures ANOVA), recurrent wheeze was additionally associated with high Th1-associated chemokines at 2 years of age, significantly so for CXCL11 (Table 3) and a statistical trend for CXCL10 (p=0.07). In contrast to the other Th2-associated chemokines, CCL18 was also elevated at 6, 12 and 24 months in non-sensitized infants with recurrent wheeze (p=0.01 for all ages).



**Figure 1.** The CCL22/CXCL10 ratio (c) in cord blood and in peripheral blood at 6, 12 and 24 months in infants developing eczema (a), sensitization (b) and IgE-associated eczema (c) (closed bars) and infants without any allergic manifestation (open bars), including both probiotic- and placebo-treated children. The 10th, 25th, 50th, 75th and 90th percentiles are indicated. \* $P < 0.05$ , \*\* $P < 0.01$ .

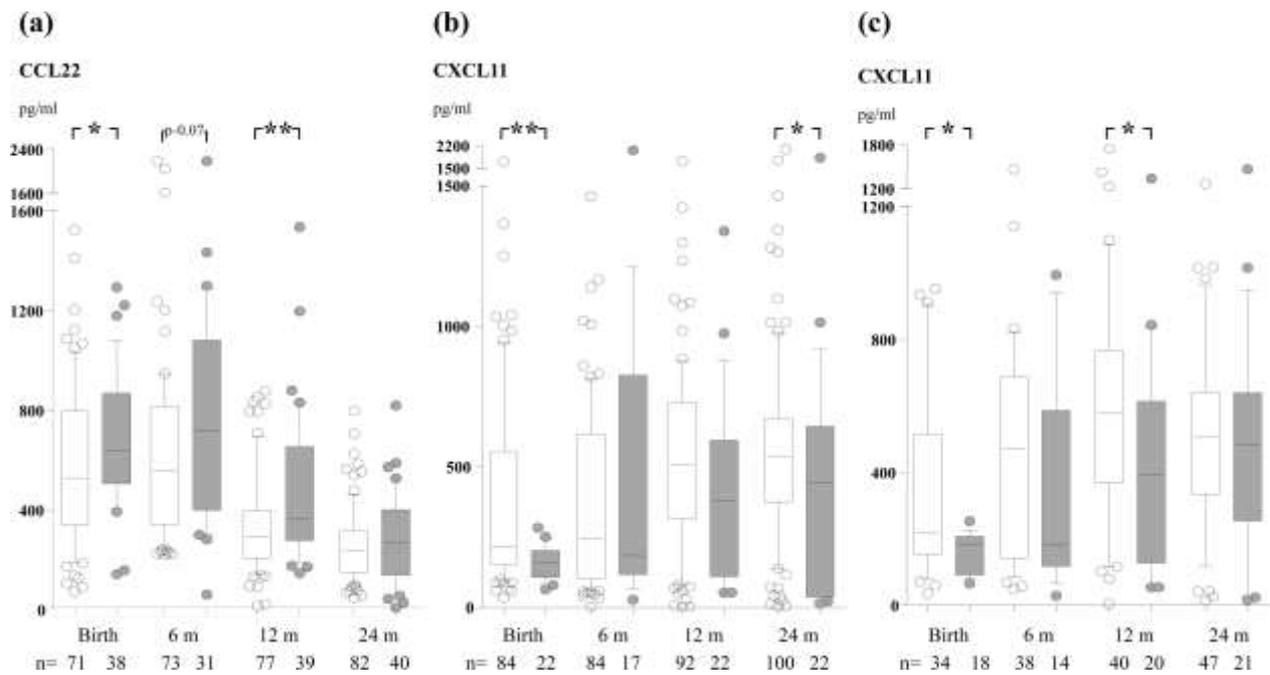
#### *Sensitization and IgE-associated allergic disease*

High CCL22 levels at birth and during the first year of life were associated with sensitization (circulating IgE to food allergens and/or positive SPT) (Fig. 2a;  $p = 0.03$  with repeated-measures ANOVA), while there was no significant association with the other chemokines (data not shown). Furthermore, SPT positive, as compared to negative, infants had low levels of the Th1-associated chemokine CXCL11 at birth and 24 months of age (Fig. 2b; 0.005 with repeated-measures ANOVA). Interestingly, none of the neonates with CXCL11 levels above the upper quartile at birth developed a positive SPT. High CCL22 levels was also associated with SPT positivity ( $p = 0.009$  with repeated-measures ANOVA, data not shown).

