

## Neonates with reduced neonatal lung function have systemic low-grade inflammation

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1 **Reduced Neonatal Lung Function Associates with Systemic Low-grade**

2 **Inflammation in Early Life**

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46 **Abbreviations:** COPSAC<sub>2000</sub> = COpenhagen Prospective Study on Asthma in Childhood; CXCL8  
47 (IL-8) = Chemokine (C-X-C motif) Ligand 8; FEV<sub>0.5</sub> = Forced Expiratory Volume at 0.5 seconds;  
48 FEF<sub>50</sub> = Forced Expiratory Flow at 50% of the forced vital capacity; hs-CRP = high-sensitivity C-  
49 reactive protein; IL-1 $\beta$  = Interleukin-1 $\beta$ , IL-6 = Interleukin-6; MMEF = Maximal Mid-Expiratory  
50 Flow; PtcO<sub>2</sub> = transcutaneous oxygen saturation; PD<sub>15</sub> = Provocative Dose of methacholine causing  
51 a 15% drop in PtcO<sub>2</sub>; PD<sub>20</sub> = Provocative Dose of methacholine causing a 20% drop in FEV<sub>1</sub> from  
52 baseline; TNF- $\alpha$  = tumor necrosis factor- $\alpha$ ; TROLS = TROublsome Lung Symptoms.

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54 table of content online at [www.atsjournals.org](http://www.atsjournals.org).

55 **At a Glance Commentary:**

56 *Scientific Knowledge on the Subject*

57 Elevated hs-CRP as a proxy of systemic low-grade inflammation has been demonstrated in  
58 asthmatic children and adults with diminished pulmonary function. It is however unknown whether  
59 asymptomatic reduced neonatal lung function is associated with systemic inflammation.

60 *What this Study Adds to the Field*

61 This study shows that children with impaired respiratory capacity as neonates are characterized by  
62 elevated hs-CRP and an up-regulated blood inflammatory profile suggesting presence of systemic  
63 low-grade inflammation in early life.

64 **Data from this manuscript has not been presented before in abstract or any other form.**

65 **ABSTRACT**66 *Rationale*

67 Previous studies indicate presence of systemic inflammation in children and adults with asthma and  
68 impaired lung function, but it is unknown whether asymptomatic reduced infant lung function is  
69 associated with low-grade inflammation in early life.

70 *Objective*

71 To investigate the possible association between infant lung function indices and biomarkers of  
72 systemic inflammation in early life.

73 *Methods*

74 Serum levels of high-sensitivity C-reactive protein (hs-CRP), interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, tumor  
75 necrosis factor- $\alpha$  (TNF- $\alpha$ ) and CXCL8 (IL-8) were measured at age 6 months in 300 children of the  
76 Copenhagen Prospective Study on Asthma in Childhood<sub>2000</sub> (COPSAC<sub>2000</sub>) birth cohort, who  
77 completed infant lung function testing at age 1 month, spirometry at 7yrs, and fulfilled a respiratory  
78 day-to-day diary from 0-7yrs. Associations between lung function indices, asthmatic symptoms and  
79 inflammatory biomarkers were investigated by conventional statistics and unsupervised principle  
80 component analysis.

81 *Measurements and Main Results*

82 Infant's forced expiratory volume at 0.5s (FEV<sub>0.5</sub>) was inversely associated with hs-CRP ( $\beta$ -  
83 coefficient, -0.12; 95% CI, -0.21 to -0.04; p=0.004) and with a uniform up-regulated inflammatory  
84 signature (p=0.02). hs-CRP at 6mo was elevated in children with asthmatic symptoms at 0-6mo  
85 compared to children without asthmatic symptoms (median, 1.79mg/L vs. 1.19mg/L; p=0.05), but  
86 was not associated with asthma or lung function at age 7yrs. Adjusting for older children in the  
87 home, infections 14d prior to blood sampling, birth BMI, and maternal smoking did not affect the  
88 associations.

89 *Conclusion*

90 Diminished infant lung function associates with elevated hs-CRP and an up-regulated blood

91 inflammatory response suggesting linkage between lung function and systemic low-grade

92 inflammation in early life.

93 **Abstract Word Count:** 252 words

94 **Key-words:** Asthma, Children, high-sensitivity C-reactive protein, pro-inflammatory cytokines,

95 spirometry.

96 **INTRODUCTION**

97 C-reactive protein (CRP) is an acute-phase reactant found in the blood in response to acute and  
98 chronic inflammatory conditions and has a broad clinical application in the screening for infectious  
99 and immune-mediated diseases<sup>1</sup>. CRP harbors important innate immunity properties and is released  
100 from the liver triggered by pro-inflammatory cytokines such as interleukin 6 (IL-6), IL-1 $\beta$ , and  
101 tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )<sup>2</sup>. Newer CRP assays<sup>3</sup> has enabled assessment of previously  
102 immeasurable low levels of CRP, termed high sensitivity CRP (hs-CRP), which is now increasingly  
103 recognized as a marker of low-grade inflammation in e.g. cardiovascular disease<sup>4</sup>, obesity<sup>5</sup>, and  
104 diabetes mellitus<sup>6</sup>.

105 Recently, elevated hs-CRP has also been demonstrated in manifest airway diseases such as asthma<sup>7</sup>  
106 and chronic obstructive pulmonary disease<sup>8</sup>. In addition, previous studies indicate that impaired  
107 lung function in asthmatic children and adults is associated with presence of systemic low-grade  
108 inflammation<sup>9,10</sup>. It is however unknown whether asymptomatic neonates with reduced pulmonary  
109 function are characterized by systemic low-grade inflammation in early life.

110 We hypothesized that children with reduced neonatal lung function may have biochemical signs of  
111 systemic low-grade inflammation in infancy. The objective of the current study was therefore to  
112 investigate the possible association between lung function indices measured in asymptomatic  
113 neonates of the Copenhagen Prospective Study on Asthma in Childhood<sub>2000</sub> (COPSAC<sub>2000</sub>) birth  
114 cohort and serum levels of hs-CRP, IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and CXCL8 (formerly IL-8) at age 6  
115 months.



