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Citation for published version (APA):

Snijders, B. E., Damoiseaux, J. G. M. C., Penders, J., Kummeling, I., Stelma, F. F., Van Ree, R., van den Brandt, P. A., & Thijs, C. (2006). Cytokines and soluble CD14 in breast milk in relation with atopic manifestations in mother and infant (KOALA Study). *Clinical and Experimental Allergy*, 36(12), 1609-1615. <https://doi.org/10.1111/j.1365-2222.2006.02613.x>

Document status and date:

Published: 01/01/2006

DOI:

[10.1111/j.1365-2222.2006.02613.x](https://doi.org/10.1111/j.1365-2222.2006.02613.x)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

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Cytokines and soluble CD14 in breast milk in relation with atopic manifestations in mother and infant (KOALA Study)

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Clinical and Experimental Allergy

Summary

Background Conflicting evidence exists concerning the protective role of breastfeeding in allergy and atopic disease aetiology. Breast milk contains biologically active molecules influencing the innate immune system of newborns.

Objective We aim to assess whether cytokines (TGF- β 1, IL-10 and IL-12) and soluble CD14 (sCD14) in breast milk are influenced by maternal atopic constitution and modify the development of atopic manifestations in infants.

Methods Milk samples were collected at 1 month post-partum of 315 lactating mothers participating in the ongoing KOALA Birth Cohort Study. The cytokines and sCD14 were analysed by ELISA in the aqueous fraction. We compared the concentrations of cytokines and sCD14 in breast milk between mothers with and without an allergic history and also with and without allergic sensitization (specific IgE). Associations of cytokines and sCD14 with the development of eczema, wheezing in the first 2 years of life and allergic sensitization of infants at the age of 2 years were analysed by multivariate logistic regression analyses to correct for confounders.

Results We found higher sCD14 levels in mothers with a positive vs. negative allergic history (7.6 vs. 7.0 $\mu\text{g}/\text{mL}$; $P = 0.04$) and in mothers who were sensitized vs. non-sensitized (7.8 vs. 7.1 $\mu\text{g}/\text{mL}$; $P = 0.03$). None of the studied immune factors were associated with infant's atopic outcomes. IL-10 was not detected above the detection limit of 0.2 pg/mL .

Conclusion Taking together the results of the present and previous studies, we conclude that there is no convincing evidence for a relation between TGF- β 1, sCD14, IL-10 or IL-12 in breast milk and atopic manifestations in infants.

Keywords cytokines, eczema, human milk, immunoglobulin E, soluble CD14

Submitted 23 June 2006; revised 4 August 2006; accepted 20 September 2006

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Introduction

Atopic diseases such as atopic eczema, allergic rhinitis and allergic asthma have increased in the developed world during recent decades [1]. Breastfeeding has been considered to afford protection against atopic disorders. Protection may be conveyed by biologically active molecules that are present in human milk such as cytokines, chemokines, hormones, growth factors, secretory antibodies and essential fatty acids [2, 3]. Through several mechanisms, immune-modulating factors in human milk can actively stimulate the newborn's immune system, which is rapidly maturing during the first years of life [4]. In the current

study, we focus on four immune factors [TGF- β 1, IL-10, IL-12 and soluble CD14 (sCD14)] that may influence the innate immune system.

TGF- β 1 and IL-10 are anti-inflammatory cytokines produced by several cell types, including regulatory T cells, which are currently in the focus of allergy and asthma research [5]. Regulatory T cells suppress both T-helper type-1 (Th1)- and Th2-mediated immune responses [6] and are thought to play a role in the development of allergic disease [7]. Especially, TGF- β 1 is an abundant cytokine in human milk [8]. Oddy et al. [9] demonstrated a positive association between low concentrations of TGF- β 1 in human milk and infant wheezing.

