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Chawes, Bo L.K.; Stokholm, Jakob; Bønnelykke, Klaus; Pedersen, Susanne Brix; Bisgaard, Hans Flinker

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Reduced Neonatal Lung Function Associates with Systemic Low-grade Inflammation in Early Life

Authors: Bo L K Chawes, MD, PhD[1]; Jakob Stokholm MD, PhD[1+3]; Klaus Bønnelykke, MD, PhD[1]; Susanne Brix, MSc, PhD[2]; Hans Bisgaard, MD, DMSc[1]

(1) Copenhagen Prospective Studies on Asthma in Childhood
Health Sciences, University of Copenhagen &
Danish Pediatric Asthma Center
Copenhagen University Hospital, Gentofte; Denmark

(2) Center for Biological Sequence Analysis,
Department of Systems Biology
Technical University of Denmark; Lyngby; Denmark

(3) Department of Pediatrics, Naestved Hospital, Naestved; Denmark.

Corresponding author:

Professor Hans Bisgaard
Copenhagen Prospective Studies on Asthma in Childhood
Health Sciences, University of Copenhagen &
Danish Pediatric Asthma Center
Copenhagen University Hospital, Gentofte
Ledreborg Allé 34
2820 Gentofte

21 Denmark

22 Tel: +45 39777360

23 Fax: +45 39777129

24 E-mail: bisgaard@copsac.com

25 Website: www.copsac.com

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Abbreviations: $COPSAC_{2000}$ = COpenhagen Prospective Study on Asthma in Childhood; CXCL8 (IL-8) = Chemokine (C-X-C motif) Ligand 8; $FEV_{0.5}$ = Forced Expiratory Volume at 0.5 seconds; FEF_{50} = Forced Expiratory Flow at 50% of the forced vital capacity; hs-CRP = high-sensitivity C-reactive protein; IL-1β = Interleukin-1β, IL-6 = Interleukin-6; MMEF = Maximal Mid-Expiratory Flow; $PtcO_2$ = transcutaneuos oxygen saturation; PD_{15} = Provocative Dose of methacholine causing a 15% drop in $PtcO_2$; PD_{20} = Provocative Dose of methacholine causing a 20% drop in FEV_1 from baseline; TNF-α = tumor necrosis factor-α; TROLS = TROublsome Lung Symptoms.

Online Repository: This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

At a Glance Commentary:

Scientific Knowledge on the Subject

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

What this Study Adds to the Field

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

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

ABSTRACT

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

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

Methods
Serum levels of high-sensitivity C-reactive protein (hs-CRP), interleukin-1β (IL-1β), IL-6, tumor necrosis factor-α (TNF-α) and CXCL8 (IL-8) were measured at age 6 months in 300 children of the Copenhagen Prospective Study on Asthma in Childhood$_{2000}$ (COPSAC$_{2000}$) birth cohort, who completed infant lung function testing at age 1 month, spirometry at 7yrs, and fulfilled a respiratory day-to-day diary from 0-7yrs. Associations between lung function indices, asthmatic symptoms and inflammatory biomarkers were investigated by conventional statistics and unsupervised principle component analysis.

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

Conclusion
Diminished infant lung function associates with elevated hs-CRP and an up-regulated blood inflammatory response suggesting linkage between lung function and systemic low-grade inflammation in early life.

Abstract Word Count: 252 words

Key-words: Asthma, Children, high-sensitivity C-reactive protein, pro-inflammatory cytokines, spirometry.

INTRODUCTION

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

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

We hypothesized that children with reduced neonatal lung function may have biochemical signs of systemic low-grade inflammation in infancy. The objective of the current study was therefore to investigate the possible association between lung function indices measured in asymptomatic neonates of the Copenhagen Prospective Study on Asthma in $Childhood_{2000}$ ($COPSAC_{2000}$) birth cohort and serum levels of hs-CRP, IL-1β, IL-6, TNF-α, and CXCL8 (formerly IL-8) at age 6 months.

METHODS

Study Cohort

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

Ethics

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

Inflammatory Biomarkers

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

Lung Function

Infant spirometry was measured at age 1 month applying the raised volume rapid thoraco-abdominal "squeeze"-jacket compression technique[14]. Repeated ventilations to predefined mouth-pressures assured expansion of the lung volume before an instant inflation of the jacket caused a full exhalation during which the flow was measured by a pneumotachograph with an aircushion facemask[15,16]. The software identified the Forced Vital Capacity (FVC) as the first plateau on the volume-time curve and measurements with FVC appearing after 0.5s and with the Forced Expiratory Volume at 0.5s ($FEV_{0.5}$) being smaller than or equal to FVC were accepted. Three to five acceptable curves were obtained for each infant and the curve containing the median value of $FEV_{0.5}$ was used for the analyses of $FEV_{0.5}$ and Forced Expiratory Flow at 50% of FVC (FEF_{50}).

