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Neonates colonized with pathogenic bacteria in the airways have a low-grade systemic inflammation

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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, KK, MK and SS have all been involved in laboratory work and processing of biological data samples. NRF, JT, BC, JS and KB have done all preliminary analysis, all final analysis and interpretation of data have been discussed and reviewed by co-authors SB, KK, MK and SS. All co-authors have provided important intellectual input to the manuscript. NRF has written the first draft of the manuscript. All authors have agreed 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. No honorarium, grant, or other form of payment was given to anyone to produce the manuscript.

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Conflict of interest:

Authors declare no potential, perceived, or real conflict of interest regarding the content of this manuscript.

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 Harmonization'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:

COPSAC₂₀₀₀ = COpenhagen Prospective Studies on Asthma in Childhood₂₀₀₀ COPSAC₂₀₁₀ =

COpenhagen Prospective Studies on Asthma in Childhood₂₀₁₀

H. influenzae = *Haemophilus influenzae*

M. catharralis = *Moraxella catharralis*

S. pneumoniae = *Streptococcus pneumoniae*

hs-CRP = High sensitivity CRP

IL-6 = Interleukin-6

TNF- α = Tumor Necrosis Factor alpha

qPCR = Quantitative Polymerase Chain Reaction

ABSTRACT

Background and Objectives: The development of childhood asthma is associated with neonatal colonization with pathogenic bacteria in hypopharynx. Furthermore, established asthma is associated with systemic low-grade inflammation. We here report on the association between neonatal colonization with pathogenic bacteria in hypopharynx and the development of systemic low-grade inflammation.

Methods: Bacterial colonization of the hypopharynx with *M. catharralis*, *H. influenzae* and/or *S. pneumoniae* was assessed in asymptomatic children from the Copenhagen Prospective Studies on Asthma in Childhood₂₀₀₀ (COPSAC₂₀₀₀) cohort at age 1 month by culturing technique (N=238) and by quantitative polymerase chain reaction (qPCR) technique (N=249) and in the COPSAC₂₀₁₀ cohort by culturing at age 1 month (N=622) and again at age 3 months (N=613). Systemic low-

grade inflammation was determined in both cohorts at age 6 months by measuring plasma levels of high-sensitivity C-reactive protein (hs-CRP), tumor necrosis factor- α (TNF- α) and interleukine-6 (IL-6).

Results: In both cohorts, bacterial colonization was associated with increased levels of hs-CRP: COPSAC₂₀₀₀, 1 month culturing (geometric mean ratio of colonized/non-colonized [95% CI]), 1.39 [0.97–2.01], $p=0.08$, 1 month qPCR, 1.55 [1.14–2.10], $p<0.01$; COPSAC₂₀₁₀, 1 month, 1.52 [1.23–1.87], $p<0.01$, and 3 month, 1.57 [1.30–1.90], $p<0.01$. A multi-parametric principal component analysis incorporating hs-CRP, TNF- α and IL-6 confirmed a systemic inflammatory profile in children colonized with *M. catharralis*, *H. influenzae* and/or *S. pneumoniae* in the hypopharynx compared to non-colonized children (p -values <0.05).

Conclusion: The composition of the upper airway microbiome in early life may cause systemic low-grade inflammation.

Keywords: asthma, clinical immunology, environment and hygiene hypothesis, innate immunity, pediatrics.

INTRODUCTION

Chronic inflammatory diseases such as asthma (1), allergy (2), chronic obstructive pulmonary disease (3), cardiovascular disease (4), obesity (5), and diabetes mellitus (6) have been reported to be associated with a low-grade systemic inflammation as reflected in elevated biomarkers such as high-sensitivity C-reactive protein (hs-CRP). This suggests a shared disease mechanism in chronic inflammatory disorders, which is proposed to be caused by alterations of the gut microbiome (7–9), and possibly the airway microbiome (10–12).

The airway microbiome of the hypopharynx in neonates has been associated with the development of childhood asthma (13,14). The airway microbiome is believed to play a key role regulating chronic airway inflammation (10,12), but it is unknown whether the early life airway microbiome is also associated with systemic low-grade inflammation.