Human milk also contains IL-12 and the soluble form of CD14 (sCD14). Both are lipopolysaccharide (LPS) [10]-associated immune factors that promote Th1 development, thereby preventing excessive IgE production and allergic inflammation [11]. CD14 is an innate immune receptor for LPS, a component of gram-negative bacterial cell walls [12]. The complex interplay between CD14, toll-like receptor (TLR) 4 and MD-2 is responsible for recognition of LPS [13]. Subsequently, intracellular signalling results in secretion of IL-12. An association was found between reduced sCD14 levels in breast milk and a diagnosis of eczema in children aged 6 months [14]. Recently, increased levels of sCD14 were found to be associated with a lower incidence of doctor's diagnosed asthma at the age of 2 years, especially in children of mothers without a history of atopic diseases [15]. Also, a deficit of IL-12 production was proposed to occur in atopic individuals [11].

There are two possible explanations for the relation between levels of immune factors in breast milk and the development of atopy in the infant: first, maternal atopic constitution (genetic or acquired) determines levels of cytokines in breast milk and infant's atopy by separate mechanisms; second, immune factors have a direct effect on mucosal immunity in the child.

This study had two objectives. First, we aimed to investigate whether the mother's atopic constitution influences milk levels of TGF- β 1, IL-10, IL-12 and sCD14. Second, we examined whether these immune factors are associated with the development of infant's atopy (eczema, wheezing) in the first 2 years of life and allergic sensitization at age 2.

Methods

Design

This study is part of the KOALA Birth Cohort Study ($N=2834$), an ongoing prospective birth cohort study in the Netherlands. The design of the study has been described, in detail, elsewhere [16]. Briefly, we recruited participants with diverse lifestyles (conventional and alternative) at 34 weeks of gestation. Pregnant women with a conventional lifestyle ($n=2343$) were recruited from an ongoing prospective cohort study on pregnancy-related pelvic girdle pain in the Netherlands. Additionally, pregnant women with an alternative lifestyle ($n=491$) were recruited through several 'alternative' channels: organic food shops, anthroposophic doctors and midwives, Steiner Schools and magazines. During the first 2 years post-partum, information on breastfeeding, atopic outcomes and their determinants was collected for all members of the cohort by repeated questionnaires at 3, 7, 12 and 24 months. During home visits at 34–36 weeks of gestation, maternal venous blood was collected. From

October 2002, we started recruiting participants for breast milk sampling by asking pregnant mothers who intended to start breastfeeding to consent for breast milk collection. This resulted in breast milk being sampled in 315 breastfeeding mothers between December 2002 and September 2003, including a short questionnaire at the time of breast milk collection (1 month post-partum). The study (including biosampling) was approved by the medical ethics committee of the Maastricht University.

Collection and processing of breast milk

Mothers received a sterile 50 mL tube (Cellstar PP-test tubes, Greiner bio-one, Kremsmünster, Austria) and were instructed to collect the milk sample in the morning, before breastfeeding their child, from the contra-lateral breast (since the last feeding) and to keep the tube in the refrigerator ($\pm 4^\circ\text{C}$) until it was collected by one of the researchers. If the mother was not able to collect the milk sample by herself (with or without a pumping regimen), an electric breast pump (Medela, Baar, Switzerland) was used with the help of one of the researchers (within the same day). During transport, the milk samples were stored in a cooler (Coleman Company Inc., Breda, the Netherlands) on packed ice ($\pm 4^\circ\text{C}$) until processing on the same day. The sample was centrifuged (400 g, 12 min, no brake, 4°C) to separate the lipid and aqueous fraction. The lipid layer was trimmed off with a pipette and released in plastic storage vials (Sarstedt, Nümbrecht, Germany). The aqueous fraction was poured in other vials with another pipette. The remaining debris was not used to avoid contamination with cell fragments. All fractions were stored at -80°C in the European Biobank, Maastricht.

