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Published in:
Allergy

Link to article, DOI:
[10.1111/all.14651](https://doi.org/10.1111/all.14651)

Publication date:
2021

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Carlsson, C. J., Rasmussen, M. A., Pedersen, S. B., Wang, N., Stokholm, J., Chawes, B. L., Bønnelykke, K., & Bisgaard, H. (2021). Airway immune mediator levels during asthmalike symptoms in young children and their possible role in response to azithromycin. *Allergy*, 76(6), 1754-1764. <https://doi.org/10.1111/all.14651>

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Article type : Original Article: Asthma and Lower Airway Disease

Title:

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

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/ALL.14651](#)

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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.

Source of Funding: All funding received by COPSAC is listed on www.copsac.com. The Lundbeck Foundation (Grant no R16-A1694); The Ministry of Health (Grant no 903516); Danish Council for Strategic Research (Grant no 0603-00280B) and The Capital Region Research Foundation have provided core support to the COPSAC research center.

Running head: Airway immune mediators and azithromycin treatment

Word count main manuscript: 4.244

This article has an online data supplement.

Article type: Original research article

Tables: 10 (in online data supplement)

Figures: 11 (8 in online data supplement)

Conflict of interest:

All authors declare no potential, perceived, or real conflict of interest regarding the content of this manuscript. The funding agencies did not have any role in design and conduct of the study; collection, management, and interpretation of the data; or preparation, review, or approval of the manuscript. No pharmaceutical company was involved in the study.

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:

COPSAC₂₀₁₀ = Copenhagen Prospective Studies on Asthma in Childhood₂₀₁₀

CRP = C-reactive protein

FDR = False discovery rate

IFN = Interferon

IL = Interleukin

ILC = Innate lymphoid cell

IQR = interquartile range

PC = Principal Component

PCA = Principal Component Analysis

RCT = Randomized Controlled Trial

RSV = Respiratory syncytial virus

SD = standard deviation

1 **ABSTRACT**

2 **Background**

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

7 **Objectives**

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

11 **Methods**

12 535 children aged 0–3 years from the Copenhagen Prospective Studies of Asthma in Childhood-
13 2010 mother-child cohort were examined for immune mediator levels in samples of nasal
14 epithelial lining fluid during episodes with asthma-like symptoms as well as in the asymptomatic
15 state. In a sub-study, children with recurrent asthma-like symptoms were randomized to either a 3-
16 day course of oral azithromycin (10 mg/kg)(n=32) or placebo (n=38). In the current study, we
17 compared the pre-treatment immune mediator levels with the clinical response to treatment with
18 azithromycin in an exploratory posthoc analysis.

19 **Results**

20 The immune mediator concentrations during vs outside episodes were significantly upregulated for
21 IFN- γ (ratio 1.73), TNF- α (ratio 2.05), IL-1 β (ratio 1.45), IL-10 (ratio 1.97), while CCL22 (ratio
22 0.65) was downregulated. Low levels of TNF- α and IL-10 and high levels of CCL22 predicted
23 better treatment response to azithromycin (p-values < 0.05).

24 **Conclusion**

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

27
28 **Word count abstract:** 264

29
30 **Keywords, MeSH:** Asthma, cytokines, chemokines, allergy and immunology, pediatrics

31 INTRODUCTION

32 Episodes of asthma-like symptoms are prevalent among preschool children in westernized
33 countries, with one in three children suffering at least one episode in their first three years of
34 life.^{1,2} Despite the high prevalence, there is little evidence regarding the pathophysiology and
35 treatment of such episodes.³ We recently demonstrated a significant reduction by 60% in the
36 duration of episodes with asthma-like symptoms in a randomized controlled trial (RCT) of oral
37 azithromycin compared to placebo, in children aged 1–3 years with recurrent asthma-like
38 symptoms.⁴ Interestingly, the treatment effect was largely independent of the presence of the most
39 common pathogenic airway bacteria or viruses, suggesting that the effect may partly be mediated
40 by immune-modulatory properties of the drug and that the individual child's immune response
41 could determine and predict the clinical response. Point-of-care tools to predict treatment response
42 are needed to limit the use of antibiotics to the children who are likely to benefit from it.
43 The immune response of the airways is mediated by cytokines and chemokines, but current
44 knowledge on topical immune mediator release during episodes of asthma-like symptoms in
45 childhood relies mainly on stimulated *in vitro* airway models and airway lavage techniques
46 introducing an unknown dilution of airway secretions.^{5–9}
47 The aim of this study was to compare undiluted immune mediator levels *in vivo* in the upper
48 airway epithelial lining fluid during episodes of asthma-like symptoms and in the asymptomatic
49 state. Furthermore, to assess the value of these mediators for predicting treatment response to
50 azithromycin.

