High cord blood levels of the T-helper 2-associated chemokines CCL17 and CCL22 precede allergy development during the first 6 years of life

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High cord blood levels of the Th2-associated chemokines CCL17 and CCL22 precede allergy development during the first 6 years of life

Running title: Cord blood chemokines and allergy

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Abstract

Exposure to a strong T-helper 2 (Th2)-like environment during foetal development may promote allergy development. Increased cord blood (CB) levels of the Th2-associated chemokine CCL22 were associated with allergy development during the first 2 years of life. The aim of the present study was to determine if CB Th1- and Th2-associated chemokine levels are associated with allergy development during the first 6 years of life, allowing assessment of respiratory allergic symptoms usually developing in this period. The CB levels of cytokines, chemokines and total IgE were determined in 56 children of 20 women with and 36 women without allergic symptoms. Total and allergen specific IgE antibody levels were quantified at 6, 12, 24 months and 6 years of age. Increased CB CCL22 levels were associated with development of allergic sensitization and asthma and increased CCL17 levels with development of allergic symptoms, including asthma. Sensitized children with allergic symptoms showed higher CB CCL17 and CCL22 levels and higher ratios between these Th2-associated chemokines and the Th1-associated chemokine CXCL10 than non-sensitized children without allergic symptoms. A pronounced Th2 deviation at birth, reflected by increased CB CCL17 and CCL22 levels, and increased CCL22/CXCL10 and CCL17/CXCL10 ratios might promote allergy development later in life.

Keywords: allergy, CCL17, CCL22, chemokines, cord blood

Abbreviations

AD: Atopic dermatitis

ARC: Allergic rhinoconjuntivitis

CB: Cord blood

SPT: Skin prick tests

Th: T-helper

Introduction

Maternal allergy may be a more significant risk factor for development of allergic diseases in the offspring than paternal allergy(1, 2). The immunological mechanisms behind this phenomenon are unknown, but indicate an impact of the maternal immunity on allergy development, besides the contribution of the genes. The maternal immunity during pregnancy and lactation might influence the neonatal immune development, and the T-helper 2 (Th2)-biased immunity of allergic mothers could possibly modulate the immune responses in their offsprings, to an IgE favouring, Th2-like phenotype. In line with this, several studies have reported higher cord blood (CB) IgE levels in children of allergic mothers as compared to children with paternal or no allergic history(1, 3, 4).

The discrepant immune response to allergens at birth, observed in children who develop allergic diseases later in life, might be related to exposure to a strong Th2 environment during gestation. For example, a decreased production of allergen-induced IFN-γ by cord blood mononuclear cells (CBMC:s) is associated with allergy development(5, 6). Furthermore, the Th1/Th2 balance *in vivo,* has shown to be Th2-biased at birth in children who develop allergic disease later in life(7, 8). Increased CB plasma levels of CCL22 were associated with questionnaire-reported wheezing during infancy(7) and development of sensitization and allergic disease during the first 2 years of life(8). Atopic dermatitis (AD) was the predominant symptom of allergic disease during the first 2 years of life in this cohort while the time period between 2 and 6 years of age allows other allergic symptoms, such as asthma and allergic rhinoconjuntivitis (ARC), to develop. Thus, it might not be sufficient to follow the study participants during early infancy only, when searching for predictive factors in cord blood.

Elevated serum levels of the IL-4 and IL-13 induced chemokines CCL11, CCL17, CCL18 and CCL22(9-12) have previously been associated with allergic manifestations, in particular atopic dermatitis(13-15). The amplification of the allergic response is partly driven by CCL17 and CCL22 as they attract CCR4 receptor expressing Th2 lymphocytes, mast cells, dendritic cells and natural killer T (NKT) lymphocytes to the site of inflammation(16). CCL11 binds selectively to the CCR3 receptor, which is expressed on Th2 lymphocytes, mast cells, basophils and eosinophils(16). CCL18 binds to T lymphocytes(17), but its receptor is not yet known. The IFN-γ induced chemokines CXCL10 and CXCL11(18, 19) on the other hand, bind the CXCR3 receptor expressed on the surface of Th1 lymphocytes, NKT and mast cells(16). Accordingly, CXCL10 and CXCL11 have been associated with Th1-like diseases like sarcoidosis(20) and Crohn´s disease(21).

Although chemokines have been used as markers for Th1/Th2 immunity in immune-mediated disorders such as allergic disease, little is known about the predictive value of circulating chemokines, before disease onset. Established allergic disease is characterized by a Th2 dominant immunity, but the timing of the development of this Th2 skewing is not known. As this Th2 skewing preceding allergic disease is believed to develop in very early life, we aimed to investigate whether Th1- and Th2-associated cytokine and chemokine levels at birth, could serve as markers for future allergy development. To address this question, CB concentrations of the cytokines IL-4, IL-5, IL-9, IL-10, IL-12(p70), IL-13, IFN-γ and the chemokines CXCL10, CXCL11, CCL11, CCL17, CCL18, and CCL22 were analysed in relation to allergy development during the first 6 years of life.

