Circulating Chemokine Levels and the Development of Allergic Phenotypes from Infancy to Adolescence: A Population-Based Birth Cohort Study

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Research

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Abstract

**Background:** Chemokines are important mediators in immune cell recruitment, contributing to allergy development. However, extensive studies of chemokines in the circulation in relation to the presence and development of allergic diseases remain scarce.

**Objective:** To investigate associations of circulating allergy-related chemokines with development of asthma and sensitisation cross-sectionally and longitudinally in a population-based cohort.

**Methods:** The chemokines CCL17, CCL22, CXCL10, CXCL11 and CCL18 were measured in plasma samples from children in a population-based birth cohort. Samples were available from cord blood at birth (n=376) and age 1 (n=195) and 8 years (n=334). Cross-sectional and longitudinal association analyses were performed in relation to asthma and allergic sensitisation, as well as allergic phenotype clusters previously derived using machine learning in the same study population.

**Results:** In children with asthma and/or allergic sensitisation, CCL18 levels at ages 1 and/or 8 were consistently elevated. In a longitudinal model which included information on asthma from 4 time-points (ages 5, 8, 11 and 16 years), we observed a significant association between increasing levels of CCL18 at age 1 year and a higher risk of asthma from early school age to adolescence (OR=2.9, 95% CI 1.1-7.6, p=0.028). We observed similar associations in longitudinal models for allergic sensitisation. Asthma later in life was preceded by increased CXCL10 levels after birth, and decreased CXCL11 levels at birth.

**Conclusion:** Elevated CCL18 levels throughout childhood foreshadow the development of asthma and allergic sensitisation. The Th1-associated chemokines CXCL10 and CXCL11 were also associated with development of both outcomes, with differential temporal effects.

Introduction

Among children and adolescents, allergic diseases such as eczema, allergic rhinoconjunctivitis and asthma affect their development and well-being, posing a considerable socioeconomic burden worldwide.\(^1\)\(^,\)\(^2\) Allergic diseases are heterogenous and may present with similar symptoms but different underlying causes, the features of which are just beginning to be characterised.\(^3\)\(^–\)\(^5\)

Both sensitisation and asthma have been recognised as heterogeneous traits, with distinct clusters being unveiled using unsupervised machine learning techniques.\(^6\)\(^,\)\(^7\) The term atopy describes the heritable propensity to produce IgE-antibodies towards innocuous antigens, hence the process of sensitisation.\(^1\) Being sensitised, however, does not imply allergy by default, as allergic symptoms may or may not coincide with sensitisation.\(^8\) Numerous immune biomarkers have been investigated as potential determinants of heterogeneity and development of allergic phenotypes, but thus far none has proven unequivocal utility in a clinical setting.
Allergic inflammation depends on recruitment of Th2 cells, eosinophils and mast cells to the allergic reaction site\(^9\), a process facilitated by concentration gradients of chemokines attracting target cells by binding to specific G-protein coupled receptors.\(^{10,11}\) As the detection of circulating T-helper cell related cytokines is occasionally difficult, chemokines have emerged as promising biomarkers for corresponding immune responses.\(^{12,13}\) Several chemokines are involved in allergy development. For instance, the Th2-associated chemokines CCL17 and CCL22 bind to the primarily Th2 and Treg cell expressed receptor CCR4.\(^{14}\) Both chemokines are expressed by the thymus\(^{15}\), and may be further induced by IL-4 and IL-13 in myeloid cells\(^{15,16}\), epithelia\(^{10,17}\) and T cells.\(^{16}\) Indeed, development of allergic symptoms and sensitisation in the first six years of life is preceded by elevated cord blood levels of CCL17 and CCL22, respectively.\(^{12,18}\) Similar findings from sensitised children with allergic symptoms\(^{12,18}\), and children developing recurrent wheeze\(^{19}\) and asthma\(^{18}\), indicate involvement of these chemokines in allergic immune responses.

In contrast, the chemokines CXCL10 and CXCL11 are induced by IFN-\(\gamma\) and bind to the preferentially Th1 cell expressed receptor CXCR3.\(^{14,20}\) The main producers of CXCL10 are epithelia in the lung\(^{21}\) and thymus\(^{22}\), as well as endothelial cells, fibroblasts and monocytes.\(^{22}\) CXCL11, however, is primarily expressed by peripheral blood leukocytes, thymus, spleen and lung epithelium.\(^{23,24,25}\) Previously believed to be mainly involved in autoimmunity\(^{26}\), the Th1-associated chemokines are nowadays also known to counteract Th2 responses driving allergic inflammation.\(^9\) Increased levels of CXCL10 and/or CXCL11 in viral-induced\(^{27,28}\), moderate to severe\(^{29-31}\) and allergen challenged asthma\(^{32}\), emphasise this complex regulation. In line with previous findings, children developing recurrent wheezing present with higher circulating levels of CXCL10, with a similar tendency for CXCL11 at two years of age.\(^{19}\) Furthermore, children developing wheeze by age three and allergic asthma by age six show elevated levels of CXCL10, CCL17 and CCL22.\(^{33}\) In contrast, sensitised children revealed lower circulating levels of CXCL11 at birth and two years of age\(^{19}\), suggesting a more complicated mechanism of action in allergy development.

