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# Cytokine levels in children and adults with wheezing and asthma show specific patterns of variability over time

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## Summary

**Levels of cytokines are used for in-depth characterization of patients with asthma; however, the variability over time might be a critical confounder. To analyze the course of serum cytokines in children, adolescents and adults with asthma and in healthy controls and to propose statistical methods to control for seasonal effects. Of 532 screened subjects, 514 (91·5%) were included in the All Age Asthma Cohort (ALLIANCE). The cohort included 279 children with either recurrent wheezing bronchitis (more than two episodes) or doctor-diagnosed asthma, 75 healthy controls, 150 adult asthmatics and 31 adult healthy controls. Blood samples were collected and 25 μl serum was used for analysis with the Bio-Plex Pr human cytokine 27-Plex assay. Mean age, body mass index and gender in the three groups of wheezers, asthmatic children and adult asthmatics were comparable to healthy controls. Wheezers (34·5%), asthmatic children (78·7%) and adult asthmatics (62·8%) were significantly more often sensitized compared to controls (4·5, 22 and 22·6%, respectively). Considering the entire cohort, interleukin (IL)-1ra, IL-4, IL-9, IL-17, macrophage inflammatory protein (MIP)-1-$\alpha$ and tumor necrosis factor (TNF)-$\alpha$ showed seasonal variability, whereas IL-1β, IL-7, IL-8, IL-13, eotaxin, granulocyte colony-stimulating factor (G-CSF), interferon gamma-induced protein (IP)-10, MIP-1β and platelet-derived growth factor (PDGF)-BB did not. Significant differences between wheezers/asthmatics and healthy controls were observed for IL-17 and PDGF-BB, which remained stable after adjustment for the seasonality of IL-17. Seasonality has a significant impact on serum cytokine levels in patients with asthma. Because endotyping has achieved clinical importance to guide individualized patient-tailored therapy, it is important to account for seasonal effects.**

**Keywords:** adults, asthma, cytokines, season: variability, wheeze: children

## Introduction

Asthma is increasingly recognized as an umbrella term of a complex syndrome characterized by variable airflow limitation, chronic inflammation and respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough [1]. In addition to heterogeneous clinical manifestations comprising fluctuating pulmonary symptoms, as well as different cellular and molecular patterns, the pathophysiological and clinical properties vary over time and intensity [2]. Asthma symptoms can start early in life, with nearly every third child having at least one episode of wheeze before their third birthday [3]. While many of these children become symptom-free during school age, some progress to persistent asthma. Similarly, asthma is also newly diagnosed in a number of adolescents and adults, with epidemiological studies now indicating that new-incident asthma can even occur in patients older than 60 years, accompanied by a significantly higher rate of co-morbidities, mortality and medical costs [4].

While chronic but reversible airway obstruction of different magnitude is a diagnostic feature of asthma, early wheeze at any age and inflammatory patterns of the airways are highly diverse [5]. Assessment of cytokine levels in serum, supernatants of cell cultures, induced sputum and nasal or bronchoalveolar lavage has become increasingly important for a clearer understanding of asthma endotypes, disease course, pathophysiology and response to treatment. Type 2 (T2) inflammation is an important molecular mechanism in a substantial number of asthmatics [5]. During the last two decades various monoclonal antibodies [i.e. anti-interleukin (IL)-5 antibody, anti-IL-5r antibody, anti-IL-4/IL-13R antibody] have been developed and evaluated in large clinical trials for a personalized treatment approach [6]. However, a significant subgroup of asthmatics has severe symptoms but a T2-low inflammation pattern, and do not respond to monoclonal antibodies directed against IL-5 or IL-4/IL-13. Therefore, the characterization of different asthma phenotypes and endotypes in cohorts of asthmatics is still a challenge. Recently, Brown and colleagues enrolled 235 asthmatic children and quantified 38 serum cytokine levels using a multiplex assay. They identified chemokine (C-X-C motif) ligand (CXCL)-1, IL-5, IL-8 and IL-17 in inner-city African American children, which were positively associated with 'easy-to-control' asthma [7]. In adults with atopic asthma, elevated T2 cytokine responses (IL-4, IL-5, IL-13) to allergenic and antigenic stimuli were observed compared to non-atopic asthmatics [8]. In the Unbiased BIOmarkers in PREDiction of respiratory disease outcomes (U-BIOPRED) project, Kuo *et al.* [9] identified three transcriptome-associated clusters (TACs) analyzing sputum cell transcriptomics from moderate-to-severe asthmatic subjects and healthy controls: TAC 1 comprises an IL-13/T helper (Th) type 2-high predominantly eosinophilic cluster, while TAC 2 [associated with interferon (IFN)/tumor necrosis factor-α (TNF-α)/inflammasome] and TAC 3 are non-Th2 phenotypes. Eosinophilic asthma was predominantly associated with TAC 1 but also present in TAC 3, whereas neutrophilic asthma was mainly present with TAC 2.

All these examples illustrate the importance of cytokine measurements to characterize different asthma endotypes. Generally, in all these cohort studies, asthmatic patients are recruited at different time-points over a distinct period. Therefore, variability over time might be a critical confounder in all these studies. To date, there has been no specification of seasonal changes in serum cytokine patterns in asthmatics or healthy controls. However, looking at a high number of different cytokines in multiplex assays, as made possible by multiplex assays, the issue of variability over time becomes even more important, as different cytokines might have different patterns of seasonal variability. Therefore, we analyzed the course of serum cytokines in children, adolescents and adults with a doctor's diagnosis of asthma and in healthy controls separately for each cytokine in our All Age Asthma Age Cohort (ALLIANCE) [10]. We applied statistical measures to control for seasonal effects and revealed that seasonality might have a relevant impact on several cytokines to be taken into account for the interpretation of specific serum cytokines in patients with asthma.

## Patients and methods

### Study population

Of the 532 subjects screened for eligibility, 514 (91·5%) were included in the ALLIANCE cohort, and blood samples were taken for subsequent cytokine analyses (Fig. 1). A total of 279 children with either recurrent wheezing bronchitis (more than two episodes in the past) or doctor-diagnosed asthma based on the current Global Initiative for Asthma (GINA) guidelines [1] and 75 healthy control children were recruited at three study sites in Hannover, Lübeck and Munich, Germany from 2013 to 2016 for the pediatric arm of ALLIANCE. The adult arm of this cohort comprised 150 asthmatics and 31 healthy controls and was recruited in Grosshansdorf and Borstel, Germany. Details of the study design as well as the inclusion and exclusion criteria are given in the Appendix and in detail in Fuchs *et al.* [10]. Healthy controls were defined as children or adults without wheeze or asthma, applying the same inclusion and exclusion criteria as cases. Healthy controls and asthmatics with fever (> 38·5°C) or signs of upper or lower respiratory tract infection during the last 2 weeks prior to the planned visit were excluded. Physical examination, questionnaires (assessment of health conditions, respiratory and atopic symptoms, infections, medication, socio-demographic data and environmental

exposures), lung function tests [forced expiratory volume in 1 s ($FEV_1$)] and blood tests were performed. To achieve comparability across the cohort arms and study centers, standard operating procedures (SOPs) for each procedure were introduced [10]. The study was approved by the local ethics committees and are registered at clinicaltrials.gov (pediatric arm: NCT02496468; adult arm: NCT02419274).