The aim of this study was to analyze systemic inflammation in neonates colonized in the upper airways with *M. catharralis*, *H. influenzae* and/or *S. pneumonia*. The analyses were performed in children in the at-risk mother-child cohort Copenhagen Prospective Studies on Asthma in Childhood, COPSAC₂₀₀₀, and replication was sought in the unselected COPSAC₂₀₁₀ mother-child cohort.

METHODS

Study Populations

Data was obtained from the two ongoing clinical mother-child cohorts, COPSAC₂₀₀₀ and COPSAC₂₀₁₀, both from Zealand, Denmark. COPSAC₂₀₀₀ is a high-risk cohort including 411 children of asthmatic mothers, where exclusion criteria were any congenital abnormality, a gestational age below 36 weeks, and any respiratory symptoms before the inclusion visit at age one month (15). COPSAC₂₀₁₀ is a population-based cohort enrolling 738 pregnant women from 24 weeks of gestation and their 700 children. Exclusion criteria were women with any endocrine, heart, or kidney disorder or a vitamin D intake above 600 IU/d (16).

The Local Ethics Committee (COPSAC₂₀₀₀: KF 01-289/96, COPSAC₂₀₁₀: H-B-2008-093) and the Danish Data Protection Agency (COPSAC₂₀₀₀ and COPSAC₂₀₁₀: 2015-41-3696) approved the studies.

Both parents gave oral and written informed consent before enrolment.

Assessments of Bacterial Colonization of the Hypopharynx

COPSAC₂₀₀₀: At the age of one month, an airway aspirate was sampled in asymptomatic neonates with a soft suction catheter passed through the nose into the hypopharynx (13–15). At the same visit, swaps were obtained from the nasopharynx by sterile ESwab (Copan Diagnostic Inc., CA USA) through the nasal cavity.

COPSAC₂₀₁₀: Airway aspirates were collected at age one month and again at age three months from the hypopharynx using the same standard operating procedure as in COPSAC₂₀₀₀ described above.

Microbial identification of *M. catarrhalis*, *H. influenzae* and *S. pneumoniae* was performed by culturing using standard methods using with non-selective and selective media, combined with characteristics of colonies, and cellular morphology (13,17,18). All bacterial identifications were confirmed biochemically by automated identification system VITEK 2 (Bio Mérieux, France).

Swaps were analyzed by quantitative polymerase chain reaction (qPCR) for presence of *M. catarrhalis*, *H. influenzae* and/or *S. pneumoniae*, by targeting specific genes with primers (see **Online Supplement** for details). In brief, qPCR assay results were analysed in Applied BioSystem 7500 Software v2.0.6 (Life Technologies, Waltham, MA, USA) and considered positive at a threshold value of 0.2 genome equivalents per microliter (geq/ μ L) template DNA.

Assessment of Systemic Inflammation

Systemic inflammation was assessed at age 6 months in both cohorts by measuring plasma levels of blood inflammatory markers: hs-CRP, tumor necrosis factor- α (TNF- α) and interleukin-6 (IL-6). These biomarkers were chosen *a priori* as they are established measures of systemic inflammation (19,2). Blood was drawn from a cubital vein into an EDTA tube, centrifuged to

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separate plasma and cells, and immediately stored at -80°C until analysis. After thawing, biomarker levels were determined by a high sensitivity ELISA technique based on electrochemiluminescence. Samples were read in duplicate by using the Sector Image 2400A (Meso Scale Discovery, Gaithersburg, MD). Lower limit of detection (LLOD) was 0.007 ng/mL for hs-CRP, 0.093 pg/mL for TNF- α and 0.178 pg/mL for IL-6.

Covariates

Infections: Evaluation of infections was based on doctor diagnosis and symptoms documented in diaries. Symptoms monitored included upper and lower respiratory tract infections, gastroenteritis and fever with unknown cause. Current infection was defined as any such infection in a period of two weeks prior to blood sampling (14,15,20).

Siblings included biologically or non-biologically related children, living at the child's address during the child's first year of life.

