Overlapping patterns and unique differences: a study into immunological variation within and between populations

Dorst, M.M.A.R. van

Chapter 3

Differences in bacterial colonization and mucosal responses between high and low SES children in Indonesia.

van Dorst, Marloes M.A.R.¶; Azimi, Shohreh¶; Wahyuni, Sitti; Amaruddin, Aldian I.; Sartono, Erliyani; Wammes, Linda J.; Yazdanbakhsh, Maria¶; Jochems, Simon P¶.
¶ Contributed equally

Adapted from:
The Pediatric Infectious Disease Journal 2022. doi: 10.1097/INF.0000000000000352

ABSTRACT

Background. Increased nasopharyngeal carriage of pathogenic bacteria is found in low socioeconomic status (SES) settings. How SES affects local immune responses, important for controlling colonization, is currently unknown.

Objective. Examining bacterial colonization and cytokine response in the nasal mucosa of children from high and low SES.

Results. *H. influenzae and S. pneumoniae* densities were increased in low SES settings compared to the high SES settings (p=0.006, p=0.026), with 6 and 67 times higher median densities, respectively. Densities of *H. influenzae and S. pneumoniae* were positively associated with levels of IL-1beta and IL-6. After correcting for bacterial density, IL-6 levels were higher in colonized children from high SES than low SES for *H. influenzae and S. pneumoniae* (both p=0.039).

Conclusion. Increased densities of *H. influenzae and S. pneumonia*e were observed in low SES children, whereas IL-6 levels associated with colonization were reduced in these children, indicating that immune responses to bacterial colonization were altered by SES.

INTRODUCTION

Lower respiratory tract infections (LRTIs) remain the leading cause of mortality amongst children under five, accounting for 2.4 million deaths in 2016 most of them in low- and middle income countries (LMICs) (1, 2).

A number infectious agents can establish LRTI, prevalent bacterial causes include *Streptococcus pneumoniae* (*S. pneumoniae*), *Haemophilus influenzae* (*H. influenzae*), *Moraxella catarrhalis* (*M. catarrhalis*) and *Staphylococcus aureus* (*S. aureus*). Nasopharyngeal carriage of these bacteria is common in infants and colonization by these bacteria is considered an essential first step in development of disease (3-5). Moreover, nasopharyngeal carriage and increased densities of S. pneumoniae, *H. influenzae* and *M. catarrhalis* have been associated with pneumonia in Tanzanian children and increased *H. influenzae* densities were found among children with (very) severe pneumonia in seven LMICs (6, 7).

Control of nasopharyngeal bacterial colonization is mediated by local immune responses and impaired innate cytokine responses have been associated with persistent bacterial colonization. A human inoculation study showed that clearance of nasal *S. aureus* requires an upregulation of chemokines, growth factors and inflammatory cytokines and that a low IL-1RA/IL-1beta ratio associates with *S. aureus* persistence (8). Furthermore, an increased prevalence of nasopharyngeal colonization with *M. catarrhalis* was observed infants with anemia, while the pro-inflammatory cytokine responses were reduced upon *in vitro* stimulation (9). Finally, murine models show that IL-1 signaling is essential for clearance of *S. pneumoniae* and suggest that reduced IL-1 responses might be permissive for persistent colonization during infancy (10).

Increased carriage rates have been observed in various settings and are associated with lower socio-economic status (SES). High nasopharyngeal bacterial colonization rates have been observed in low-income countries (11), rural areas (12) and indigenous populations (4). Furthermore, a study in Israel showed that SES rather than ethnicity drove these differences in pneumococcal carriage (13). Finally, socio-economic factors were identified as risk factors for carriage of potential pathogenic bacteria in Indonesian children (14).

Since increased bacterial colonization has been observed in low SES settings and local immune responses play an important role in the control of the bacterial colonization, reduced mucosal immune responses in low

SES populations could play a role. Studies comparing immune responses between socio-economic settings are scarce and often focus on the systemic immune system. To our knowledge, no studies have compared the immune responses to bacterial colonization between different socio-economic settings. Moreover, most studies that found an association between SES and colonization have been performed in a heterogenous population. Therefore, this study aimed to examine the cytokine response to nasopharyngeal bacterial colonization of children from high and low SES schools in a single urban area.