Enzyme linked immunosorbent assays in breast milk

Quantitative colorimetric cytokine ELISA kits were used to assess the concentrations of TGF- β 1, sCD14 (R&D Systems Europe Ltd., Abingdon, UK), IL-10 and IL-12 (Biosource Int., Camarillo, CA, USA) in human aqueous milk fractions according to the manufacturer's instructions. The aqueous milk fractions were assayed at dilution factors of 1.4 for TGF- β 1 and 2000 for sCD14. The minimum detectable dose of TGF- β 1 and sCD14 was 7 pg/mL and 125 pg/mL, respectively. For the activation of latent TGF- β 1 to the active form, a standard activation procedure was used. Briefly, 0.1 mL 1 M HCl was added to 0.5 mL aqueous milk fraction and subsequently mixed to incubate for 10 min at room temperature. Neutralization of the acidified milk sample was performed by adding 0.1 mL 1.2 M NaOH/0.5 M 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid. Undiluted aqueous milk fractions were used to determine the IL-10 and IL-12 concentrations. The minimum detectable dose was 0.2 pg/mL for IL-10 and 0.8 pg/mL for IL-12. All ELISA assays were performed in duplicate. We conducted

a pilot study for all parameters. We could not detect IL-10 concentrations in our pilot study ($n = 16$).

Determination of maternal and infant's total and specific Immunoglobulin E

Maternal venous blood samples were obtained during a home visit at 34–36 weeks of gestation. Serum samples were analysed for total IgE levels as described earlier [17, 18]. For values < 150 IU/mL a sandwich RIA was used [17], and for values > 150 IU/mL a competitive RIA was used [18]. Maternal venous blood samples were analysed for specific IgE against 13 common respiratory and food allergens. Calculation was performed by means of a standard curve that was obtained by RAST with a dilution series of a chimeric monoclonal IgE antibody against the major allergen Der p 2 and Sepharose-coupled recombinant Der p 2 [19].

In order to collect venous blood of the infants, home visits were made at 2 years post-partum. Infants' venous blood samples were analysed for specific IgE against hen's eggs, cow's milk, peanuts, birch, grass pollen, cat, dog and mite using RAST as described earlier [18]. The detection limit for total and specific IgE was 0.50 and 0.10 IU/mL, respectively.

Definition of maternal allergic history and maternal sensitization

A positive maternal allergic history was defined if, in her self-reported questionnaire, the mother positively confirmed that a physician had at least once diagnosed asthma, eczema, allergy for house dust mite/pets or allergic rhinoconjunctivitis (such as hayfever).

Maternal sensitization was considered as present if serum-specific IgE against one or more of the tested inhalant or food allergens was positive (> 0.3 IU/mL). High total IgE for mothers was arbitrarily defined as total IgE level > 100 IU/mL.

Definition of infant's atopic manifestations and infant's allergic sensitization

Information on the development of eczema (based on ISAAC questions) was obtained in the 3-, 7- and 12-month questionnaires. Parents were asked; 'has your child ever had an itchy rash that was coming and going in the past months'? If this question was answered affirmatively, infants were defined as having developed eczema in the first 2 years of life. Cases of only diaper rash, rash around the eyes and/or scalp scaling were excluded. 'Ever wheezing' was defined as parentally reported presence of wheezing, with at least one attack, in the 7-, 12- or 24-month post-partum questionnaires. Infants were considered to be sensitized if specific serum IgE-levels were

> 0.3 IU/mL against one or more of the tested food or inhalant allergens. High total IgE for infants was arbitrarily defined as total IgE level > 10 IU/mL.

Atopic dermatitis by UK Working Party criteria

To specify eczema reported by parents as described above, we defined atopic dermatitis according to UK Working Party criteria [20] for all infants who were visited at home at age 2 years. The probability of the presence of atopic dermatitis was derived from the presence of four clinical symptoms: (1) presence of itchy rash (PIR, coded as 0 = absent, 1 = present), (2) history of flexural dermatitis (HFD, 0 = absent, 1 = present), (3) visible flexural dermatitis (VFD, 0 = absent, 1 = present) and (4) onset before age of 2 years (OB2, 0 = absent, 1 = present). The UK working party (UK-WP) probability score of atopic dermatitis (AD) is then computed as: probability AD = odds (AD)/[odds (AD)+1], where odds (AD) = $\exp[-4.36 + 1.84(\text{HFD}) + 3.46(\text{OB2}) + 2.09(\text{VFD}) + 1.71(\text{PIR})]$ [20]. In this study, infants with a UK-WP probability score of atopic dermatitis (AD) > 0.9 were regarded as infants with 'probable presence of atopic dermatitis'.