Statistical Analysis

Levels of hs-CRP, TNF- α and IL-6 were log-transformed prior to analysis and reported as median levels (interquartile range, IQR) (see **online Table E1** for details). Before log-transformation, the lower limit of detection was added to all measurements as a pseudo-count to avoid zero values.

Upper airway colonization with *M. catarrhalis*, *H. influenzae* and/or *S. pneumoniae* was analyzed as a dichotomous variable (colonized vs. non-colonized). Associations between bacterial colonization and inflammatory markers were examined separately in each cohort using general linear models for analyses adjusted for siblings (13,17) and current infections(21). All results are reported as adjusted geometric mean ratio (GMR) of colonized/non-colonized with 95%

confidence interval [CI]. Wilcoxon rank sum test was used for skewed data. In addition, we did a combined analysis of the bacterial culturing results from age one month in both cohorts with an inverse variance meta-analysis using the R-package “meta” (22).

We also conducted a supplementary multi-parametric principal component analysis (PCA) in both cohort studies, including all biomarker levels of hs-CRP, IL-6 and TNF- α , and analyzed the relationship between bacterial colonization of the hypopharynx and principal component 1 (PC-1).

All analyses were done with SAS, version 9.4 (SAS institute, Cary, NC) or R version 3.2.3 (The R Foundation for Statistical Computing) (23) and visualized with the “ggplot2” package (24). Further details are given in the **online repository**.

RESULTS

Baseline Characteristics

In COPSAC₂₀₀₀, 238 (58%) of the 411 children had data both on inflammatory biomarker levels and on bacterial colonization of hypopharynx determined by culturing technique, and 248 (60%) had data both on inflammatory biomarker levels and on bacterial colonization determined by qPCR technique.

In COPSAC₂₀₁₀, 622 (89%) of the 700 children had data both on inflammatory biomarker levels and bacterial colonization at age 1 month, and 613 (88%) had data both on inflammatory biomarker levels and airway colonization at age 3 months (**online Figure E1**).

Infection in the 2-weeks before the time of blood sampling for assessment of inflammatory biomarker levels was reported for 122 (30%) of the included children in COPSAC₂₀₀₀ and 174 (31%) of the children in COPSAC₂₀₁₀. Children with infection vs. without infection in the 2-weeks before blood sampling had increased levels of hs-CRP, median (IQR): COPSAC₂₀₀₀, 0.43 mg/L [0.17–0.53] vs. 0.09 mg/L [0.04–0.32], $p < 0.01$, and COPSAC₂₀₁₀, 0.04 mg/L [0.08–0.57] vs. 0.09 mg/L [0.05–0.27], $p < 0.01$.

Baseline characteristics of included and excluded children from the two cohorts are described in **Table 1**, showing no significant differences between included and excluded children in the COPSAC₂₀₀₀ cohort. In the COPSAC₂₀₁₀ cohort, the included vs. excluded children were less often exposed to smoking during pregnancy (42 (7%) vs. 12 (15%), $p = 0.01$) and had a higher gestational age (mean 279 days (SD, 11) vs. 276 (16), $p = 0.01$).

Bacterial Colonization and Systemic Low-grade Inflammation

COPSAC₂₀₀₀: *M. catarrhalis*, *H. influenzae* and/or *S. pneumoniae* was detected in 52 (22%) of the 238 cultured samples and in 86 (35%) of the 248 samples analyzed by qPCR. A total of 222 children had samples analyzed by both qPCR and conventional culturing technique; 87 (39%) were positive by either method, 39 (17%) by both methods, whereas 38 (17%) were positive only by qPCR contrasting 10 (5%) only by culturing technique.

Children who were colonized at age 1 month determined by culturing had a median [IQR] hs-CRP level at age 6 months of 0.30 mg/L [0.08–0.51] compared to 0.13 mg/L [0.05–0.47] for non-colonized children: GMR of colonized/non-colonized [95% CI], 1.4 [0.9–2.0], $p = 0.08$.

