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Airway immune mediator levels during asthma-like symptoms in young children and their possible role in response to azithromycin

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Authors Contributions: The guarantor of the study is HB, from conception and design to conduct of the study and acquisition of data, data analysis, and interpretation of data. SB was responsible for measurement of immune mediators in upper airway epithelial lining fluid extracted from filter

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papers. CJC has written the first draft of the manuscript. KB contributed to design of the study. All co-authors have provided important intellectual input and contributed considerably to the analyses and interpretation of the data. All authors guarantee that the accuracy and integrity of any part of the work has been appropriately investigated and resolved and all have approved the final version of the manuscript. The corresponding author had full access to the data and had final responsibility for the decision to submit for publication. The corresponding author attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted. No honorarium, grant, or other form of payment was given to any of the authors to produce this manuscript.

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Governance: We are aware of and comply with recognized codes of good research practice, including the Danish Code of Conduct for Research Integrity. We comply with national and international rules on the safety and rights of patients and healthy subjects, including Good Clinical Practice (GCP) as defined in the EU’s Directive on Good Clinical Practice, the International Conference on Harmonisation’s (ICH) good clinical practice guidelines and the Helsinki Declaration. We follow national and international rules on the processing of personal data, including the Danish Act on Processing of Personal Data and the practice of the Danish Data Inspectorate.

Abbreviations:
COPSAC_2010 = Copenhagen Prospective Studies on Asthma in Childhood

CRP = C-reactive protein

FDR = False discovery rate

IFN = Interferon

IL = Interleukin

ILC = Innate lymphoid cell

IQR = interquartile range

PC = Principal Component

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ABSTRACT

Background
Asthma-like symptoms in young children are orchestrated by the local airway immune response, but current knowledge largely relies on in vitro airway models. Azithromycin has been shown to reduce the duration of episodes with asthma-like symptoms but efficacy may depend on the individual child's immune response.

Objectives
To investigate in vivo upper airway immune mediator levels during episodes with asthma-like symptoms in young children and their ability to predict the clinical response to azithromycin treatment.

Methods
535 children aged 0–3 years from the Copenhagen Prospective Studies of Asthma in Childhood-2010 mother-child cohort were examined for immune mediator levels in samples of nasal epithelial lining fluid during episodes with asthma-like symptoms as well as in the asymptomatic state. In a sub-study, children with recurrent asthma-like symptoms were randomized to either a 3-day course of oral azithromycin (10 mg/kg)(n=32) or placebo (n=38). In the current study, we compared the pre-treatment immune mediator levels with the clinical response to treatment with azithromycin in an exploratory posthoc analysis.

Results
The immune mediator concentrations during vs outside episodes were significantly upregulated for IFN-γ (ratio 1.73), TNF-α (ratio 2.05), IL-1β (ratio 1.45), IL-10 (ratio 1.97), while CCL22 (ratio 0.65) was downregulated. Low levels of TNF-α and IL-10 and high levels of CCL22 predicted better treatment response to azithromycin (p-values < 0.05).

Conclusion
Upper airway immune mediator levels were altered during episodes of asthma-like symptoms, and levels of TNF-α, CCL22 and IL-10 may predict the response to azithromycin treatment.

Word count abstract: 264

Keywords, MeSH: Asthma, cytokines, chemokines, allergy and immunology, pediatrics
INTRODUCTION
Episodes of asthma-like symptoms are prevalent among preschool children in westernized countries, with one in three children suffering at least one episode in their first three years of life.\textsuperscript{1,2} Despite the high prevalence, there is little evidence regarding the pathophysiology and treatment of such episodes.\textsuperscript{3} We recently demonstrated a significant reduction by 60% in the duration of episodes with asthma-like symptoms in a randomized controlled trial (RCT) of oral azithromycin compared to placebo, in children aged 1–3 years with recurrent asthma-like symptoms.\textsuperscript{4} Interestingly, the treatment effect was largely independent of the presence of the most common pathogenic airway bacteria or viruses, suggesting that the effect may partly be mediated by immune-modulatory properties of the drug and that the individual child’s immune response could determine and predict the clinical response. Point-of-care tools to predict treatment response are needed to limit the use of antibiotics to the children who are likely to benefit from it.

The immune response of the airways is mediated by cytokines and chemokines, but current knowledge on topical immune mediator release during episodes of asthma-like symptoms in childhood relies mainly on stimulated in vitro airway models and airway lavage techniques introducing an unknown dilution of airway secretions.\textsuperscript{5–9} The aim of this study was to compare undiluted immune mediator levels in vivo in the upper airway epithelial lining fluid during episodes of asthma-like symptoms and in the asymptomatic state. Furthermore, to assess the value of these mediators for predicting treatment response to azithromycin.