Statistical analysis

Extreme values of concentrations of cytokines and sCD14 were not excluded as these did not influence our results. The mean values of concentrations of cytokines and sCD14 were compared between groups using analysis of variance (ANOVA). To assess the association between cytokines and infant's atopic manifestations, we conducted a multivariate logistic regression analysis in order to adjust for possible confounders ('recruitment group' (conventional vs. alternative), maternal age (years), maternal allergic history (yes/no), number of older siblings (no siblings, one, two or more), season of breast milk collection (winter 2002–2003, spring 2003, summer 2003), use of probiotics in capsules or yoghurts at 1 month post-partum (yes/no), maternal infection in week of breast milk collection (yes; that is, vomiting, diarrhoea, having a cold, sore throat, mastitis, fever or urinary tract infection), time-interval between birth and breast milk collection (in days) and total storage time in freezer until analysis (in days)). Based on sample sizes of previous studies [9, 14], we estimated that 300 breast milk samples were sufficient to detect differences of interest. We consider P -values ≤ 0.05 as statistically significant. All analyses are presented for both recruitment groups (conventional and alternative cohort) together, because stratified analyses showed similar results for both groups.

Results

A total of 315 mothers donated a breast milk sample at 1 month post-partum. This subcohort consisted of 60% of

breastfeeding mothers from the KOALA study who were visited for venous blood collection at 34–36 weeks of gestation (from October 2002 onwards). The main reasons for not donating breast milk were unsuccessful breastfeeding or logistic reasons. The characteristics of these mothers and their infants are summarized in Table 1. We could determine TGF- β 1 and sCD14 in 307 (98%) breast milk samples, whereas IL-12 was determined in 293 (93%) samples. No data of IL-10 in breast milk are presented as we did not detect IL-10 above the detection limit of 0.2 pg/mL.

We found no difference in the mean concentrations of TGF- β 1 in breast milk between mothers with or without an allergic history (Table 2). Also, the mean concentrations of TGF- β 1 in breast milk were not different between mothers with and without allergic sensitization ($P=0.15$; Table 2) or between mothers with or without high levels of total IgE (not shown). For sCD14, we found a somewhat higher concentration in breast milk in mothers with an allergic history compared with mothers without (7.6 vs. 7.0 μ g/mL; $P=0.04$) (Table 2). Also, we found a higher concentration of sCD14 in mothers who were sensitized compared with non-sensitized mothers (7.8 vs. 7.1 μ g/mL, $P=0.03$; Table 2). The levels of sCD14 were similar in

Table 1. Characteristics of participants (with conventional versus alternative lifestyle) with breast milk sample ($N=315$)

	Conventional $N=146$	Alternative $N=169$
Maternal age in years (mean \pm SD)	32.4 \pm 3.3	34.0 \pm 4.2
Breastfeeding duration in months (mean \pm SD)*	4.2 \pm 3.0	6.5 \pm 3.1
Sex of infant (no. of boys, percentage)	71 (48.6%)	83 (49.1%)
Infant's atopic outcome, at age 2 years		
Eczema by questionnaire (yes, percentage)	42 (28.8%)	51 (30.2%)
Atopic dermatitis by UK-WP (yes, percentage)	16 (11.0%)	15 (8.9%)
Wheeze (yes, percentage)	41 (28.1%)	42 (24.9%)
Both eczema + wheeze (yes, percentage)	11 (7.5%)	12 (7.1%)
Cytokines and sCD14 in breast milk		
TGF- β 1 in pg/mL (mean \pm SD) $N=307^\dagger$	225.6 \pm 178.3	212.5 \pm 117.8
Range TGF- β 1 in pg/mL	12.0–1536.8	2.0–647.9
IL-10 in pg/mL	ND ‡	ND ‡
sCD14 in μ g/mL (mean \pm SD) $N=307^\dagger$	7.7 \pm 2.9	7.1 \pm 2.2
Range sCD14 in μ g/mL	1.7–23.8	2.3–20.1
IL-12 in pg/mL (mean \pm SD) $N=293^\dagger$	10.6 \pm 6.5	10.8 \pm 6.0
Range IL-12 in pg/mL	0.7–52.8	1.5–51.6

*Exclusive and non-exclusive breastfeeding.