Level of hs-CRP was significantly elevated among children who were colonized at age 1 month determined by qPCR; median [IQR] hs-CRP level was 0.23 mg/L [0.08–0.50] for colonized children

compared to 0.11 mg/L [0.04–0.38] for non-colonized children: GMR, 1.55 [1.1–2.1], $p < 0.01$.

Adjusting these analyses for infections and siblings resulted in similar results though with slightly lower estimates, particularly after adjustment for older siblings (**Table 2**).

COPSAC₂₀₁₀: *M. catarrhalis*, *H. influenzae* and/or *S. pneumoniae* was detected by culturing in 182 (29%) of 622 children at age 1 month and in 264 (43%) of 613 children at age 3 months. A total of 319 (53%) children were colonized at one or both time points of whom 106/319 (33%) were colonized at both time points, 64/319 (20%) were only colonized at age 1 month, and 149/319 (47%) were only colonized at age 3 months (**online Table E2**). Colonization at age 1 month was associated with increased hs-CRP, colonized vs. non-colonized, 0.23 mg/L [0.07–0.53] vs. 0.10 mg/L [0.05–0.38], GMR, 1.52 [1.2–1.9], $p < 0.01$, with similar findings for colonization at age 3 months, 0.22 mg/L [0.07–0.52] vs. 0.09 mg/L [0.05–0.37]; GMR, 1.57 [1.30–1.90], $p < 0.01$ (**Figure 1**). Analyses adjusted for infections and siblings yielded similar results (**Table 2**).

A sensitivity analysis excluding all samples from children with infections in the 2-weeks before the time of blood sampling for assessment of inflammatory biomarker levels showed a similar association between bacterial colonization and elevated levels of hs-CRP in both cohorts (**Table 3**).

Analyzing the hs-CRP data with non-parametric statistical tests yielded similar results (**online Table E3**).

Adjusting the association between colonization at age 1 month and hs-CRP for colonization at age 3 month and vice versa, still revealed significant associations from colonization at both time points, showing independent contribution to systemic low-grade inflammation from each time point.

A combined analysis of the age 1 month conventional culturing results from COPSAC₂₀₀₀ and COPSAC₂₀₁₀ showed that bacterial colonization was associated with elevated hs-CRP levels: GMR 1.49, CI [1.3–1.8], $p < 0.01$ (**Table 2 and Figure 2**).

TNF- α and IL-6 blood levels showed similar results as hs-CRP, with increased levels in colonized vs. non-colonized children, but the associations were less significant (see **online Figure E2**).

Analyzing the pathogen-specific associations between colonization with *M. catarrhalis*, *H. influenzae* or *S. pneumoniae* and hs-CRP levels showed similar trends of elevated hs-CRP, but less pronounced as the signal from colonization with either of the pathogens, which is presumably due to lower power in these subgroup analysis (**online Table E4**). **Table E5** shows pathogen-specific associations with IL-6 and TNF- α level.

Multi-parametric PCA

The multi-parametric PCA approach is a data-driven way to reduce the variance across the three different cytokines captured in PC-1, which is a proxy of the overall inflammatory response.

COPSAC₂₀₀₀: PC-1 from the multi-parametric PCA incorporating hs-CRP, IL-6 and TNF- α (**Figure 3**), explained 55% of the total variance in the biomarker data set. All three markers were positively correlated in PC-1, and especially IL-6 and hs-CRP were highly correlated. Bacterial colonization of hypopharynx determined by culturing at age 1 month was significantly associated with PC-1: GMR, 1.4 [1.0–1.9], $p = 0.04$, with a similar association for qPCR results: GMR, 1.5 [1.2–1.9], $p < 0.01$.

COPSAC₂₀₁₀: PC-1 from the PCA on the COPSAC₂₀₁₀ dataset also explained 55% of the variance.

Colonization at age 1 month and 3 months were both significantly associated with the inflammatory profile represented by PC-1: GMR, 1.4 [1.2–1.7], $p < 0.01$) and 1.5 [1.2–1.7], $p < 0.01$, respectively (**Table 4**).