METHODS
The COPSAC\textsubscript{2010} cohort
COPSAC\textsubscript{2010} is an ongoing, population-based, prospective mother-child cohort with 700 children enrolled at one-week of age during 2009-2010, as previously described in detail.\textsuperscript{10–12} During the first three years of life the children attended the COPSAC research clinic at nine scheduled visits (i.e. at 1 week, 1, 3, 6, 12, 18, 24, 30, and 36 months), as well as for acute care visits during acute asthma-like symptoms. At the acute care visits, the children were examined by study physicians.
with pediatric training, who were solely responsible for the diagnosis and treatment strictly adherent to predefined standard operating procedures.\(^4\)

Randomized controlled trial of azithromycin for episodes of asthma-like symptoms in children aged 1-3

In a randomized, double-blind, placebo-controlled trial,\(^4\) we included children from the COPSAC\(_{2010}\) cohort, aged 1–3 years who had recurrent episodes of asthma-like symptoms, as described in the online data supplement. Each episode of asthma-like symptoms that occurred through age 1–3 years (or up to a maximum of seven treatments per child) was randomized at each episode to either a 3-day course of azithromycin oral solution, 10 mg/kg per day or a matching placebo of similar look and taste. Episodes with clinical signs of pneumonia as described in the online data supplement or blood CRP level above 476 nmol/L (50 mg/L) were not included in the study. The primary outcome, which was also used in the current study, was diary-verified duration of symptoms after initiation of treatment. The current study is based on a posthoc analysis of the dataset from the original trial.

Measurements of cytokines and chemokines in upper airway epithelial lining fluid

Upper airway epithelial lining fluid from the nasal mucosa was sampled at the planned visits at 2 years of age, where the child was without any respiratory symptoms, as well as during episodes of asthma-like symptoms at acute care visits from age 0–3 years i.e. potentially both before and/or after the sample obtained in asymptomatic periods.\(^10\) Samples from episodes included in the azithromycin vs. placebo trial were obtained before randomization and initiation of treatment. We used a filter-paper sampling technique previously successfully used to evaluate immune mediator profiles in the upper airway epithelial lining fluid of neonates at 1 month of age.\(^13,14\) Strips of filter-paper (Accuwik Ultra, fibrous hydroxylated polyester sheets, cat no.SPR0730, Pall Life Sciences, Portsmouth, Hampshire, UK) were inserted bilaterally into the anterior part of the inferior turbinate of the nasal cavity. After 2 minutes of absorption the filter-papers were removed and immediately frozen at -80°C. Sex, age and season at sampling was recorded as seasonal fluctuations in upper airway immune mediator levels has previously been documented.\(^15\) Epithelial lining fluid samples were analyzed using MesoScale Discovery multiplexed array system (MesoScale Discovery, Gaithersburg, Md) as described in the online data supplement.
During the process of laboratory analyses we recorded date of extraction from filter-papers, date and batch of analysis.

The epithelial lining fluid samples were analyzed for the following protein immune mediators: IL-12p70, CXCL10 (IP-10), Interferon-gamma (IFN-γ), Tumor necrosis factor-alpha (TNF-α), CCL4 (MIP-1β), CCL2 (MCP-1), CCL13 (MCP-4), IL-4, IL-5, IL-13, CCL11 (eotaxin-1), CCL26 (eotaxin-3), CCL17 (TARC), CCL22 (MDC), IL-1β, CXCL8 (IL-8), IL-10, and IL-2.

Selection of the immune mediators was decided *a priori* to represent different types of immune responses: Type 1 (Th1/CD8+/NK cells/innate lymphoid cells (ILC) 1), Type 2 (Th2, eosinophils, ILC2), Type 17 (neutrophils, ILC3), and regulatory type responses. Moreover, both acute and planned samples from children participating in the azithromycin vs. placebo trial were analyzed for levels of C-reactive protein (CRP), an acute phase reactant and activator of the complement system, in the epithelial lining fluid as described in the online data supplement.

At the time of immune mediator sampling we also collected hypopharyngeal aspirates for routine bacterial cultures of *H influenzae*, *S pneumoniae* and *M catarrhalis* and nasopharyngeal aspirates for PCR identification of a range of viral pathogens as detailed in the online data supplement.