CCL18 is another allergy related chemokine, which is under dual regulation of both Th2 cells and Treg cells, as it may be induced by IL-4, IL-13 as well as IL-10.\(^{34}\) This contrasts to CCL17 and CCL22, which both are inhibited by IL-10.\(^{35,36}\) The preferential receptor for CCL18 is CCR8\(^{37}\), which is expressed on e.g. Th2, Treg and skin homing T cells.\(^{34}\) This chemokine is produced primarily by tissue resident antigen presenting cells, with constitutive expression in the lung and circulation, and increased expression upon allergen challenge. Being mainly a regulatory chemokine at steady state, CCL18 is up-regulated in allergic conditions such as allergic rhinitis, atopic dermatitis and asthma\(^{34}\), possibly compensating for lessened binding abilities to immune cells in allergic disease.\(^{38,39}\) Indeed, our previous study showed elevated levels of CCL18 in children developing eczema and recurrent wheeze in the first years of life.\(^{19}\)

We hypothesised that allergy-related chemokines, namely CXCL10, CXCL11, CCL17, CCL22 and CCL18, foreshadow the development of different allergic phenotypes throughout childhood. To test our hypothesis, we measured circulating levels of these chemokines at three time points throughout
childhood (at birth, 1 year and 8 years of age) in a population-based birth cohort, and related these chemokines to allergic outcomes from infancy to age 16 years. Furthermore, we ascertained the relationship between these chemokines and previously described clusters of allergic diseases derived using machine learning in this cohort.

Materials And Methods

Study design, setting, participants and data sources

The Manchester Asthma and Allergy Study (MAAS) is a population-based birth cohort from the greater Manchester area, consisting of a mixed urban-rural population within 50 square miles of South Manchester and Cheshire, United Kingdom located within the maternity catchment area of Wythenshawe and Stepping Hill Hospitals. Validated questionnaires were interviewer-administered to collect information on parentally-reported symptoms and physician-diagnosed illnesses. We assessed allergic sensitisation by skin prick tests (SPT). The study was approved by the South Manchester Local Research Ethics Committee and parents gave written informed consent.

Screening and recruitment

All pregnant women were screened for eligibility at antenatal visits (8-10th week of pregnancy) between October 1st 1995 and July 1st 1997. Of the 1499 couples who met the inclusion criteria (≤ 10 weeks of pregnancy, maternal age ≥ 18 years, and questionnaire and skin prick data test available for both parents), 288 declined to take part in the study and 27 were lost to follow-up between recruitment and the birth of a child. A total of 1184 children born into the study had at least some evaluable data.

Allergic sensitisation

Sensitisation was ascertained by SPT at ages 1, 3, 5, 8, 11 and 16 years for 7 allergens (Dermatophagoides pteronyssinus, cat, dog, grass pollen, mixed moulds, milk, and egg [Bayer, Elkahrt, Ind, US]). From age 8 years, SPTs were additionally performed for tree pollen (birch) and peanut (total of 9 allergens tests). We defined sensitisation as a mean wheal diameter 3 mm larger than that elicited by the negative control to at least 1 of the allergens tested.

Allergy development

Children were followed prospectively, and attended review clinics at ages 1, 3, 5, 8, 11, and 16 years of age. At age 1 year, only children with either both atopic parents, or no atopic parents who lived in homes without a pet were invited to attend clinical follow up. At all other time points for all other measures all children were invited to participate.

Variable definition
Sensitisation

Sensitisation was defined as having a positive skin prick test to any of the above-mentioned allergens at ages 1, 3, 5, 8, 11 and 16 years of age.

Asthma

Asthma was defined by fulfilling at least two out of three following criteria: current wheeze, current use of asthma medication, or physician-diagnosed asthma ever at ages 1, 3, 5, 8, 11 and 16 years of age.

Outcomes derived using machine learning and data-driven methodologies

In this study population, we have previously described clusters of allergic sensitisation (using SPTs and IgE to whole allergen extracts and component-resolved diagnostics (CRD), allergic diseases (eczema, wheeze and rhinitis) and asthma exacerbations. In the current analysis, we used the following multivariable outcomes

**Atopy clusters**: (1) non-dust mite atopic vulnerability, (2) dust mite atopic vulnerability, (3) multiple late atopic vulnerability, (4) multiple early atopic vulnerability and (5) no latent atopic vulnerability.

**Atopic march clusters**: (1) no disease, (2) atopic march, (3) persistent eczema and wheeze, (4) persistent eczema with later-onset rhinitis, (5) persistent wheeze with later-onset rhinitis, (6) transient wheeze, (7) eczema only and (8) rhinitis only.

**IgE clusters**: (1) Multiple sensitisation, (2) predominantly house dust mite sensitisation, (3) predominantly grass and tree sensitisation and (4) lower-grade sensitisation.