Spirometry at age 7yrs was performed as previously detailed[17] using a pneumotachograph, Masterscope Pneumoscreen, system 754,916 spirometer (Erich Jaeger, Wurtzburg, Germany) for assessing FEV_1 and maximal mid-expiratory flow (MMEF).

Infant bronchial responsiveness: After an initial saline inhalation, methacholine was given in quadrupling dose-steps via a dosimeter attached to a nebulizer (SPIRA 08 TSM 133; Respiratory Care Center; Hämeenlinna, Finland)[16]. Bronchial responsiveness was determined by continuous assessment of transcutaneuos oxygen saturation ($PtcO_2$) (TCM3; Radiometer; Copenhagen, Denmark). The provocative dose causing a 15% drop in $PtcO_2$ (PD_{15}) was estimated from the dose response curves fitted with a logistic function.

Bronchial responsiveness at age 7yrs was defined as the provocative dose of methacholine causing a 20% drop in FEV_1 from baseline (PD_{20})[17].

Clinical Investigator-diagnosed End-points

Troublesome lung symptoms (TROLS) were defined as significant cough or wheeze or dyspnea severely affecting the well-being of the child and recorded by the parents in a daily diary chart as a

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

Asthma at age 7yrs was diagnosed according to international guidelines and was based on recurrent TROLS as defined above, symptoms judged by the COPSAC pediatricians to be typical of asthma, in need of intermittent inhaled β_2-agonist, responding to a 3-month trial of inhaled corticosteroids and relapsing when stopping treatment[12,13].

Covariates

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

Statistics

Biomarker null values were set to half of the lowest detected value for the specific biomarker, values were log-transformed, and the mean of the duplicate measurements were used for association analyses. Z-scores were calculated for $FEV_{0.5}$, FEV_1, FEF_{50} and MMEF, and PD_{15} and PD_{20} were log-transformed to obtain normality. The associations between lung function, asthmatic symptoms, and inflammatory biomarkers were tested by conventional statistics and by unsupervised pattern recognition using principal component analysis (PCA).

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

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

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

RESULTS

Inflammatory Biomarker Assessments

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

The median hs-CRP level was 1.39 mg/L (inter-quartile range [IQR], 0.46-4.61), IL-1β was 0.01 ng/L (0.001-0.04), IL-6 was 0.20 ng/L (0.11-0.31), TNF-α was 2.34 ng/L (1.92-2.88), and CXCL8 was 3.04 ng/L (2.19-4.37). The IL-6 and TNF-α levels were strongly positively correlated with hs-CRP levels ($p<0.001$ for both) whereas IL-1β and CXCL8 levels were not correlated with hs-CRP ($p\geq0.62$). The measured values of hs-CRP, IL-6, TNF-α and CXCL8 were within the expected range[22] with very few null values, whilst IL-1β levels were much lower than expected[22] with null values for 72 of 308 children (23%). Due to that and the fact that IL-1β has been shown to significantly degrade over time even at -80° C[23], IL-1β was not included in further analyses.

Determinants of hs-CRP

Children with older children in the home at birth had significantly higher hs-CRP level at age 6 months compared to children without older children in the home: median hs-CRP level 2.20 mg/L (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 who had suffered an infectious episode within 14 days prior to biomarker assessment compared to children without apparent infections: 4.29 mg/L (1.71-5.34) vs. 0.84 mg/L (0.36-2.67), $p<0.0001$. We did not detect associations between hs-CRP level and paternal history of asthma, eczema or allergy, child gender, birth BMI, household income, maternal smoking during 3rd pregnancy

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

Lung Function and Systemic Low-grade Inflammation

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

Increasing $FEV_{0.5}$ was also significantly associated with decreasing levels of IL-6 (β-coefficient, -0.10; 95% CI, -0.18 to -0.01; p=0.03) (Figure 2). Confounder adjustment did not modify the association: β-coefficient, -0.09; 95% CI, -0.18 to 0.00; p=0.04. We did not detect a significant association between FEF_{50} and IL-6 levels.

$FEV_{0.5}$ and FEF_{50} measurements were not associated with CXCL8 or TNF-α levels although the β-coefficients suggested an inverse association between lung function indices and TNF-α (Table 2).