53 METHODS

54 *The COPSAC₂₀₁₀ cohort*

55 COPSAC₂₀₁₀ is an ongoing, population-based, prospective mother-child cohort with 700 children
56 enrolled at one-week of age during 2009-2010, as previously described in detail.^{10–12} During the
57 first three years of life the children attended the COPSAC research clinic at nine scheduled visits
58 (i.e. at 1 week, 1, 3, 6, 12, 18, 24, 30, and 36 months), as well as for acute care visits during acute
59 asthma-like symptoms. At the acute care visits, the children were examined by study physicians

60 with pediatric training, who were solely responsible for the diagnosis and treatment strictly
61 adherent to predefined standard operating procedures.⁴

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

64 In a randomized, double-blind, placebo-controlled trial,⁴ we included children from the
65 COPSAC₂₀₁₀ cohort, aged 1–3 years who had recurrent episodes of asthma-like symptoms, as
66 described in the online data supplement. Each episode of asthma-like symptoms that occurred
67 through age 1–3 years (or up to a maximum of seven treatments per child) was randomized at
68 each episode to either a 3-day course of azithromycin oral solution, 10 mg/kg per day or a
69 matching placebo of similar look and taste. Episodes with clinical signs of pneumonia as described
70 in the online data supplement or blood CRP level above 476 nmol/L (50 mg/L) were not included
71 in the study. The primary outcome, which was also used in the current study, was diary-verified
72 duration of symptoms after initiation of treatment. The current study is based on a posthoc
73 analysis of the dataset from the original trial.

74 *Measurements of cytokines and chemokines in upper airway epithelial lining fluid*

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

89 During the process of laboratory analyses we recorded date of extraction from filter-papers, date
90 and batch of analysis.

91 The epithelial lining fluid samples were analyzed for the following protein immune mediators:

92 IL(interleukin)-12p70, CXCL10 (IP-10), Interferon-gamma (IFN- γ), Tumor necrosis factor-alpha
93 (TNF- α), CCL4 (MIP-1 β), CCL2 (MCP-1), CCL13 (MCP-4), IL-4, IL-5, IL-13, CCL11 (eotaxin-
94 1), CCL26 (eotaxin-3), CCL17 (TARC), CCL22 (MDC), IL-1 β , CXCL8 (IL-8), IL-10, and IL-2.

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

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

104

105 *Daily prospective monitoring of asthma-like symptoms*

106 Diary cards were used from birth to monitor burden of asthma-like symptoms between visits as
107 previously validated.¹⁶ Asthma-like symptoms were explained to the parents as any symptom
108 significantly affecting the child's breathing, such as noisy breathing (wheeze or whistling sounds),
109 shortness of breath, or persistent troublesome cough affecting the child's sleep or activity.

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

115 *Statistics*

116 Raw levels of all immune mediators (pg/mL) obtained during asymptomatic periods and during
117 asthma-like symptoms were summarized using median and interquartile ranges (IQR). Before
118 further analyses, immune mediator concentrations were z-scored to make mediators with different