116 **METHODS**117 *Study Cohort*

118 The study participants were 411 infants born of mothers with a history of asthma enrolled at 1  
119 month of age in the COPSAC<sub>2000</sub> prospective birth cohort study<sup>11-13</sup>. Exclusion criteria were any  
120 respiratory symptoms or respiratory support prior to inclusion, gestational age <36 weeks, and any  
121 congenital abnormality or systemic illness. The children attended the COPSAC research clinic at  
122 age 1 month for assessment of infant lung function and subsequently at 6-monthly intervals till age  
123 7 years for scheduled clinical investigations, collection of medical history since last visit supported  
124 by a day-to-day lung symptom diary, and for detailed exposure assessments. Additional acute visits  
125 were arranged upon occurrence of any respiratory symptoms.

126 *Ethics*

127 The study was conducted in accordance with the guiding principles of the Declaration of Helsinki  
128 and was approved by the Local Ethics Committee (KF 01-289/96), and the Danish Data Protection  
129 Agency (2008-41-1754). Both parents gave written informed consent before enrolment.

130 *Inflammatory Biomarkers*

131 At age 6 months blood was drawn from a cubital vein, centrifuged to separate serum and serum  
132 cells, and immediately stored at -80° C until analyses. The samples were transported on dry ice to  
133 the laboratory, where levels of the selected biomarkers were determined by high-sensitivity ELISA  
134 assays based on electrochemiluminescence in a 4-plex setting for IL-1 $\beta$ , IL-6, CXCL8 and TNF- $\alpha$   
135 and as a single assay for hs-CRP. Samples were read in duplicates using the Sector Imager 6000  
136 (MesoScale Discovery®, Gaithersburg, MD, USA). The limit of detection (mean signal from blanks  
137 +3SD) was 9.54 pg/mL for hs-CRP, 0.15 pg/mL for IL-1 $\beta$ , 0.17 pg/mL for IL-6, 0.09 pg/mL for  
138 CXCL8 and 0.08 pg/mL for TNF- $\alpha$ .

139 *Lung Function*

140 Infant spirometry was measured at age 1 month applying the raised volume rapid thoraco-  
141 abdominal “squeeze”-jacket compression technique<sup>14</sup>. Repeated ventilations to predefined mouth-  
142 pressures assured expansion of the lung volume before an instant inflation of the jacket caused a full  
143 exhalation during which the flow was measured by a pneumotachograph with an aircushion  
144 facemask<sup>15,16</sup>. The software identified the Forced Vital Capacity (FVC) as the first plateau on the  
145 volume-time curve and measurements with FVC appearing after 0.5s and with the Forced  
146 Expiratory Volume at 0.5s (FEV<sub>0.5</sub>) being smaller than or equal to FVC were accepted. Three to  
147 five acceptable curves were obtained for each infant and the curve containing the median value of  
148 FEV<sub>0.5</sub> was used for the analyses of FEV<sub>0.5</sub> and Forced Expiratory Flow at 50% of FVC (FEF<sub>50</sub>).

149 Spirometry at age 7yrs was performed as previously detailed<sup>17</sup> using a pneumotachograph,  
150 Masterscope Pneumoscreen, system 754,916 spirometer (Erich Jaeger, Wurtzburg, Germany) for  
151 assessing FEV<sub>1</sub> and maximal mid-expiratory flow (MMEF).

152 Infant bronchial responsiveness: After an initial saline inhalation, methacholine was given in  
153 quadrupling dose-steps via a dosimeter attached to a nebulizer (SPIRA 08 TSM 133; Respiratory  
154 Care Center; Hämeenlinna, Finland)<sup>16</sup>. Bronchial responsiveness was determined by continuous  
155 assessment of transcutaneous oxygen saturation (PtcO<sub>2</sub>) (TCM3; Radiometer; Copenhagen,  
156 Denmark). The provocative dose causing a 15% drop in PtcO<sub>2</sub> (PD<sub>15</sub>) was estimated from the dose  
157 response curves fitted with a logistic function.

158 Bronchial responsiveness at age 7yrs was defined as the provocative dose of methacholine causing a  
159 20% drop in FEV<sub>1</sub> from baseline (PD<sub>20</sub>)<sup>17</sup>.

160 *Clinical Investigator-diagnosed End-points*

161 Troublesome lung symptoms (TROLS) were defined as significant cough or wheeze or dyspnea

162 severely affecting the well-being of the child and recorded by the parents in a daily diary chart as a

163 dichotomized score (yes/no) from birth till age 7yrs<sup>18</sup>. *Recurrent TROLS* was defined from the  
164 diaries as five episodes within 6 months, each episode lasting at least three consecutive days, or  
165 daily symptoms for four consecutive weeks<sup>19,20</sup>.

166 Asthma at age 7yrs was diagnosed according to international guidelines and was based on recurrent  
167 TROLS as defined above, symptoms judged by the COPSAC pediatricians to be typical of asthma,  
168 in need of intermittent inhaled  $\beta_2$ -agonist, responding to a 3-month trial of inhaled corticosteroids  
169 and relapsing when stopping treatment<sup>12,13</sup>.

#### 170 *Covariates*

171 Covariates included *heredity* (father's history of asthma, eczema or allergy [yes/no]);  
172 *anthropometrics* (birth BMI [7-12, 12-13, 13-14, 14-17m/kg<sup>2</sup>]); *demographics* (gender, older  
173 children in the home at birth [yes/no], yearly household income [low (<53.000 €), medium (53.000-  
174 80.000 €), high (>80.000 €)]; *pre- and antenatal exposures* (maternal smoking during 3<sup>rd</sup>  
175 pregnancy trimester [yes/no], caesarean section [yes/no]); *postnatal exposures* (solely breastfeeding  
176 length [0-3, 3-6, >6mo], age at start in daycare [0-9, 9-12, >12mo], pets in the home in the 1<sup>st</sup> year  
177 of life: cat [yes/no], dog [yes/no]); and *infections 14 days prior to biomarker assessment* (upper and  
178 lower respiratory tract infections, gastroenteritis or fever with unknown cause [yes/no]).

#### 179 *Statistics*

180 Biomarker null values were set to half of the lowest detected value for the specific biomarker,  
181 values were log-transformed, and the mean of the duplicate measurements were used for association  
182 analyses. Z-scores were calculated for FEV<sub>0.5</sub>, FEV<sub>1</sub>, FEF<sub>50</sub> and MMEF, and PD<sub>15</sub> and PD<sub>20</sub> were  
183 log-transformed to obtain normality. The associations between lung function, asthmatic symptoms,  
184 and inflammatory biomarkers were tested by conventional statistics and by unsupervised pattern  
185 recognition using principal component analysis (PCA).