**Table 4.** The levels of Th1- and Th2-associated chemokines in cord blood and in peripheral blood at 6, 12 and 24 months in infants developing recurrent wheeze and infants without any allergic manifestation until two years of age, including both probiotic and placebo treated children [median (25-75th percentiles)]

	<b>cord blood</b>		<b>6 months</b>		<b>12 months</b>		<b>24 months</b>	
	<b>recurrent wheeze</b>		<b>recurrent wheeze</b>		<b>recurrent wheeze</b>		<b>recurrent wheeze</b>	
	no	yes	no	yes	no	yes	no	yes
<b>n=</b>	51	10	53	7	53	10	56	11
<b><u>Th1- associated chemokines</u></b>								
<b>CXCL9</b>	104	120	169	289	238	301	225	223
<b>pg/ml</b>	(71-136)	(51-159)	(124-290)	(131-384)	(165-323)	(182-477)	(170-347)	(184-472)
<b>CXCL10</b>	21	16	54	67	83	59	82	114
<b>pg/ml</b>	(14-35)	(12-24)	(32-98)	(26-86)	(48-141)	(37-105)	(54-121)	(72-210)
<b>CXCL11</b>	214	166	445*	109*	526	529	490*	687*
<b>pg/ml</b>	(156-471)	(90-242)	(133-644)	(60-334)	(330-725)	(118-744)	(317-631)	(593-1101)
<b><u>Th2- associated chemokines</u></b>								
<b>CCL17</b>	412*	615*	230*	402*	129**	375**	95*	211*
<b>pg/ml</b>	(227-660)	(463-973)	(129-376)	(232-662)	(47-232)	(199-450)	(31-143)	(72-380)
<b>CCL18</b>	28	38	105*	172*	130**	231**	153*	183*
<b>ng/ml</b>	(22-37)	(26-62)	(76-158)	(97-208)	(96-188)	(142-303)	(96-191)	(142-232)
<b>CCL22</b>	535	535	458*	776*	279**	409**	240	321
<b>pg/ml</b>	(338-789)	(360-1104)	(328-732)	(559-1237)	(195-350)	(333-759)	(146-304)	(153-425)

\*p<0.05. \*\* p<0.01 with Mann-Whitney U-test.



**Figure 2.** The levels of CCL22 (a) and CXCL11 (b and c) in peripheral blood at birth, 6, 12 and 24 months in infants with (closed bars) and without (open bars) sensitization (a), a positive skin prick test (b) or IgE-associated disease (c) until 2 years of age, including both probiotic- and placebo-treated children. \*P<0.05, \*\*P<0.01.