### Blood collection and analysis

Whole blood was collected by venipuncture (S-Monovette, Sarstedt, Germany) at the study visits. Total numbers of eosinophils and neutrophils were determined by the product of the differential and total cell count. We used 25 µl of serum, which was isolated by centrifugation (2000 ***g***, 10 min) and stored at −80°C until the analysis with the Bio-Plex Pro human cytokine 27-Plex assay (Biorad, Feldkirchen, Germany; lot/cn #M500KCAF0Y). The medical laboratory assistant was present during the blood sampling procedure. After blood-drawing, processing of the sample with the centrifugation and freezing procedure started immediately. There were no cycles of freeze/thaw before analyzing our serum samples. Plotting cytokine levels *versus* storage time revealed no trend of activity loss of one of the tested cytokines (data not shown). The assay was performed following the manufacturer's protocol, with a sample dilution of 1 : 4, a standard curve and a blank value. Instrument calibration and verification runs were applied before each measurement using the MAGPIX calibration kit (Biorad; lot/cn 171213001) and the MAGPIX Verification Kit (Biorad; lot/cn 171213002). The measurements were achieved using the Bio-Plex MAGPIX multiplex reader (Biorad) controlled by the software Bio-Plex Manager MP (Biorad; version 1.0.0.03). Standard curve was fitted for eight standards for each cytokine with known concentrations using Biorad's five-parameter logistic curve with logarithmic variation coefficient weighting, available from the Bio-Plex Manager Software version 6.1 package (Biorad). Samples were measured in duplicate and exported to tab-delimited files for further analysis. Replicate pairs were averaged to produce a single result for analysis. The cytokines included in the analysis were fibroblast growth factor (FGF) basic, eotaxin, granulocyte–colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-γ, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, IFN gamma-induced protein (IP-)10, monocyte chemoattractant protein (MCP)-1 (MCAF), macrophage inflammatory protein (MIP)-1α, MIP-1β, platelet-derived growth factor (PDGF)-BB, regulated upon activation, normal T cell expressed and secreted (RANTES), TNF-α and vascular endothelial growth factor (VEGF).

### Statistical analysis

Because cytokine levels were not normally distributed, Box–Cox transformations were applied before further analyses, leading to log-transformations of the original values. We then compared values of children and adults graphically to evaluate whether the values were in similar ranges so that a pooling of the data would be adequate.

The log-transformed cytokine levels were compared between wheeze/asthma cases and controls in three different generalized linear models (GLMs), accounting for different kinds of seasonal variation. Age was added to all models as an additional covariate.

The first GLM tested group differences without any kind of seasonality. The second approach estimated a cosinor regression model [11]. The cosinor model is commonly applied for detecting seasonality in annual data. It covers a seasonal pattern using a sinusoid and is suitable

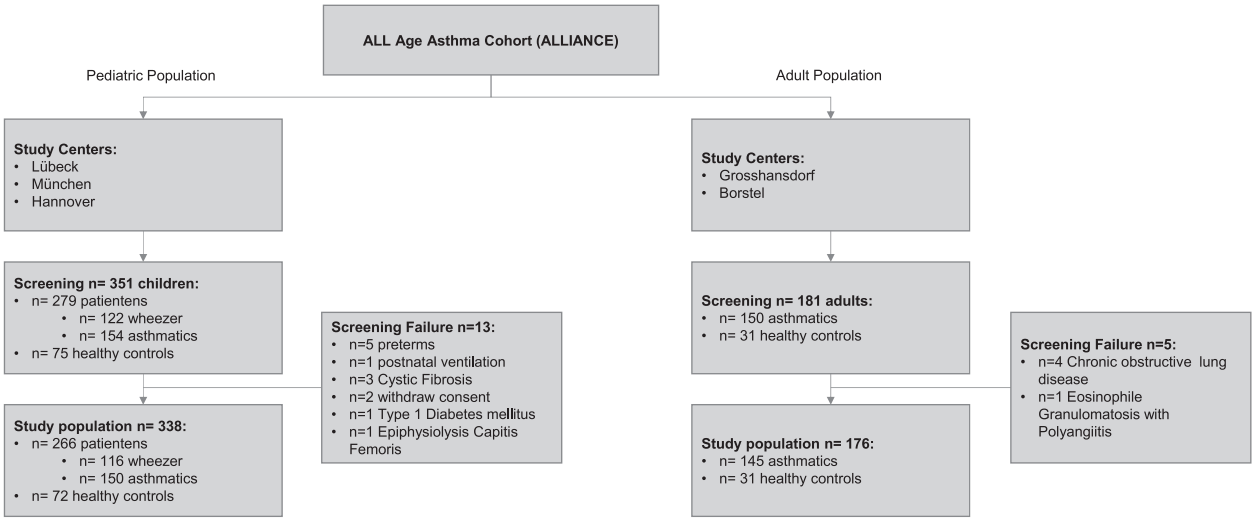


**Fig. 1.** All age asthma study population.

for relatively simple seasonal patterns that are symmetric and stationary. A sinusoid is a mathematical curve describing a smooth periodic oscillation and consists of a sine and cosine term.

A linear representation of this model is given by:

$$Y_t = c \cdot \cos\omega_t + s \cdot \sin\omega_t,$$

where the log-transformed cytokine level $Y_t$, depending on a time variable $t$, was modeled by a linear combination of cos and sin with a fixed frequency ω estimating the standardized time information. From the estimated parameters $c$ and $s$, the amplitude and phase of a sinusoidal function could be derived. At a significance level of $\alpha/2$, $c$ and $s$ were tested to be equal to zero, meaning no presence of a seasonal effect. As the cosinor model is based on a GLM we added age and case–control status as additional covariates in the models showing seasonality. Using this approach we could test for group differences when accounting for age and the seasonality throughout the year with a sinusoidal function, which is the most likely form of a seasonal effect. However, if the seasonal effects are different, the model loses power. We therefore employed a third approach, in which – in contrast to the cosinor regression model – we do not assume a specific form of the seasonality, but merely assume that the cytokine values differ between months [11]. For this, we used GLMs with the log-transformed cytokine levels as dependent variable and case–control status, age and month of measurement as independent categorical variables.

After obtaining the test results of the group differences, we adjusted the *P*-values for multiple testing with the Bonferroni–Holm method. Each *P*-value was adjusted with regard to the number of cytokines tested for that model.