† Missing due to insufficient aqueous fraction available.

‡ Not detectable i.e. under the detection limit of 0.2 pg/mL.

Table 2. Mean concentrations of TGF- β 1, sCD14 and IL-12 in breast milk related to maternal allergic history and specific serum IgE

	TGF- β 1 (pg/mL)		sCD14 (μ g/mL)		IL-12 (pg/mL)	
	n	Mean (SD)	n	Mean (SD)	n	Mean (SD)
Maternal allergic history						
No	125	221.1 (140.1)	124	7.0 (2.4)	120	10.3 (6.2)
Yes	182	216.9 (155.1)	183	7.6 (2.6)	173	11.0 (6.3)
P^\ddagger		0.81		0.04		0.35
Maternal sensitization						
No sensitization*	164	207.9 (127.3)	164	7.1 (2.8)	164	10.4 (5.2)
Sensitization	123	238.1 (180.2)	123	7.8 (2.4)	123	11.1 (7.4)
P^\ddagger		0.15		0.03		0.35

SD, standard deviation.

*Specific IgE > 0.3 IU/mL for any of the 13 measured food or inhalant allergens (missing $n=6$).

‡ P value based on ANOVA.

mothers with high vs. low total IgE levels (7.3 vs. 7.2 μ g/mL; $P=0.84$; results not shown in table). Finally, we did not detect differences in the mean concentrations of IL-12 in breast milk between mothers with and without an allergic history or between sensitized and non-sensitized mothers (Table 2), nor between mothers with and without high levels of total IgE (not shown).

The results for the relationships between the concentrations of breast milk immune factors (in tertiles) and the infant's atopic outcome are presented in Table 3. The risk of infant's eczema (by questionnaires) and wheeze was not associated with breast milk levels of TGF- β 1, sCD14 and IL-12 (odds ratios show no trend over the tertiles, and 95% confidence intervals include the value 1.0). Also, no association was present between the presence of allergic sensitization and TGF- β 1, sCD14 and IL-12 (Table 3). Neither did we find an association between high total serum IgE (> 10 IU/mL) or atopic dermatitis by UK-WP criteria and TGF- β 1, sCD14 and IL-12 (results not shown).

Discussion

In the present study, we found higher levels of sCD14 in mothers with a maternal allergic history and sensitization. No such associations between TGF- β 1 and IL-12 in breast milk and maternal allergic status were found. None of the studied immune factors (TGF- β 1, sCD14 and IL-12) in breast milk were associated with infant's atopic outcomes.

Our first aim was to assess whether milk levels of TGF- β 1, IL-10, IL-12 and sCD14 reflect the mother's allergic history. A few studies have compared the concentrations of immune factors in breast milk between atopic and non-atopic mothers. In a small study, Rigotti et al. [21] found that TGF- β 1 was significantly less in the mature milk of allergic mothers. In agreement with the present study,

Table 3. Adjusted odds ratios for the presence of eczema and wheezing in the first 2 years of life and allergic sensitization at age 2 years, depending on the concentrations of TGF- β 1, sCD14 and IL-12 (in tertiles) in breast milk