119 ranges of concentration directly comparable. Concentrations were also total-sum normalized per
120 sample (immune mediator concentration/sum of all immune mediator concentrations in sample) to
121 account for a high within sample collinearity of immune mediator levels, likely due to varying
122 amounts of nasal secretion collected on the filter papers, as described in the online data
123 supplement.¹⁷ The correlation between the various immune mediator levels were calculated and
124 plotted in hierarchical clustering heatmaps. Differences in immune mediator levels during
125 episodes of asthma-like symptoms compared to asymptomatic periods were analyzed using linear
126 mixed effect models with mediator levels as response variables and clinical state (i.e. during or
127 outside episodes) as the explanatory variable. Sampling covariates (i.e. sex, age and season), date
128 of extraction from filter-papers, date of analysis, and batch of analysis were included in the model,
129 as described in the online data supplement. The individual participant identifier was also included
130 in the model as a random effect to adjust for repeated measurements in the samples obtained
131 during episodes of asthma-like symptoms. Results are reported as ratios of the mediator
132 concentration during episodes of asthma-like symptoms compared to asymptomatic periods with
133 95% confidence intervals (CI) and both nominal and false discovery rate (FDR) adjusted p-values.
134 To circumvent a potential multiple testing issue we applied a principal component analysis (PCA)
135 which decomposes the signal from the 18 mediators into three independent principal components
136 (PCs) that capture the overall variation in the mediator levels and compared the PC scores during
137 episodes of asthma-like symptoms vs. asymptomatic periods. PCs were also included as the
138 explanatory variable in linear mixed effect models.

139 The overall treatment effect of azithromycin on episode duration was analyzed using a Poisson
140 regression model with a log link in accordance with methods used in the original study.⁴ The
141 effect of immune mediator levels on the treatment response to azithromycin was analyzed using
142 linear mixed effect models with duration of episodes as outcome variable and treatment arm (i.e.
143 azithromycin or placebo) as the explanatory variable with mediator levels as an interaction term.
144 Analyses were adjusted for findings of viral and/or bacterial airway infections as described in the
145 online data supplement. Results are reported as factor change in treatment effect per increase of 1
146 standard deviation (SD) of immune mediator concentration in samples obtained during episodes of
147 asthma-like symptoms.

148 Plots showing symptom duration after initiation of treatment in relation to immune mediator levels
149 (SDs of concentration) for both treatment arms are shown as well as graphs showing the derived

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

159

160

161 **RESULTS**

162 Of the 700 children included in the COPSAC₂₀₁₀ cohort, 535 (76%) had available samples for
163 immune mediator analyses obtained during episodes of asthma-like symptoms and/or during
164 asymptomatic periods (Table E1). The sample study base (Figure 1) included 522 samples from
165 292 children obtained during episodes of asthma-like symptoms and 441 samples from 441
166 children obtained in asymptomatic periods as detailed in the online data supplement. Ninety-four
167 children provided only one or more samples obtained during asthma-like symptoms, 243 provided
168 only a sample obtained during asymptomatic periods and 198 children provided both types of
169 samples.

170 Thirty-two samples obtained during episodes were excluded due to a diagnosis of pneumonia
171 while seven samples obtained in asymptomatic periods were excluded due to adjacent diary
172 recordings of asthma-like symptoms. Hence, 490 samples from 282 children (mean age 1.3 years
173 +/- 0.6 SD) obtained during episodes of asthma-like symptoms and 434 samples from 434 children
174 (mean age 2.1 years +/- 0.2 SD) obtained in asymptomatic periods were included in the analyses.
175 Sampling time points for included samples obtained during and outside of symptoms are shown in
176 Figure E1. The median number of samples obtained during symptomatic episodes was 1 [IQR 1-
177 2]; (min. 1, max. 9). Distribution of sex, age, and season between the two sample groups are
178 shown in Table E2. Raw levels of immune mediators obtained during asthma-like symptoms and
179 in asymptomatic periods are shown in Table 1.

180 The sample study base for measurement of CRP levels were 68 samples from 42 children obtained
181 during episodes of asthma-like symptoms and 27 samples from 27 children obtained outside of
182 episodes as described in the online data supplement and shown in Figure E2.