METHODS

Data collection

The data was obtained as part of a larger study into high and low SES school-aged children in Makassar, Indonesia. The main objective of this larger study was to study the effect of SES on the immune responses (15). This study had a cross-sectional design and included children attending two primary schools in Makassar, the capital of South Sulawesi. These schools were selected based on the socio-economic background of the children attending the school. One of the schools, SD Athirah, is a privately funded school with extensive facilities and therefore attended by children from High SES families. The other school, SD Baraya, is a public school and has very limited facilities and is predominantly attended by low SES children. Both schools are located in the center of Makassar, 2km apart. Within the selected schools and selected grades, all children were invited to participate in the study and all children with parental/guardian informed consent were included in the study. We excluded children that were using antihistamines or corticosteroids. From this larger study including 360 children in total, nasosorption samples were collected from a subgroup of 98 children for this current study, based on order of children's availability indicated by teachers.

In the current study, we aimed to compare i) microbial presence and density, and 2) mucosal responses to microbes between low SES and high SES. We expected the microbial prevalence in our study population to be 20-50%, thus including 10-25 children per SES group for a given microbe. Samples were collected in October 2019. Informed written consent was obtained from primary caregivers.. Ethical approval was obtained from the Health Research Ethical Committee, Faculty of Medicine, Hasanuddin University (No:703/H4.6.4.5.31/PP36/2019).

Socio-demographic information was obtained from all participants via questionnaires. To determine skin reactivity to house dust mite, a common

aeroallergen, a skin prick test (SPT) was performed in children using histamine chloride (10 mg/ml) as a positive control, saline as a negative control and two extracts; *Dermatopagoides pteronyssinus* and *D. farinae* (HAL Allergen BV, Leiden). Skin test reactivity was considered positive if the longest diameter plus the diameter perpendicular of wheal size divided by two was 3 mm or larger as measured fifteen minutes after application. Finally, all children were asked to fill the stool container (Sarstedt Inc, Nümbrecht, Germany) and the Kato-Katz methods were used to quantify the eggs of intestinal helminths such as *Ascaris lumbricoides*, *Trichuris trichiura* and hookworm in these stool samples. All these parameters have been measured for the entire cohort as described earlier (15).

Nasosorption samples preparation

Nasosorption samples were collected by inserting an absorptive matrix strip (Nasosorption™, Hunt Developments) into one nostril and pushed against the nasal lining for 30-60 seconds (16). These strips were then placed on ice until storage at -80°C within 8 hours after collection. The nasosorption samples were eluted by adding 100µL sterilized PBS with 1%BSA+0.05%Triton-X100 to the filter and spun down at 3600xg for 10 minutes at 4°C. The supernatant was transferred to a new tube and centrifuged at 16000xg, 4°C, 10 minutes. The supernatant was moved to a new tube for cytokine analysis and the pellet was stored at -20°C until DNA extraction.

Cytokine concentration measurement

Of the supernatant, 12 µl was used to measure the concentration of 25 cytokines, by the Human Cytokine 25-plex ProcartaPlex Panel (Invitrogen, ThermoFisher No:EXP250-12166-901) according to manufacturer's instructions, but additional washing steps and alcohol flushes were performed to account for the mucus present in such nasal samples, which can cause beads to aggregate. The samples were measured by the Luminex200 device at normal RP1 target and concentrations were obtained by using the xPonent3.1 software in pg/mL. Children for who >90% of the cytokines could not be measured in their nasosorption sample were excluded from further analysis. This was the case for 15% of the children, 6 high and 9 low SES children.

Generation of standard curves

The DNA concentrations of *S. aureus* (ATCC43300), *H. influenzae* (ATCC49766), *S. pneumoniae* 6305 and clinical isolate of *M. catarrhalis* were determined spectrophotometrically with the NanoDrop1000 (Thermo scientific). The copy numbers of template were calculated using the genome length computed with URI Genomics & Sequencing Center (http://cels.uri.edu/

gsc/cndna.html). A standard curve was generated of 10-fold serial dilution scheme ranging from 10^7-10^1 copy/mL

Bacterial colonization measurement
To determine nasal bacterial colonization, qPCR was performed on the bacterial pellet. DNA extraction was performed using magnetic beads, as previously published (17). To determine the colonization of *S. pneumoniae* and *H. influenzae*, qPCR was performed using the *LytA* and *IgA1* gene respectively, as previously described (17, 18). qPCR for the *copB* gene was performed to determine colonization of *M. catarrhalis* and for *S. aureus* the *nuc* gene was used as previously described (6). Using the standard curves, the genome length and Avogadro's number, the number of genome copies/µL was determined in the samples per bacterium. Children negative for all four bacteria and with 16S Ct values that did not exceed the background signal (19), were excluded from further analysis. This excluded 4 children from high SES and 4 children from low SES. Details regarding the primers and probes utilized in this study can be found in **Table 1**.