The unsupervised PCA showed that hs-CRP, IL-6, TNF-α and CXCL8 were positively correlated in the first principal component (PC_1) which explained 41% of the total variation in the data. The PCA approach is illustrated in the biplot (Figure 3) showing scores for PC_1 and PC_2 and loadings for the biomarkers. Because of the univocal pattern in PC_1, we focused on PC_1 in the further analyses. Confirming the findings from the conventional statistics, we found that $FEV_{0.5}$ was inversely associated with PC_1 (p=0.02) and remained significant after confounder adjustments (p=0.03). The

β-coefficients also suggested an inverse association between FEF_{50} and PC_1, but the model was not significant (Table 2).

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

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

Lung Symptoms, Asthma and Systemic Low-grade Inflammation
Children experiencing TROLS at any time-point from birth till biomarker assessment (0-6mo) compared to children without TROLS had significantly elevated levels of hs-CRP: median 1.79 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 (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 PCA approach confirmed an up-regulated blood inflammatory profile in children experiencing TROLS at age 0-6mo (p=0.01). The findings were unaffected by adjustment for older children in the home and infectious episodes within 14 days prior to biomarker assessment (Table 3).

Elevated hs-CRP showed a trend of a 1.5-fold increased risk of recurrent TROLS till age 1yr (hazard ratio, 1.5; 95% CI, 0.9-2.5, p=0.10), but was not associated with recurrent TROLS after age 1yr or asthma at age 7yrs. Similar associations were detected with IL-6 and PC_1 (Table 3).

DISCUSSION

Key Findings

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

Strengths and Limitations of the Study

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

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

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

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

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

Meaning of the Study

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

Hitherto, only very few childhood studies have investigated hs-CRP level in relation to pulmonary function outcomes[9,26,27]. In line with our findings, a study of 63 asthmatic children aged 2-12 years with and without acute exacerbations[27] and a study of 60 school-aged children treated with inhaled corticosteroids as well as steroid-naïve children[9] showed a reciprocal relationship between FEV_1 and hs-CRP. In contrast, another similar study of 62 school-aged children with controlled and uncontrolled asthma[26] did not detect association between hs-CRP and FEV_1, but found that hs-CRP was higher in uncontrolled vs. controlled asthma which may reflect degree of airway inflammation. All these studies are significantly hampered by low numbers and wide age-ranges and solely investigate children with manifest asthma. Our study extends the current knowledge by demonstrating an association between hs-CRP and infant lung function measured at age 1 month in asymptomatic neonates prior to onset of any respiratory symptoms.

In support of our findings, a number of recent larger cross-sectional analyses in adult and adolescent studies have shown that increased hs-CRP is associated with respiratory impairment in both population-based settings and in asthmatic and non-asthmatic strata[10,28,29]. Longitudinal lung function follow-up performed 6-9 years after baseline in these studies and in another similar study showed no association between baseline hs-CRP and follow-up FEV_1[28-30]. In line with those findings, we found no association between hs-CRP in early life and lung function at age 7 years suggesting that low-grade systemic inflammation mainly reflects current airway inflammation and does not predict subsequent decline in lung function. This hypothesis aligns with our finding of elevated hs-CRP being associated with a recent history of asthma-like symptoms and an increased risk of developing recurrent asthma-like symptoms in the first year of life but not thereafter.

A possible explanation of the identified association between reduced infant lung function and 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-α and IL-1β in
337 the pathophysiology of obstructive airway inflammation[31,32]. 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[1].

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[33].

348 *Conclusion*
349 Children of the Danish $COPSAC_{2000}$ 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.

			hs-CRP (mg/L) at age 6 months	
Characteristic		**N**	**Median (IQR)**	**P-value**
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^2	78	1.06 (0.45-4.46)	0.56
	12-13m/kg^2	73	1.80 (0.56-4.69)	
	13-14m/kg^2	74	1.41 (0.48-4.98)	
	14-17m/kg^2	76	1.25 (0.39-3.75)	
Older children in the home at birth	Yes	114	2.20 (0.63-5.05)	**0.005**
	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 3rd 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 1st 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 1st 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)	**<0.0001**
	No	206	0.84 (0.36-2.67)	

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

**Infections include any upper or lower respiratory tract infection, gastroenteritis or fever with unknown cause within 14 days before the blood sampling for hs-CRP measurement.