	Infant's eczema (by questionnaires)		Infant's wheeze		Infant's sensitization	
	<i>n</i>	aOR (95% CI) [†]	<i>n</i>	aOR (95% CI) [†]	<i>n</i>	aOR (95% CI) [†]
TGF-β1 (pg/mL)						
Low (2.0 – 166.9)	30	1.0 (reference)	24	1.0 (reference)	17	1.0 (reference)
Middle (166.9 – 248.4)	32	1.14 (0.59-2.10)	28	1.21 (0.61-2.93)	21	1.19 (0.52-2.74)
High (248.5 – 1536.8)	28	1.00 (0.53-1.91)	28	1.13 (0.57-2.23)	12	0.51 (0.21-1.24)
<i>P</i> for trend		1.00 (<i>N</i> = 299)		0.73 (<i>N</i> = 299)		0.13 (<i>N</i> = 200)
sCD14 (μg/mL)						
Low (1.7 – 6.3)	29	1.0 (reference)	25	1.0 (reference)	16	1.0 (reference)
Middle (6.4 – 8.0)	38	1.70 (0.90-3.23)	26	1.38 (0.69-2.77)	18	1.11 (0.49-2.54)
High (8.1 – 23.8)	26	1.00 (0.50-2.00)	32	1.85 (0.92-3.69)	17	1.06 (0.46-2.47)
<i>P</i> for trend		0.98 (<i>N</i> = 307)		0.08 (<i>N</i> = 307)		0.89 (<i>N</i> = 207)
IL-12 (pg/mL)						
Low (0.6 – 7.8)	24	1.0 (reference)	24	1.0 (reference)	18	1.0 (reference)
Middle (7.9 – 11.8)	35	1.60 (0.83-3.11)	29	1.35 (0.68-2.69)	13	0.77 (0.32-1.89)
High (11.9 – 52.8)	26	1.18 (0.59-2.38)	22	1.06 (0.51-2.21)	15	0.77 (0.33-1.83)
<i>P</i> for trend		0.61 (<i>N</i> = 285)		0.84 (<i>N</i> = 285)		0.54 (<i>N</i> = 190)

aOR, adjusted odds ratio.

*Sensitization is defined as specific serum IgE-levels > 0.3 IU/mL against one or more of the tested food or inhalant allergens.

[†]From logistic regression analysis, adjusting for: recruitment group (conventional versus alternative), maternal age, maternal allergic history, number of older siblings, season of breast milk collection, maternal use of probiotics, maternal infection (during week of breast milk collection), time interval between birth and breast milk collection (in days) and total storage time in freezer until analysis (in days).

Böttcher et al. [22] reported no differences for TGF- β . In agreement with our findings, a small study found no associations between IL-12 in breast milk and a maternal atopic status [23]. Our findings of higher levels of sCD14 in mothers with a maternal allergic history ($P=0.04$) or mothers with sensitization ($P=0.03$) have not been reported earlier. However, the results of a German study presented a slightly higher prevalence of maternal atopic history in the upper quartile of breast milk sCD14, directed towards borderline statistical significance ($P=0.07$) [15]. Savilahti et al. [24] found lower levels of sCD14 in the colostrum of atopic vs. non-atopic mothers when the definition of atopy was based on a single symptom but when a more rigid definition was used, the difference disappeared. For sCD14 levels in breast milk, our results tend towards a relation with both a maternal allergic history and maternal sensitization but not with high total IgE in the mother. We speculate that disease activity (at the moment of breast milk sampling) in atopic mothers may be reflected in higher sCD14 levels. Higher levels of serum sCD14 are seen in subjects with disease activity for instance after exacerbation of asthma in children [25, 26]. However, we did not assess the asthmatic status or the presence of rhinoconjunctivitis at the moment of breast milk sampling. Alternatively, atopic vs. non-atopic mothers could differ in genetic constitution, leading to different sCD14 levels. Guerra et al. [27] found that sCD14 levels in milk differed significantly by CD14/–1619 and CD14/–550 genotypes and haplotypes. The biologic

relevance for the infant of such small differences in sCD14 concentrations between atopic and non-atopic mothers can be argued.

Our second aim was to assess whether TGF- β 1, IL-10, IL-12 and sCD14 levels in breast milk were associated with the development of infant's atopic manifestations. We consider infant's eczema (by questionnaire, adapted from ISAAC) and sensitization as separate outcome parameters instead of atopic eczema because sensitized infants do not necessarily show symptoms of eczema or vice versa [28]. Consequently, we use the term eczema instead of atopic eczema. However, we use the term 'atopic dermatitis' for infants fulfilling the UK-WP criteria according to the criteria of Williams et al. [20]. If we used more stringent criteria for wheeze such as proposed by others [29], the number of cases was too small for a multivariate analysis.