**Exacerbations clusters**: represented by the following clusters at age 8; (1) no wheeze, (2) wheeze no exacerbations, (3) infrequent exacerbations and (4) early onset frequent exacerbations.

Quantification of chemokines in plasma samples

A total of 905 plasma samples originating from the time of birth (cord blood, n = 376), 1 year (n = 195) and 8 years (n = 334), were analysed for their chemokine content (Figure S1). Circulating plasma levels of CCL18 were measured using an in-house DuoSet ELISA kit (R&D Systems) and an in-house multiplex bead assay was setup for analysis of circulating CCL17, CCL22, CXCL10 and CXCL11, as described in detail below.

Elisa (ccl18)

Due to its high concentration in the circulation, the CCL18 chemokine was measured with a separate DuoSet ELISA kit (DY394, R&D systems). On the first day, Corning® 96 Well Half Area Clear Flat Bottom Polystyrene High Bind Microplates (Corning Life Sciences, Kennebunk, ME, USA) were coated with
4 µg/ml monoclonal anti-human CCL18/PARC antibody (MAB 394, clone: 65407 R&D systems, Minneapolis, MN, USA) diluted in carbonate-bicarbonate buffer (28 mM Na$_2$CO$_3$ 72 mM NaHCO$_3$, pH 9.6, Sigma Aldrich). The plate was shaken for 1 h at RT, and thereafter left in RT without shaking overnight. The following day, the plate was washed 4 times in PBS-T (Medicago, Uppsala, Sweden), and subsequently blocked by adding skimmed cow’s milk diluted in PBS (Medicago, Uppsala, Sweden). One hour of incubation on a plate shaker at RT followed. Next, a seven-point standard curve, diluted in steps of 1:2, was prepared (125 – 1.9 pg/ml) in PBS + 1% BSA (Probumin, Merck Millipore, Darmstadt, Germany). After washing the plate, standards, samples and blanks were added in duplicates to the plate, which was followed by a one-hour incubation at RT while shaking. Washing was thereafter followed by addition of 200 ng/ml biotinylated anti-human CCL18/PARC antibody (polyclonal goat IgG, BAF394, R&D systems Minneapolis, MN, USA) diluted in HPE buffer (High Performance ELISA buffer, Sanquin Plesmalaan, The Netherlands), and one hour incubation at RT while shaking. Upon washing the plate, streptavidin-poly-horse radish peroxidase-conjugate (SA-poly-HRP, Sanquin Plesmalaan, The Netherlands) was added and let incubate for 30 min at RT while shaking in the dark. Another wash step later, the substrate for SA-poly HRP, TMB (3,3’,5,5’-Tetramethylbenzidine, Sigma Aldrich, St. Louis, MO, USA) was added to the plate and 30 min of shaking incubation at RT in the dark followed. The reaction was stopped using 1.8 M H$_2$SO$_4$, and the plate was read at 450 nm in the microplate absorbance reader (Tecan Sunrise, Austria) and subsequently analysed. The standard curve was calculated by means of five parametric statistics. In general, data from duplicate samples were considered reliable when the CV was below 15%. However, in total 4 samples (2 samples at birth and 2 samples at 8 years of age) were included in further analyses despite their higher CV (all below 17%). The detection limit was 3.9 pg/ml, and the inter-assay variation was below 30%.

**Multiplex Luminex bead assay (CCL17, CCL22, CXCL10 and CXCL11)**

An in-house multiplex bead assay was setup for CCL17, CCL22, CXCL10 and CXCL11 for analysis in plasma samples. In preparation for the in house multiplex bead assay, four bead sets were coupled with 5 µg capture antibody (Clones; CCL17: 54026, CCL22: 57226 and CXCL11: 87328 (R&D Systems, Abingdon, UK) and CXCL11: 4D5/A7/C5, BD Pharmingen, NJ, USA) per million beads for the respective chemokine of interest. The coupling procedure was validated by running standard curves with some control samples. For the main analyses, 1.2 µm pore-size filter plates Millipore multiscreen, Millipore corporation, Bedford, MA, USA) were moistened by addition of PBS + 1% BSA (Probumin, Merck Millipore, Darmstadt, Germany), while shaking for a while at RT. Dilution of the seven point standard curves (in steps of 1:3) for the respective chemokines; 1400 – 1.9 pg/ml for CXCL10 (Cat.no: 266-IP), CCL17 (Cat.no: 364-DN) and CCL22 (Cat.no: 336-MD), and 4000 – 5.5 pg/ml for CXCL11 (Cat.no: 672-IT) followed (all standards: R&D Systems, Abingdon, UK). Upon removal of the buffer from the plate using vacuum manifold, standards, samples and blanks were added to the plates in duplicates. Thereafter, a mixture of beads diluted to a concentration of 2000 beads per bead set and well, was added to the plate. After one hour of incubation on a plate shaker in RT, the plate was shaken over night at 4 °C. The following day, a
mixture of the detection antibodies for CCL17 (500 ng/ml, Cat.no: BAF364, R&D systems), CCL22 (200 ng/ml, Cat.no: BAF336 R&D systems), CXCL11 (500 ng/ml, Cat.no: BAF672, R&D systems) and CXCL10 (1000 ng/ml, clone: 6D4/D6/G2 BD Pharmingen, NJ, USA), was added to the plate upon washing and removal of fluid by vacuum manifold. The plate was incubated for 1 h in the dark, while shaking at RT. Upon washing and removal of the fluid, SA-PE (Life Technologies, Oregon, US) was added to the plate to a concentration of 1 µg/ml, and a 30 min incubation at RT while shaking followed. Prior to analysis of the plate in a Luminex 200 instrument (Luminex Corporation, Austin, Texas, US), the plate was washed again, and the beads were resuspended in 75 µl PBS + 1% BSA. The acquired data were evaluated utilizing the Masterplex software (Version 2.0.0.68, Hitachi, South San Francisco, CA, US). Standard curves were calculated by means of five parametric statistics. The standard curve from the Th1-associated chemokine CXCL11 was weighted (1/y) for all plates, as to increase the resolution at the lower end of the curve when retrieving the sample data from the Luminex analyses. Data from duplicate samples were considered reliable when the CV was below 15%. For CXCL11, three samples were included in further analyses despite their higher CV (a CV of less than 16%, two samples at 1 year and one sample at 8 years of age). The detection limits of the chemokines were as follows; CCL17: 1.0 pg/ml, CCL22: 1.9 pg/ml, CXCL10: 5.8 pg/ml and CXCL11: 16.5 pg/ml. Undetectable samples were given half cut-off levels, represented by half of the previously mentioned detection limits. Inter-assay variation was below 30%.