186 The relation between continuous lung function indices and continuous levels of inflammatory  
187 biomarkers at age 6 months was tested with general linear models. The association between  
188 biomarker levels and time to recurrent TROLS was modeled using Cox-regression. Logistic  
189 regression was used to compute the odds ratio of asthma at age 7yrs.

190 For the pattern recognition analyses, we extracted underlying orthogonal components that described  
191 the systematic part of the variation across the biomarkers using centered and scaled (equal variance)  
192 mediator levels. Scree plots of the Eigen values were used to select the number of components for  
193 subsequent association analyses.

194 All results are presented as raw estimates with 95% CI and as estimates adjusted for covariates  
195 associated with levels of hs-CRP using a cut-off at  $p \leq 0.10$ . Birth BMI and maternal smoking during  
196 3<sup>rd</sup> trimester were retained in the multivariable models with infant lung function independently of  
197 their association with hs-CRP as these are important determinants of infant lung function<sup>21</sup>. A p-  
198 value  $\leq 0.05$  was considered significant. All analyses were done using SAS version 9.3 (SAS  
199 Institute, Cary, NC).

200 **RESULTS**201 *Inflammatory Biomarker Assessments*

202 Measurements of IL-1 $\beta$ , IL-6, TNF- $\alpha$  and CXCL8 were performed on 309 and hs-CRP on 301  
203 serum samples collected at age 6 months. One sample was lost for technical reasons while  
204 performing the 4-plex assay, resulting in 300 children (73% of the original 411 cohort children)  
205 with available measurements for all five biomarkers. We found no significant differences in  
206 baseline characteristics between children with and without available biomarker assessments (Table  
207 E1).

208 The median hs-CRP level was 1.39 mg/L (inter-quartile range [IQR], 0.46-4.61), IL-1 $\beta$  was 0.01  
209 ng/L (0.001-0.04), IL-6 was 0.20 ng/L (0.11-0.31), TNF- $\alpha$  was 2.34 ng/L (1.92-2.88), and CXCL8  
210 was 3.04 ng/L (2.19-4.37). The IL-6 and TNF- $\alpha$  levels were strongly positively correlated with hs-  
211 CRP levels ( $p < 0.001$  for both) whereas IL-1 $\beta$  and CXCL8 levels were not correlated with hs-CRP  
212 ( $p \geq 0.62$ ). The measured values of hs-CRP, IL-6, TNF- $\alpha$  and CXCL8 were within the expected  
213 range<sup>22</sup> with very few null values, whilst IL-1 $\beta$  levels were much lower than expected<sup>22</sup> with null  
214 values for 72 of 308 children (23%). Due to that and the fact that IL-1 $\beta$  has been shown to  
215 significantly degrade over time even at -80° C<sup>23</sup>, IL-1 $\beta$  was not included in further analyses.

216 *Determinants of hs-CRP*

217 Children with older children in the home at birth had significantly higher hs-CRP level at age 6  
218 months compared to children without older children in the home: median hs-CRP level 2.20 mg/L  
219 (IQR, 0.63-5.05) vs. 1.16 mg/L (0.41-3.40),  $p = 0.005$ . In addition, hs-CRP was elevated in children  
220 who had suffered an infectious episode within 14 days prior to biomarker assessment compared to  
221 children without apparent infections: 4.29 mg/L (1.71-5.34) vs. 0.84 mg/L (0.36-2.67),  $p < 0.0001$ .  
222 We did not detect associations between hs-CRP level and paternal history of asthma, eczema or  
223 allergy, child gender, birth BMI, household income, maternal smoking during 3<sup>rd</sup> pregnancy

224 trimester, birth by caesarean section, breastfeeding, daycare attendance or pets in the home (Table  
225 1).

### 226 *Lung Function and Systemic Low-grade Inflammation*

227 The conventional statistical approach showed a strong linear inverse association between FEV<sub>0.5</sub> at  
228 age 1 month and hs-CRP level at age 6 months ( $\beta$ -coefficient, -0.12; 95% CI, -0.21 to -0.04;  
229  $p=0.004$ ) suggesting increasing grade of inflammation by diminished neonatal lung volume (Figure  
230 1). The association was unaffected by adjustment for older children in the home, infections 14 days  
231 prior to biomarker assessment, birth BMI and maternal smoking in 3<sup>rd</sup> trimester:  $\beta$ -coefficient, -  
232 0.13; 95% CI, -0.22 to -0.04;  $p=0.005$ . FEF<sub>50</sub> also seemed inversely associated with hs-CRP, but  
233 was not significant:  $\beta$ -coefficient, -0.06; 95% CI, -0.15 to 0.02;  $p=0.14$ .

234 Increasing FEV<sub>0.5</sub> was also significantly associated with decreasing levels of IL-6 ( $\beta$ -coefficient, -  
235 0.10; 95% CI, -0.18 to -0.01;  $p=0.03$ ) (Figure 2). Confounder adjustment did not modify the  
236 association:  $\beta$ -coefficient, -0.09; 95% CI, -0.18 to 0.00;  $p=0.04$ . We did not detect a significant  
237 association between FEF<sub>50</sub> and IL-6 levels.

238 FEV<sub>0.5</sub> and FEF<sub>50</sub> measurements were not associated with CXCL8 or TNF- $\alpha$  levels although the  $\beta$ -  
239 coefficients suggested an inverse association between lung function indices and TNF- $\alpha$  (Table 2).

240 The unsupervised PCA showed that hs-CRP, IL-6, TNF- $\alpha$  and CXCL8 were positively correlated in  
241 the first principal component (PC<sub>1</sub>) which explained 41% of the total variation in the data. The PCA  
242 approach is illustrated in the biplot (Figure 3) showing scores for PC<sub>1</sub> and PC<sub>2</sub> and loadings for the  
243 biomarkers. Because of the univocal pattern in PC<sub>1</sub>, we focused on PC<sub>1</sub> in the further analyses.

244 Confirming the findings from the conventional statistics, we found that FEV<sub>0.5</sub> was inversely  
245 associated with PC<sub>1</sub> ( $p=0.02$ ) and remained significant after confounder adjustments ( $p=0.03$ ). The

246  $\beta$ -coefficients also suggested an inverse association between FEF<sub>50</sub> and PC<sub>1</sub>, but the model was not  
247 significant (Table 2).