**Daily prospective monitoring of asthma-like symptoms**

Diary cards were used from birth to monitor burden of asthma-like symptoms between visits as previously validated. Asthma-like symptoms were explained to the parents as any symptom significantly affecting the child’s breathing, such as noisy breathing (wheeze or whistling sounds), shortness of breath, or persistent troublesome cough affecting the child’s sleep or activity.

Symptoms were recorded daily as individual components of wheeze, dyspnea and cough with any one component signifying a day with asthma-like symptoms. An episode of asthma-like symptoms was defined as at least 3 consecutive days during which the child had symptoms. Samples from episodes with clinical signs indicative of pneumonia or croup were excluded from this study, as described in the online data supplement.

**Statistics**

Raw levels of all immune mediators (pg/mL) obtained during asymptomatic periods and during asthma-like symptoms were summarized using median and interquartile ranges (IQR). Before further analyses, immune mediator concentrations were z-scored to make mediators with different
ranges of concentration directly comparable. Concentrations were also total-sum normalized per sample (immune mediator concentration/sum of all immune mediator concentrations in sample) to account for a high within-sample collinearity of immune mediator levels, likely due to varying amounts of nasal secretion collected on the filter papers, as described in the online data supplement. The correlation between the various immune mediator levels were calculated and plotted in hierarchical clustering heatmaps. Differences in immune mediator levels during episodes of asthma-like symptoms compared to asymptomatic periods were analyzed using linear mixed effect models with mediator levels as response variables and clinical state (i.e. during or outside episodes) as the explanatory variable. Sampling covariates (i.e. sex, age and season), date of extraction from filter-papers, date of analysis, and batch of analysis were included in the model, as described in the online data supplement. The individual participant identifier was also included in the model as a random effect to adjust for repeated measurements in the samples obtained during episodes of asthma-like symptoms. Results are reported as ratios of the mediator concentration during episodes of asthma-like symptoms compared to asymptomatic periods with 95% confidence intervals (CI) and both nominal and false discovery rate (FDR) adjusted p-values. To circumvent a potential multiple testing issue we applied a principal component analysis (PCA) which decomposes the signal from the 18 mediators into three independent principal components (PCs) that capture the overall variation in the mediator levels and compared the PC scores during episodes of asthma-like symptoms vs. asymptomatic periods. PCs were also included as the explanatory variable in linear mixed effect models.

The overall treatment effect of azithromycin on episode duration was analyzed using a Poisson regression model with a log link in accordance with methods used in the original study. The effect of immune mediator levels on the treatment response to azithromycin was analyzed using linear mixed effect models with duration of episodes as outcome variable and treatment arm (i.e. azithromycin or placebo) as the explanatory variable with mediator levels as an interaction term. Analyses were adjusted for findings of viral and/or bacterial airway infections as described in the online data supplement. Results are reported as factor change in treatment effect per increase of 1 standard deviation (SD) of immune mediator concentration in samples obtained during episodes of asthma-like symptoms. Plots showing symptom duration after initiation of treatment in relation to immune mediator levels (SDs of concentration) for both treatment arms are shown as well as graphs showing the derived
treatment effect (i.e. the difference between the two treatment arms at any concentration) in relation to immune mediator levels.

We finally performed unadjusted sub-analyses using raw (non-z-scored, non-normalized) immune mediator levels in order to test the predictive performance in a situation closer to a clinical setting, where only data from a single individual would be available. We tested the association with treatment response for a subset of single mediator levels as well as ratios between up- and down-regulated immune mediators, as a simple alternative to normalization. All data analyses were conducted using the statistical software R v3.4.0 (R Core Team, 2015) and the add-on package lme4.

RESULTS

Of the 700 children included in the COPSAC2010 cohort, 535 (76%) had available samples for immune mediator analyses obtained during episodes of asthma-like symptoms and/or during asymptomatic periods (Table E1). The sample study base (Figure 1) included 522 samples from 292 children obtained during episodes of asthma-like symptoms and 441 samples from 441 children obtained in asymptomatic periods as detailed in the online data supplement. Ninety-four children provided only one or more samples obtained during asthma-like symptoms, 243 provided only a sample obtained during asymptomatic periods and 198 children provided both types of samples.