**Statistical analyses**

Descriptive analyses of demographic variables and allergy outcomes (at ages 1, 3, 5, 8, 11 and 16) in the children of the sub-study, compared to the excluded children or the entire MAAS cohort, were performed using a Chi-square test or Student’s t-test, for discrete and continuous variables, respectively (Tables S1-S3).

To assess the normality of the chemokine data, Shapiro-Wilk's test was applied to the measurements. As most of the chemokine concentrations were non-normally distributed, univariate comparisons of chemokine levels at the different ages were performed using the non-parametric Kruskal-Wallis test with Dunn’s post hoc test for multiple comparisons, whereas Mann-Whitney U tests were used for cross-sectional comparisons. Correlations between the chemokines were assessed using the Spearman's rank correlation.

The chemokine levels were thereafter assessed in relation to asthma and sensitisation outcomes at ages 1, 3, 5, 8, 11 and 16 years of age. Furthermore, CCL18 levels at all ages were related to previously machine learning derived clusters, more specifically sensitisation clusters, atopic diseases clusters, CRD IgE clusters and exacerbations clusters.

Binomial logistic regression analyses were performed on the natural log transformed chemokine data in relation to binary sensitisation and asthma variables, to study whether chemokine levels at birth, 1 year or 8 years of age predicted outcomes in terms of sensitisation or asthma at ages 8, 11 and 16 years of age, respectively. All chemokines were included at each age, as significant outcomes were consistent
regardless if the models included one chemokine or all chemokines at once (results not shown). The models were adjusted for parental atopy at recruitment, parental smoking at recruitment and sex. These variables were chosen on the basis of differences seen in the demographic data and previous knowledge from the cohort. As not to over-fit the models, we chose to merge the maternal and paternal variables on atopy and asthma into one parental variable each. We did not correct for pet ownership separately or as a merged variable, as only cat-ownership was significantly different, and as not to over-fit the models. Coefficients represent the increased/decreased odds of the respective outcome per log-unit increase in chemokine levels.

The longitudinal analyses were performed by using generalised estimating equations (GEE), which takes into account the correlations reported for the chemokines. Population-averaged GEE models were developed to investigate whether the effect of natural log-transformed chemokine levels on the development of asthma (ages 5–16 and 8–16) or sensitisation (ages 3–16 and 8–16) changed over time. This was investigated in different time intervals to study the role of the chemokines both contemporaneously as well as later in life. For asthma, the interval 5–16 years was chosen, as we excluded the development of pre-school wheeze as a surrogate for asthma development. For sensitisation, we chose to use the interval 3–16 years of age, as the prevalence of sensitisation at age 1 was generally lower than later in life and the sample size was smaller than at the other ages. For both outcomes, the 8–16 years interval was chosen, as chemokine measurements at age 8 were studied. The models were adjusted for parental atopy at recruitment, parental smoking at recruitment and sex. Coefficients represent the increased/decreased odds of sensitisation per log-unit increase in chemokine levels.

Analyses were conducted in GraphPad Prism 8, IBM SPSS Statistics version 25, and Stata 15 software.

**Results**

**Demographic characteristics of the study population**

Participant flow, and the number of children with measured chemokines at each age, is shown in Figure S1. Comparisons of demographic characteristics revealed no significant differences between children included in this analysis (n = 653) from those excluded (n = 531), except for slightly higher proportions of atopy and asthma among both parents, as well as fewer being cat owners, in the chemokine study (Table S1). The cross-sectional prevalence of allergic sensitisation and asthma among children who attended each of the 6 follow-ups, and in those with chemokines measured in cord blood, and at ages 1 and 8 years, is shown in Tables S2 and S3.