Methods

Study group

56 children of 20 women with allergic symptoms and 36 women without allergic symptoms were included in the study (Fig 1). Due to practical reasons, it was not possible to perform this study, with additional detailed follow-up of the mothers during pregnancy, with a larger number of participants. An experienced allergy research nurse interviewed the mothers regarding their allergic status. Seventeen mothers had allergic rhinoconjuntivitis (ARC), 4 had asthma (of whom 1 also had ARC) and 2 had AD (both of them also had ARC).
Umbilical CB (n=46) was collected at birth and the plasma and serum samples were frozen and stored at -20°C. Maternal and neonatal characteristics are described in detail elsewhere(22).

The children were followed with questionnaires at 3, 6, 12, 18, 24 months and 6 years of age regarding environmental factors and allergic symptoms in the children. At 6 and 12 months of age, a medical examination was performed by an experienced allergy research nurse and at 24 months and 6 years by a paediatric allergologist. Blood samples were collected at the time of the clinical examinations. The plasma samples were frozen and stored at -20°C.

Three children did not attend the clinical examinations. All of the other children (n=53) attended the 6 and 12 months examinations, 47 children to the 24 month examination and 37 children to the 6 year. Nine children choose to participate with questionnaires only at the 6 year follow-up.

The diagnosis of AD was established using the criteria suggested by Hanifin and Rajka(23), i.e. pruritic, chronic or chronically relapsing non-infectious dermatitis with typical features

and distribution. Asthma, at 6 years of age, was defined as one or more episodes of bronchial obstruction after two years of age, at least once verified by a physician. At 2 years of age, asthma was defined as three or more episodes of bronchial obstruction since birth, at least once verified by a physician or two episodes of bronchial obstruction combined with **AD** or food allergy. Five children were diagnosed with asthma between 0 and 2 years of age, with at least 3 bronchial obstruction episodes. All of the 3 children diagnosed with asthma between 2 and 6 years of age had experienced more than one episode of bronchial obstruction during this time period. All 8 asthmatic children used inhalant corticosteroids, intermittently or continuously. ARC was defined as rhinitis and conjunctivitis appearing at least twice after exposure of an inhalant allergen and not related to infection. Urticaria was defined as allergic if it appeared within one hour after exposure to a particular allergen, at least at two separate occasions. Symptoms of food allergy were defined as vomiting and/or diarrhoea on at least two separate occasions after intake of certain offending food. Oral allergy syndrome was defined as allergic if it appeared at least at two separate occasions after intake of certain offending food. Nineteen children reported allergic symptoms, as described in detail in table 1. Twenty-seven children reported no symptoms of allergic disease (Fig 1).

Skin prick tests (SPT) were performed on the volar aspects of the forearms, with thawed egg white, fresh skimmed cow´s milk (lipid concentration 0.5%) (6, 12, 24 months and 6 years), cat (12, 24 months, 6 years), and birch and timothy (24 months and 6 years). All extracts were standardised allergen extracts from Allergologisk Laboratorium A/S, (ALK, Soluprick®, Hørsholm, Denmark). Histamine hydrochloride (10 mg/ml) was used as positive control and albumin diluent (ALK) was included as a negative control. The test was regarded as positive when the mean wheal diameter was at least 3 mm. Sixteen of the children had at least 1 positive SPT, 11 to egg, 5 to cat, 5 to timothy, 3 to milk, 3 to birch. Twenty-five children were

not sensitized according to SPT.

The total and allergen specific IgE concentrations in plasma samples at 6, 12, 24 months and 6 years of age were analysed by ImmunoCAP (Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions. The total IgE levels were also quantified in the CB samples using ImmunoCAP Total IgE Low Range (Phadia, Uppsala, Sweden). The lower detection limit was 0.35 kU/l for the Low Range assay and 2 kU/l for the conventional total IgE assay. Specific IgE antibodies directed to common food allergens (egg, milk, fish, wheat, peanut, soybean) were measured at 6, 12, 24 months and 6 years of age with the PhadiatopInfant® (Phadia) test. At 6 years of age, specific IgE antibodies to a mix of common inhalant allergens from birch, mugwort, timothy, cat, dog, horse, house-dust mite, (Dermatophagoides pteronyssinus and farinae), Cladosporium was measured with the Phadiatop® (Phadia) test. The cut-off for positivity was 0.35 kU_A/l for the PhadiatopInfant® and the Phadiatop® test. Eighteen children were sensitized according to the PhadiatopInfant® (n=17) and the Phadiatop® test (n=11, of whom 10 were also sensitized according to the PhadiatopInfant® test). Twenty-one children showed allergen specific IgE levels below the cut-off for positivity.