All analyses were performed in R [12]. We used the R package season [13] for estimation of the cosinor model. The Box–Cox transformations were calculated with the R package caret [14].

## Results

Baseline characteristics of the study population are given in Table 1. Basically, children with recurrent wheeze, children and adolescents with asthma and adult asthmatics showed comparable baseline characteristics compared to healthy controls, except for the items allergic sensitization and treatment with inhaled corticosteroids, which were more prevalent in wheezers and asthmatics (Table 1).

Mean values of serum cytokine levels are summarized in Supporting information, Table S1 for the different groups. Plotting log-transformed cytokine levels of children and adults showed that both groups had cytokine levels in a comparable range (Fig. 2). Therefore, we pooled data from children and adults to analyze seasonality and differences between asthmatics and controls.

For in-depth analysis of seasonal effects, only cytokines with at least 80% of the obtained values within the defined detection range of the standard curve of the pertinent cytokine were further explored. Consequently, IL-2, IL-5, IL -6, IL -10, IL -12, IL -15, FGF basics, GM-CSF, IFN-γ, MCP-1, RANTES and VEGF were excluded from further analysis. For the remaining 15 of 27 cytokines, values below or above the limit of quantification were set to the value of the lower or upper limit of quantification.

First, we looked at group differences taking no seasonality but the age into account. IL-17 and PDGF-BB showed significant differences between the case–control status after adjusting for the 15 cytokines for multiple testing (Table 2 and Supporting information, Tables S2 and S3). Next, we analyzed the seasonal variability of serum cytokine levels in our cohort applying the cosinor model (Table 2 and Supporting information, Tables S2 and S3). In total, the model revealed seasonal variability for IL-1ra, IL-4, IL-9, IL-17, MIP-1α and TNF-α. The lowest estimated concentrations were observed during January (IL-4, TNF-α), February (IL-1ra) and March (IL-9, IL-17, MIP-1α), and the highest concentrations during July (TNF-α), August (IL-1ra, IL-4) and September (IL-9, IL-17, MIP-1α). The cytokines IL-1 β, IL-7, IL-8, IL-13, eotaxin, G-CSF, IP-10, MIP-1 β and PDGF-BB exhibited no significant variability over time.

We next analyzed whether the sinusoidal seasonality or any kind of monthly variation influences the group differences. Four potential outcomes might occur: (1) serum cytokine levels differ between groups and correction of seasonality does not alter the results; (2) serum cytokine levels differ between groups, but they disappear after correction for seasonality; (3) serum cytokine levels do not differ between groups, but correction of seasonality reveals group differences; and (4) serum cytokine levels do not differ between groups and correction of seasonality does not alter the results. For IL-17, group differences for asthmatics/wheezers and healthy controls were observed, which did not change after correction for seasonality. Serum cytokine levels of PDGF-BB were significantly higher in asthmatics/wheezers compared to healthy controls. Showing no sinusoidal seasonality, this effect did not disappear when taking the month as categorical variable into account. Interpreting the nominal *P*-values as signals, adjustment for both kinds of seasonal variation revealed group differences in TNF-α and after adjustment for month as categorical variable group differences in IP-10 remained. All other cytokines showed no group differences. When

**Table 1.** Baseline characteristics of study population

| | < 6 years | | | > 6 to < 18 years | | | > 18 years | | |
|---|---|---|---|---|---|---|---|---|---|
| | Wheezer | Healthy controls | *P*-value | Asthmatic children | Healthy controls | *P*-value | Asthmatics | Healthy controls | *P*-value |
| *n* | 116 | 22 | | 150 | 50 | | 145 | 31 | |
| Age (mean ± s.d., years) | 3·1 (±1·5) | 3·7 (±1·6) | 0·186 | 11·3 (±3·2) | 11·1 (±3·1) | 0·684 | 51 (±13·9) | 40·3 (±17·9) | < 0·001 |
| Male gender; *n*, % | 79 (68·1%) | 13 (59%) | 0·761 | 101 (67·3%) | 26 (52%) | 0·075 | 66 (45·5%) | 20 (64%) | 0·085 |
| BMI (mean ± s.d.) | 16·3 (±1·5) | 15·7 (±1·5) | 0·137 | 20·2 (±5·8) | 18·5 (±3·8) | 0·053 | 27·3 (±5·3) | 24·6 (±3·7) | 0·008 |
| Active smoker; *n*, % | 0 | 0 | n.a. | 2 (1.3%) | 0 | n.a. | 12 (8·3%) | 0 (0%) | 0·205 |
| Allergic sensitization, *n*, % | 40 (34·5%) | 1 (4·5%) | 0·013 | 118 (78·7%) | 11 (22·0%) | < 0·001 | 91 (62·8%) | 7 (22·6%) | < 0·001 |
| ICS and steroid-naive; *n*, % | 51 (44·0%) | n·a· | n·a· | 36 (17·5%) | n.a. | n.a. | 17 (11.7%) | n.a. | n.a. |
| OCS; *n*, % | 0 | n.a. | n.a. | 0 | n.a. | n.a. | 25 (17.2%) | n.a. | n.a. |
| $FEV_1$ (l; mean ± s.d.) | 1·04 (±0·21) | 1·08 (±0·17) | 0·645 | 2·32 (±0·83) | 2·5 (±0·84) | 0·192 | 2·56 (±0·85) | 3·90 (±0·83) | < 0·001 |
| FeNO (ppb; mean ± s.d.) | 18·5 (±3·1) | 7·0 (±3·5) | 0·104 | 29·0 (±30·6) | 12·9 (±5·4) | 0·002 | 30·8 (±24·9) | 15·5 (±5·7) | 0·001 |
| Eosinophils (% leukocyte counts) | 4·9 (±4·6) | 2·9 (±1·3) | 0·051 | 6·4 (± 4·4) | 4·0 (±4·0) | 0·002 | 4·6 (±3·2) | 2·6 (±1·5) | 0·001 |
| Neutrophils (% leukocyte counts) | 37·6 (±11·0) | 41·5 (±12·3) | 0·150 | 46·7 (±11·2) | 50·6 (±8·9) | 0·037 | 59·9 (±10·3) | 53·6 (±8·6) | 0·002 |

*P*-values of two-sided *t*-test for continuous data and $\chi^2$ test for categorical data.

ICS = inhalative corticosteroids; s.d. = standard deviation; OCS = oral corticosteroids; BMI = body mass index; $FEV_1$ = forced expiratory volume in 1 s; n.a. = not applicable.

adjusting for any kind of seasonality, these results did not alter.

An overview of seasonal effects and group differences of the tested cytokines with > 80% values within the quantification limit is given in Table 2. The full results of the GLMs are shown in Supporting information, Tables S2 and S3. Exemplary presentation of the monthly course of cytokine levels is given in Fig. 3 for IL-4 and IL-17.