Thirty-two samples obtained during episodes were excluded due to a diagnosis of pneumonia while seven samples obtained in asymptomatic periods were excluded due to adjacent diary recordings of asthma-like symptoms. Hence, 490 samples from 282 children (mean age 1.3 years +/- 0.6 SD) obtained during episodes of asthma-like symptoms and 434 samples from 434 children (mean age 2.1 years +/- 0.2 SD) obtained in asymptomatic periods were included in the analyses. Sampling time points for included samples obtained during and outside of symptoms are shown in Figure E1. The median number of samples obtained during symptomatic episodes was 1 [IQR 1-2]; (min. 1, max. 9). Distribution of sex, age, and season between the two sample groups are shown in Table E2. Raw levels of immune mediators obtained during asthma-like symptoms and in asymptomatic periods are shown in Table 1.
The sample study base for measurement of CRP levels were 68 samples from 42 children obtained during episodes of asthma-like symptoms and 27 samples from 27 children obtained outside of episodes as described in the online data supplement and shown in Figure E2.

Upper airway immune mediator levels during episodes of asthma-like symptoms compared to asymptomatic periods

Several immune mediators were significantly up- or downregulated during episodes of asthma-like symptoms compared to asymptomatic periods (Figure 2 and Table 2). IFN-γ (ratio episode of asthma-like symptoms/asymptomatic period, 1.73, 95% CI [1.33;2.26], p < 0.01, FDR adjusted p=0.06), TNF-α (2.05, [1.55;2.72], p < 0.01, FDR adjusted p=0.02), IL-1β (1.45, [1.13;1.87], p = 0.01, FDR adjusted p=0.06), IL-10 (1.97, [1.39;2.79], p = 0.02, FDR adjusted p=0.10) were upregulated, while CCL22 (0.65, [0.43;0.99], p = 0.05, FDR adjusted p=0.18) was downregulated. Level of CRP in upper airway epithelial lining fluid samples, where such assessment was available, was also upregulated during episodes (1.74, [1.23;2.45], p < 0.01) as shown in Figure E3. Sensitivity analyses comparing only paired samples from children who contributed both samples obtained during symptoms and in asymptomatic periods (Table E3) or including only samples obtained during symptomatic episodes occurring after the age of two years, i.e. after the asymptomatic sampling, showed similar results. Adjustment for allocated interventions in randomized trials with antenatal dietary fish oil and high-dose vitamin D supplementations carried out the cohort did not change the results (data not shown). Hierarchical clustering heatmaps showed positive correlation and clustering of several immune mediators attributed to the same immune response classes during episodes of asthma-like symptoms, as shown in Figure E4 and detailed in the online data supplement.

Viral and bacterial findings during episodes of asthma-like symptoms are shown in Table E4.

Stratifying analyses for episodes with purely viral (N=61), purely bacterial (N=100) or co-infection (N=275) generally showed similar immune mediator patterns (Figure E5). I

Stratifying analyses by a history of recurrent asthma-like symptoms (N=204 samples obtained during episodes and N=130 in asymptomatic periods) and no such diagnosis (N=286 samples obtained during episodes and N=304 during asymptomatic periods) did not significantly change the results (Figure E6 and Table E6).

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We studied the first three PCs from the PCA model, where PC1 explained 25%, PC2 16% and PC3 10% of the variation in the immune mediator levels. A statistically significant response during episodes of asthma-like symptoms compared to asymptomatic periods were seen for PC1 (-0.39 [-0.72; -0.06], p=0.02) and PC3 (0.59 [0.28; 0.89], p<0.01), while it was borderline significant for PC2 (-0.37 [-0.70; -0.04], p=0.07). Score plots showing separation in several dimensions of samples obtained during episodes (blue ellipses) compared to asymptomatic periods (red ellipses) are presented in Figure E7. As seen from the corresponding loading plots (Figure E7, panels B and D), IFN-γ, TNF-α, IL-1β, IL-10 and CCL22 all contributed to this separation (vector arrows), with the latter contributing in the opposite direction of the other immune mediators, which aligns with the results from the traditional statistical approach. The correlation between specific immune mediator levels and PCs are shown in Figure E3.

**Upper airway immune mediator profiles for prediction of treatment response to azithromycin for episodes of asthma-like symptoms**

Of the 148 episodes of asthma-like symptoms randomized to azithromycin or placebo, filter-paper samples were available from 70 episodes (azithromycin n=32; placebo n=38) in 42 children. Numbers of missing episodes were similar in the two treatment arms (i.e. N=42 in the azithromycin, and N=36 in the placebo group). In this subset of samples, we found an average duration of symptoms after treatment of 3.2 days in the azithromycin group and 8.1 days in the placebo group corresponding to a reduction in duration of episodes of 65.7% (95% CI, 54.3%;74.4%, p<0.01) due to azithromycin treatment.