Table 1. Primers and probes qPCR bacteria

Target gene		Sequence (5' - 3')	Bacteria	Reference
lytA	Forward	ACGCAATCTAGCAGATGAAGCA	*Streptococcus pneumoniae*	[16]
	Reverse	TCGTGCGTTTTAATTCCAGCT		
	Probe	FAM-TGCCGAAAACGCTTGATACAGGGAG-BHQ-1		
IgA1	Forward	CAAAATTGCCAAGATTAAATGCTT	*Hemophilus influenza*	[17]
	Reverse	TGCTCGCCATACTGCACAA		
	Probe	FAM-CCTGCGGTTAAACC-MGB		
CopB	Forward	CGTGTTGACCGTTTTGACTTT	*Moraxella catarrhalis*	[5]
	Reverse	TAGATTAGGTTACCGCTGACG		
	Probe	HEX-ACCGACATCAACCCAAGCTTTGG-BHQ1		
Nuc	Forward	GTTGCTTAGTGTTAACTTTAGTTGTA	*Staphylococcus aureus*	[5]
	Reverse	AATGTCGCAGGTTCTTTATGTAATTT		
	Probe	HEX AAGTCTAAGTAGCTCAGCAAATGCA-BHQ1		

Statistical analysis
The standardized z-scores of body mass index (z-BMI) were determined according to the WHO guidelines (20). To obtain approximately normally distributed data, cytokine concentrations and bacterial loads were $\log_{10}$-transformed. A Student's t-test was performed for continuous data and for binary and categorical data Pearson's Chi-square was used. Pearson's correlation was used to examine the correlation of two continuous variables. To assess what the main drivers are of the correlation between bacterial colonization and cytokines, we performed a canonical correlation.

Similarly to PCA analysis, canonical correlation allows the integration of two multi-dimensional datasets to find common drivers of variation maximizing covariance between the datasets, allowing us to compare bacteria and cytokines on a global level (21). To investigate the effect of SES on the bacterial loads and to estimate the effect of bacterial density and SES on cytokine levels, a regression model was performed. For all regression models in this study age, sex and z-BMI were considered *a priori* confounders and adjustment was performed accordingly. To identify the main drivers of the correlation between bacterial density and cytokine concentrations, a canonical correlation analysis was performed by using Wilk's Lambdas including the density of the four bacteria and the concentrations of all widely measurable cytokines. The two bacteria and two cytokines that showed the largest coefficient in the canonical correlation analysis were selected for further analysis. P-values smaller than 0.05 were considered statistically significant. Data was analyzed and visualized using RStudio and R software.

RESULTS

Bacterial density of *H. influenzae* and *S. pneumoniae* increased in low SES
A total of 98 school-age children were included: 50 children attended the low SES school and 48 children the high SES school (**Table 2**). There was no significant difference in age or sex, but z-BMI and parental income were between lower in low SES compared with high SES children. Finally, the presence of some of the factors that could affect the bacterial colonization and cytokine levels was examined. However, only 2 children, both high SES, had self-reported atopic asthma and 3 children, all low SES, had a current helminth infection; thus, these factors were not taken along in the rest of the analysis.

Table 2. Characteristics of sample population

	Low SES (n= 50)	High SES (n=48)	p-value
Age (in years, mean, SD)	8.34 ± 1.40	7.85 ± 1.17	0.061
Sex (female %, n/N)	50.0 (25/ 50)	45.8 (22/48)	0.680
z-BMI (mean, SD)	-1.74 ± 1.51	0.44 ± 1.82	<0.001
Education father (high %, n/N)	8.3 (4/48)	84.4 (38/45)	<0.001
Maternal education (high %, n/N)	8.3 (4/48)	73.9 (34/46)	<0.001
Floor material (ceramics %, n /N)	36.7 (18/49)	95.7 (44/46)	<0.001
Wall material (brick or concrete %, n/N)	53.1 (26/49)	100 (46/46)	<0.001
Toilet (private inside %, n/N)	83.3 (40/48)	100 (46/46)	0.004
Helminth infection by microscope (%, n/N)	6.0 (3/43)	0.0 (0/33)	0.122
Bacterial colonization (carrier % , n/N)			
H. Influenzae	63.0 (29/46)	70.5 (31/44)	0.456
S. Pneumoniae	56.5 (26/46)	47.7 (21/44)	0.404
M. Catarrhalis	80.4 (37/46)	81.8 (36/44)	0.867
S. Aureus	47.8 (22/46)	36.4 (16/44)	0.271
Number of bacteria (%, n/N)			
No bacteria	0.0 (0/46)	2.3 (1/44)*	0.865
One bacterium	21.7 (10/46)	20.5 (9/44)	
Two bacteria	28.3 (13/46)	31.8 (14/44)	
Three bacteria	30.4 (14/46)	29.5 (13/44)	
All four bacteria	19.6 (9/46)	15.9 (7/44)	