## Discussion

Cytokines are cell-signaling proteins that initiate and modulate various immune responses. Quantification of cytokines in serum, supernatants of cell cultures, induced sputum and bronchoalveolar lavage has decisively improved our insight in asthma pathophysiology. We showed that serum cytokine levels of asthmatic children, adolescents and adults as well as healthy controls are critically influenced by seasonality. Moreover, adjusting for seasonal effects critically might affect the correct allocation of group differences between wheezers/asthmatics and healthy controls. Because endotyping has gained clinical importance to guide patient-tailored therapy, it is important to apply statistical methods to control for seasonal effects.

The unique and novel approach from our cohort is that we have obtained a standardized and detailed collection of epidemiological and clinical data as well as molecular deep phenotyping of a comprehensive range of biomaterials in a considerable number of study participants of all ages. Of note, the ALLIANCE Cohort of the German Center for Lung Research (DZL) includes preschool children aged as young as 6 months to adults beyond the age of 70 years [10]. This approach offers a unique framework not only to assess the potential role of molecular markers for distinct asthma endotypes in a prospective fashion, but also to disentangle confounding variables.

Information considering natural variability of potential biomarkers in asthmatics is sparse. As well as genetic factors, cytokine levels might be additionally influenced by age [15], gender [16], body weight [17] and environmental variables such as seasonality. It is worthy of note that there are few studies regarding seasonal effects in patients with asthma or allergy.

Recently, Spath and colleagues aimed to study chronobiological variations of the circulating cytokines in patients affected by primary chronic venous insufficiency (CVD) [18]. Cytokines of 17 patients in the autumn group and 15 subjects in the spring group were analyzed. Significant seasonal differences were observed for 11 of 22 proinflammatory analytes. In line with these results, TNF-α and MIP-1α also showed significant seasonal variability in our cohort while, in contrast, eotaxin, G-CSF and PDGF-BB

appeared not to be influenced by seasonality in our cohort or were not analyzed due to < 80% of values within our detection range. Different findings between our cohort and the data of patients with CVD might be explained by the relatively small number of patients analyzed by Spath *et al.* and in differences of the underlying disease. Cytokines may have pleiotropic effects, which are a prime impetus for pathophysiological changes in different diseases. As such, eotaxin is a powerful driver of eosinophilic inflammation and T2 shift but is also important for ischemia-induced remodeling of vessel walls [19]. Similarly, IL-8 is a key molecule for chemotaxis of neutrophils but is also a determining factor in promoting angiogenesis and is increased as a result of oxidative stress [20]. These aspects suggest that seasonal effects observed in a specific group of patients might be confounded by disease status as well as by disease-specific effects.

With regard to serially performed tuberculin skin tests, plasma samples from people with discordant results were recently multiplexed to determine the association between circulating cytokines and antigen-stimulated IFN-γ levels [21]. The authors reported a seasonal effect, with up-regulation of IL-1β and IL-3 during spring, and this up-regulation was observed with the amount of IFN-γ measured in discordant results [21]. Beyond that, the presence of annual seasonality was recently demonstrated in gene expression profiles of human immune cells in healthy subjects [22]. Based on these results, Ter Horst and colleagues analyzed the effect of environmental factors on the measured cytokine responses in humans [23]. Similar to our results, TNF-α and IL-1β were reported to be influenced by season. Moreover, Ter Horst *et al.* revealed that age is a critical confounder for cytokine levels and measured higher concentrations of IL-6 and IL-1ra in

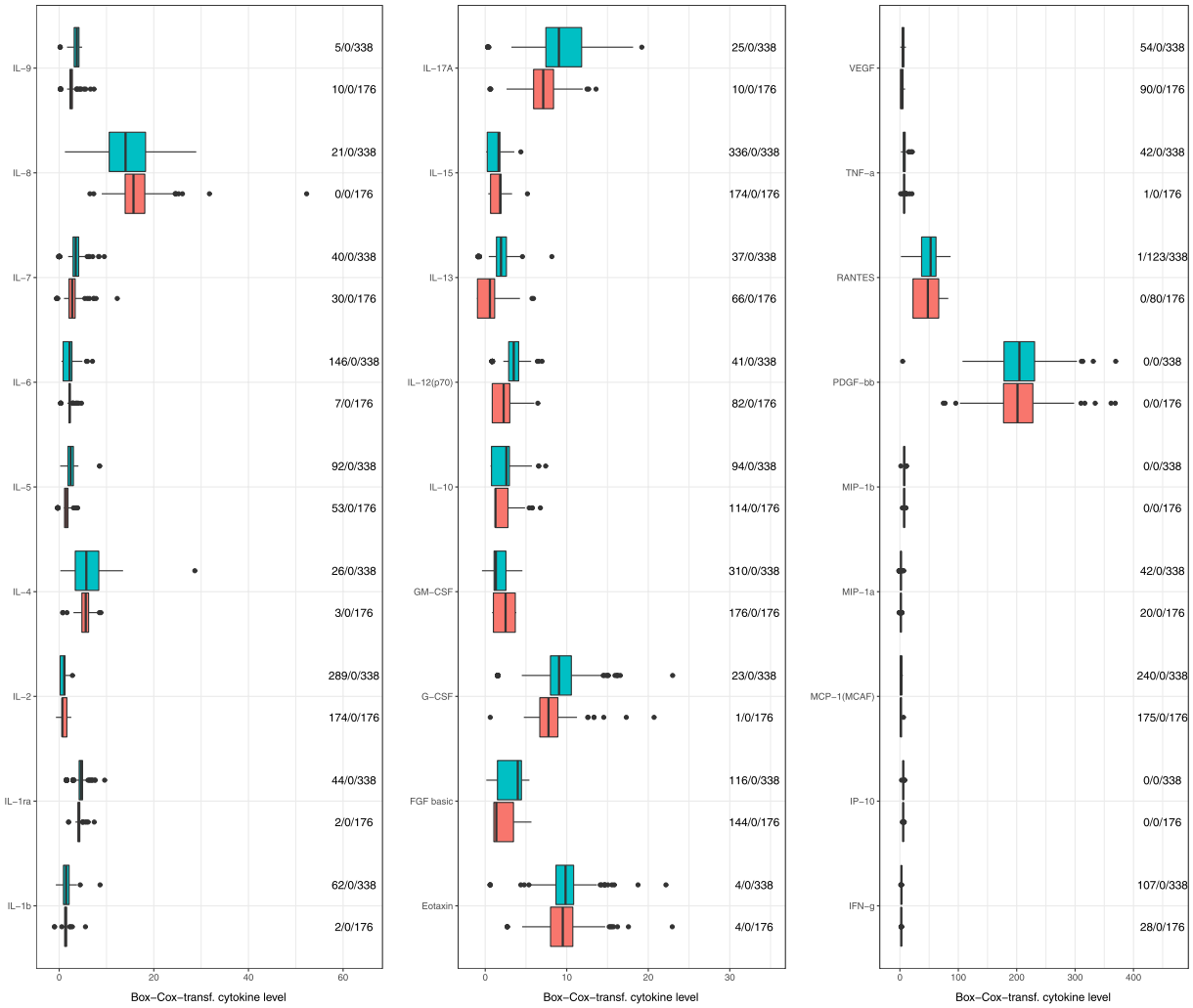


**Fig. 2.** Log-transformed cytokine levels of the pediatric (green) and adult part (red) of the cohort. Boxes show the median, 25th and 75th percentiles; bars indicate the range. Numbers in the right column indicate number of values below the lower limit of quantification(LOQ) /values above the upper LOQ and total samples in children and adults.