Of the five immune mediators significantly (nominal p-values) up- or downregulated during episodes of asthma-like symptoms, three mediators were associated with the effect of azithromycin on the duration of symptoms (Figure 3 and Table 3). Lower levels of TNF-α and IL-10 before treatment predicted longer duration of episodes in the placebo group (Figure 3, top panel, slope= -0.79, p=0.03 and slope=-0.75 p=0.03 respectively) while there were no significant association between immune mediator levels and symptom duration in the azithromycin group. Lower levels of TNF-α and IL-10 predicted a more pronounced treatment effect, i.e. reduction in symptom days and treatment effect decreased for higher mediator levels with a factor 0.50 (TNF-α) and 0.50 (IL-10) per increase in SD of concentration (Figure 3, bottom panel). No treatment effect was found above 0.95 SDs (TNF-α) and 0.90 SDs (IL-10) of concentration. Inversely,
higher levels of CCL22 predicted longer duration of episodes in the placebo group (Figure 3, top panel, slope=1.07, p<0.01) while there was no significant association between immune mediator level and symptom duration in the azithromycin group. Higher levels of CCL22 predicted a better treatment effect with a factor 2.36 increase per increase in SD of concentration (Figure 3, bottom panel). No treatment effect was found below -0.71 SDs of concentration for CCL22. Importantly, the three mediators modified the treatment effect so that better response was seen for children with mediator levels opposite to the change typically observed during episodes of asthma-like symptoms (Figure 2), i.e. at low levels of TNF-α and IL-10 and high levels of CCL22. There was no apparent effect of IFN-γ or IL-1β levels on the treatment effect of azithromycin. In the original RCT, presence of H. Influenzae during the symptomatic episode was associated with a stronger treatment response, but sensitivity analyses stratified for presence of analysis of H. Influenzae (H. Influenzae positive n=18 and negative n=49), and adjusted for presence of H. Influenzae showed that this did not affect the results of this study (data not shown).

Lower levels of CRP before treatment predicted longer duration of episodes (Figure E8, top panel, p=0.05). There was no significant modification of the treatment effect from levels of CRP although there was a tendency in the same direction as for TNF-α and IL-10 with a factor 0.75 [0.52:1.07] decrease in treatment effect per increase in SD of concentration (p=0.12).

Unadjusted sub-analyses using raw (non-z-scored, non-normalized) immune mediator levels showed that raw levels of TNF-α and IL-10 were also associated with the effect of azithromycin on the duration of symptoms (factor 0.78 and 0.74 per doubling in concentration [pg/mL] respectively), although the latter was only borderline significant (p=0.05) (Table E7). Using a ratio of the raw levels of TNF-α/CCL22 or IL-10/CCL22 resulted in even stronger associations with the effect of azithromycin on the duration of symptoms (factor 0.68 and 0.66 per doubling in ratio respectively) (Table E7).

DISCUSSION

IFN-γ, TNF-α, IL-1β and IL-10 as well as the acute phase reactant CRP were upregulated in the upper airway mucosa whereas CCL22 was relatively downregulated during episodes of asthma-like symptoms compared to asymptomatic periods.
TNF-α, IL-10 and CCL22 predicted treatment effect of azithromycin for episodes of asthma-like symptoms, with improved responses in episodes where the child was unable to mount a sufficient response of TNF-α and IL-10 while having a relatively increased expression of CCL22 (i.e. mediator levels opposite to what was typically observed during episodes of asthma-like symptoms).

Strengths and Limitations

A major strength of the current study is the nasal epithelial lining fluid sampling method, providing undiluted in vivo levels of immune mediators from the upper airways. Importantly, the current sampling technique was performed without a stimulation of the immune system, thereby enabling us to quantify the physiological concentrations of immune mediators both during symptomatic episodes and in asymptomatic periods. Even though we do not have samples from lung epithelium, the epithelial lining fluid in upper airways has been suggested to reflect that of the lower airways.18,19

The close clinical single-center surveillance of the COPSAC2010 birth cohort is another strength of this study. COPSAC served de facto as the primary health-care center for the children in the cohort, ensuring a standardized approach to sampling following predefined standard operating procedures, and to diagnosis and treatment according to predefined and validated algorithms. This approach reduced the risk of misclassification of illness and variation in sampling quality.

Not all children provided both samples obtained during episodes of asthma-like symptoms and in asymptomatic periods and the majority of samples obtained during symptoms were taken before the scheduled sample at age two years. However, sensitivity analyses including only paired observations and only samples obtained during episodes of asthma-like symptoms occurring after the age of two years respectively confirmed the results of the primary analyses.