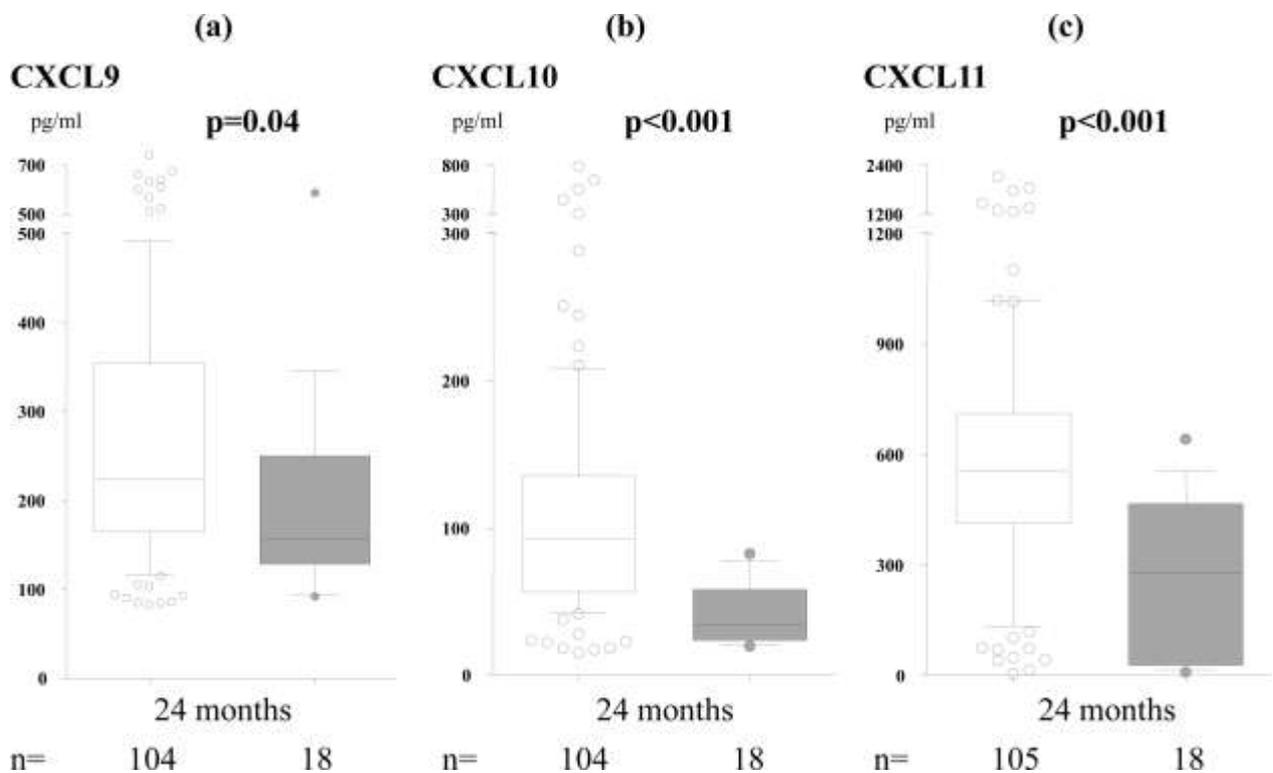
The development of IgE-associated allergic disease was associated with low levels of CXCL11 ( $p=0.003$  with repeated-measures ANOVA), significantly so already at birth (Fig. 2c), while CCL17 and CCL22 levels were significantly higher only at 12 months of age (data not shown,  $p=0.01$  and  $p=0.003$ , respectively). Furthermore, eczematous infants with, as compared with without, sensitization had low CXCL11 and high CCL22 in cord blood (data not shown,  $p=0.02$  and  $p=0.03$ , respectively). The Th2/Th1 ratio (CCL22/CXCL10) was also high already at birth in infants developing sensitization and IgE-associated eczema (Fig. 1b and c).

#### *Nutrition and environmental factors*

Infants who were still breastfed at 6 months had a lower incidence of SPT positivity than infants who were not (21% vs. 38%,  $p=0.049$ ). Yet, breastfeeding at 6 months was related with subsequent elevated CCL22 levels ( $p=0.02$  with repeated-measures ANOVA for 6, 12

and 24 months, data not shown). Day-care attendance was associated with reduced SPT reactivity (20% vs. 44%,  $p=0.003$ ) and IgE-associated eczema (19% vs. 39%,  $p=0.02$ ), but tended to be related to an increased incidence of recurrent wheeze (11% vs. 0%,  $p=0.05$ ). Furthermore, day-care attendance the second year of life was clearly associated with elevated levels of all Th1-associated chemokines (Fig 3). Infections were more common in infants attending day-care (5.7 vs. 4.3 times,  $p=0.03$ , t-test), but the number of infections and prescription of antibiotics did not correlate with the chemokine levels.

Adjustments with potential confounders possibly affecting the relationship between the chemokines and allergic manifestations or other factors such as treatment group, *L. reuteri* colonization, breastfeeding and day-care did not affect the result significantly with one exception: CXCL11 was not significantly lower at 24 months of age in SPT positive, as compared to negative infants after adjustment with day-care attendance.



**Figure 3.** Levels of CXCL9 (a), CXCL10 (b) and CXCL11 (c) in peripheral blood at 24 months in infants attending (open bars) and not attending (closed bars) day-care the second year of life. The 10th, 25th, 50th, 75th and 90th percentiles as well as outliers are indicated.