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

	Log-hs-CRP		**Log-IL-6**		**Log-TNF-α**		**Log-CXCL8**		**PC1**	
	β-coefficient (95% CI)	p	β-coefficient (95% CI)	p	β-coefficient (95% CI)	p	β-coefficient (95% CI)	p	β-coefficient (95% CI)	p
	UNADJUSTED ANALYSIS									
z-$FEV_{0.5}$	-0.12 (-0.21 to -0.04)	**0.004**	-0.10 (-0.18 to -0.01)	**0.03**	-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)	**0.02**
z-FEF_{50}	-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_{15}	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
	ADJUSTED ANALYSIS*									
z-$FEV_{0.5}$	-0.13 (-0.22 to -0.04)	**0.005**	-0.09 (-0.18 to 0.00)	**0.04**	-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)	**0.03**
z-FEF_{50}	-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_{15}	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

PC1 = Principal Component 1; $FEV_{0.5}$ = Forced Expiratory Volume at 0.5 seconds; FEF_{50} = Forced Expiratory Flow at 50% of the forced vital capacity; PD_{15} = Provocative Dose of methacholine causing a 15% drop in transcutaneuos oxygen saturation.

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

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

	Log-hs-CRP		**Log-IL-6**		**Log-TNF-α**		**Log-CXCL8**		**PC_1**	
	Estimate (95% CI)	p	Estimate (95% CI)	p	Estimate (95% CI)	p	Estimate (95% CI)	p	Estimate (95% CI)	p
	UNADJUSTED ANALYSIS									
Any TROLS, 0-6mo[1]	0.04 (0.00-0.08)	**0.05**	0.04 (0.00-0.09)	**0.05**	0.09 (-0.05-0.23)	0.18	0.09 (0.00-0.17)	**0.04**	0.06 (0.01-0.10)	**0.01**
Recurrent TROLS, 0-1yr[2]	1.5 (0.9-2.5)	**0.10**	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[2]	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[3]	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
	ADJUSTED ANALYSIS*									

Any TROLS, 0-6mo[1]	0.05 (0.00-0.09)	**0.04**	0.04 (-0.01-0.08)	**0.08**	0.06 (-0.08-0.21)	0.40	0.07 (-0.01-0.15)	0.11	0.05 (0.01-0.10)	**0.03**
Recurrent TROLS, 0-1yr[2]	1.6 (0.9-2.7)	**0.09**	1.4 (0.8-2.3)	0.20	1.3 (0.3-5.4)	0.76	1.1 (0.6-2.0)	0.79	1.3 (0.9-1.9)	0.21
Recurrent TROLS, 0-yrs[2]	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)	0.30	1.0 (0.8-1.2)	0.68
Asthma, 7yrs[3]	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)	0.15	0.9 (0.7-1.2)	0.43

369 PC_1 = 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 [1]Occurence of any TROLS from birth till age 6 months: general linear model (estimate=β-coefficent).

373 [2]Time to onset of recurrent TROLS: Cox regression (estimate=hazard ratio).

374 [3]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.

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

Dog in the home in 1st year of life, % (N)	15% (44)	10% (10)	0.16[c]

[*]Yearly household income at birth of infant: low (<53.000 €), medium (53.000-80.000 €), high (>80.000 €), [c]Chi-square test, [t]t-test, [w]Wilcoxon rank sum test