The analyses of treatment response to azithromycin for episodes of asthma-like symptoms in relation to immune mediator levels were based on an RCT with clearly defined and previously validated endpoints.4 However, it is a limitation to this part of the study that we only had samples from 70 of 148 episodes included in the RCT. Immune mediator sampling was often omitted due to the extensive program of investigations performed in relation to the acute visits at the research clinic. However, numbers of missing episodes were similar in the two treatment arms, and the

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incomplete sampling of episodes included in the RCT did not bias the results of this study. The average duration of episodes in the two treatment arms, and the overall effect of azithromycin on episode length were comparable to the findings of the main study.\cite{4} An important limitation is that only children with a history of recurrent asthma-like symptoms were included in the RCT, and the findings cannot be generalized beyond this phenotype.

We analyzed 18 cytokines and chemokines, which were chosen *a priori* to represent various major pathways of the immune system. To circumvent a multiple testing problem, we also applied a dimension reducing multivariate approach using a PCA model, which in an unsupervised fashion decomposes the immune signal from the 18 mediators into a few independent components, capturing the overall variation in the immune mediators. The PCA model confirmed that samples obtained during episodes of asthma-like symptoms were distinctly different from those collected in asymptomatic periods, and further replicated the findings from the conventional statistics showing that IFN-γ, TNF-α, IL-1β, IL-10 and CCL22 contributed to this distinction. Concordance between results obtained from the conventional statistics and the multivariate approach further enhances confidence in the findings, even though the result for only one immune mediator (TNF-α) was significant while the results of further two (IFN-γ and IL-1β) were borderline significant after FDR adjustment of p-values.

CRP has earlier been reported to be expressed in respiratory epithelium in quantities sufficient for antimicrobial effect and levels are regulated by several upstream immune mediators including TNF-α, IL-1b and IL-10.\cite{20-22} Therefore, the fact that CRP was upregulated along with the immune mediators TNF-α and IL-1b strengthens our belief in the findings.

**Interpretation**

This study showed a distinct upper airway immune mediator profile during episodes of asthma-like symptoms in young children when compared to asymptomatic periods. This is to our knowledge the first study to investigate unstimulated *in vivo* airway immune mediator levels in the epithelial lining fluid during acute asthma-like symptoms in children. The identified upregulated mediators are mainly part of an activated Type 1 and inflammasome-based Type 17 response, while the reduced CCL22 Type 2 response results in less recruitment of Treg and Th2 cells and less eosinophilic infiltration of the airway mucosa. This observation is in
line with the hypothesis that the neutrophil, rather than the eosinophil, is the key effector cell in early childhood lung symptoms. Hierarchical clustering of correlations between various immune mediators showed some grouping of immune mediators according to attributed immune response class, supporting this classification in vivo. The immune profile was independent of a prior diagnosis of recurrent asthma-like symptoms, i.e. long term symptom load, and thus appears to represent an acute response independent of the underlying propensity for asthma-like symptoms.

Of particular relevance to clinicians, we found that the levels of TNF-α, IL-10 and CCL22 in the upper airway mucosa during acute symptoms may predict response to treatment with azithromycin. Interestingly, the treatment response was most marked in episodes with mediator levels opposite to what was observed in the analysis comparing episodes of asthma-like symptoms with asymptomatic periods (Figure 2). This suggests that the changes in upper airway immune mediator levels observed during acute symptoms may in fact represent a “normal” and beneficial immune response, the absence of which warrants therapeutic intervention with azithromycin. However, this study does not provide any evidence as to the mechanism underlying this observation.

The finding that prediction of treatment effect was independent of concurrent viral or bacterial airway infections is in line with the original RCT which showed that the treatment effect of azithromycin was largely independent of such infections.

Our finding that specific immune mediators may predict treatment response to azithromycin proposes a possible use of these mediators as predictive biomarkers for targeted therapy. This is important since no other predictive biomarkers are known. As the measurements of immune mediator levels are amenable to point-of-care testing such applications could be used to guide clinical decision making and restrict use of antibiotics to the children who will benefit the most. However, several steps are needed before potential use in clinical practice. First, the results presented in this study are exploratory post-hoc analyses based on a small subset of episodes and should be viewed as hypothesis generating. Second, a robust method for use in individual patients without need for population-based z-scoring of data must be developed. In this respect it is promising that the findings were confirmed using raw immune mediator levels and ratios of raw levels, the latter representing a practical approach to adjust for varying amounts of secretion.
Conclusion

This study shows a distinct upper airway immune mediator profile during episodes of asthma-like symptoms in early childhood, consistent with neutrophilic inflammation as the dominating acute disease process. Levels of TNF-α, IL-10 and CCL22 may predict response to oral azithromycin for such episodes, but this needs replication in larger studies.