The number of positives (n) of the total number (N). SD: standard deviation. SES: socio-economic status. CI: confidence interval. Student's t-test for continuous variables and Pearson's Chi-square for binary/categorical variables. *qPCR with 16S was performed for this child to confirm DNA extraction was successful

The carriage rate was highest for *M. catarrhalis* (81.1%, 73/90), more than 50% for *H. influenzae* (66.7% 60/90) and *S. pneumoniae* (52.2%, 47/90), lowest for *S. aureus* (42.2%, 38/90) and most common was a combination of two (30.0%, 27/90) or three (30.0%, 27/90). Neither the carriage rates of the bacteria nor the number of co-colonizing bacteria differed significantly between high and low SES.

Although the carriage rates and combinations did not differ between high and low SES, differences in the densities of bacteria were observed (**Figure 1**). The median bacterial load of *H. influenzae* was 59,075 copies per

nasosorption sample in low (IQR: 6,330 – 381,417) and 9,804 in high SES (IQR: 960-41,675), indicating a 6-times higher median density. For *S. pneumoniae*, the median bacterial load was 97,422 in the low (IQR: 580 – 1,008,292) and 1,446 in the high SES (IQR: 366 – 87,704), corresponding to a 67-times higher median density. No significant differences were observed in the bacterial loads of *M. catarrhalis* and *S. aureus* between high and low SES. To further examine the relation between the bacterial load and SES, a multivariate regression analysis was performed adjusted for *a priori* confounders. These results showed that SES significantly affected the *H. influenzae* (β_{SES}=-1.19, p=0.006) and *S. pneumoniae* density (β_{SES} =-1.25, p=0.026).

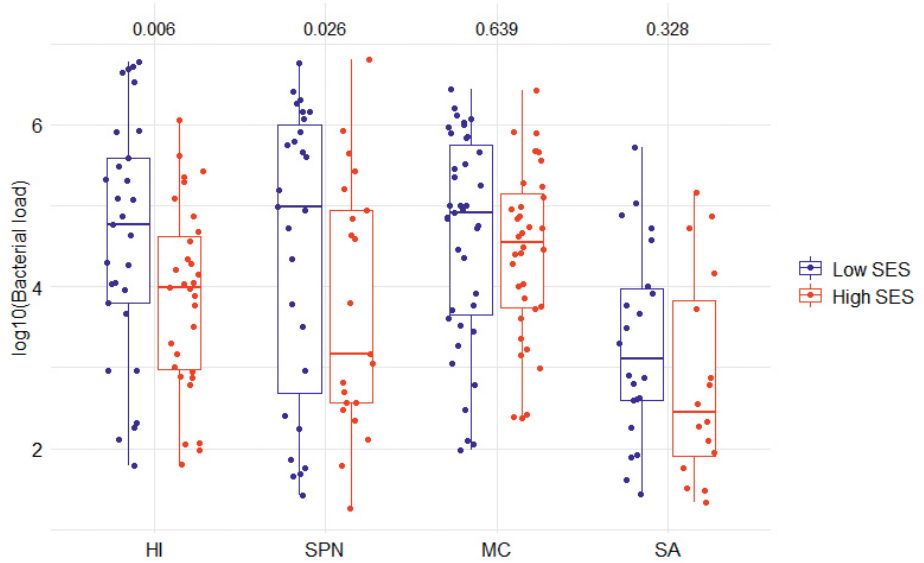

Figure 1. Bacterial loads in high and low SES school-aged children.
All children for which bacterial colonization could be determined were included, thus n = 90 of which 46 low SES (blue) and 44 high SES (red) children. The boxes represent the interquartile range and the line within represents the median. The whiskers represent the 1.5 IQ of the upper and lower quartile bacterial load in each group. P-values are depicted above the corresponding boxplot and are derived with a multivariate regression model, correcting for age, sex and z-BMI. HI: *Hemophilus influenzae,* MC: *Moraxella catarrhalis,* SA: *Staphylococcus aureus,* SES: Socio-economic status, SPN : *Streptococcus pneumoniae*.