**Table 2.** Overview of the tested cytokine levels with > 80% values within the quantification limits. Four different GLMs, all accounting for age, were applied: (a) testing group difference without adjusting for seasonality; (b) testing for sinusoidal seasonality; (c) testing group difference accounting for sinusoidal seasonality; (d) testing group difference accounting for any monthly variation. y(es) (marked green) and n(o) mean that the *P*-value that is adjusted for multiple testing is less or higher than the significance level $\alpha = 0 \cdot 05$ Season (marked green) and no (marked red) season mean that the *P*-value of one of the cosinor coefficients is less than the significance level $\alpha = 0 \cdot 025$.

| | (a) | (b) | (c) | (d) |
|---|---|---|---|---|
| Statistical model | GLM case–control + age | GLM age + cosinor | GLM case-control + age + cosinor | GLM case-control + age + month |
| Description | Testing group difference | Testing for sinusoidal seasonality | Testing group difference accounting for sinusoidal seasonality | Testing group difference accounting for any monthly variation |
| Interleukins | | | | |
| IL-1β | N | No season | N | N |
| IL 1ra | N | Season | N | N |
| IL-4 | N | Season | N | N |
| IL-7 | N | No season | NA | N |
| IL-9 | N | Season | N | N |
| IL-13 | N | No season | NA | N |
| IL-17 | Y | Season | Y | Y |
| Tumor necrosis factor | | | | |
| TNF-α | N | Season | N | N |
| Growth factors | | | | |
| G-CSF | N | No season | n.a. | N |
| PDGF-BB | Y | No season | NA | Y |
| Chemokines | | | | |
| MIP-1α | N | Season | N | N |
| MIP-1β | N | No season | n.a. | N |
| IL-8 | N | No season | n.a. | N |
| Eotaxin | N | No season | n.a. | N |
| IP-10 | N | No season | n.a. | N |

IL = interleukin; G-CSF = granulocyte-colony stimulating factor; IP-10 = interferon gamma-induced protein 10; MIP = macrophage inflammatory protein; PDGF = platelet-derived growth factor; TNF = tumor necrosis factor; VEGF = vascular endothelial growth factor.

elderly patients. To date, data on age-dependent changes in immune responses are conflicting. While impaired TLR-induced cytokine responses in dendritic cells were reported as a consequence of aging [24], Janssen *et al.* have not observed age-dependent effects [25]. Our study was not designed to elucidate age-dependent effects and the age groups of our patients were not evenly distributed. However, cytokine levels of children and adults showed comparable results, so we combined both groups for further analysis. Looking specifically at IL-6 and TNF-α, cytokine values in children were not lower compared to adults but showed a broader range. Of note, all calculated models were controlled for age as a potential confounder.

The variability of cytokine levels over time might be influenced by several factors. In patients with allergic sensitizations, seasonal effects might be also triggered by pollen counts in the ambient air, as reported in several studies. Ulmer and colleagues reported seasonal effects of eosinophilic cationic protein (ECP), with a significantly higher increase in sensitized children during pollination compared to healthy controls [26]. As ECP is highly correlated with eosinophil counts and eosinophil differentiation as well as activation *per se* is regulated by IL-5 and IL-13, these data suggest that pollen count might also account for seasonal effects on cytokine level. In a small group of 30 asthmatics, Batmaz *et al.* observed seasonal variability of vitamin D levels, $FEV_1$ and the asthma control test score, with low values during winter compared to the summer season [27]. Of note, vitamin D showed a positive correlation with regulatory T cell ($T_{reg}$) counts and a negative correlation with T2 type cytokines. Therefore, it is possible that the observed variability for certain cytokines might, at least in part, be attributable to seasonal variability of vitamin D.

One weakness of our trial is that we did not measure vitamin D levels in our cohort. While the most important source of vitamin D is endogenous skin synthesis following sunlight exposure, vitamin D levels show a seasonal variation with peak values during the summer months [27–29]. There is a complex relationship between vitamin D insufficiency and the pathology of asthma, with increasing evidence that vitamin D modulates diverse immunological pathways in different asthma endotypes. That is, vitamin D alleviates the inflammatory response