Eleven of the 19 children with allergic symptoms were sensitized (according to SPT and/or circulating allergen specific IgE antibodies). Eight of these sensitized children with allergic symptoms had **AD**, 6 of them also had asthma and 3 of these 6 children also had urticaria, and 1 child had **AD** and urticaria. Three children had ARC of whom 1 child also had **AD** and 1 child had **AD**, asthma and urticaria combined with ARC. One child had symptoms of food allergy and one child experienced obstructive discomfort after intake of certain offending food. Two of the 3 children with allergic symptoms who participated with questionnaires only

at the 6 year follow up are included in the group of sensitzed children with allergic symptoms as well. These children visited the allergy clinic very often. The diagnosis of these 2 children were based on notes in the medical records and SPT:s performed within the clinical practice. One child had AD at the age of 4, although without any sensitization. At 6 years of age, the AD had regressed and the child was sensitized to inhalant allergens (Phadiatop test). This child is included in the group of sensitized children and in the group of children with allergic symptoms but not in the group of sensitized children with allergic symptoms, as the allergic symptom and sensitization was completely unrelated to each other. Fifteen children were non-sensitized without allergic symptoms.

Determination of CB cytokine and chemokine concentrations

The CB levels of IL-4, IL-5, IL-9, IL-10, IL-12(p70), IL-13, IFN-γ, CCL11, CXCL10 and CCL22 were quantified by a multiplex assay (Luminex ^{100}, Biosource, Nivelles, Belgium) using the Beadlyte® Human Multi-Cytokine Beadmaster™ Kit (Upstate, CA, USA), as described in detail elsewhere(8). All measurements were blinded to the clinical symptoms.

Determination of CB CCL17, CCL18 and CXCL11 concentrations by ELISA

An in-house double-antibody sandwich ELISA (VersaMax, Molecular Devices, Sunnyvale, CA, USA) was used for quantification of CB chemokines, as described in detail elsewhere(8).

Statistics

Non-parametric tests, corrected for ties, were used. The correlations were analysed with Spearman's rank order correlation coefficient test. Comparisons between unpaired groups were done with the Mann-Whitney *U*-test. The calculations were made with the statistical

package SPSS 15.0 for Windows (SPSS Inc, Chicago, IL, USA). Undetectable levels were given the value of half the cut-off.

Logistic regression was used to investigate if CB IgE, CXCL10, CCL17 and CCL22 predicted the cumulative occurrence of allergic symptoms, sensitization (SPT and/or presence of allergen specific IgE antibodies) and allergic symptoms combined with sensitization during the first 6 years of life. The logistic regression was performed using Minitab 15 (Minitab Inc, State College, PA, USA).

Ethics

The Regional Ethics Committee for Human Research at the University Hospital of Linköping approved the study. All families gave their informed consent.

Results

Increased CB CCL22 levels, but not CCL17 levels, are associated with development of allergic sensitization later in life

The CB levels of IL-4, IL-5, IL-9, IL-10, IL-12(p70), IL-13, IFN-γ, CXCL10, CXCL11, CCL11, CCL17, CCL18 and CCL22 were analysed in relation to development of allergic sensitization during the first 6 years of life. The cytokines were not detectable, or only sporadically detectable, in the CB samples.

Sensitized children (with positive SPT and/or presence of circulating allergen specific IgE antibodies) had higher CB CCL22 levels (Fig 2A) and CCL22/CXCL10 ratios (Fig 2B) than non-sensitized children. The levels of CCL17 (Fig 2C) and the other chemokines were similar between the 2 groups. Furthermore, CB CCL22 levels predicted development of allergic sensitization during the first 6 years of life, Odds Ratio (OR) 1.14, 95% confidence interval (CI) 1.03-1.26, p=0.02, based on 100-pg/ml intervals.

Neonatal IgE, CCL17 and, in particular, CCL22 levels, were correlated to the total IgE levels later in life (Table 2).

Increased CB CCL17 levels, but not CCL22 levels, are associated with development of allergic symptoms later in life

Development of allergic symptoms during the first 6 years of life was associated with high CB CCL17 levels (Fig 3A) and high CCL17/CXCL10 ratios (p=0.01). Even though a weak relationship between CB CCL22 levels and development of allergic symptoms was seen (Fig 3B), a significantly increased CCL22/CXCL10 ratio (p=0.03) was observed in the group of children who developed allergic symptoms. The CB CCL17 levels predicted development of allergic symptoms during the first 6 years of life OR 1.27, (95% CI 1.01-1.59) p=0.04, for a 100 pg/ml increase in CCL17.