**Kinetics of circulating chemokines**

Initial statistical analyses of the chemokine measurements in plasma samples revealed distinctive expression patterns for the five analytes (Fig. 1). The Th2-associated chemokine CCL17 displayed
significantly lower levels at 1 and 8 years of age, compared to at birth (Fig. 1A). In contrast, the Th2-associated chemokine CCL22 revealed higher concentrations at age 8 compared to earlier in life (Fig. 1B). For the Th2/Treg-associated chemokine CCL18 and the Th1-associated chemokine CXCL10, significantly higher levels were evident at 1 and 8 years of age compared to at birth (Fig. 1C-D). However, the expression was significantly lower at age 8 than at age 1 for both chemokines. The Th1-associated chemokine CXCL11 showed significantly higher levels at age 8 compared to the earlier time points (Fig. 1E).

**CXCL10, CXCL11 and CCL18 levels associate with asthma and sensitisation**

As several of the investigated chemokines have previously been related to allergy development, we set out to investigate possible associations of the chemokine levels in this cohort to different allergy outcomes. To this end, we performed cross-sectional analyses in relation to outcomes of asthma and sensitisation in childhood and adolescence. The results of these analyses are shown in Figs. 2 and S2 (asthma) and Figs. 3 and S3 (sensitisation).

**Asthma**

CXCL10 levels were significantly increased at age 1 year in children with asthma at the same age (Fig. 2A). Furthermore, children with asthma at ages 8 and 11 years exhibited elevated CXCL10 levels at age 8 years (Fig. 2B, Figure S2A). In contrast, CXCL11 was significantly decreased in cord blood of children having asthma at age 16 (Figure S2B). For CCL18, higher levels at age 8 associated with asthma at ages 8 and 16 years (Fig. 2C-D).

*Sensitisation:* CXCL10 levels in cord blood were significantly lower than in non-sensitised children at ages 1 and 16 years (Figure 3A-B). Similarly, CXCL11 levels at 1 year and at birth were significantly elevated in non-sensitised children at 1 and 16 years of age, respectively (Figure S3A-B). Children who were sensitised at ages 1, 8, 11 and 16 years consistently displayed significantly elevated concentrations of CCL18 at 1 and/or 8 years compared to non-sensitised children (Figure 3C-E, Figure S3C). Increased levels of the Th2-associated chemokine CCL17 at birth associated with sensitisation at age 8 (Figure S3D).

**CXCL10, CXCL11 and CCL18 predict asthma and sensitisation**

As some of the chemokines indeed associated with the development of asthma and sensitisation later in childhood, we were interested in how these associations changed over time. Hence, we tested the ability of the chemokine levels to predict asthma and sensitisation later in childhood and adolescence using binomial logistic regression models. In the adjusted models, all five chemokines at the studied age points were included, as the outcomes did not differ from when studying models using one chemokine at the time (results not shown). This remained true, although the chemokines revealed low to moderate correlations with each other (Table S4).
Asthma

Results of models relating the chemokines to asthma development are illustrated in Fig. 4A. Having higher CXCL11 levels at birth constituted lower odds of being asthmatic at age 16 (OR = 0.4, 95% CI 0.2–0.8, p = 0.012). Furthermore, higher CCL18 levels at 8 years of age markedly increased the odds of being asthmatic at ages 8 (OR = 3.8, 95% CI 1.5–9.8, p = 0.006) and 16 years (OR = 5.3, 95% CI 1.7–16.1, p = 0.003).

Sensitisation: Similar patterns were revealed in relation to odds of becoming sensitised (Figure 4B). Presenting with elevated CCL18 levels at age 8, posed a three times or higher odds ratio for developing sensitisation at ages 8 (OR=3.3, 95% CI 1.5-7.1, p=0.002), 11 (OR=3.0, 95% CI 1.3-6.7, p=0.008) and 16 years of age, (OR=4.2, 95% CI 1.7-10.5, p=0.002). In contrast, having high levels of CXCL10 at age 8 seemed protective against becoming sensitised at age 11 (OR=0.4, 95% CI 0.2-0.8, p=0.012).

Association between CCL18 levels and the development of asthma and sensitisation

Proceeding with the chemokines showing the strongest consistent associations cross-sectionally, we wanted to examine whether the cross-sectional trends of these chemokine levels would translate into longitudinal relationships on clinical outcomes over time. Therefore, we set up GEE models studying each chemokine at each age separately in relation to various intervals of asthma and sensitisation development. These models take into account correlations within individuals, and as the data are not independent over time, this provides an advantage of running both the GEE and logistic regression models. Consistent longitudinal patterns were only revealed for circulating levels of CCL18 at ages 1 and 8 (Fig. 5).