## Discussion

This prospective study revealed that an imbalance in circulating Th1- and Th2-associated chemokines may precede the onset of sensitization, eczema and recurrent wheeze from birth, implicating that these chemokines may sometimes be primarily involved in the pathogenesis of allergic diseases and not only secondary to a general immune deviation after disease onset. Th1 and Th2 cytokines are likely important upstream mediators of these effects, as they induce production of the respective chemokines. The subsequent delay in maturation of the immune system with a prolonged Th2-deviation in children developing allergic disease supports and extends previous reports [2, 3]. Our findings extend previous reports of elevated levels of Th2-associated chemokines in patients with eczema and recurrent wheeze [8, 9, 12, 13, 20] showing that elevated levels also precede clinical manifestations. Determination of circulating chemokine levels in epidemiological studies may be a tool for identification of factors associated with the development of sensitization or allergic disease.

Eczema did not associate significantly with any chemokine the second year of life, whereas recurrent wheeze was significantly associated with high CCL17 and CCL18 also at 24 months. Moreover, in contrast to eczema and sensitization, recurrent wheeze was related to elevated Th1-associated chemokines the second year of life, which is in concordance with previous reports. Elevated CXCL10 levels have been reported in BAL from asthmatic patients after allergen exposure [21]. Although CXCL10 favored Th1-like response in lymph nodes in a mouse model, it attracted Th2-cells and eosinophils locally in lung at a late stage of airway inflammation [22]. Thus, when the subject once has become asthmatic, Th1-inducing factors might aggravate the asthmatic inflammation [22, 23].

It has been suggested that CCL18, previously named pulmonary- and activation-regulated chemokine, has a key role in asthma [13], and it was strongly associated with recurrent wheeze in our material. It is preferentially expressed in the lung by antigen presenting cells and is induced by Th2-associated chemokines [13]. It is not strictly a Th2-associated chemokine, however, since its receptor is still not known, and its expression seems to be differently regulated than CCL17 and CCL22. Thus, while IL-4 and IL-13 induce CCL17, CCL22 and CCL18, the anti-inflammatory cytokine IL-10 enhances CCL18 expression and reduces CCL17 and CCL22 secretion [24-26]. In contrast to the other Th2-associated chemokines, the CCL18 levels increased during infancy in our study and were also associated to non-sensitized recurrent wheeze.

The Th2-associated chemokine CCL17 was elevated already at birth in infants who subsequently developed recurrent wheeze. Recently, it was suggested that DCs are the primary source of CCL17 and CCL22 in the lung, and that CCL17 and CCL22 are critical for the recruitment of Th2 cells into the airways during allergic inflammation [27]. Furthermore, the CCR4 receptor is required for efficient entry of both iNKT [28] and antigen-specific Th2 cells [29] into the lung in murine models of pulmonary allergic inflammation. Thus, infants developing recurrent wheeze may be predisposed for pulmonary allergic inflammation already from birth.

Interestingly, only two of the chemokines were related to sensitization, CCL22 and CXCL11. Although CCL22 share the same Th2-associated receptor, CCR4, with CCL17, there are differences in the regulation of the expression of these chemokines. While CCL22 is not produced by endothelium, CCL17 is expressed on dermal vascular endothelial cells in inflamed skin [30, 31]. Circulating CCL17 levels may therefore relate more strongly to skin

symptoms, which was supported by our result. In contrast, as dendritic cells produce higher levels of CCL22 than CCL17 [32], particularly after CD40L ligation [33], it is tempting to speculate that CCL22 may be involved in the allergen sensitization process. Also, we hypothesize that the higher CXCR3 affinity and potency of CXCL11 compared with CXCL9 and CXCL10 [34] may possibly explain why reduced neonatal levels of this chemokine were related to sensitization. The fact, that subsequent sensitization was related to reduced CXCL11 and elevated CCL22 levels in cord blood, reflecting the *in utero* condition, supports the theory that sensitization might be associated with immune programming during the fetal period [35, 36].