248 We did not detect any association between inflammatory biomarkers at age 6 months and lung  
249 function at age 7 years neither by conventional statistics nor by PCA approach (Table E2).

250 *Bronchial Responsiveness and Systemic Low-grade Inflammation*

251 Bronchial responsiveness to methacholine in neonatal life and at age 7 years was not associated  
252 with biomarkers of low-grade inflammation at age 6 months (Tables 2 and E2).

253 *Lung Symptoms, Asthma and Systemic Low-grade Inflammation*

254 Children experiencing TROLS at any time-point from birth till biomarker assessment (0-6mo)  
255 compared to children without TROLS had significantly elevated levels of hs-CRP: median 1.79  
256 mg/L (IQR,0.50-4.72) vs. 1.19 mg/L (0.46-4.14), p=0.05; IL-6: 0.21 ng/L (0.13-0.42) vs. 0.19 ng/L  
257 (0.11-0.29), p=0.05; and CXCL8: 3.37 ng/L (2.18-5.31) vs. 2.90 ng/L (2.22-3.85), p=0.04. The  
258 PCA approach confirmed an up-regulated blood inflammatory profile in children experiencing  
259 TROLS at age 0-6mo (p=0.01). The findings were unaffected by adjustment for older children in  
260 the home and infectious episodes within 14 days prior to biomarker assessment (Table 3).

261 Elevated hs-CRP showed a trend of a 1.5-fold increased risk of recurrent TROLS till age 1yr  
262 (hazard ratio, 1.5; 95% CI, 0.9-2.5, p=0.10), but was not associated with recurrent TROLS after age  
263 1yr or asthma at age 7yrs. Similar associations were detected with IL-6 and PC<sub>1</sub> (Table 3).

264 **DISCUSSION**265 *Key Findings*

266 This study shows that children with reduced pulmonary capacity as neonates are characterized by  
267 elevated levels of hs-CRP and a generally up-regulated blood inflammatory response suggesting  
268 presence of systemic low-grade inflammation in early childhood. These findings indicate that  
269 reduced infant lung function reflects an ongoing asymptomatic airway inflammation with a  
270 measurable systemic component early in life.

271 *Strengths and Limitations of the Study*

272 A major strength of the study is the unique assessment of neonatal lung function with the state-of-  
273 the-art raised volume rapid thoraco-abdominal compression technique performed strictly in  
274 coherence with recognized guidelines<sup>14</sup>. The infant spirometry measurements were obtained in a  
275 large sample of asymptomatic children prior to presence of any respiratory symptoms and are thus  
276 unbiased from preexisting or concurrent airway disease. Another significant strength of the study is  
277 the availability of a range of environmental exposure assessments enabling robust confounder  
278 adjustment for factors with possible influence on infant lung function and low-grade inflammation.

279 There were strong linear correlations between IL-6 and TNF- $\alpha$  and hs-CRP levels. As IL-6 and  
280 TNF- $\alpha$  are main triggers of CRP release from the liver<sup>2</sup>, these expected correlations serve as a  
281 biological validation of the data. The lack of correlation between CXCL8 and hs-CRP levels was  
282 not surprising because CXCL8 primarily has a neutrophilic chemotactic function in the innate  
283 immune system and does not directly induce CRP release<sup>24</sup>. The finding of significantly elevated  
284 hs-CRP levels in children experiencing an infectious episode within 14 days prior to biomarker  
285 assessment further assures a high signal-to-noise ratio as CRP is a reliable biomarker of ongoing  
286 infection<sup>1</sup>. Even after adjusting for this potentially strong confounder, the association between  
287 infant lung function and hs-CRP persisted with largely unchanged effect estimates. Furthermore,



288 both the standard statistical approach and the unsupervised data driven approach revealed identical  
289 associations enhancing our confidence in the findings of the study.

290 It is a limitation of the study that we were unable to detect a biologically meaningful signal from IL-  
291  $1\beta$  which is presumably partly due to the sample storage time of up to 13 years. It is well known  
292 that circulating IL- $1\beta$  levels are approximately x5 lower than TNF- $\alpha$  in healthy adults<sup>22</sup>, but in our  
293 case the median IL- $1\beta$  level was x200 lower than the median TNF- $\alpha$  level (0.01 vs. 2.34ng/L) and  
294 we were unable to detect association between IL- $1\beta$  and hs-CRP. This was not unexpected as IL- $1\beta$   
295 is particularly sensitive to freeze-thaw cycles and degrades >50% over time, even when samples are  
296 stored at -80 degree C<sup>23</sup>.

297 Another limitation of the study is the at-risk nature of the cohort, as all children are born to mothers  
298 with a history of asthma. We recently demonstrated that the offspring of mothers with a history of  
299 asthma, allergy or eczema in an unselected mother-child cohort has a topical down-regulated  
300 immune signature in the airway mucosa compared to children of mothers without such disorders<sup>25</sup>.  
301 The at-risk nature of the studied cohort may have impacted the measured biomarker levels but  
302 should not hamper our ability to explore the association between infant spirometry incentives and  
303 evident markers of systemic low-grade inflammation within the cohort.

#### 304 *Meaning of the Study*

305 The strong linear inverse association between infant lung function and hs-CRP proposes that  
306 neonates with diminished lung function are characterized by manifest systemic low-grade  
307 inflammation very early in life. This suggests that airway inflammation accompanies reduced lung  
308 function even in asymptomatic neonates and that such airway inflammation is not a local  
309 phenomenon but has a measurable systemic component. To our knowledge, no other previous study  
310 has investigated the relationship between infant lung function and low-grade inflammation in early  
311 life.

312 Hitherto, only very few childhood studies have investigated hs-CRP level in relation to pulmonary  
313 function outcomes<sup>9,26,27</sup>. In line with our findings, a study of 63 asthmatic children aged 2-12 years  
314 with and without acute exacerbations<sup>27</sup> and a study of 60 school-aged children treated with inhaled  
315 corticosteroids as well as steroid-naïve children<sup>9</sup> showed a reciprocal relationship between FEV<sub>1</sub>  
316 and hs-CRP. In contrast, another similar study of 62 school-aged children with controlled and  
317 uncontrolled asthma<sup>26</sup> did not detect association between hs-CRP and FEV<sub>1</sub>, but found that hs-CRP  
318 was higher in uncontrolled vs. controlled asthma which may reflect degree of airway inflammation.  
319 All these studies are significantly hampered by low numbers and wide age-ranges and solely  
320 investigate children with manifest asthma. Our study extends the current knowledge by  
321 demonstrating an association between hs-CRP and infant lung function measured at age 1 month in  
322 asymptomatic neonates prior to onset of any respiratory symptoms.