Asthma

In a longitudinal model including information on asthma from four time-points (ages 5, 8, 11 and 16), we observed a significant association between increasing CCL18 levels at age 1 year and the higher risk of asthma from early school age to adolescence (OR = 2.9, 95% CI 1.1–7.6, p = 0.028). Similarly, higher CCL18 levels at ages 1 (OR = 3.5, 95% CI 1.3–9.8, p = 0.01) and 8 years (OR = 3.0, 95% CI 1.6–5.9, p = 0.001) were associated with an increased risk of asthma between ages 8 and 16 years.

Sensitisation

In a longitudinal model including information on SPTs from five time-points (ages 3–16 years), the odds of becoming sensitised increased significantly with increased CCL18 concentrations at age 1 (OR = 3.1, 95% CI 1.2–8.3, p = 0.022). Similarly, the odds ratios for developing sensitisation between the ages 8 and 16 years increased significantly with increasing CCL18 levels at ages 1 (OR = 3.5, 95% CI 1.3–9.9, p = 0.018) and 8 years (OR = 3.0, 95% CI 1.5–5.9, p = 0.002).
Association of CCL18 levels with clusters of allergic diseases

As we previously have derived clusters of allergic sensitisation\(^{41,42}\), allergic diseases\(^{43}\) and asthma exacerbations\(^{44}\) from children in the MAAS cohort using machine learning, we sought to study the relationship between the chemokine levels and these putative endotypes of allergic diseases.

Children belonging to the multiple early sensitisation cluster\(^{41}\) had significantly higher levels of CCL18 at age 8 compared to non-atopic subjects (Fig. 6A). In relation to CRD sensitisation patterns\(^{42}\), circulating levels of CCL18 at age 8 were elevated in individuals sensitised towards multiple allergens as compared to children with predominant grass and tree sensitisation, and as a trend compared to children who were sensitised to a lesser degree (Fig. 6B).

There was a trend towards higher levels of CCL18 at age 8 both in the eczema only and the atopic march clusters compared to the cluster consisting of healthy children\(^{43}\) (Fig. 6C).

Associating chemokine levels to developmental pattern of asthma exacerbations in the first 8 years of life, children presenting with exacerbations\(^{44}\) showed significantly higher levels of CCL18 at age 8 compared to non-wheezers (Fig. 6D).

None of the other chemokines revealed any differences between the investigated clusters.

Discussion

In this study, we show that levels of the chemokines CCL18, CXCL10 and CXCL11 in early life and childhood may predict outcomes of allergic disease later in childhood and adolescence. To our knowledge, this is the first study to show that childhood circulating CCL18 levels may affect allergy-related outcomes longitudinally until adolescence.

The main finding was that the dually Th2/Treg regulated chemokine CCL18 predicted development of both asthma and sensitisation, with consistent effects over time. Cross-sectional analyses revealed increased circulating levels at ages 1 and 8 years in relation to sensitisation and asthma, both contemporaneously and later in life. Further investigations to assess whether these associations also translated into predictions of outcomes confirmed that augmented CCL18 levels at age 8 predicted asthma at ages 8 and 16, and sensitisation at ages 8, 11 and 16. As established by the GEE models, these effects were also persistent over time, revealing longitudinal relationships of the CCL18 concentrations at ages 1 and 8 to outcomes of asthma and sensitisation in the age ranges 5–16/8–16 and 3–16/8–16 years, respectively.

Being constitutively expressed in the lung and lymphoid tissues during homeostatic conditions\(^{34}\), CCL18 exhibits both chemotactic and immunoregulatory properties. It promotes tolerogenic differentiation of
dendritic cells\textsuperscript{38}, which in turn may polarise T cells into Tregs, and may polarise memory T cells into FoxP3 + T cells \textit{in vitro}.\textsuperscript{39} In allergic subjects, however, the tolerogenic effects of CCL18 are seemingly abrogated\textsuperscript{38,39}, despite being upregulated in allergic conditions such as atopic dermatitis\textsuperscript{19,50,51} and asthma.\textsuperscript{52,53} Described being due to less efficient binding of the protein on immune cells\textsuperscript{38,39}, this possibly could partly explain the loss of tolerance in allergic individuals. Furthermore, CCL18 induces production of collagen both in the skin and lung\textsuperscript{54}, implying a role in remodelling of the airways typically seen in asthmatic subjects. As alveolar macrophages are the main producers of CCL18 in the lung\textsuperscript{34}, where its expression is constitutive, it is tempting to speculate that these levels are augmented in asthmatic individuals owing to dysregulation of these cells. However, as our measurements were performed in plasma samples, and CCL18 may originate from one of many bodily sources, we cannot draw conclusions on tissue specific effects of the observed elevation without performing functional studies. Furthermore, whether heightened CCL18 responses in asthmatic and sensitised children constitute causative mechanisms of allergy induction, or compensatory immune dampening responses, remains to be elucidated.