Asthma development was associated with increased CB CCL17, CCL22 and CCL22/CXCL10 ratio (p=0.04 for all comparisons). The same pattern was shown for the development of asthma and/or ARC, p=0.003 for CB CCL17 and p=0.007 for the CB CCL22 levels, p=0.01 for the CCL17/CXCL10 and p=0.03 for the CCL22/CXCL10 ratios. Increased CB IgE levels tended to be associated with development of asthma and/or ARC (p=0.07). Development of atopic dermatitis was associated with high CB CCL17 (p=0.02) levels, CCL17/CXCL10 (p=0.01) and CCL22/CXCL10 (p=0.02) ratios.

Increased CB CCL17 and CCL22 levels are associated with development of allergic symptoms and sensitization during the first 6 years of life

The sensitized children with allergic symptoms had higher CB CCL17 and CCL22 levels than non-sensitized children without allergic symptoms (Fig 4).

The Th1/Th2 balance was shifted towards a more Th2-like profile as well, as the ratios of CCL17/CXCL10 and CCL22/CXCL10 were higher in sensitized children with allergic symptoms than non-sensitized children without allergic symptoms (p=0.04 and p=0.005, respectively). Increased CB IgE levels tended to be associated with development of allergic symptoms combined with sensitization as well (p=0.09). The levels of CXCL10, CXCL11, CCL11 and CCL18 were similar in the two groups.

Possible confounders, *i.e.* older siblings, gender and smoking during pregnancy, did not affect the CB chemokine levels in this cohort (Mann Whitney U-test). CCL11, CCL17, CCL18, CCL22 and CXCL11 levels were not affected by the mode of delivery, but children delivered by caesarean section showed lower CXCL10 levels as compared to the children which were born vaginally (p=0.04). Fifty % of the children delivered by caesarean section (n=10) and 39% of the children delivered vaginally developed allergic symptoms during the first 6 years of life.

Discussion

Circulating levels of the Th2-associated chemokines CCL17 and CCL22 at birth might be important for the immune development later in life. Thus, increased CB CCL17 levels were associated with development of allergic symptoms, with and without accompanying sensitization during the first 6 years of life, whereas elevated CB CCL22 levels were seen in children who develop sensitization, with and without accompanying allergic symptoms. Our results clearly indicate that high CCL17 and CCL22 levels at birth could affect the offspring postnatally. CB CCL17 levels predicted development of allergic symptoms and CB CCL22 levels predicted development of allergic sensitization later in life. If CCL17 and CCL22 are actively involved in the initiation of the disease, or if increased CCL17 and CCL22 levels are markers for a general, stronger Th2 shift at birth in these children, remains to be settled.

A possible mechanism for the contribution of CCL22 in allergy development could be the increased IgE production seen up to 2 years of age. Children with a more marked Th2 deviation at birth might experience difficulties in the downregulation of Th2 responses, possibly causing a delayed maturation of the immune system. A continued Th2 dominance during infancy might stimulate IgE synthesis and promote allergy development. A prolonged Th2 dominance in the immune responses to allergens has been associated with allergy development (24, 25). We did observe a relationship between CB CCL22 levels and total IgE levels during the first 2 years of age, whilst a corresponding relationship between CB IgE, CB CCL17 and future total IgE levels was observed at 6 months of age only. The rho-values indicated moderate correlations. As CB CCL17 levels were associated with development of allergic symptoms, but not sensitization only, it is tempting to speculate that CB CCL17 and CCL22 contribute to development of allergic disease through different mechanisms, despite the similarities of these two chemokines. CCL17 and CCL22 share 32% sequence

288 homology(26) and are both induced by IL-4 and IL-13(9, 10). They also bind to the same
289 receptor, CCR4(16).
290
291 The present study confirms and extends our previous data on CB CCL22 and development of
292 sensitization and allergic disease up to 2 years of age. **AD** is the predominant symptom of
293 allergic disease during the first 2 years of life and the time period between 2 and 6 years of
294 age allows other allergic symptoms such as asthma and allergic rhinoconjuntivitis, to develop.
295 Thus, it is very interesting to demonstrate a relationship between a pronounced Th2 deviation
296 at birth, shown as increased CB CCL17 and CCL22 levels, and development of allergic
297 symptoms and sensitization up to 6 years of age. Our study did not reveal any relationship
298 between CCL11, CCL18, CXCL10 and CXCL11 levels and allergy development. We can not
299 exclude the possible influence of the present study size on these negative findings, as our
300 population may have been too small to reveal such relationship.
301
302 Cord blood IgE has been evaluated as a potential predictor of elevated IgE levels and
303 development of allergic disease later in life(27, 28). However, the use of CB IgE as a
304 predictor has been limited, due to poor sensitivity(27-29). Although our findings need to be
305 confirmed in a larger number of samples, CB CCL22 may possibly be an attractive candidate
306 as a predictor of elevated IgE levels and future allergy development. We did observe
307 correlations between CB CCL22 and total IgE levels up to 2 years of age, and a corresponding
308 correlation between CB IgE and total IgE levels up to 6 months of age. Furthermore, CCL22,
309 in contrast to IgE, is easily detected in CB. In the present study, CCL22 was detected in all
310 CB samples whilst only 12 (26%) of the 46 CB samples had detectable levels of total IgE.
311 The CB levels of CCL22 are, in fact, approximately 20 times higher than adult levels
312 (unpublished data), thereby also reducing the impact of contamination of the CB samples with