323 In support of our findings, a number of recent larger cross-sectional analyses in adult and adolescent  
324 studies have shown that increased hs-CRP is associated with respiratory impairment in both  
325 population-based settings and in asthmatic and non-asthmatic strata<sup>10,28,29</sup>. Longitudinal lung  
326 function follow-up performed 6-9 years after baseline in these studies and in another similar study  
327 showed no association between baseline hs-CRP and follow-up FEV<sub>1</sub><sup>28-30</sup>. In line with those  
328 findings, we found no association between hs-CRP in early life and lung function at age 7 years  
329 suggesting that low-grade systemic inflammation mainly reflects current airway inflammation and  
330 does not predict subsequent decline in lung function. This hypothesis aligns with our finding of  
331 elevated hs-CRP being associated with a recent history of asthma-like symptoms and an increased  
332 risk of developing recurrent asthma-like symptoms in the first year of life but not thereafter.

333 A possible explanation of the identified association between reduced infant lung function and  
334 elevated hs-CRP is that diminished forced volume is accompanied by airway inflammation with a

335 systemic component. Thus, in vitro murine and human lung cell studies have established a possible  
336 role of the pro-inflammatory cytokines stimulating CRP release such as IL-6, TNF- $\alpha$  and IL-1 $\beta$  in  
337 the pathophysiology of obstructive airway inflammation<sup>31,32</sup>. Persistently elevated CRP may induce  
338 an increased vulnerability to changes in the early life environment through its actions as a general  
339 scavenger protein with important innate immune functions in the recognition and elimination of  
340 bacteria and damaged human cells via opsonization, phagocytosis, and cell-mediated cytotoxicity<sup>1</sup>.

341 Alternatively, reduced neonatal lung function does not per se trigger systemic inflammation, but is  
342 rather an independent characteristic of infants with a less efficient inflammatory regulation leading  
343 to a cycle of sustained low-grade inflammation in early life. Such inefficient immune-regulation  
344 might be driven by the infant's genotype interacting with the intra uterine and early-in-life  
345 environment, thereby affecting the plasticity of the developing immune system. In support of the  
346 latter theory, higher baseline CRP levels has been demonstrated in westernized populations where  
347 obstructive airway disorders are more prevalent compared to rural societies<sup>33</sup>.

#### 348 *Conclusion*

349 Children of the Danish COPSAC<sub>2000</sub> at-risk cohort with reduced infant lung function are  
350 characterized by elevated hs-CRP level and an up-regulated blood inflammatory response  
351 suggesting that reduced lung function reflects an ongoing asymptomatic airway inflammation with a  
352 measurable systemic component early in life.

353 **TABLES**

354 **Table 1:** Heredity, anthropometrics, demographics, pre-, peri- and postnatal exposures, and  
 355 infectious episodes prior to assessment of low-grade inflammation in relation to hs-CRP level at age  
 356 6 months.

			<b>hs-CRP (mg/L) at age 6 months</b>	
<b>Characteristic</b>		<b>N</b>	<b>Median (IQR)</b>	<b>P-value</b>
Paternal asthma, allergy or eczema	Yes	135	1.52 (0.46-4.61)	0.54
	No	153	1.31 (0.46-4.44)	
Gender	Male	155	1.37 (0.37-4.44)	0.62
	Female	146	1.51 (0.49-4.81)	
Body mass index (BMI) at birth	7-12m/kg <sup>2</sup>	78	1.06 (0.45-4.46)	0.56
	12-13m/kg <sup>2</sup>	73	1.80 (0.56-4.69)	
	13-14m/kg <sup>2</sup>	74	1.41 (0.48-4.98)	
	14-17m/kg <sup>2</sup>	76	1.25 (0.39-3.75)	
Older children in the home at birth	Yes	114	2.20 (0.63-5.05)	<b>0.005</b>
	No	177	1.16 (0.41-3.40)	
Household income at birth (yearly)*	Low	77	0.83 (0.38-3.57)	0.17
	Average	144	1.31 (0.46-4.63)	
	High	70	2.27 (0.67-4.92)	
Maternal smoking during 3 <sup>rd</sup> trimester	Yes	51	1.40 (0.41-4.46)	0.33
	No	250	1.22 (0.64-5.02)	
Cesarean section	Yes	60	1.81 (0.52-5.13)	0.20
	No	205	1.31 (0.46-3-93)	
Solely breastfeeding period	0-3mo	64	2.16 (0.49-5.35)	0.43

	3-6mo	160	1.51 (0.46-4.31)	
	>6mo	40	1.02 (0.55-3.87)	
Age at start in daycare	0-9mo	89	1.80 (0.50-5.13)	0.26
	9-12mo	77	1.13 (0.36-3.40)	
	>12mo	123	1.59 (0.50-4.82)	
Cat in the home in 1 <sup>st</sup> year of life	Yes	46	1.74 (0.67-3.87)	0.42
	No	248	1.41 (0.44-4.67)	
Dog in the home in 1 <sup>st</sup> year of life	Yes	44	1.15 (0.50-3.65)	0.56
	No	249	1.52 (0.49-4.69)	
Infection 14 d before hs-CRP assessment**	Yes	95	4.29 (1.71-5.34)	<b>&lt;0.0001</b>
	No	206	0.84 (0.36-2.67)	

357 \*Yearly household income at birth of infant: low (<53.000 €), medium (53.000-80.000 €), high

358 (>80.000 €).

359 \*\*Infections include any upper or lower respiratory tract infection, gastroenteritis or fever with

360 unknown cause within 14 days before the blood sampling for hs-CRP measurement.

361 **Table 2:** Association between infant lung function and inflammatory biomarkers at age 6 months: conventional and principal component  
 362 analysis approach.