183

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

186 Several immune mediators were significantly up- or downregulated during episodes of asthma-like
187 symptoms compared to asymptomatic periods (Figure 2 and Table 2). IFN- γ (ratio episode of
188 asthma-like symptoms/asymptomatic period, 1.73, 95% CI [1.33;2.26], $p < 0.01$, FDR adjusted
189 $p=0.06$), TNF- α (2.05, [1.55;2.72], $p < 0.01$, FDR adjusted $p=0.02$), IL-1 β (1.45, [1.13;1.87], $p =$
190 0.01 , FDR adjusted $p=0.06$), IL-10 (1.97, [1.39;2.79], $p = 0.02$, FDR adjusted $p=0.11$) were
191 upregulated, while CCL22 (0.65, [0.43;0.99], $p = 0.05$, FDR adjusted $p=0.18$) was downregulated.
192 Level of CRP in upper airway epithelial lining fluid samples, where such assessment was
193 available, was also upregulated during episodes (1.74, [1.23;2.45], $p < 0.01$) as shown in Figure
194 E3. Sensitivity analyses comparing only paired samples from children who contributed both
195 samples obtained during symptoms and in asymptomatic periods (Table E3) or including only
196 samples obtained during symptomatic episodes occurring after the age of two years, i.e. after the
197 asymptomatic sampling, showed similar results. Adjustment for allocated interventions in
198 randomized trials with antenatal dietary fish oil and high-dose vitamin D supplementations carried
199 out the cohort did not change the results (data not shown). Hierarchical clustering heatmaps
200 showed positive correlation and clustering of several immune mediators attributed to the same
201 immune response classes during episodes of asthma-like symptoms, as shown in Figure E4 and
202 detailed in the online data supplement.

203

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

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

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

211 We studied the first three PCs from the PCA model, where PC1 explained 25%, PC2 16% and
212 PC3 10% of the variation in the immune mediator levels. A statistically significant response
213 during episodes of asthma-like symptoms compared to asymptomatic periods were seen for PC1 (-
214 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
215 for PC2 (-0.37 [-0.70:-0.04], $p=0.07$). Score plots showing separation in several dimensions of
216 samples obtained during episodes (blue ellipses) compared to asymptomatic periods (red ellipses)
217 are presented in Figure E7. As seen from the corresponding loading plots (Figure E7, panels B and
218 D), IFN- γ , TNF- α , IL-1 β , IL-10 and CCL22 all contributed to this separation (vector arrows), with
219 the latter contributing in the opposite direction of the other immune mediators, which aligns with
220 the results from the traditional statistical approach. The correlation between specific immune
221 mediator levels and PCs are shown in Figure E3.

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

224 Of the 148 episodes of asthma-like symptoms randomized to azithromycin or placebo, filter-paper
225 samples were available from 70 episodes (azithromycin $n=32$; placebo $n=38$) in 42 children.
226 Numbers of missing episodes were similar in the two treatment arms (i.e. $N=42$ in the
227 azithromycin, and $N=36$ in the placebo group). In this subset of samples, we found an average
228 duration of symptoms after treatment of 3.2 days in the azithromycin group and 8.1 days in the
229 placebo group corresponding to a reduction in duration of episodes of 65.7% (95% CI,
230 54.3%;74.4%, $p<0.01$) due to azithromycin treatment.

231 Of the five immune mediators significantly (nominal p -values) up- or downregulated during
232 episodes of asthma-like symptoms, three mediators were associated with the effect of
233 azithromycin on the duration of symptoms (Figure 3 and Table 3). Lower levels of TNF- α and IL-
234 10 before treatment predicted longer duration of episodes in the placebo group (Figure 3, top
235 panel, slope= -0.79, $p=0.03$ and slope=-0.75 $p=0.03$ respectively) while there were no significant
236 association between immune mediator levels and symptom duration in the azithromycin group.
237 Lower levels of TNF- α and IL-10 predicted a more pronounced treatment effect, i.e. reduction in
238 symptom days and treatment effect decreased for higher mediator levels with a factor 0.50 (TNF-
239 α) and 0.50 (IL-10) per increase in SD of concentration (Figure 3, bottom panel). No treatment
240 effect was found above 0.95 SDs (TNF- α) and 0.90 SDs (IL-10) of concentration. Inversely,