maternal blood. It should also be noted that cytokine levels were too low to be safely detected in CB and therefore not suitable for prediction of allergy development.

In conclusion, children who develop allergic symptoms and sensitization during the first 6 years of life showed increased CCL17 and CCL22 levels already in CB as compared to children that remained non-allergic, indicating that the Th2 deviation preceding established allergy takes place very early in life.

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426
427

Figure legends

Figure 1, Flow-chart of the study participants.

Fifty-six women were included in the study. Twenty women reported allergic symptoms of whom 13 were also sensitized whereas 36 women reported no allergic symptoms of whom 30 were non-sensitized. Nineteen of the 56 children reported allergic symptoms during the first 6 years of life and 27 children reported no symptoms of allergic disease. Ten children dropped out at various time points during childhood and 9 of the remaining 46 children choose to participate with questionnaires only at the 6 year follow up (marked with Q in the figure). Of the 19 children with allergic symptoms, 11 were also sensitized and 15 of the 27 children without allergic symptoms were non-sensitized. The following numbers of CB samples were available from the analysed groups, children with allergic symptoms n=15, children without allergic symptoms n=22, sensitized children with allergic symptoms n=8, non-sensitized children without allergic symptoms n=12. Abbreviations used, all symp: allergic symptoms, no all symp: no allergic symptoms, sens: sensitized, not sens: not sensitized.

Figure 2, CB CCL22, CCL22/CXCL10 ratio and CCL17 levels in sensitized and non-sensitized children.

A, Sensitized children (with positive SPT and/or circulating allergen specific IgE antibodies, n=15) during the first 6 years of life showed increased CB CCL22 levels and **B,** CCL22/CXCL10 ratios as compared to non-sensitized children (n=17). SPT and circulating IgE antibodies were performed/measured at 6, 12, 24 months and 6 years of age. **C,** The CB levels of CCL17 were similar in the sensitized and non-sensitized children. *=$p<0.05$, **=$p<0.01$

Figure 3, CB CCL17 and CCL22 levels in children with and without allergic symptoms. **A,** Increased CB CCL17 levels were shown in the group of children with allergic symptoms (n=15) compared to children without allergic symptoms (n=22). **B,** Children who reported allergic symptoms during the first 6 years of life showed a trend to higher levels of CB CCL22 as the children without allergic symptoms. ‡=$p<0.1$ **=$p<0.01$

Figure 4, CB CCL17 and CCL22 levels in sensitized children with allergic symptoms and non-sensitized children without allergic symptoms.

Increased CB CCL17 and CCL22 levels are associated with development of allergic symptoms and sensitization. The sensitized children with allergic symptoms (n=8) showed increased **A,** CB CCL17 and **B,** CCL22 levels compared to non-sensitized children without allergic symptoms (n=12). *=$p<0.05$

Table 1. Allergic manifestations and sensitization in the 19 children with allergic symptoms, who were followed prospectively for the first 6 years of life.

Children	Symp and sens 0-2 years	Symp and sens 2-6 years
1	AD	AD
2		ARC, *SPT+birch, timothy, Phinf+, Phad+*
3		OAS
4	AD	
5	AD, *SPT+ egg, milk, Phinf+*	AB, U
6	AD, AB	AB, U, ARC, *SPT+ cat, Phinf+, Phad+*
7	AD, *SPT+ egg, cat, Phinf+*	AB
8		AB
9	AD, AB, U, *SPT+ egg, Phinf+*	AD, AB, *SPT+ egg, timothy, Phinf+, Phad+*
10	Obst.Dis. *SPT+ egg, Phinf+*	*SPT+ egg, Phinf+*
11	AD	AD
12	AD, *SPT+ egg, milk, Phinf+*	ARC, *SPT+ birch, timothy, cat, Phinf+, Phad+*
13	*SPT+ egg, Phinf+*	FA, *Phinf+*
14	AD, AB, *SPT+ egg, cat, Phinf+*	*SPT+ egg, Phinf+, Phad+*
15		AD, ARC
16	AD, AB, *SPT+ egg, Phinf+*	AD, AB, *SPT+ egg, Phinf+, Phad+*
17[†]		AD, *Phad+*
18	AD, AB	
19	U, *SPT+ egg*	AD