Interesting findings also appeared for the Th1-associated chemokines CXCL10 and CXCL11. Elevated levels of CXCL10 at ages 1 and 8 associated with development of asthma at ages 1, 8 and 11, in line with a study revealing increased circulating levels of CXCL10 in children with wheezing at age 3, subsequently developing asthma at age 6.\textsuperscript{33} Additionally, CXCL10 levels are increased in viral-induced asthma\textsuperscript{27,28}, suggesting that viral infections may induce Th1-chemokine responses in asthmatic individuals. On the contrary, low cord blood CXCL10 levels associated with sensitisation at ages 1 and 16, and increased levels at age 8 inversely associated with being sensitised at age 11. Similarly, decreased CXCL11 levels at birth and age 1 associated with development of sensitisation at ages 16 and 1, respectively. This corroborates findings from our previous studies, where SPT-positive children had lower levels of CXCL11 at birth and 24 months.\textsuperscript{19} As sensitisation is a Th2-driven process, and Th1-responses were lessened in sensitised children, diminished neonatal Th1-responses seemingly paves way for development of sensitisation in these children. Additionally, reduced cord blood CXCL11 levels associated with asthma at age 16 and translated into a predicted lower risk with high CXCL11 levels at birth, supporting previous results where children with the highest quartile CXCL11 levels at birth did not become sensitised throughout the first two years of life.\textsuperscript{19} No long-term effects of CXCL10 and CXCL11 on allergy development could be demonstrated in this study. Possibly, the function of Th1 cells, and their expression of IFN-\(\gamma\), may become attenuated due to immunoregulatory effects of the highly expressed CCL18 on Tregs, although the findings may also constitute altered patterns of expression in allergic conditions. Collectively, this indicates that although these chemokines are induced by the same cytokine, downstream effects seem to be differentially regulated both in terms of allergy outcome and how levels reflect temporal development of disease.

For the Th2-associated chemokines, only CCL17 demonstrated augmented cord blood levels associating to sensitisation at age 8, in line with previous findings, revealing elevated levels of both CCL17 and CCL22 at birth in children with IgE-associated allergies at ages 2 and 6.\textsuperscript{12,18} While cord blood CCL17 and
CCL22 levels predict development of allergic symptoms and sensitisation, respectively\textsuperscript{12,18}, neither showed predictive abilities in the present study.

We further examined chemokine expression within allergy clusters previously derived from our cohort.\textsuperscript{41–44,55} Indeed, CCL18 levels at age 8 were higher in the multiple early allergic sensitisation cluster. Moreover, children with asthma exacerbations had higher levels of CCL18 at age 8 compared to children without wheeze. Taken together, this suggests that increased CCL18 levels later in childhood may reflect allergic disease severity, although further studies should elaborate on this matter.

Regarding kinetics of the chemokines, differences to previous measurements unfolded. In two Swedish cohorts\textsuperscript{12,18,19} and a Taiwanese cohort\textsuperscript{56} of children, Th2-associated chemokine levels were highest in cord blood, and decreased with age, whereas Th1-associated chemokines revealed the opposite pattern in the Swedish children.\textsuperscript{12,18,19} Principally, CCL17, CXCL10 and CXCL11 adhered to this pattern in this study, whereas CCL22 surprisingly increased with age. CCL18 revealed similar kinetics to earlier findings\textsuperscript{19}, being lower at birth and increasing with age. Discrepancies in the findings could be due to several reasons. One being substantial differences in sample size, another being differential allergen exposures between Sweden and the UK. Swedish children are mainly sensitised towards grass and birch pollen, furred pets and food allergens, whereas house dust mite sensitisation is uncommon in the subarctic, non-humid climate.\textsuperscript{57,58} Generally in the UK, however, and as shown specifically in our cohort, house dust mites are major contributors to sensitisation.\textsuperscript{57} This may explain the increasing levels of CCL22 in our study, as Taiwanese children who were sensitised towards house dust mites at 1.5 years of age, revealed increasing levels of CCL22 from birth until that time point.\textsuperscript{56}

There are both limitations and strengths to the present study. We evaluated circulating chemokine levels but did not have the opportunity to evaluate functional aspects of the same mediators in different tissues. This would have added mechanistic insights into the findings presented here. Also, the generalisability of these data may be limited, as children in the cohort originate from the Greater Manchester region, with rather homogenous populations. A strength of this study includes the substantial sample size, as few studies have surveyed circulating chemokines at this magnitude. Furthermore, the consistency of the methodologies used compared to previous studies provides another advantage. Moreover, by performing both cross-sectional logistic regression and longitudinal GEE models we have taken into account different temporal perspectives throughout childhood, which is a strength of this study.

**Conclusions**

In conclusion, we have shown that elevated levels of CCL18 throughout childhood foreshadow the development of asthma and sensitisation, findings that remained solid longitudinally. The Th1-associated chemokines CXCL10 and CXCL11 also predicted development of sensitisation and asthma, with differential regulation at different time points in life. This motivates further investigations of
chemokines as biomarkers for allergy development, with putative clinical utility in the prediction of allergic outcomes.