241 higher levels of CCL22 predicted longer duration of episodes in the placebo group (Figure 3, top
242 panel, slope=1.07, $p<0.01$) while there was no significant association between immune mediator
243 level and symptom duration in the azithromycin group. Higher levels of CCL22 predicted a better
244 treatment effect with a factor 2.36 increase per increase in SD of concentration (Figure 3, bottom
245 panel). No treatment effect was found below -0.71 SDs of concentration for CCL22. Importantly,
246 the three mediators modified the treatment effect so that better response was seen for children with
247 mediator levels opposite to the change typically observed during episodes of asthma-like
248 symptoms (Figure 2), i.e. at low levels of TNF- α and IL-10 and high levels of CCL22. There was
249 no apparent effect of IFN- γ or IL-1 β levels on the treatment effect of azithromycin. In the original
250 RCT, presence of H. Influenzae during the symptomatic episode was associated with a stronger
251 treatment response, but sensitivity analyses stratified for presence of analysis of H. Influenzae (H.
252 Influenzae positive $n=18$ and negative $n=49$), and adjusted for presence of H. Influenzae showed
253 that this did not affect the results of this study (data not shown).

254 Lower levels of CRP before treatment predicted longer duration of episodes (Figure E8, top panel,
255 $p=0.05$). There was no significant modification of the treatment effect from levels of CRP
256 although there was a tendency in the same direction as for TNF- α and IL-10 with a factor 0.75
257 [0.52:1.07] decrease in treatment effect per increase in SD of concentration ($p=0.12$).

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

265

266 DISCUSSION

267 IFN- γ , TNF- α , IL-1 β and IL-10 as well as the acute phase reactant CRP were upregulated in the
268 upper airway mucosa whereas CCL22 was relatively downregulated during episodes of asthma-
269 like symptoms compared to asymptomatic periods.

270 TNF- α , IL-10 and CCL22 predicted treatment effect of azithromycin for episodes of asthma-like
271 symptoms, with improved responses in episodes where the child was unable to mount a sufficient
272 response of TNF- α and IL-10 while having a relatively increased expression of CCL22 (i.e.
273 mediator levels opposite to what was typically observed during episodes of asthma-like
274 symptoms).

275 *Strengths and Limitations*

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

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

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

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

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.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-1 β , 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-1 β) 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-1 β and IL-10.^{20–22} Therefore, the fact that CRP was upregulated along with the immune mediators TNF- α and IL-1 β 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.^{23–26} 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 testing³⁰ 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.

358 *Conclusion*

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

363

364 **ACKNOWLEDGEMENTS**

365 We express our deepest gratitude to the children and families of the COPSAC₂₀₁₀ cohort study for
366 all their support and commitment. We acknowledge and appreciate the unique efforts of the
367 COPSAC research team, and of technicians Vibeke Holt and Lisbeth Buus Rosholm, Department
368 of Biotechnology and Biomedicine, Technical University of Denmark for expert assistance in
369 extraction and measurements of immune mediators in upper airway lining fluid collected onto
370 filter papers.

371 Dr. Carlsson has nothing to disclose. Mr. Rasmussen has nothing to disclose. Ms. Pedersen has
372 nothing to disclose. Ms. Wang has nothing to disclose. Dr. Stockholm has nothing to disclose. Dr.
373 Chawes has nothing to disclose. Professor Bønnelykke has nothing to disclose. Professor Bisgaard
374 has nothing to disclose.