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REFERENCES


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**Table 1.** Raw immune mediator levels measured during episodes of asthma-like symptoms (N=490) and in asymptomatic periods (N=434).

<table>
<thead>
<tr>
<th>Immune mediator</th>
<th>Asymptomatic (n=434) median [IQR] (pg/mL)</th>
<th>Asthma-like symptoms (n=490) median [IQR] (pg/mL)</th>
</tr>
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<tbody>
<tr>
<td>IL-12p70</td>
<td>1.1 [0.3, 2.0]</td>
<td>1.6 [0.8, 2.3]</td>
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<tr>
<td>CXCL10</td>
<td>3369.8 [1184.5, 5740.2]</td>
<td>4999.9 [2401.2, 7364.3]</td>
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<tr>
<td>IFN-γ</td>
<td>3.2 [1.3, 7.7]</td>
<td>7.7 [3.7, 16.3]</td>
</tr>
<tr>
<td>TNF-α</td>
<td>19.3 [6.0, 82.1]</td>
<td>74.6 [24.4, 203.7]</td>
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<tr>
<td>CCL4</td>
<td>346.8 [96.9, 1163.2]</td>
<td>870.7 [312.3, 2047.1]</td>
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<td>CCL2</td>
<td>103.6 [44.1, 206.3]</td>
<td>153.9 [84.0, 278.5]</td>
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<td>CCL11</td>
<td>151.4 [67.7, 272.9]</td>
<td>208.5 [116.6, 333.7]</td>
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<td>CCL13</td>
<td>13.8 [7.1, 20.7]</td>
<td>13.2 [8.2, 18.2]</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.7 [0.2, 1.5]</td>
<td>1.1 [0.2, 2.5]</td>
</tr>
<tr>
<td>IL-5</td>
<td>1.7 [0.8, 3.3]</td>
<td>2.1 [1.2, 3.6]</td>
</tr>
<tr>
<td>IL-13</td>
<td>15.3 [8.5, 21.8]</td>
<td>15.3 [10.4, 21.2]</td>
</tr>
<tr>
<td>CCL22</td>
<td>482.6 [315.2, 689.3]</td>
<td>477.2 [304.5, 668.9]</td>
</tr>
<tr>
<td>IL-18</td>
<td>208.6 [50.8, 820.3]</td>
<td>485.7 [154.0, 1179.9]</td>
</tr>
<tr>
<td>CXCL8</td>
<td>11982.3 [3563.2, 16969.7]</td>
<td>14846.8 [8625.5, 18807.9]</td>
</tr>
<tr>
<td>IL-2</td>
<td>1.0 [0.3, 2.0]</td>
<td>1.1 [0.5, 2.2]</td>
</tr>
</tbody>
</table>
Table 2. Upper airway immune mediator levels during episodes of asthma-like symptoms (N=490) compared to asymptomatic periods (N=434). CI95: 95% confidence interval. *False discovery rate adjusted p-value.
<table>
<thead>
<tr>
<th>Immune mediator</th>
<th>Immune response class</th>
<th>Ratio of immune mediator levels (asthma-like symptoms / asymptomatic)</th>
<th>CI95</th>
<th>p-value</th>
<th>Corrected p-value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12</td>
<td>Type 1</td>
<td>1.17</td>
<td>[0.76 : 1.79]</td>
<td>0.48</td>
<td>0.79</td>
</tr>
<tr>
<td>CXCL10</td>
<td>Type 1</td>
<td>1.06</td>
<td>[0.81 : 1.39]</td>
<td>0.68</td>
<td>0.86</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Type 1</td>
<td>1.73</td>
<td>[1.33 : 2.26]</td>
<td>&lt;0.01</td>
<td>0.06</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Type 1</td>
<td>2.05</td>
<td>[1.55 : 2.72]</td>
<td>&lt;0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>CCL4</td>
<td>Type 1</td>
<td>1.23</td>
<td>[0.93 : 1.62]</td>
<td>0.15</td>
<td>0.37</td>
</tr>
<tr>
<td>CCL2</td>
<td>Type 1</td>
<td>0.99</td>
<td>[0.73 : 1.34]</td>
<td>0.96</td>
<td>0.96</td>
</tr>
<tr>
<td>CCL11</td>
<td>Type 2</td>
<td>0.95</td>
<td>[0.66 : 1.36]</td>
<td>0.77</td>
<td>0.86</td>
</tr>
<tr>
<td>CCL13</td>
<td>Type 2</td>
<td>0.74</td>
<td>[0.51 : 1.06]</td>
<td>0.11</td>
<td>0.34</td>
</tr>
<tr>
<td>IL-4</td>
<td>Type 2</td>
<td>0.84</td>
<td>[0.62 : 1.15]</td>
<td>0.29</td>
<td>0.53</td>
</tr>
<tr>
<td>IL-5</td>
<td>Type 2</td>
<td>0.83</td>
<td>[0.47 : 1.48]</td>
<td>0.54</td>
<td>0.81</td>
</tr>
<tr>
<td>IL-13</td>
<td>Type 2</td>
<td>0.89</td>
<td>[0.56 : 1.40]</td>
<td>0.60</td>
<td>0.84</td>
</tr>
<tr>
<td>CCL26</td>
<td>Type 2</td>
<td>0.76</td>
<td>[0.54 : 1.07]</td>
<td>0.16</td>
<td>0.37</td>
</tr>
<tr>
<td>CCL17</td>
<td>Type 2</td>
<td>1.07</td>
<td>[0.75 : 1.53]</td>
<td>0.72</td>
<td>0.86</td>
</tr>
<tr>
<td>CCL22</td>
<td>Type 2/Treg</td>
<td>0.65</td>
<td>[0.43 : 0.99]</td>
<td>0.05</td>
<td>0.18</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Type 17</td>
<td>1.45</td>
<td>[1.13 : 1.87]</td>
<td>0.01</td>
<td>0.06</td>
</tr>
<tr>
<td>CXCL8</td>
<td>Type 17</td>
<td>1.03</td>
<td>[0.71 : 1.49]</td>
<td>0.87</td>
<td>0.92</td>
</tr>
<tr>
<td>IL-10</td>
<td>Regulatory type</td>
<td>1.97</td>
<td>[1.39 : 2.79]</td>
<td>0.02</td>
<td>0.11</td>
</tr>
<tr>
<td>IL-2</td>
<td>Regulatory type</td>
<td>0.73</td>
<td>[0.43 : 1.22]</td>
<td>0.25</td>
<td>0.49</td>
</tr>
<tr>
<td>------</td>
<td>----------------</td>
<td>------</td>
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<td>------</td>
<td>------</td>
</tr>
</tbody>
</table>