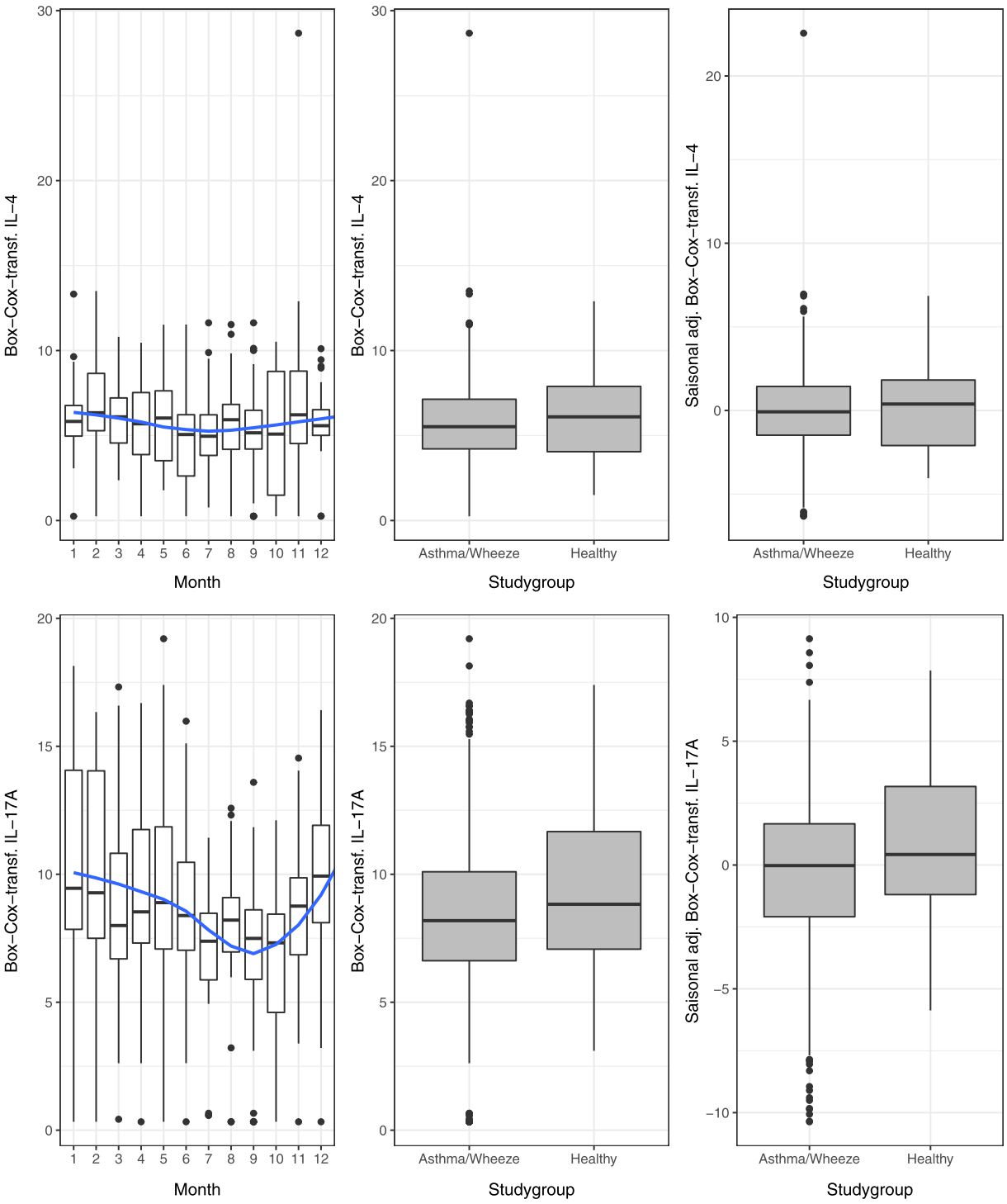


**Fig. 3.** Exemplary presentation of the monthly course of cytokine levels. The cosinor model revealed a seasonal variability, i.e. for the cytokines interleukin (IL)-4 and IL-17. No differences were observed between asthmatics and controls for IL-4, while IL-17 was significantly lower in wheezers/asthmatics. Boxes show the median, 25th and 75th percentiles; bars indicate the range.

in asthmatics by up-regulating the number of forkhead box protein 3 $(FoxP3)^+$ regulatory T lymphocytes and IL-10 expression in asthmatics [30,31]; it suppresses the immunoglobulin (Ig)E production of B lymphocytes; suppresses mast cell activation [32]; promotes tolerogenic dendritic cells [33]; and stimulates bronchial epithelial

cells to produce soluble ST2, a decoy blocker for the inflammatory cytokine IL-33 [34]. In contrast to this substantial evidence, clinical trials showed contradictory results. While some trials reported preventive [35] or even therapeutic effects [36] of vitamin D supplementation, others did not [37,38]. However, this is not surprising, as these trials varied greatly in clinical design. A recently published meta-analysis showed that vitamin D supplementation reduced the rate of asthma exacerbations requiring treatment with systemic corticosteroids [39]. Taken together, the obvious effect of vitamin D on various immunological parameters reveals that information regarding vitamin D levels would greatly add to our data and would allow better classification of our findings. Further studies addressing seasonal variability of immunological parameters should, therefore, include this parameter in the analysis.

During the past decade, the role of IL-17 family of cytokines has focused upon asthma pathophysiology. IL-17 maintains epithelial integrity and responds to microbial stimuli through the recruitment of neutrophils and the release of anti-microbial peptides [40]. IL-17 induces several cytokines, i.e. IL-6 and G-CSF, as well as chemokines, including CXCL1, CXCL2 and CXCL8 (IL-8), promoting myeloid-driven inflammation [40]. Several groups reported an association between IL-17 and human asthma. While some authors reported elevated IL-17$^+$ cells in bronchoalveolar lavage fluid or sputum, which correlated with neutrophil inflammation, disease severity or bronchial hyper-reactivity [7,41–43], these results were not replicated consistently. Other authors reported no evidence of increased IL-17A protein in asthmatics in serum, sputum or bronchoalveolar lavage (BAL) [44]. We observed no significant differences in serum cytokine levels of IL-17 in children, adolescents and adults. In contrast, children below the age of six with recurrent wheeze showed significant lower IL-17 serum levels compared to healthy controls. The reason for the inconsistency in the literature and the discrepancy in our results might be explained by the different pathophysiology of early wheeze and adolescent or adult asthma. Moreover, serum cytokine levels might not be the best proxy for bronchial cytokine levels. Finally, IL-17 might be differentially regulated in different asthma endotypes. To date, it obviously remains unclear whether IL-17 is causing asthma pathology in a subset of asthma patients or whether it is present as a response to some externally mediated epithelial stimuli – i.e. acute viral infection or bacterial colonization – related to the underlying asthma [40].

Our study has several strengths and limitations. A strength of our approach is that we have obtained data for children and adults with a wide age range from early childhood until late adulthood. Moreover, we have confirmed our findings by several statistical models addressing seasonal effects over time. A limitation of our study is that the results are only conclusive for serum cytokine measurements. We assume that cytokine levels in cell supernatants or in nasal or bronchial lavage fluid show also seasonal fluctuations, but our results cannot be extrapolated to other biomaterial samples. Therefore, we recommend that potential effects of seasonality should be considered separately for different biomaterials and statistically controlled in further studies. Even though we have included a reasonably large cohort, breaking down our sample analysis to different age groups in different months reduces the number of patients during certain periods. Therefore, our results should be interpreted with caution, and reproduction in larger cohorts is clearly necessary. The cytokine levels in the sera of asthmatics and healthy controls can be additionally influenced by external factors, i.e. acute respiratory illness caused by viral infections. To minimize this potential source of bias we excluded patients with symptoms of acute respiratory infection (i.e. cough, runny nose) during the last 2 weeks prior to our study visit. Moreover, acute fever within a period of 2 weeks prior to the study visit was an exclusion criterion. Finally, disease status and asthma medication might also influence the seasonal variability of cytokine levels over time. This might be notably important for the group of adult asthmatics taking OCS ($n$ = 17, 11%). However, stratifying our analysis for these additional factors would lead to smaller groups, which could undermine precise assessment of seasonality.

Collectively, cytokine analysis in asthmatics may contribute to the identification of certain endotypes and might help us identifying, for example, difficult-to-control asthma in different populations. As endotyping will obtain increasing clinical importance guiding a patient-tailored therapy, accurate analysis of seasonal effects for potential biomarkers is mandatory. Moreover, it is essential to review appropriate statistical methods to control for seasonal effects in cohorts who are recruited over a prolonged period.

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## Data Availability Statement

Data are available for members of the ALLIANCE cohort and for non-members with a granted proposal. We used the core dataset V3_0 and serum cytokine data set V2_0 from the pediatric arm of the cohort and the baseline data set V2_0 and serum cytokine data set V2_0 from the adult arm for the analysis.