	Log-hs-CRP		Log-IL-6		Log-TNF- $\alpha$		Log-CXCL8		PC1	
	$\beta$ -coefficient (95% CI)	p	$\beta$ -coefficient (95% CI)	p	$\beta$ -coefficient (95% CI)	p	$\beta$ -coefficient (95% CI)	p	$\beta$ -coefficient (95% CI)	p
<b>UNADJUSTED ANALYSIS</b>										
z-FEV <sub>0.5</sub>	-0.12 (-0.21 to - 0.04)	<b>0.004</b>	-0.10 (-0.18 to - 0.01)	<b>0.03</b>	-0.11 (-0.38 to 0.17)	0.44	0.02 (-0.15 to 0.19)	0.83	-0.10 (-0.19 to - 0.01)	<b>0.02</b>
z-FEF <sub>50</sub>	-0.06 (-0.15 to 0.02)	0.14	-0.02 (-0.11 to 0.06)	0.61	-0.09 (-0.37 to 0.18)	0.52	-0.06 (-0.22 to 0.11)	0.49	-0.06 (-0.14 to 0.03)	0.17
Log-PD <sub>15</sub>	0.04 (-0.12 to 0.21)	0.60	-0.03 (-0.21 to 0.15)	0.75	-0.02 (-0.56 to 0.52)	0.94	0.15 (-0.17 to 0.46)	0.36	0.03 (-0.14 to 0.19)	0.76
<b>ADJUSTED ANALYSIS*</b>										
z-FEV <sub>0.5</sub>	-0.13 (-0.22 to - 0.04)	<b>0.005</b>	-0.09 (-0.18 to 0.00)	<b>0.04</b>	-0.13 (-0.43 to 0.18)	0.41	0.02 (-0.15 to 0.20)	0.79	-0.10 (-0.20 to - 0.01)	<b>0.03</b>
z-FEF <sub>50</sub>	-0.06 (-0.16 to 0.03)	0.18	-0.03 (-0.12 to 0.06)	0.49	-0.11 (-0.42 to 0.19)	0.46	-0.06 (-0.23 to 0.12)	0.50	-0.06 (-0.15 to 0.03)	0.19
Log-PD <sub>15</sub>	0.02 (-0.16 to 0.20)	0.83	-0.03 (-0.22 to 0.15)	0.72	-0.06 (-0.61 to 0.50)	0.84	0.15 (-0.18 to 0.48)	0.35	-0.02 (-0.19 to 0.16)	0.85

363 PC1 = Principal Component 1; FEV<sub>0.5</sub> = Forced Expiratory Volume at 0.5 seconds; FEF<sub>50</sub> = Forced Expiratory Flow at 50% of the forced  
 364 vital capacity; PD<sub>15</sub> = Provocative Dose of methacholine causing a 15% drop in transcutaneous oxygen saturation.

365 \* Adjusted for birth BMI, maternal smoking during 3<sup>rd</sup> pregnancy trimester, older children in the home at birth and infectious episodes  
 366 within 14days prior to blood sampling for inflammatory biomarkers assessment.

367 **Table 3:** Association between inflammatory biomarkers at age 6 months and asthma-related outcomes at 0-7 years: conventional and  
 368 principal component analysis approach.

	Log-hs-CRP		Log-IL-6		Log-TNF- $\alpha$		Log-CXCL8		PC <sub>1</sub>	
	Estimate (95% CI)	p	Estimate (95% CI)	p	Estimate (95% CI)	p	Estimate (95% CI)	p	Estimate (95% CI)	p
	<b>UNADJUSTED ANALYSIS</b>									
Any TROLS, 0-6mo <sup>1</sup>	0.04 (0.00-0.08)	<b>0.05</b>	0.04 (0.00-0.09)	<b>0.05</b>	0.09 (-0.05-0.23)	0.18	0.09 (0.00-0.17)	<b>0.04</b>	0.06 (0.01-0.10)	<b>0.01</b>
Recurrent TROLS, 0-1yr <sup>2</sup>	1.5 (0.9-2.5)	<b>0.10</b>	1.5 (0.9-2.4)	0.11	1.5 (0.4-6.6)	0.56	1.2 (0.6-2.3)	0.63	1.4 (0.9-2.0)	0.12
Recurrent TROLS, 0-yrs <sup>2</sup>	1.0 (0.8-1.2)	0.95	1.0 (0.8-1.2)	0.79	1.0 (0.6-1.9)	0.88	0.9 (0.6-1.3)	0.43	1.0 (0.8-1.2)	0.97
Asthma, 7yrs <sup>3</sup>	1.0 (0.8-1.3)	0.99	1.0 (0.7-1.2)	0.73	0.7 (0.3-1.6)	0.36	0.6 (0.3-1.3)	0.17	0.9 (0.7-1.2)	0.62
	<b>ADJUSTED ANALYSIS*</b>									

Any TROLS, 0-6mo <sup>1</sup>	0.05 (0.00-0.09)	<b>0.04</b>	0.04 (-0.01-0.08)	<b>0.08</b>	0.06 (-0.08-0.21)	0.40	0.07 (-0.01-0.15)	0.1 1	0.05 (0.01-0.10)	<b>0.03</b>
Recurrent TROLS, 0-1yr <sup>2</sup>	1.6 (0.9-2.7)	<b>0.09</b>	1.4 (0.8-2.3)	0.20	1.3 (0.3-5.4)	0.76	1.1 (0.6-2.0)	0.7 9	1.3 (0.9-1.9)	0.21
Recurrent TROLS, 0-yrs <sup>2</sup>	1.0 (0.8-1.2)	0.97	1.0 (0.8-1.1)	0.62	0.9 (0.5-1.6)	0.66	0.8 (0.5-1.2)	0.3 0	1.0 (0.8-1.2)	0.68
Asthma, 7yrs <sup>3</sup>	1.0 (0.8-1.3)	0.97	0.9 (0.7-1.2)	0.66	0.6 (0.3-1.4)	0.26	0.6 (0.3-1.2)	0.1 5	0.9 (0.7-1.2)	0.43

369 PC<sub>1</sub> = Principal Component 1; TROLS = TRoublesome Lung Symptoms.

370 \* Adjusted for older children in the home at birth and infectious episodes within 14days prior to blood sampling for inflammatory  
371 biomarkers assessment.

372 <sup>1</sup>Occurence of any TROLS from birth till age 6 months: general linear model (estimate= $\beta$ -coefficient).

373 <sup>2</sup>Time to onset of recurrent TROLS: Cox regression (estimate=hazard ratio).

374 <sup>3</sup>Asthma at age 7 years (yes/no): logistic regression (estimate=odds ratio).



375 **Table E1 Online:** Comparison of baseline characteristics between children with and without  
 376 complete assessment of early-life low-grade inflammation.