PC-1 was stronger and more significantly associated with airway colonization than analyzing the cytokines separately with traditional univariate statistical approaches (**online table E5 and Figure 3**).

DISCUSSION

Primary Findings

Asymptomatic neonates colonized with *M. catarrhalis*, *H. influenzae* and/or *S. pneumoniae* in the upper airways have systemic low-grade inflammation. This was shown in a mother-child cohort at-risk of asthma and subsequently replicated in an unselected mother-child cohort, where asymptomatic bacterial colonization of hypopharynx at both 1 and 3 months was associated with increased hs-CRP levels, suggesting that a perturbed upper airway microbiome may cause a systemic low-grade inflammation.

Strengths and Limitations

A major advantage of the study is the unique data on bacterial upper airway colonization in asymptomatic neonates and assessment of blood inflammatory markers in infancy measured at similar age points in two independent birth cohorts. While COPSAC₂₀₀₀ is a high-risk birth cohort of children born to mothers with asthma, COPSAC₂₀₁₀ is a population-based cohort. The similar findings in these two cohorts confirm that the association between neonatal bacterial airway

colonization and a systemic low-grade inflammatory process is a true relation. The association in COPSAC₂₀₁₀ between colonization at age 3 months and systemic low-grade inflammation further supports a link between the upper airway microbiome and systemic inflammation.

Both conventional culturing and qPCR techniques were applied in COPSAC₂₀₀₀ at age 1 month, whereas colonization data from COPSAC₂₀₁₀ at both age 1 month and 3 months were obtained by culturing, which is less sensitive than qPCR. This was illustrated by a stronger association between qPCR data and hs-CRP vs. culturing data and hs-CRP in COPSAC₂₀₀₀, whilst the culturing results from both age 1 month and 3 months in COPSAC₂₀₁₀ were strongly associated with elevated hs-CRP, probably because of the higher numbers.

The panel of bacteria assessed in this study only represents a small fraction of the complex biological composition of the diverse airway ecosystem of bacteria, fungi and viruses (25,26). We restricted the study to the three most common pathogenic airway bacteria (*M. catarrhalis*, *H. influenzae* and/or *S. pneumoniae*) because we previously demonstrated that colonization with either of these bacteria in the hypopharyngeal region was associated with development of childhood asthma (13). We assume that these bacteria originate from the lower airways, but the possibility of contamination from the oral/upper gastrointestinal microbiome cannot be ruled out.

The measurement of inflammatory biomarkers was done three to five months after assessment of bacterial colonization, which can be seen as both strength and a limitation. Simultaneous assessments of microbes and systemic low-grade inflammation would have allowed a more direct evaluation of this relationship and ruled out that other factors led to or contributed to increased biomarker levels. Conversely, this temporal asynchronicity may suggest a more permanent inflammatory phenotype in the colonized children.

Detailed information from daily diary cards of common infections in early childhood allowed us to robustly adjust the analyses for infections in a 2-week time period before measurement of blood inflammatory biomarker levels, which did not alter the findings. The frequency of infections was high, but similar in the two independent cohorts, and was associated with increased levels of hs-CRP.

Adjusting the analyses for siblings, which in our data was associated with both bacterial colonization (13) and hs-CRP levels (19), resulted in slightly smaller effect sizes and loss of significance in some of the analyses, but did not affect the overall conclusions. This is interesting given the fact that siblings has been shown to decrease the risk of developing allergic rhinitis (27), which we have shown is associated with elevated hs-CRP levels at school-age (2). We speculate that the observation of association between siblings and hs-CRP levels in our study is driven by a relationship between siblings, an altered microbial exposure, and susceptibility to develop infections in early life.

Interpretation

Asymptomatic bacterial colonization in hypopharynx with *M. catarrhalis*, *H. influenzae* and/or *S. pneumoniae* at age one month and at age three months was independently associated with a systemic low-grade inflammation at six months of age. This may reflect an intermediary process between a perturbed upper airway microbiome and development of childhood asthma as systemic low-grade inflammation has previously been associated with asthma and decreased lung function in asthmatics (28).