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Table 3. Treatment effect of azithromycin for episodes of asthma-like symptoms depending on upper airway immune mediator levels.
CI95: 95% confidence interval. SD: Standard deviation. *p-value for interaction.

<table>
<thead>
<tr>
<th>Immune mediator</th>
<th>Treatment effect (factor / increase SD of concentration)</th>
<th>CI95</th>
<th>p-value*</th>
<th>Concentration of no treatment effect (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>1.06</td>
<td>[0.58 : 1.95]</td>
<td>0.84</td>
<td>-</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.50</td>
<td>[0.28 : 0.90]</td>
<td>0.03</td>
<td>&gt; 0.95</td>
</tr>
<tr>
<td>CCL22</td>
<td>2.36</td>
<td>[1.15 : 4.84]</td>
<td>0.02</td>
<td>&lt; -0.71</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1.03</td>
<td>[0.58 : 1.84]</td>
<td>0.92</td>
<td>-</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.50</td>
<td>[0.27 : 0.94]</td>
<td>0.04</td>
<td>&gt; 0.90</td>
</tr>
</tbody>
</table>
Figure 1. Study base selection of upper airway epithelial lining fluid samples obtained from young children during episodes of asthma-like symptoms and in asymptomatic periods. Samples were analyzed for levels of various immune mediators (i.e. cytokines and chemokines). *Stokholm et al.
Figure 2. Immune mediator levels during episodes of asthma-like symptoms (N=490 samples) compared to in asymptomatic periods (N=434 samples).

Figure 3. Top panel: Duration of episodes of asthma-like symptoms with various immune mediator levels in episodes treated with azithromycin (N=32) and placebo (N=38). Green lines: placebo group; orange lines: azithromycin group. Bottom panel: Treatment effect of azithromycin for episodes of asthma-like symptoms with various immune mediator levels. Treatment effect is the calculated difference in symptom duration between the placebo and the azithromycin group for any immune mediator level. X-axis indicators signifies observations.