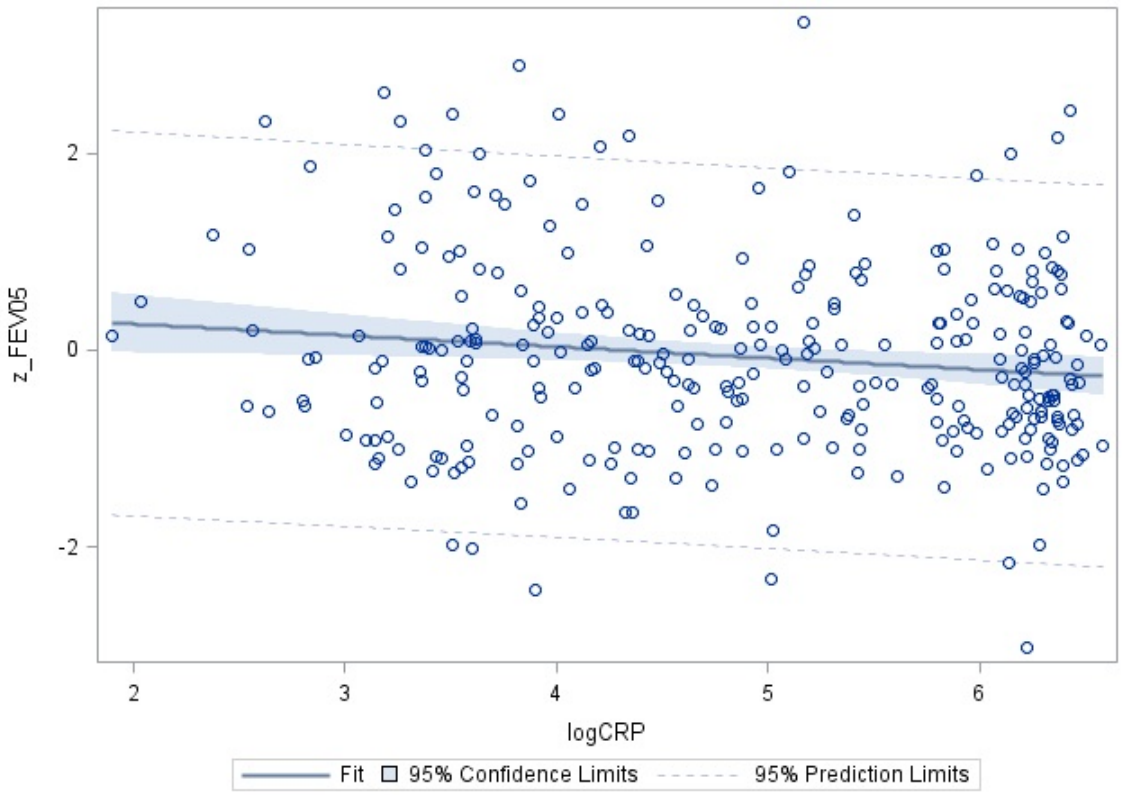
Table E2: Association between inflammatory biomarkers at age 6 months and lung function at age 7 years: conventional and principal component analysis approach.

	Log-hs-CRP		**Log-IL-6**		**Log-TNF-α**		**Log-CXCL8**		**PC1**	
	β-coefficient (95% CI)	p	β-coefficient (95% CI)	p	β-coefficient (95% CI)	p	β-coefficient (95% CI)	p	β-coefficient (95% CI)	p
z-FEV_1	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_{20}	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

PC1 = Principal Component 1; FEV_1 = Forced Expiratory Volume at 0.5 seconds; MMEF = Maximal Mid-Expiratory Flow; PD_{20} = Provocative Dose of methacholine causing a 20% drop in FEV_1 from baseline.

FIGURES

Figure 1: Scatter plot illustrating the relationship between neonatal lung function (z-score of $FEV_{0.5}$) and hs-CRP at age 6 months (log-transformed values).



388 **Figure 2:** Scatter plot illustrating the relationship between neonatal lung function (z-score of

389 $FEV_{0.5}$) and IL-6 at age 6 months (log-transformed values).