Definition of abbreviations: Symp = symptoms, Sens = sensitization, AD = atopic dermatitis, AB = asthma bronchiale, ARC = allergic rhinoconjunctivitis, U= urticaria, OAS = oral allergy syndrome, FA = Food Allergy, Obst.Dis = obstructive discomfort, SPT = skin prick test,

Phinf = Phadiatop Infant test, Phad = Phadiatop test. †= This child had AD at the age of 4, although without any sensitization. At 6 years of age, the AD had regressed and the child was sensitized to inhalant allergens (Phadiatop test). This child is included in the group of sensitized children and in the group of children with allergic symptoms but not in the group of sensitized children with allergic symptoms, as the allergic symptom and sensitization was completely unrelated to each other.

Table 2. Correlations between CB IgE, CCL17, CCL22 levels and total IgE levels at 6, 12, 24 months and 6 years of age (Spearman's rank order correlation coefficient test, Rho, p).

	Total IgE 6 mo	**Total IgE 12 mo**	**Total IgE 24 mo**	**Total IgE 6 y**
CB IgE	0.49 **	0.28 ‡	0.22 **NS**	0.15 **NS**
CB CCL17	0.38 *	0.13 **NS**	0.02 **NS**	0.09 **NS**
CB CCL22	0.43 **	0.28 ‡	0.46 **	0.34 ‡

Definition of abbreviations: **mo**=months, y=years, ‡=p<0.1 *=p<0.05, **=p<0.01 **NS**=not significant