<b>Baseline characteristic</b>	<b>Children with biomarker assessment N=300</b>	<b>Children without biomarker assessment N=111</b>	<b>p</b>
Paternal asthma, allergy or eczema, % (N)	47% (135)	46% (50)	0.84 <sup>c</sup>
Male gender, % (N)	51% (154)	44% (49)	0.20 <sup>c</sup>
BMI at birth, mean (SD)	12.79m/kg <sup>2</sup> (1.34)	12.84m/kg <sup>2</sup> (1.22)	0.63 <sup>t</sup>
Older children in the home at birth, % (N)	39% (114)	40% (38)	0.91 <sup>c</sup>
Household income at birth <sup>*</sup> , % (N)			0.12 <sup>c</sup>
Low	27% (77)	38% (35)	
Average	49% (143)	41% (39)	
High	24% (70)	21% (20)	
Maternal smoking during 3 <sup>rd</sup> trimester, % (N)	17% (51)	11% (12)	0.12 <sup>c</sup>
Cesarean section, % (N)	23% (60)	27% (25)	0.45 <sup>c</sup>
Solely breastfeeding length, median (IQR)	122days (90-155)	122days (74-164)	0.90 <sup>w</sup>
Age at start in daycare, median (IQR)	345days (240-415)	307days (216-412)	0.27 <sup>w</sup>
Cat in the home in 1 <sup>st</sup> year of life, % (N)	16% (46)	14% (14)	0.61 <sup>c</sup>

Dog in the home in 1 <sup>st</sup> year of life, % (N)	15% (44)	10% (10)	0.16 <sup>c</sup>
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377 \*Yearly household income at birth of infant: low (<53.000 €), medium (53.000-80.000 €), high

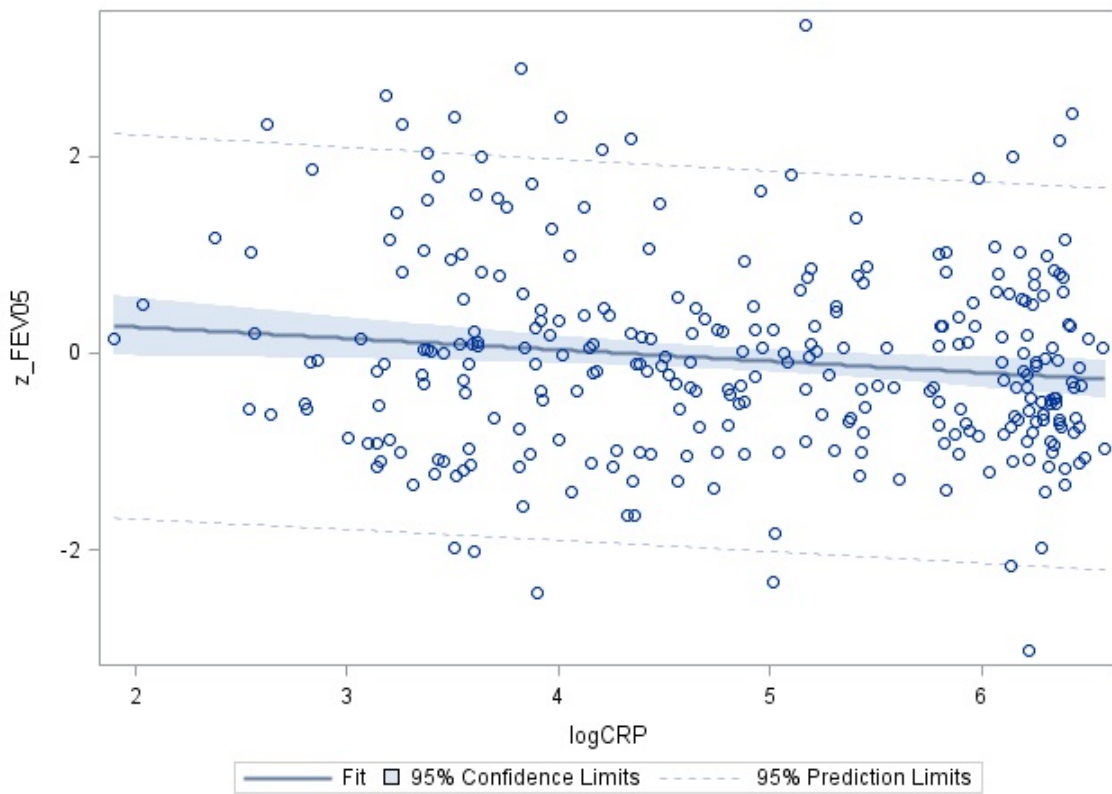
378 (>80.000 €), <sup>c</sup>Chi-square test, <sup>t</sup>t-test, <sup>w</sup>Wilcoxon rank sum test

379 **Table E2:** Association between inflammatory biomarkers at age 6 months and lung function at age 7 years: conventional and principal  
 380 component analysis approach.

	<b>Log-hs-CRP</b>		<b>Log-IL-6</b>		<b>Log-TNF-<math>\alpha</math></b>		<b>Log-CXCL8</b>		<b>PC1</b>	
	$\beta$ -coefficient (95% CI)	p	$\beta$ -coefficient (95% CI)	p	$\beta$ -coefficient (95% CI)	p	$\beta$ -coefficient (95% CI)	p	$\beta$ -coefficient (95% CI)	p
z-FEV <sub>1</sub>	0.04 (-0.06-0.15)	0.45	0.02 (-0.08-0.12)	0.68	0.20 (-0.12-0.52)	0.21	0.18 (-0.03-0.40)	0.09	0.05 (-0.05-0.15)	0.32
z-MMEF	0.01 (-0.10-0.11)	0.92	-0.01 (-0.11-0.10)	0.91	0.11 (-0.23-0.44)	0.53	0.14 (-0.09-0.36)	0.23	0.03 (-0.08-0.14)	0.57
Log-PD <sub>20</sub>	0.13 (-0.15-0.29)	0.08	0.09 (-0.06-0.25)	0.24	0.30 (-0.15-0.75)	0.19	0.02 (-0.30-0.34)	0.90	0.07 (-0.07-0.21)	0.34

381 PC1 = Principal Component 1; FEV<sub>1</sub> = Forced Expiratory Volume at 0.5 seconds; MMEF = Maximal Mid-Expiratory Flow; PD<sub>20</sub> =