**Abbreviations**

CB
cord blood
CCL17
C-C motif chemokine ligand 17
CCL18
C-C motif chemokine ligand 18
CCL22
C-C motif chemokine ligand 22
CCR4
C-C motif chemokine receptor 4
CCR8
C-C motif chemokine receptor 8
CI
confidence interval
CRD
component resolved diagnostics
CXCL10
C-X-C motif chemokine ligand 10
CXCL11
C-X-C motif chemokine ligand 11
CXCR3
C-X-C motif chemokine receptor 3
GEE
generalised estimating equations
IFN-γ
Interferon-γ
IgE
immunoglobulin E
IL
interleukin
MAAS
Manchester Asthma and Allergy Study
OR
odds ratio
SPT
skin prick test
Th cells
T helper cells
Treg
regulatory T cells

**Declarations**

*Ethics approval and consent to participate*

Informed consent was provided in written form by parents or legal guardians, and the study was approved by the South Manchester Local Research Ethics Committee. Ethical approvals for this study compose the start of the study (including the 1 year follow-up, Dnr ERP/94/032), the amendment for cord blood analyses (ERP/95/137) and the follow-up at age 8 years (Dnr 03/SM/400).

*Consent for publication*

Not applicable

*Availability of data and materials*

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

*Competing interests*

AS has received grants from MRC and Manchester Biomedical Research centre during conduct of the study. CSM reports lecture fees from GSK, Novartis, Astra Zeneca and Thermo Fisher Scientific. AC has received consultancy and/or speaker fees from Novartis, Thermo Fisher Scientific, Philips, Sanofi and Stallergenes Greer. JH, SH and MCJ report no conflicts of interest.

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None of the above-mentioned supporting funders have neither been involved in the sample collection, planning, execution and interpretation of the data, nor in the preparation of this manuscript.
Author’s contributions

AS, CSM and AC designed the study and were responsible for sample collection and clinical evaluation within the MAAS study. AC and MJ designed the experimental work in this paper. JH performed the experimental work. JH and SH performed statistical analyses. JH presented the data and drafted the manuscript. All authors interpreted and discussed the results. All authors contributed to and approved the final draft for publication.

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Author’s information

Not applicable

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**Figures**
Figure 1

Circulating levels of chemokines from children in the MAAS cohort. Plasma concentrations of A. CCL17, B. CCL22, C. CCL18, D. CXCL10 and E. CXCL11 were measured by means of Luminex and ELISA methodology at birth (in cord blood), age 1 and age 8. The data are displayed as medians with interquartile ranges. Statistical differences were ascertained using a Kruskal-Wallis test with a Dunn's post hoc test for multiple comparisons. *** p < 0.001. CB – cord blood.
Figure 2

Associations of the chemokines CXCL10 and CCL18 to asthma development. Plasma concentrations of the Th1-associated chemokine CXCL10 are displayed in relation to development of asthma at age 1 in A and age 8 in B. Circulating levels of the Th2/Treg-associated chemokine CCL18 in relation to asthma development at age 8 and 16 years are illustrated in C and D, respectively. Asthma was defined as fulfilling at least two out of three criteria at the investigated time point: current wheeze, current use of asthma medication, or physician-diagnosed asthma. The data are presented as medians with interquartile ranges. Mann-Whitney U tests were performed to survey statistical signicance. * p < 0.05, *** p < 0.001. CB – cord blood.
Figure 3

Associations of the chemokines CXCL10 and CCL18 to allergic sensitisation. Plasma concentrations of the Th1-associated chemokine CXCL10 are displayed in relation to development of sensitisation, at age 1 in A and age 16 years in B. Circulating levels of the Th2/Treg-associated chemokine CCL18 in relation to sensitisation at age 1, 8 and 16 years are illustrated in C, D and E, respectively. Sensitisation status was determined by means of skin prick testing. The data are displayed as medians with interquartile ranges. Mann-Whitney U tests were performed. * p < 0.05, ** p < 0.01. CB – cord blood.
Figure 4

Forest plots of odds ratios from logistic regression models on predicting allergy development from circulating chemokine levels. Probabilities of developing asthma as depicted in A and sensitisation as illustrated in B from cross-sectional adjusted logistic regression models including all chemokines and correction for the confounding factors sex, parental atopy and parental smoking. Odds ratios are denoted with crosses and the corresponding values below them, and 95% confidence intervals are illustrated by the error bars. All displayed models have an adjusted p-value of <0.05.
Figure 5

Forest plot of odds ratios from generalised estimation equation (GEE) models predicting longitudinal allergy development from the measured chemokines. In the GEE-models, the predictive ability of the chemokine CCL18 on longitudinal development of asthma at ages 5-16 years and 8-16 years, as well as sensitisation at 3-16 years and 8-16 years of age, was examined. Odds ratios are denoted with crosses and the corresponding values below them, and 95% confidence intervals are illustrated by the error bars. All displayed models revealed an adjusted p-value of <0.05.
Figure 6

Associations of CCL18 levels at age 8 to previously machine learning derived clusters of allergy outcomes from the MAAS cohort. Panel A displays sensitisation clusters, B CRD IgE clusters, C atopic diseases clusters and D exacerbations clusters. The data are presented as medians with interquartile ranges. A Kruskal-Wallis test with Dunn's post hoc test for multiple comparisons was performed. * p < 0.05, ** p < 0.01. CRD – component resolved diagnostics.

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