We found no associations between TGF- β 1, sCD14 and IL-12 and infants' atopic outcomes. The Infant Immune Study (Tucson, Arizona) found a statistically significant association between low levels of TGF- β 1 and wheeze at the age of 1 year [9]. Furthermore, the British study of Jones et al. [14] found that low levels of breast milk sCD14 were associated with a diagnosis of eczema in 6-month-old infants, irrespective of skin prick testing, in a high-risk cohort. More recently, Rothenbacher et al. [15] found a lower risk for atopic eczema for breastfed infants (≥ 6 months, sCD14 in the highest quartile) vs. less than 3 months' breastfeeding. They suggested that the beneficial

effects of breastfeeding are supported by high levels of sCD14. By contrast, Laitinen *et al.* found that sCD14 tended to be higher in mature breast milk received by infants with vs. without atopic eczema with a positive skin prick test. Previous studies have investigated IL-10 [9, 22, 30–32], IL-12 [23, 33, 34] or TGF- β 1 [8, 9, 22, 24, 32, 35, 36] concentrations in breast milk with inconsistent results with respect to the mothers and/or infant's atopic outcomes. We speculate that three explanations may clarify these inconsistencies. First, differences in methodologies could be an explanation, e.g. the moment of breast milk sampling. It can be anticipated that cytokine concentrations are higher in colostrum than mature milk. This is not supported by a study with longitudinal human milk collection, reporting a slight change in the mean concentration of both TGF- β 1 and TGF- β 2 during the first 12 weeks of lactation, including colostrum [36]. However, this may not necessarily apply for sCD14 and IL-12. Second, the effects caused by colostrum factors may no longer be valid at the infant's age of 1 month because the permeability of the gut is diminishing and the efficiency of proteolysis is enhanced [37]. In addition, Blais *et al.* [38] found that sCD14 in breast milk is more susceptible to the infant's pancreatin digestion vs. pepsin digestion by *in vitro* experiments, suggesting decreased activity of sCD14 in the LPS-rich environment of the distal bowel. Third, the use of different ELISA kits may explain inconsistent results between studies [23]. It was noted in two studies that the use of ELISA assays of R&D Systems did not detect IL-12 in breast milk [33, 34]. We were also unable to detect IL-12 in breast milk with that same ELISA. However, using a high-sensitivity kit (Biosource Int.), we detected IL-12 in our breast milk samples. Previously, IL-12 was also found in breast milk samples in a study using ELISA assays of Pharmingen (San Diego, CA, USA) [23].

A limitation of our study was a restriction of complete standardization of breast milk collection. Different modes of pumping and individual variation in pumping techniques could have led to variation of the measured immune factors. The strength of this study was the number of breast milk samples available as only a few studies measured sCD14 or cytokines in a large amount of breast milk samples.

In summary, we found higher concentrations of sCD14 in breast milk in mothers with a positive allergic history and mothers with allergic sensitization but not for high total IgE. Taking together the results of the present study and other studies, we conclude that there may be no consistent effect on inter-individual differences of TGF- β 1, sCD14 and IL-12 in breast milk and the infant's atopic outcomes. Therefore, the search for the modifying effects of factors present in breast milk on the development of atopic manifestations should continue.

Acknowledgements

This study was supported by a grant from the Netherlands Organisation for Health Research and Development (Zon-Mw), programme of Innovative Prevention Research (Prevention Program 1, no. 210-00-090).

We thank Nicole Custers (assistance in breast milk collection), Chantal Delnoy/Brigitte Winants/Karen Groot (blood collection), Cobie Martens/Willeke Hendriks (assistance data collection questionnaires), Astrid van Leeuwen (Sanquin, IgE determination), Mia Vaessen (assistance in ELISA) and all mothers and their infants participating in the KOALA study.

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