Correlation between densities of the different bacteria

Correlation analysis showed that the *H. influenzae* density positively correlated with the *M. catarrhalis* density (β=0.53, p<0.001) and the bacterial load of *S. pneumoniae* negatively associated with *S. aureus* (β=-0.50, p=0.04) (**Figure 2**). Furthermore, there was a trend for positive association between

H. influenzae and *S. pneumonia*e density (β=0.34, p=0.07). No association was found between the other combinations of bacteria.

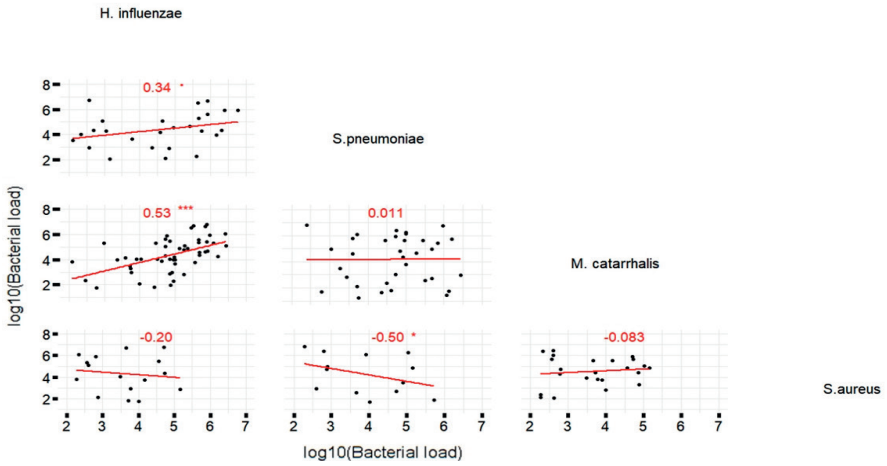

Figure 2. Correlation between the loads of different bacteria
All children for which bacterial colonization could be determined were included, thus n = 90. Left lower panel are the bivariate scatterplot of $\log_{10}$(bacterial loads) of the different bacteria with a regression line in red and for each plot the correlation (Pearson's correlation) and as stars the significance level of this correlation '***' for p-value < 0.001, '**' for p-value ≤ 0.01, '*' for p-value ≤ 0.05 and '·' for p ≤ 0.10.

IL-1beta concentrations are increased in the low SES compared to the high SES.

To understand whether impaired mucosal responses might be responsible for the higher densities of colonized bacteria in low SES children, the concentration of 25 cytokines in nasal fluid were analyzed using a multiplex assay. Nine of these cytokines were detectable in the majority of samples: IL-18, IL-1alpha, IL-1beta, IL-1RA, IL-27, IL-4, IL-6, IL-7 and TNF-alpha. The other cytokines were below the limit of reliable detection in most samples (**Table 3**). Comparison of the cytokine levels between high and low SES showed that IL-1beta levels were significantly increased in the low SES, with a geometric mean concentration of 338.8 (95% CI: 15.6 – 7.226) in low and 102.3 (95% CI: 4.7 – 2,194) in high SES after adjusting for confounders (β_{SES}=-0.518, p=0.048) (**Figure 3, Table 4**).