## References

1 Bateman ED, Becker A, Boulet LP *et al*. A summary of the new GINA strategy: a roadmap to asthma control. Eur Respir J 2015; **46**:622–39.

2 Hekking PP, Bel EH. Developing and emerging clinical asthma phenotypes. J Allergy Clin Immunol Pract 2014; **2**:671–80.

3 Spycher BD, Cochrane C, Granell R *et al*. Temporal stability of multitrigger and episodic viral wheeze in early childhood. Eur Respir J 2017; **50**:pii:1700014.

4 Yáñez A, Cho SH, Soriano JB *et al*. Asthma in the elderly: what we know and what we have yet to know. World Allergy Organ J 2014; **7**:8.

5 Fahy JV. Type 2 inflammation in asthma – present in most, absent in many. Nat Rev Immunol 2015; **15**:57–65.

6 Patel TR, Sur S. IgE and eosinophils as therapeutic targets in asthma. Curr Opin Allergy Clin Immunol 2017; **17**:42–9.

7 Brown KR, Krouse RZ, Calatroni A *et al*. Endotypes of difficult-to-control asthma in inner-city African American children. PLOS ONE 2017; **12**:e0180778.

8 Kakumanu S, Jaffee K, Visness CM *et al*. The influence of atopy and asthma on immune responses in inner-city adults. Immun Inflamm Dis 2016; **4**:80–90.

9 Kuo C, Pavlidis S, Loza M *et al*., U-BIOPRED Study Group. T-helper cell type 2 (Th2) and non-Th2 molecular phenotypes of asthma using sputum transcriptomics in U-BIOPRED. Eur Respir J 2017; **49**:1602135.

10 Fuchs O, Bahmer T, Weckmann M *et al*., ALLIANCE Study Group as part of the German Centre for Lung Research (DZL). The all age asthma cohort (ALLIANCE) – from early beginnings to chronic disease: a longitudinal cohort study. BMC Pulm Med 2018; **18**:140.

11 Barnett AG, Dobson AJ. Analysing Seasonal Health Data. Berlin/Heidelberg, Germany; Springer, 2010.

12 R Core Team. R: a language and environment for statistical computing, version 3.6.0. Vienna, Austria: R Foundation for Statistical Computing, 2020.

13 Barnett AG, Baker PJ, Dobson AJ. season: Analysing seasonal data R functions. R package version 0.3.8. Available at: https://CRAN.R-project.org/package=season (accessed 30 September 2007).

14 Kuhn M.caret: Classification and Regression Training. R package version 6.0-81. 2018. https://CRAN.R-project.org/package=caret (accessed 30 September 2007).

15 Bernstein ED, Murasko DM. Effect of age on cytokine production in humans. Age 1998; **21**:137–51.

16 Aulock SV, Deininger S, Draing C, Gueinzius K, Dehus O, Hermann C. Gender difference in cytokine secretion on immune stimulation with LPS and LTA. J Interferon Cytokine Res 2006; **26**:887–92.

17 Nielsen NO, Soborg B, Børresen M, Andersson M, Koch A. Cytokine responses in relation to age, gender, body mass index, *Mycobacterium tuberculosis* infection, and otitis media among Inuit in Greenland. Am J Hum Biol 2013; **25**:20–8.

18 Spath P, Tisato V, Gianesini S *et al*. The calendar of cytokines: seasonal variation of circulating cytokines in chronic venous insufficiency. J Roy Soc Med Cardiovasc Dis 2017; **6**:2048004017729279.

19 Gallegos AM, Bevan MJ. Driven to autoimmunity: the NOD mouse. Cell 2004; **117**:149–51.

20 Vlahopoulos S, Boldogh I, Casola A *et al*. Nuclear factor-kappa B-dependent induction of interleukin-8 gene expression by tumor necrosis factor alpha: evidence for an antioxidant sensitive activating pathway distinct from nuclear translocation. Blood 1999; **94**:1878–89.

21 Griffin ML, Agarwal S, Murphy MZ, Teeter LD, Graviss EA. Influence of seasonality and circulating cytokines on serial QuantiFERON discordances. Tuberc Res Treat 2018. https://doi.org/10.1155/2018/673207.

22 De Jong S, Neeleman M, Luykx JJ *et al*. Seasonal changes in gene expression represent cell-type composition in whole blood. Hum Mol Genet 2014; **23**:2721–8.

23 Ter Horst R, Jaeger M, Smeekens SP *et al*. Host and environmental factors influencing individual human cytokine responses. Cell 2016; **167**:1111–24.

24 Panda A, Qian F, Mohanty S *et al*. Age-associated decrease in TLR function in primary human dendritic cells predicts influenza vaccine response. J Immunol 2010; **184**:2518–27.

25 Janssen N, Derhovanessian E, Demuth I, Arnaout F, Steinhagen-Thiessen E, Pawelec G. Responses of dendritic cells to TLR-4 stimulation are maintained in the elderly and resist the effects