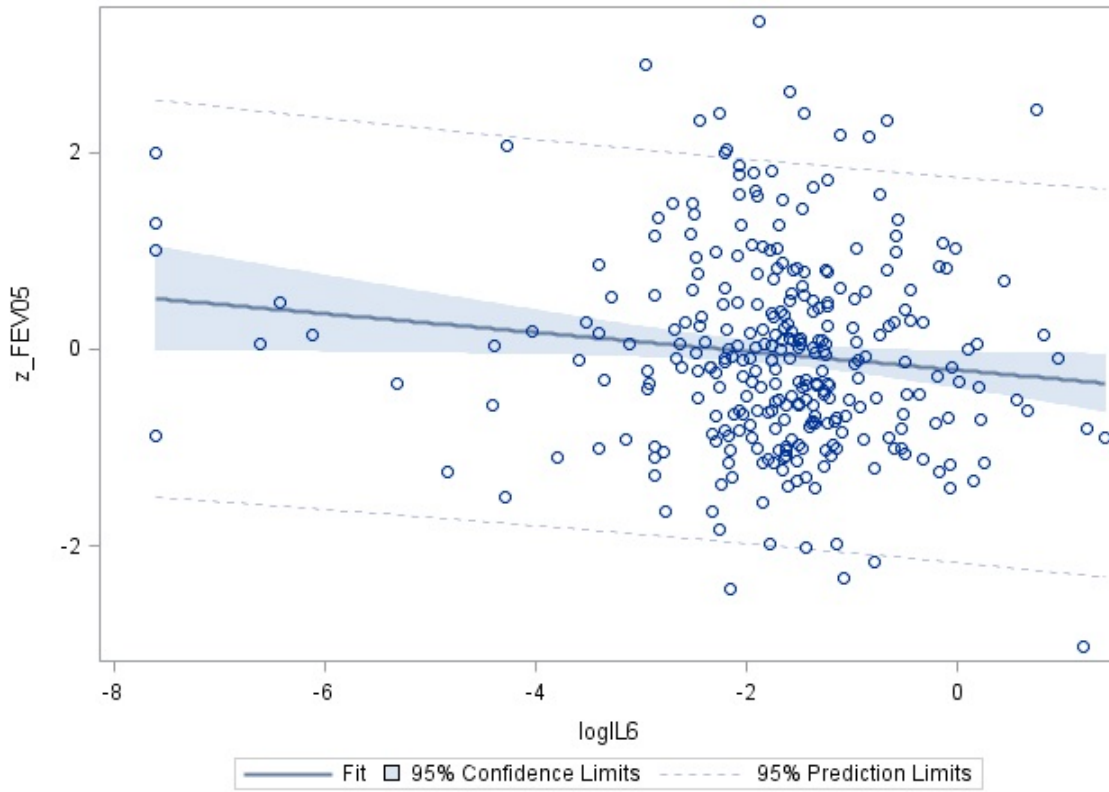
382 Provocative Dose of methacholine causing a 20% drop in FEV<sub>1</sub> from baseline.

383 **FIGURES**384 **Figure 1:** Scatter plot illustrating the relationship between neonatal lung function (z-score of385 FEV<sub>0.5</sub>) and hs-CRP at age 6 months (log-transformed values).

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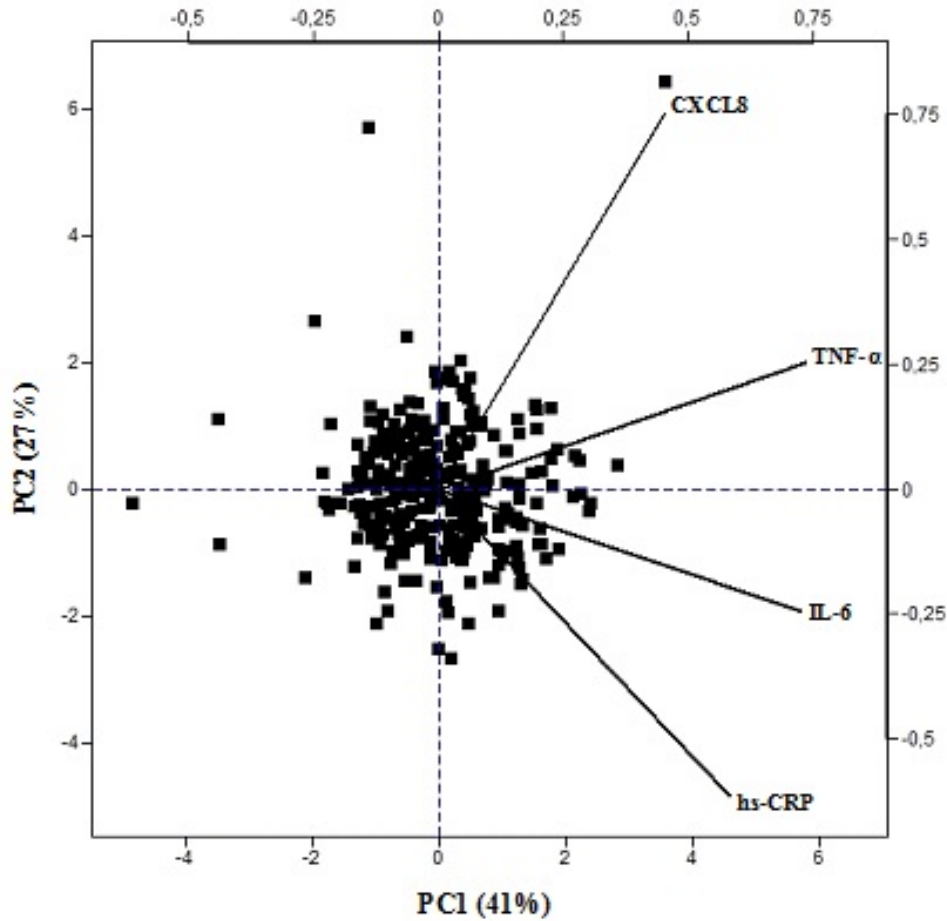
388 **Figure 2:** Scatter plot illustrating the relationship between neonatal lung function (z-score of  
389 FEV<sub>0.5</sub>) and IL-6 at age 6 months (log-transformed values).



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392 **Figure 3:** Principal component analysis biplot showing scores and loadings for hs-CRP, IL-6, TNF-  
393  $\alpha$  and CXCL8 in the first principal component (PC1) and second principal component (PC2).  
394 Percentages in parenthesis are the part of the total variation in the data set explained by the  
395 components.



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