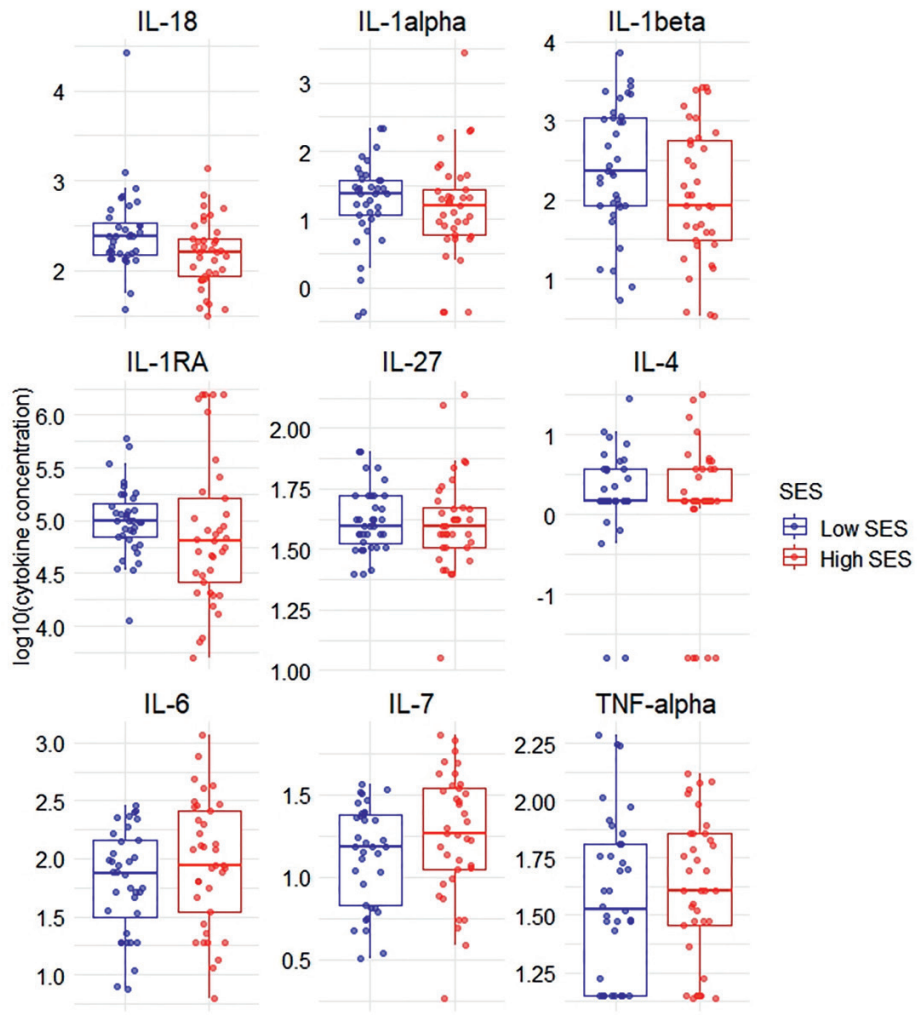

Figure 3. Nasal cytokine levels in high and low SES school-aged children.
All children for which cytokines could be measured were included, thus n = 79 of which 39 low SES (blue) and 40 high SES (red) children. The boxes represent the interquartile range and the line within represents the median. Individuals are depicted by circles. The whiskers represent the 1.5 IQ of the upper and lower quartile. SES: Socio-economic status.

Table 3. Number of samples in range and in reliable range per cytokine

Cytokine	Number of samples in range (%)[1]	Number of samples in range in high SES (%)[1]	Number of samples in range in low SES (%)[1]	Number of samples in reliable range (%) [2]	Number of samples in reliable range in high SES (%) [2]	Number of samples in reliable range in low SES (%) [2]
GM-CSF	28 (35%)	17 (43%)	11 (28%)	3 (4%)	3 (8%)	0
IFN-alpha	7 (9%)	1 (3%)	6 (15%)	0	0	0
IFN-gamma	50 (63%)	27 (68%)	23 (59%)	13 (16%)	8 (20%)	5 (13%)
IL-2	17 (22%)	13 (33%)	4 (10%)	7 (9%)	6 (15%)	1 (3%)
IL-5	17 (22%)	8 (20%)	9 (23%)	4 (5%)	3 (8%)	1 (3%)
IL-9	51 (65%)	26 (65%)	25 (64%)	4 (5%)	4 (10%)	0
IL-10	45 (57%)	25 (63%)	20 (51%)	7 (9%)	6 (15%)	1 (3%)
IL-12p70	49 (62%)	26 (65%)	23 (59%)	4 (5%)	3 (8%)	1 (3%)
IL-13	35 (44%)	16 (40%)	19 (49%)	13 (16%)	8 (20%)	5 (13%)
IL-15	32 (41%)	18 (45%)	14 (36%)	4 (5%)	4 (10%)	0
IL-17A	56 (71%)	29 (73%)	27 (69%)	7 (9%)	6 (15%)	1 (3%)
IL-21	44 (56%)	20 (50%)	24 (62%)	8 (10%)	5 (13%)	3 (8%)
IL-22	27 (34%)	12 (30%)	15 (38%)	10 (13%)	5 (13%)	5 (13%)
IL-23	7 (9%)	6 (15%)	1 (3%)	0	0	0
IL-31	5 (6%)	3 (8%)	2 (5%)	0	0	0
TNF-beta	7 (9%)	4 (10%)	3 (8%)	0	0	0