Figure 1

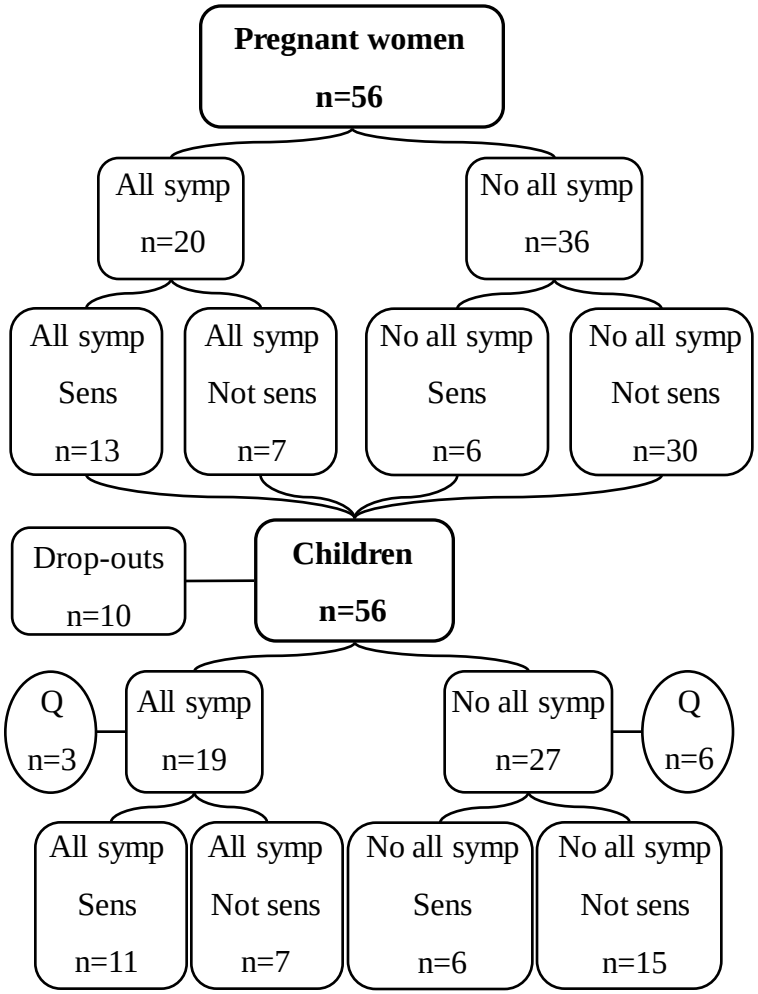

Fig 1

Figure 2

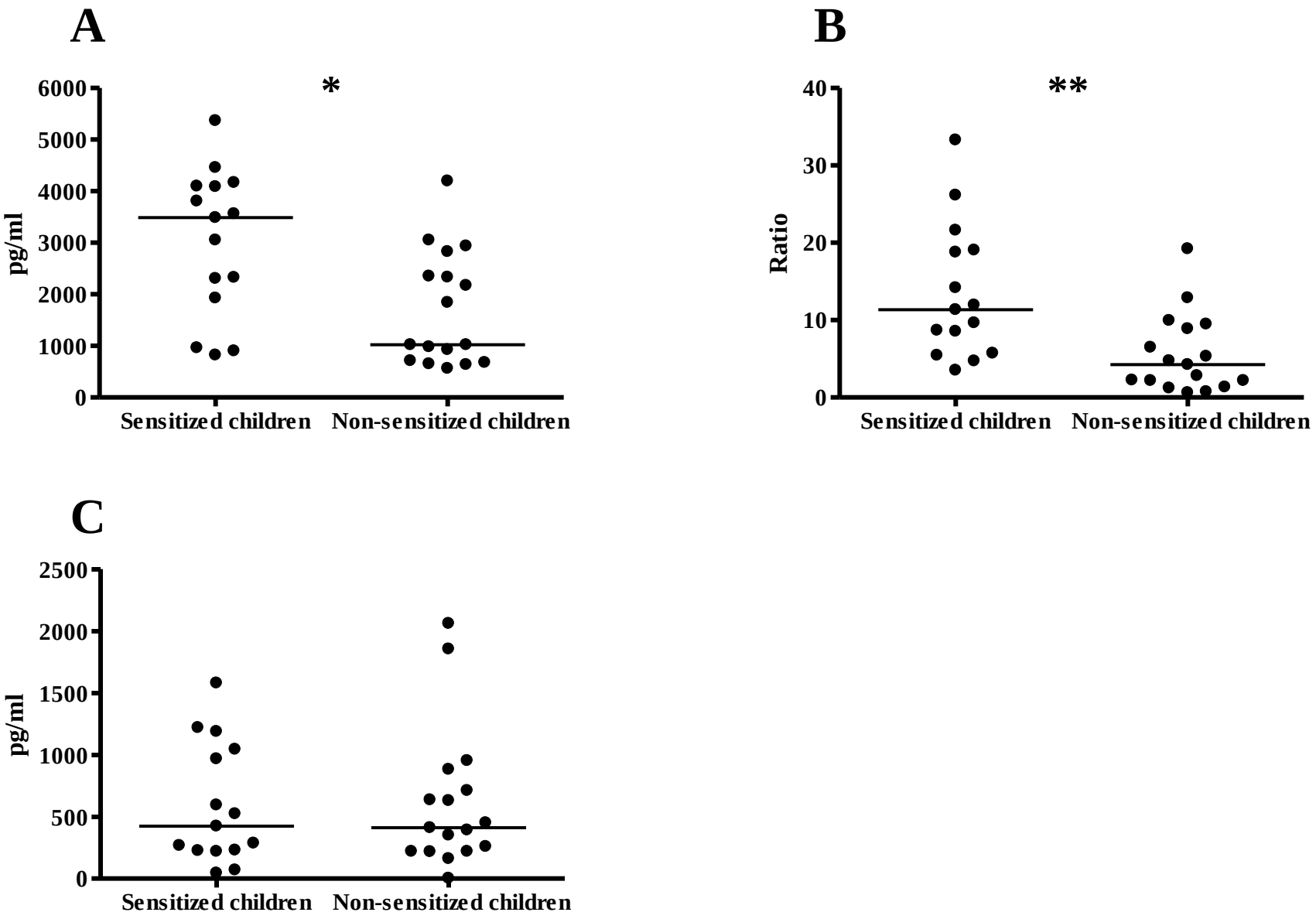

Fig 2