Although the presence of *L. reuteri* in infant stool the first week of life was associated with low CCL17 and CCL22 and high CXCL11 levels at 6 months, no differences in chemokine levels were revealed between the *L. reuteri* and placebo treated group. Therefore the previously reported effect on IgE-associated eczema by *L. reuteri* [17] seems to be primarily mediated by another mechanism. *Lactobacillus reuteri* has been associated with induction of anti-inflammatory cytokines and T regulatory cells in human *in vitro* and animal models [37] [38].

This study confirms that humans are born Th2-skewed, as found in most [39-41] but not all [42] studies, and gradually develop a Th1/Th2-balance. This process is stimulated by environmental factors, such as exposure to commensal microflora [43]. Attendance to day-care was the most Th1-stimulating factor in this study. Accordingly, this was also related to a lower incidence of sensitization and IgE-associated eczema, which is consistent with previous reports [44]. The lack of correlation between infection rate and the Th1-associated chemokines suggests that the effect of day-care most likely is mediated by enhanced exposure

to commensal microbiota [45]. Besides day-care attendance, CXCL9 was not associated with any other variable in our study. Thus, CXCL9 may not be interesting in *in vivo* studies such as this one, speculatively due to its having the lowest CXCR3-affinity of the Th1-associated chemokines [34]. Only infants with a family history of allergic disease were included in the original study, which might have influenced some results, but probably not the significant differences between infants developing allergic disease or not. In an independent cohort where children both with and without allergic heredity were included, elevated cord blood CCL22 levels preceded development of sensitisation and elevated CCL17 levels were associated with development of allergic symptoms, further supporting our findings [6].

In conclusion, the Th2-associated chemokines CCL17 and CCL22 were as highest at birth and then decreased, whereas CCL18 and the Th1-associated chemokines CXCL10 and CXCL11 increased with age. Allergic disease in infancy was associated with low circulating Th1- and high Th2-associated chemokine levels in infancy. Sensitization was preceded by low levels of CXCL11 and high levels of CCL22 already at birth. Of clinical relevance, circulating chemokines are useful for investigating the Th1/Th2 imbalance in allergic disease *in vivo*. Elucidation of the role of chemokines in allergic diseases may lead to future treatments.

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**Supplementary Table 1.** Correlation between the chemokines in cord blood and peripheral blood at 6, 12 and 24 months of age.

		<b>Cord blood</b>					
		<b>CCL17</b>	<b>CCL22</b>	<b>CXCL10</b>	<b>CXCL11</b>		
<b>6 months</b>	<b>CCL17</b>	rho	<b>X</b>	0.39	0.13	0.12	<b>CCL17</b>
		p		<0.001	ns	ns	
	<b>CCL22</b>	rho	0.25	<b>X</b>	-0.09	-0.22	<b>CCL22</b>
		p	0.04		ns	0.01	
	<b>CXCL10</b>	rho	-0.36	-0.21	<b>X</b>	0.37	<b>CXCL10</b>
		p	0.001	0.04		<0.001	
	<b>CXCL11</b>	rho	-0.46	-0.26	0.73	<b>X</b>	<b>CXCL11</b>
		p	<0.001	0.04	<0.001		
		<b>CCL17</b>	<b>CCL22</b>	<b>CXCL10</b>	<b>CXCL11</b>		
<b>6 months</b>							
		<b>12 months</b>					
		<b>CCL17</b>	<b>CCL22</b>	<b>CXCL10</b>	<b>CXCL11</b>		
<b>24 months</b>	<b>CCL17</b>	rho	<b>X</b>	0.39	-0.19	-0.30	<b>CCL17</b>
		p		<0.001	0.04	0.001	
	<b>CCL22</b>	rho	0.45	<b>X</b>	-0.36	-0.42	<b>CCL22</b>
		p	<0.001		<0.001	<0.001	
	<b>CXCL10</b>	rho	-0.03	-0.24	<b>X</b>	0.70	<b>CXCL10</b>
		p	ns	0.008		<0.001	
	<b>CXCL11</b>	rho	0.01	-0.25	0.60	<b>X</b>	<b>CXCL11</b>
		p	ns	0.006	<0.001		
		<b>CCL17</b>	<b>CCL22</b>	<b>CXCL10</b>	<b>CXCL11</b>		
<b>24 months</b>							