All children for which cytokines could be measured were included, thus n = 79 of which 39 low SES and 40 high SES children. [1] Number of samples for which the samples could be determined based on their position in relation to the standard curve or that were out of range but could be estimated based on the MFI value. [2] Reliable range (often) corresponds with above the seventh standard and is determined based on the standard curve for each cytokine. None of the cytokines had levels that were out of range above the standards

Table 4. Results of regression models cytokine levels adjusted for *a priori* confounders

	Mean cytokine concentration	Coefficient SES (95% CI)	P-value
IL-18			
Low SES	741.3	Reference	
High SES	468.8	-0.199 (-0.442 ; 0.0439)	0.113
IL-1alpha			
Low SES	9.984	Reference	0.389
High SES	6.180	-0.186 (-0.605 ; 0.234)	
IL-1beta			
Low SES	338.8	Reference	
High SES	102.3	-0.518 (-1.02 ; -0.0149)	**0.048**
IL-1RA			
Low SES	75,857	Reference	
High SES	67,608	-0.0494 (-0.374; 0.275)	0.766
IL-27			
Low SES	42.66	Reference	
High SES	49.32	0.0626 (-0.0360; 0.161)	0.218
IL-4			
Low SES	2.455	Reference	
High SES	2.786	0.0553 (-0.377; 0.488)	0.803
IL-6			
Low SES	85.11	Reference	
High SES	116.95	0.138 (-0.164; 0.439)	0.374
IL-7			
Low SES	16.60	Reference	
High SES	21.18	0.106421 (-0.0852; 0.298)	0.280
TNF-alpha			
Low SES	53.70	Reference	
High SES	64.41	0.0788 (-0.119; 0.276)	0.437

All children for which cytokines could be measured were included, thus n = 79 of which 39 low SES and 40 high SES children. Mean cytokine concentration is the geometric mean in high and low SES after adjusted for *a priori* confounders including age (in years), sex and z-BMI.

IL-6 concentrations are increased in high SES children colonized by *H. influenzae* or *S. pneumoniae*

The densities of *H. influenzae* and *S. pneumoniae* and IL-1beta and IL-6 concentrations were identified as the main drivers of the correlation between bacteria and cytokines (**Table 5**). Indeed both *H. influenzae* and *S. pneumoniae* densities positively associated with IL-1beta and IL-6 levels *(***Figure 4A-D).** To quantify these correlations, a regression model including *a priori* confounders was used. The results showed that *H. influenzae* and *S. pneumoniae* densities are positively associated with IL-1beta ($\beta_{bacteria}$ = 0.294, p=0.002, $\beta_{bacteria}$=0.221, p=0.008, respectively) and IL-6 ($\beta_{bacteria}$=0.190, p<0.001 and $\beta_{bacteria}$=0.136, p=0.006) (**Tabel 6**). After adjusting for *a priori* confounders and the bacterial densities, IL-6 levels were increased in high compared to low SES in children colonized by *H. influenzae* (β_{SES}=0.360, p=0.039) and *S. pneumoniae* (β_{SES}=0.366, p=0.039), indicating that IL-6 levels were increased in high compared to low SES at any given density of these bacteria. To examine whether SES affected the strength and direction of this association, a regression model including the interaction between these bacterial density and cytokine levels was performed, which showed that this was not significant (**Table 7**). Thus, children from both high and low SES had dose-dependent cytokine responses to *H. influenzae* or *S. pneumoniae* colonization, but these responses were stronger in high SES children for IL-6.

Table 5. Canonical correlation analysis bacterial loads

	Standardized coefficients
Bacterial load	
H. influenzae	**-0.877**
S. pneumoniae	**-0.240**
M. catarrhalis	0.109
S. aureus	-0.016
Cytokine concentration	
IL-1alpha	0.097
IL-1beta	**-0.791**
IL-1RA	0.037
IL-4	0.080
IL-6	**-0.311**
IL-7	-0.028
IL-18	0.309
IL-27	0.173
TNF-alpha	-0.141

< Description table 5. All children for which both cytokines and bacterial colonization could be measured were included, thus n = 73 of which 36 low SES and 37 high SES children. Standardized coefficient of the canonical correlation analysis first dimension with p=0.042 using Wilks' Lambda. In bold the variables that predominantly drive the correlation and that will be used for further analysis. Both bacterial loads and cytokine concentrations are log_{10} transformed.