Figure 3

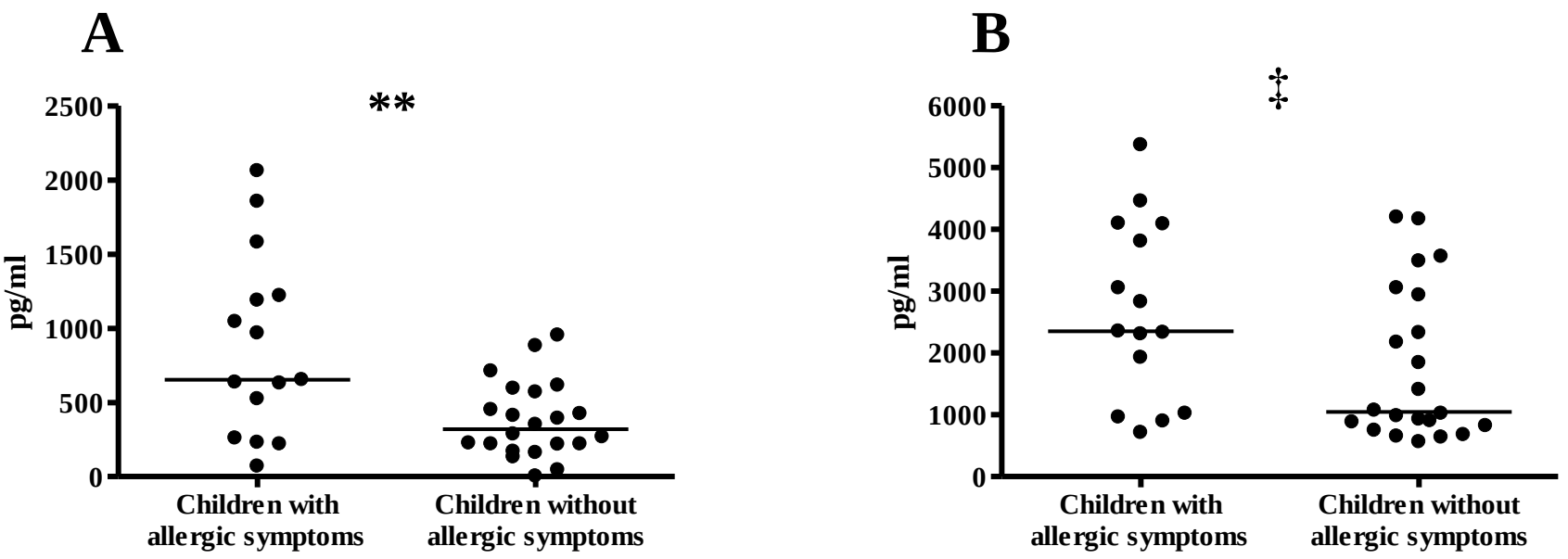

Fig 3

Figure 4

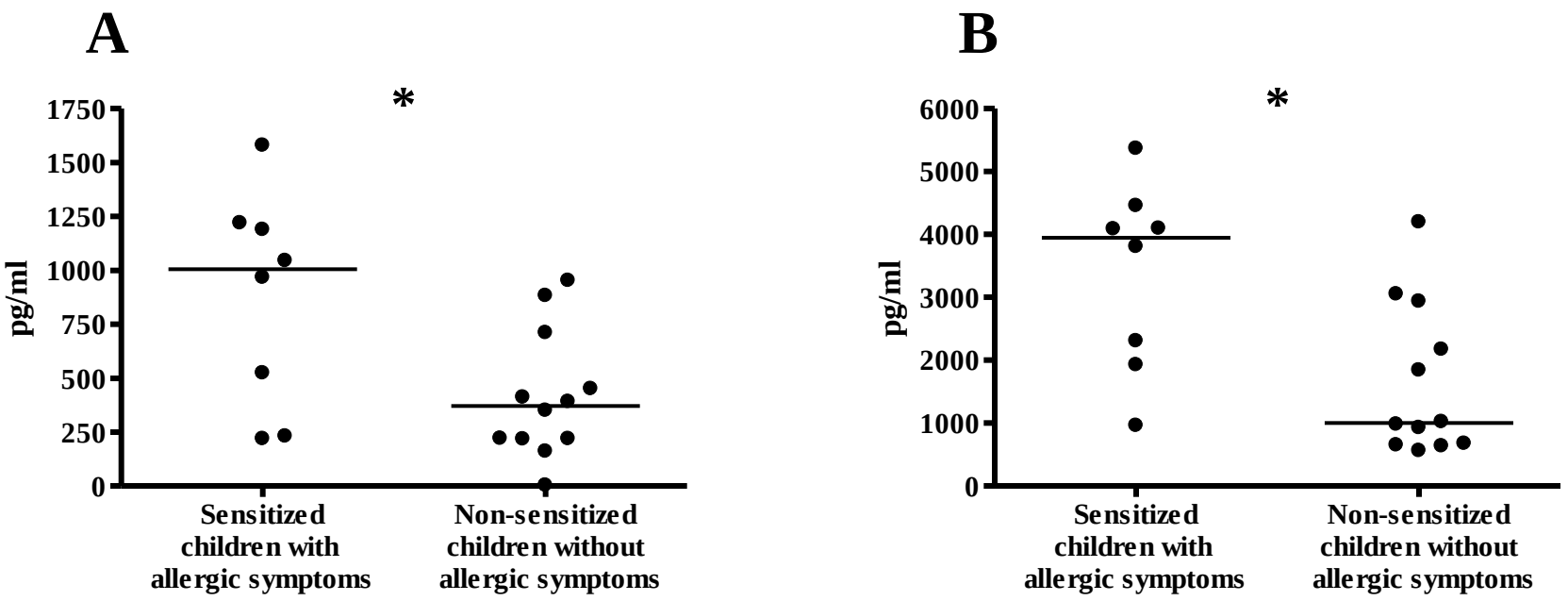

Fig 4