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TABELS

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

Immune mediator	Asymptomatic (n=434) median [IQR] (pg/mL)	Asthma-like symptoms (n=490) median [IQR] (pg/mL)
IL-12p70	1.1 [0.3, 2.0]	1.6 [0.8, 2.3]
CXCL10	3369.8 [1184.5, 5740.2]	4999.9 [2401.2, 7364.3]
IFN- γ	3.2 [1.3, 7.7]	7.7 [3.7, 16.3]
TNF- α	19.3 [6.0, 82.1]	74.6 [24.4, 203.7]
CCL4	346.8 [96.9, 1163.2]	870.7 [312.3, 2047.1]
CCL2	103.6 [44.1, 206.3]	153.9 [84.0, 278.5]
CCL11	151.4 [67.7, 272.9]	208.5 [116.6, 333.7]
CCL13	13.8 [7.1, 20.7]	13.2 [8.2, 18.2]
IL-4	0.7 [0.2, 1.5]	1.1 [0.2, 2.5]
IL-5	1.7 [0.8, 3.3]	2.1 [1.2, 3.6]
IL-13	15.3 [8.5, 21.8]	15.3 [10.4, 21.2]
CCL26	7.7 [2.4, 14.7]	13.1 [4.5, 20.8]
CCL17	12.8 [7.1, 19.5]	14.7 [9.8, 20.4]
CCL22	482.6 [315.2, 689.3]	477.2 [304.5, 668.9]
IL-1 β	208.6 [50.8, 820.3]	485.7 [154.0, 1179.9]
CXCL8	11982.3 [3563.2, 16969.7]	14846.8 [8625.5, 18807.9]
IL-10	10.1 [3.7, 20.8]	19.2 [10.4, 39.7]
IL-2	1.0 [0.3, 2.0]	1.1 [0.5, 2.2]

464 Table 2. Upper airway immune mediator levels during episodes of asthma-like symptoms (N=490) compared to asymptomatic periods (N=434). CI95:
465 95% confidence interval. *False discovery rate adjusted p-value.
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Immune mediator	Immune response class	Ratio of immune mediator levels (asthma-like symptoms / asymptomatic)		CI95	p-value	Corrected p-value*
IL-12	Type 1	1.17		[0.76 : 1.79]	0.48	0.79
CXCL10	Type 1	1.06		[0.81 : 1.39]	0.68	0.86
IFN- γ	Type 1	1.73		[1.33 : 2.26]	<0.01	0.06
TNF- α	Type 1	2.05		[1.55 : 2.72]	<0.01	0.02
CCL4	Type 1	1.23		[0.93 : 1.62]	0.15	0.37
CCL2	Type 1	0.99		[0.73 : 1.34]	0.96	0.96
CCL11	Type 2	0.95		[0.66 : 1.36]	0.77	0.86
CCL13	Type 2	0.74		[0.51 : 1.06]	0.11	0.34
IL-4	Type 2	0.84		[0.62 : 1.15]	0.29	0.53
IL-5	Type 2	0.83		[0.47 : 1.48]	0.54	0.81
IL-13	Type 2	0.89		[0.56 : 1.40]	0.60	0.84
CCL26	Type 2	0.76		[0.54 : 1.07]	0.16	0.37
CCL17	Type 2	1.07		[0.75 : 1.53]	0.72	0.86
CCL22	Type 2/Treg	0.65		[0.43 : 0.99]	0.05	0.18
IL-1 β	Type 17	1.45		[1.13 : 1.87]	0.01	0.06
CXCL8	Type 17	1.03		[0.71 : 1.49]	0.87	0.92
IL-10	Regulatory type	1.97		[1.39 : 2.79]	0.02	0.11

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IL-2	Regulatory type	0.73	[0.43 : 1.22]	0.25	0.49
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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.

Immune mediator	Treatment effect (factor / increase SD of concentration)	CI95	p-value*	Concentration of no treatment effect (SD)
IFN- γ	1.06	[0.58 : 1.95]	0.84	-
TNF- α	0.50	[0.28 : 0.90]	0.03	> 0.95
CCL22	2.36	[1.15 : 4.84]	0.02	< -0.71
IL-1 β	1.03	[0.58 : 1.84]	0.92	-
IL-10	0.50	[0.27 : 0.94]	0.04	> 0.90

474 **FIGURE LEGENDS**

475 Figure 1. Study base selection of upper airway epithelial lining fluid samples obtained from young
476 children during episodes of asthma-like symptoms and in asymptomatic periods. Samples were
477 analyzed for levels of various immune mediators (i.e. cytokines and chemokines). *Stokholm et al.

478

479 Figure 2. Immune mediator levels during episodes of asthma-like symptoms (N=490 samples) compared to in asymptomatic periods (N=434
480 samples).

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