Emerging evidence indicates that altered microbial colonization patterns are involved in the physiologic, immunologic, and metabolic deregulation seen in asthma and other chronic inflammatory diseases (7,31–34). Most studies have focused on the role of the gut microbiome in

subjects with ongoing chronic inflammatory diseases, whereas few studies have investigated the airway microbiome and systemic inflammation in chronic inflammatory diseases (33,34), and no studies have investigated the relationship between the early life airway microbiome and systemic inflammation before disease development.

We have previously demonstrated (13,35) and replicated (14) that asymptomatic bacterial colonization in hypopharynx at age one month was associated with the risk of recurrent wheeze, childhood asthma and lower respiratory tract infections. We also reported that polymorph blood mononuclear cells from children later developing asthma showed an exaggerated *ex vivo* response to the same airway bacteria at 6 months of age, i.e. before disease development (36,37). Further, we showed that bacterial colonization in hypopharynx was associated with a local up-regulation of pro-inflammatory immune mediators in the upper airways (17). The current study shows that colonization of the upper airways is also associated with a systemic low-grade inflammation.

Despite the observational nature of the study it appears most likely that the causal direction is the airway microbiome causing a systemic low-grade inflammation, rather than the other way around. Thus, the specific composition of the upper airway microbiome characterized by presence of *M. catarrhalis*, *H. influenzae* and/or *S. pneumoniae* in hypopharynx may be speculated to induce an inflammatory airway response leading to a prolonged systemic low-grade inflammation and a trajectory towards childhood asthma. However, we cannot rule out that the indications of a local as well as a systemic inflammation is a reflection of a pre-existing aberrant immune system allowing pathogenic bacteria to establish themselves in the airways.

Conclusion

Pathogenic bacterial colonization of the upper airways in asymptomatic infants is associated with a systemic low-grade inflammation. This may reflect an intermediary process between a perturbed upper airway microbiome and development of childhood asthma.

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TABELS

Table 1. Baseline characteristics and comparison of included vs. excluded children from the COPSAC₂₀₀₀ and COPSAC₂₀₁₀ birth cohorts.

	All children	Included children	Excluded children	p
COPSAC₂₀₀₀ cohort	411	248	163	
Infection at time of blood sampling (%)	122 (29.7)	78 (32.8)	44 (25.4)	0.13
Siblings at home (%)	160 (40.1)	100 (40.8)	60 (39.0)	0.79
Smoking during pregnancy (%)	99 (24.1)	65 (26.2)	34 (20.9)	0.26
Passive smoke exposure (%)	311 (83.2)	191 (81.3)	120 (86.3)	0.26
Cat in home (%)	61 (15.4)	36 (14.9)	25 (16.1)	0.85
Dog in home (%)	55 (13.9)	34 (14.1)	21 (13.5)	0.97
Natural (vaginal) birth (%)	324 (78.8)	194 (78.2)	130 (79.8)	0.80
Breastfed length, days (median [IQR])	122 [90.0- 155.0]	122 [91.0 - 153.5]	122 [78.5-155.5]	0.93
Daycare start age, days (mean (SD))	1.10 (0.80)	1.11 (0.78)	1.07 (0.83)	0.63
Sex, Male (%)	203 (49.4)	130 (52.4)	73 (44.8)	0.16
Birth weight (mean (SD))	3.52 (0.52)	3.50 (0.51)	3.54 (0.53)	0.53
Birth length (mean (SD))	52.30 (2.31)	52.22 (2.28)	52.42 (2.37)	0.38
Head circumference at birth (mean (SD))	352.17 (16.22)	351.84 (16.17)	352.66 (16.33)	0.63
Gestational age, days (mean (SD))	282.11 (10.74)	282.16 (10.74)	282.04 (10.77)	0.92
	All children	Included children	Excluded children	p
COPSAC₂₀₁₀ cohort	700	622	78	
Infection at time of blood	174 (31.2)	165 (31.6)	9 (25.0)	0.52