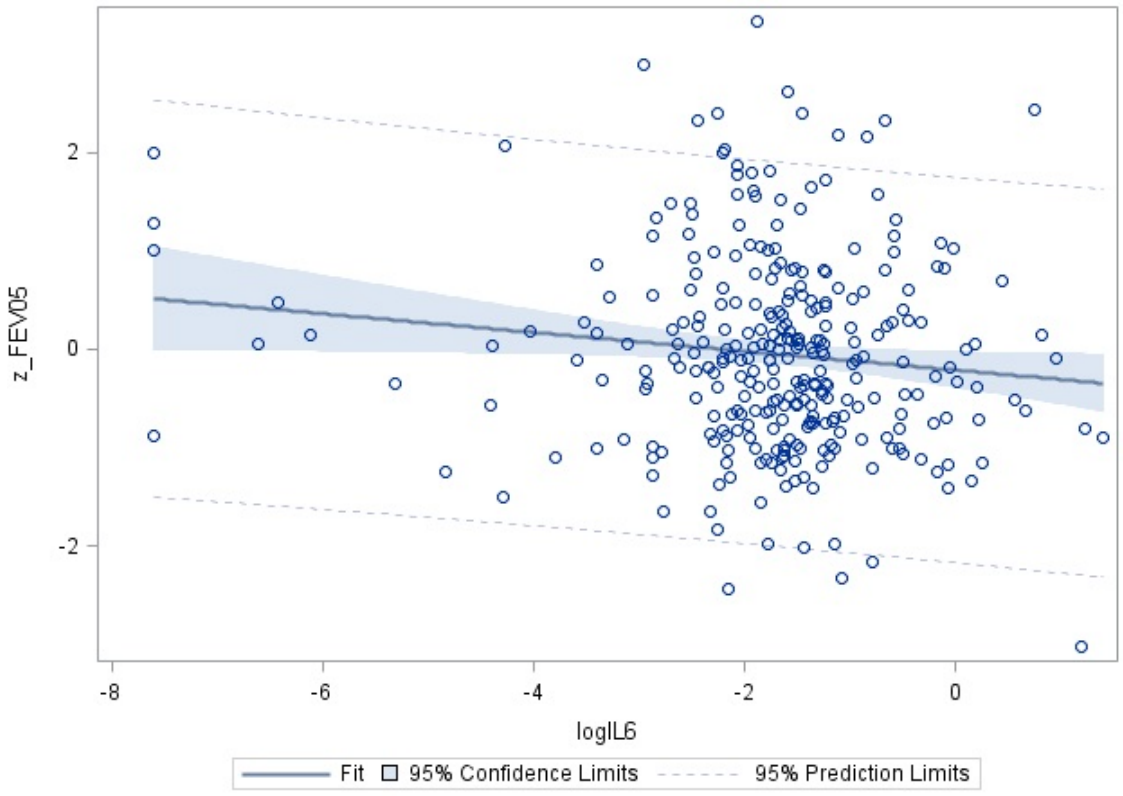


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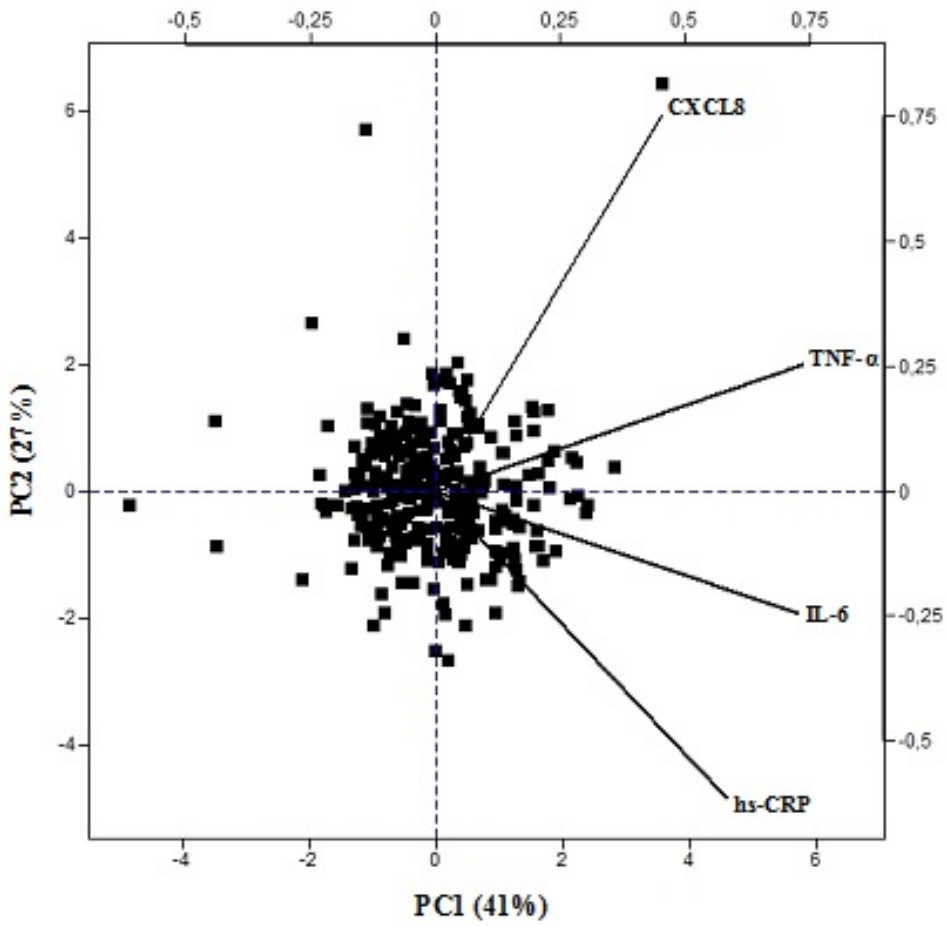
Figure 3: Principal component analysis biplot showing scores and loadings for hs-CRP, IL-6, TNF-α and CXCL8 in the first principal component (PC1) and second principal component (PC2). Percentages in parenthesis are the part of the total variation in the data set explained by the components.



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