of CMV infection seen in the young. J Gerontol A Biol Sci Med Sci 2016; **71**:1117–23.
26 Ulmer C, Kopp MV, Ihorts G, Moseler M, Forster J, Kühr J. Allergische Entzündung der Nasenschleimhaut bei pollensensibilisierten Kindern während natürlicher Pollenexposition. Soz Pädiatr Kinderärztliche Praxis. 1996;**18**:104–110.
27 Batmaz SB, Arıkoğlu T, Tamer L, Eskandari G, Kuyucu S. Seasonal variation of asthma control, lung function tests and allergic inflammation in relation to vitamin D levels: a prospective annual study. Adv Dermatol Allergol 2018; **35**:99–105.
28 Holick MF. Vitamin D deficiency. N Engl J Med 2007; **357**:266–81.
29 Karohl C, Su S, Kumari M *et al*. Heritability and seasonal variability of vitamin D concentrations in male twins. Am J Clin Nutr 2010; **92**:1393–8.
30 Urry Z, Chambers ES, Xystrakis E *et al*. The role of 1alpha, 25-dihydroxyvitamin D3 and cytokines in the promotion of distinct Foxp3(+) and IL-10(+) CD4(+) T cells. Eur J Immunol 2012; **42**:2697–708.
31 Gupta A, Dimeloe S, Richards DF *et al*. Defective IL-10 expression and in vitro steroid-induced IL-17A in paediatric severe therapy-resistant asthma. Thorax 2014; **69**:508–15.
32 Hartmann B, Heine G, Babina M *et al*. Targeting the vitamin D receptor inhibits the B cell-dependent allergic immune response. Allergy 2011; **66**:540–48.
33 Széles L, Keresztes G, Töröcsik D *et al*. 1,25-dihydroxyvitamin D3 is an autonomous regulator of the transcriptional changes leading to a tolerogenic dendritic cell phenotype. J Immunol 2009; **182**:2074–83.
34 Pfeffer PE, Chen YH, Woszczek G *et al*. Vitamin D enhances production of soluble ST2, inhibiting the action of IL-33. J Allergy Clin Immunol 2015; **135**:824–7.
35 Litonjua AA, Carey VJ, Laranjo N *et al*. Effect of prenatal supplementation with vitamin D on asthma or recurrent wheezing in offspring by age 3 years: the VDAART randomized clinical trial. JAMA 2016; **315**:362–70.
36 Tachimoto H, Mezawa H, Segawa T, Akiyama N, Ida H, Urashima M. Improved control of childhood asthma with low-dose, short-term vitamin D supplementation: a randomized, double-blind, placebo-controlled trial. Allergy 2016; **71**:1001–9.
37 Castro M, King TS, Kunselman SJ *et al*., National Heart, Lung, and Blood Institute AsthmaNet. Effect of vitamin D3 on asthma treatment failures in adults with symptomatic asthma and lower vitamin D levels: the VIDA randomized clinical trial. JAMA 2014; **311**:2083–91.
38 Litonjua AA, Carey VJ, Laranjo N *et al*. Six-year follow-up of a trial of antenatal vitamin D for asthma reduction. N Engl J Med 2020; **382**:525–33.
39 Jolliffe DA, Greenberg L, Hooper RL *et al*. Vitamin D supplementation to prevent asthma exacerbations: a systematic review and meta-analysis of individual participant data. Lancet Respir Med 2017; **5**:881–90.
40 McGeachy MJ, Cua DJ, Gaffen SL. The IL-17 family of cytokines in health and disease. Immunity 2019; **50**:892–906.
41 Molet S, Hamid Q, Davoine F *et al*. IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. J Allergy Clin Immunol 2001; **108**:430–8.
42 Barczyk A, Pierzchala W, Sozañska E. Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. Respir Med 2003; **97**:726–33.
43 Al-Ramli W, Préfontaine D, Chouiali F *et al*. T(H)17-associated cytokines (IL-17A and IL-17F) in severe asthma. J Allergy Clin Immunol 2009; **123**:1185–7.
44 Hinks TS, Zhou X, Staples KJ *et al*. Innate and adaptive T cells in asthmatic patients: Relationship to severity and disease mechanisms. J Allergy Clin Immunol 2015; **136**:323–33.

## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

**Table S1.** Mean cytokine concentration [pg/ml] in children and adults with wheeze or asthma and in healthy controls, respectively. Statistical significant differences between the groups (Mann–Whitney U test $p < 0.05$) are marked in green (corrected for multiple testing) and red (not corrected for multiple testing), respectively. bFGF: Basic Fibroblast Growth Factor; G-CSF: Granulocyte-colony stimulating factor; Granulocyte-macrophage colony-stimulating factor; IFN: Interferon; IL: interleukin; IP-10: Interferon gamma-induced protein 10; MCP-1: Monocyte Chemoattractant Protein 1; MIP: Macrophage inflammatory protein; PDGF: Platelet-derived growth factor; RANTES: Regulated upon activation, normal T-cell expressed, and secreted; TNF: Tumor necrosis factor; VEGF: Vascular endothelial growth factor.

**Table S2.** Overview of the tested cytokine associations with > 80% values within the quantification limits. All nominal *P*-values and *P*-values that are adjusted for multiple testing (in brackets) are listed. Four different GLMs were conducted, all accounting for age: A) testing group difference without adjusting for seasonality; B) testing for sinusoidal seasonality; C) testing group difference accounting for sinusoidal seasonality; D) testing group difference accounting for any monthly variation. The p-values were adjusted columnwise with the Bonferroni-Holm method. The GLM (age + cosinor) column lists the smallest p-value of the cosinor coefficients indicating sinusoidal seasonal variation. All adjusted p-values being smaller than the significance level $\alpha = 0.05$ for tests for group differences and $\alpha = 0.025$ for sinusoidal seasonality are marked green. Cytokines showing no sinusoidal seasonality are marked red.

**Table S3.** Overview of the tested cytokine effect estimates with > 80% values within the quantification limits. All estimates for the case-control coefficients and their

95%-confidence intervals are shown. Four different GLMs were conducted, all accounting for age: A) testing group difference without adjusting for seasonality; B) testing for sinusoidal seasonality; C) testing group difference accounting for sinusoidal seasonality; D) testing group difference accounting for any monthly variation. The GLM (age + cosinor) column lists the cosinor coefficient and 95%-confidence interval with the smallest p-value. All effect estimates with a confidence interval that does not include the 0 are marked green. Cytokines showing no sinusoidal seasonality are marked red.

**APPENDIX A**

Inclusion criteria – children:

- informed consent of either parent or caretaker and of the child if aged 8 years or older
- age 6 months to 18 years
- term delivery (≥ 37 weeks)
- active/passive understanding of German.

Exclusion criteria

- known inborn or perinatal pulmonary disease
- pulmonary malformation
- oxygen therapy after birth with a duration of more than 24
- support or mechanical ventilation after birth
- diagnosis of cystic fibrosis; primary ciliary dyskinesia
- heart failure diagnosed after birth affecting pulmonary circulation
- major respiratory diseases such as e.g. interstitial lung disease
- any current non-atopic comorbidities.
- Moreover, children are excluded from study visits and biomaterial collection in the case of fever of at least 38.5 °C during the last 2 weeks prior to the planned visit.

Definition of cases – children:

- either having doctor-diagnosed wheeze during at least 2 occasions during the last 12 months (age < 6 years) ***or***
- having doctor-diagnosed asthma (age ≥ 6 years) with diagnosis according to current guidelines including lung function
- Steroid–/leukotriene receptor antagonist (LTRA)-naivety is defined as no use of inhaled or systemic corticosteroids or LTRA for at least 4 months prior to inclusion.

Definition of controls – children:

- children without wheeze or asthma and otherwise applying the same in- and exclusion criteria as mentioned above.
- Adolescent cases turning 18 years of age will automatically enter transition into the adult arm of ALLIANCE, with identical data and biomaterial collection as prior to transition.

Inclusion criteria – adults:

- informed consent for participants who are newly recruited during adulthood
- age ≥ 18 years
- active/passive understanding of German
- established diagnosis of asthma according to current guidelines

Comment: Participants are allowed to be current or former smokers to avoid significant selection bias. Patients with asthma and a relevant smoking history are accurately screened for features distinguishing asthma from chronic obstructive pulmonary disease (COPD): age at onset, pattern and time course of symptoms, personal and family history, variable or persistent airflow limitation, lung function in symptom-free episodes, and severe hyperinflation. If no clear distinction is possible and patients currently present with predominant features of COPD, they are excluded from the study.

Exclusion criteria – adults:

- severe upper respiratory tract infection (URTI)
- severe exacerbation during the last 4 weeks, ensuring that patients are in a stable phase of their disease

Definition of controls – adults:

- Controls had to be without any pulmonary disease but were allowed to have concurrent allergic rhinoconjunctivitis.