sampling (%)				
Siblings at home (%)	395 (56.4)	357 (57.4)	38 (48.7)	0.18
Daycare start age, days (mean (SD))	326.29 (96.89)	327.30 (93.77)	318.10 (119.54)	0.43
Passive smoke exposure (%)	374 (54.4)	340 (55.2)	34 (47.2)	0.25
Smoking during pregnancy (%)	54 (7.7)	42 (6.8)	12 (15.4)	0.01
Natural (vaginal) birth (%)	549 (78.4)	489 (78.6)	60 (76.9)	0.84
Cat in home (%)	151 (21.9)	135 (21.9)	16 (22.2)	1.00
Dog in home (%)	150 (21.8)	134 (21.8)	16 (22.2)	1.00
Athmatic mother (%)	186 (26.6)	162 (26.0)	24 (30.8)	0.45
Breastfed length, days (median [IQR])	122 [46.0 - 150.0]	122 [57.7- 150.0]	116 [20.0-149.0]	0.09
Gestational age in days (mean (SD))	279.00 (11.65)	279.40 (10.92)	275.78 (16.05)	0.01
Birth length (mean (SD))	51.87 (2.54)	51.90 (2.44)	51.62 (3.25)	0.35
Birth weight (mean (SD))	3.54 (0.55)	3.54 (0.53)	3.50 (0.66)	0.56
Head circumference at birth (mean (SD))	35.00 (1.71)	34.99 (1.71)	35.05 (1.71)	0.76
Sex, Male (%)	360 (51.4)	319 (51.3)	41 (52.6)	0.93
Social circumstances, PCA score (mean (SD))	0.00(1.00)	0.00 (1.00)	0.03(1.03)	0.79

Table 2. Association between early life bacterial airway colonization with *M. catharralis*, *H.*

influenzae and/or *S. pneumoniae* and levels of hs-CRP at age 6 months in COPSAC₂₀₀₀, COPSAC₂₀₁₀

and combined.

COPSAC₂₀₀₀: age 1 month	Colonized (total n)	Colonized, median [IQR] mg/L	Non-colonized, median [IQR] mg/L	GMR [95%CI]	P
Culture data	52 (238)	0.30 [0.08 – 0.51]	0.13 [0.05 – 0.47]	1.39 [0.97 – 2.01]	0.08
Adjusted for current infections	52 (238)	-	-	1.30 [0.92 – 1.84]	0.12
Adjusted for siblings	52 (238)	-	-	1.11 [0.74 – 1.65]	0.60
qPCR data	86 (248)	0.23 0.08 – 0.50]	0.11 [0.04 – 0.38]	1.55 [1.14 – 2.10]	<0.01
Adjusted for current infections	86 (248)	-	-	1.48 [1.09 - 2.00]	0.01
Adjusted for siblings	86 (248)	-	-	1.33 [0.95 – 1.87]	0.09
COPSAC₂₀₁₀: age 1 month	Colonized (total n)	Colonized, median [IQR] mg/L	Non-colonized, median [IQR] mg/L	GMR [95%CI]	P
Culture data	182 (622)	0.23 [0.07 – 0.53]	0.10 [0.05 – 0.38]	1.52 [1.24 – 1.87]	<0.01
Adjusted for current infections	154 (530)	-	-	1.51 [1.22 – 1.86]	<0.01
Adjusted for siblings	182 (622)	-	-	1.28 [1.04 - 1.58]	0.02
COPSAC₂₀₁₀: age 3 months	Colonized (total n)	Colonized, median [IQR] mg/L	Non-colonized, median [IQR] mg/L	GMR [95%CI]	P

Culture data	264 (613)	0.22 [0.07 – 0.52]	0.09 [0.05 – 0.37]	1.57 [1.30 – 1.90]	<0.01
Adjusted for current infections	223(522)	-	-	1.56 [1.29 – 1.89]	<0.01
Adjusted for siblings	264 (613)	-	-	1.32 [1.08 - 1.61]	<0.01
Combined analysis of COPSAC₂₀₀₀ and COPSAC₂₀₁₀: age 1 month	Colonized (total n)	Colonized, median [IQR] mg/L	Non-colonized, median [IQR] mg/L	GMR [95%CI]	P
Culture data	234 (860)	-	-	1.49 [1.25 – 1.78]	<0.01
Adjusted for current infections	206 (772)	-	-	1.45 [1.21 – 1.74]	<0.01
Adjusted for siblings	234 (860)	-	-	1.24 [1.03 – 1.50]	0.02

Table 3. Association between bacterial airway colonization and levels of hs-CRP in children with no infections before time of blood sampling in COPSAC₂₀₀₀ and COPSAC₂₀₁₀

COPSAC₂₀₀₀	Colonized (total n)	Colonized, median [IQR] mg/L	Non-colonized, median [IQR] mg/L	GMR [95%CI]	P
Culture data, age 1 month	32 (128)	0.10 [0.05 – 0.48]	0.89 [0.05 – 0.48]	1.39 [0.89 – 2.18]	0.15
qPCR data, age 1 month	58 (113)	0.14 [0.05 – 0.46]	0.80 [0.03 – 0.19]	1.60 [1.12 – 2.29]	0.01
COPSAC₂₀₁₀	Colonized (total n)	Colonized, median [IQR] mg/L	Non-colonized, median [IQR] mg/L	GMR [95%CI]	P
Culture data, age 1 month	137 (455)	0.14 [0.06 – 0.49]	0.08 [0.04 – 0.28]	1.50 [1.18 – 1.90]	<0.01
Culture data, age 3 months	185 (348)	0.14 [0.06 – 0.45]	0.08 [0.04 – 0.53]	1.50 [1.20 – 1.88]	<0.01

Table 4. Multi-parametric analysis of the association between bacterial airway colonization and principle component 1 from a principle component analysis of hs-CRP, TNF-a and IL-6, which explains 55.4% of the variation in the data.

COPSAC₂₀₀₀: age 1 month	Colonized, culturing (total n)	Estimate	[95%CI]	P
Principal component 1	52 (238)	1.4	[1.0–1.9]	0.04
Adjusted for current infections	52 (238)	1.3	[0.9 – 1.8]	0.12
Adjusted for siblings	52 (238)	1.1	[0.7 – 1.7]	0.60
	Colonized, qPCR (total n)	Estimate	[95%CI]	P
Principal component 1	84 (246)	1.5	[1.2–1.9]	<0.01
Adjusted for current infections	84 (246)	1.5	[1.2 – 2.1]	<0.01
Adjusted for siblings	84 (246)	1.35	[0.96 – 1.9]	0.08
COPSAC₂₀₁₀: age 1 month	Colonized, culturing (total n)	Estimate	[95%CI]	P
Principal component 1	182 (622)	1.4	[1.2 – 1.7]	<0.01
Adjusted for current infections	182 (622)	1.5	[1.2 – 1.9]	< 0.01
Adjusted for siblings	182 (622)	1.3	[1.0 – 1.6]	0.02
COPSAC₂₀₁₀: age 3 months	Colonized, culturing	Estimate	[95%CI]	P

	(total n)			
Principal component 1	264 (613)	1.5	[1.2 – 1.7]	<0.01
Adjusted for current infections	224 (526)	1.6	[1.3 – 1.9]	<0.01
Adjusted for siblings	264 (613)	1.3	[1.1 – 1.6]	<0.01

FIGURE LEGENDS

Figure 1: hs-CRP levels at age 6 months in children with vs. without early life bacterial airway colonization. The horizontal lines depict group median.

Figure 2: Combined analysis of bacterial airway colonization detected by traditional culturing technique in both cohorts at age 1 month and hs-CRP level at age 6 months.

Figure 3: Principal component analysis biplots incorporating hs-CRP, IL-6 and TNF- α levels at age 6 months in relation to early life bacterial airway colonization in the COPSAC₂₀₀₀ (panel A) and COPSAC₂₀₁₀ (panel B) birth cohorts. Each point in the panel corresponds to a child. Two points closely related indicates children with similar profiles. The ellipses reflect the two-dimensional distribution of the group of children colonized (red) vs. children not colonized (blue) with pathogenic airway bacteria.