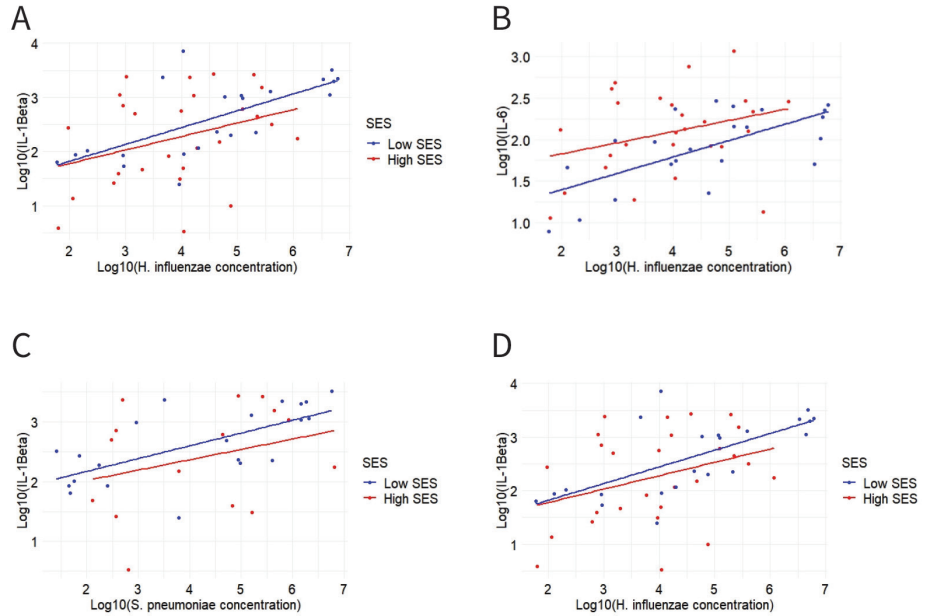

Figure 4. Association between *H. influenzae* (A and B) or *S. pneumoniae* concentration (C and D) and IL-1beta and IL-6 concentration in high and low SES school-aged children

For all models with *H. influenzae* only children colonized and with cytokine data are included thus n = 49, which includes 27 high SES children and 22 low SES children. For all models with *S. pneumoniae* only children colonized and with cytokine data are included thus n = 36, which includes 15 high SES children and 21 low SES children.

Table 6. Relation between *H. influenzae* or *S. pneumoniae* bacterial load and the IL-1beta or IL-6 concentrations

	Intercept (95% CI)	Coefficient bacterial load (95% CI)	P-value bacterial load	Coefficient SES (95% CI)	P-value SES
H. influenzae					
IL-1beta	1.12 (-0.661 – 2.91)	0.294 (0.119 – 0.470)	0.002	-0.123 (-0.742 – 0.496)	0.699
IL-6	1.14 (0.189 – 2.09)	0.190 (0.096 – 0.284)	<0.001	0.360 (0.029 – 0.284)	0.039
S. pneumoniae					
IL-1beta	1.48 (-0.413 – 3.41)	0.221 (0.069 – 0.373)	0.008	-0.154 (-0.720 – 0.411)	0.596
IL-6	1.14 (0.032 – 2.27)	0.136 (0.047 – 0.225)	0.006	0.366 (0.03 – 0.697)	0.039

Adjusted for *a priori* confounders age (in years), sex and z-BMI. Both bacterial loads and cytokine concentrations are log10 transformed. For all models with *H. influenzae* only children colonized are included thus n = 49, which includes 27 high SES children and 22 low SES children. For all models with *S. pneumoniae* only children colonized are included thus n = 36, which includes 15 high SES children and 21 low SES children.

Table 7. Regression model *H. influenzae* and *S. pneumoniae* load and the IL-1beta or IL-6 concentrations with interaction term

	Intercept (95% CI)	Coefficient bacterial load (95% CI)	P-value bacterial load	Coefficient SES (95% CI)	P-value SES	Coefficient interaction (95% CI)	p-value interaction
H. influenzae							
IL-1beta	1.01 (-0.951 – 2.96)	0.316 (0.089 – 0.542)	0.009	0.105 (-1.58 – 1.71)	0.899	-0.05 (-0.393 – 0.288)	0.764
IL-6	1.12 (0.076 – 2.17)	0.194 (0.073 – 0.314)	0.003	0.398 (-0.461 – 1.26)	0.369	-0.01 (-0.191 – 0.173)	0.924
S. pneumoniae							
IL-1beta	1.46 (-0.554 – 3.47)	0.228 (0.05 – 0.402)	0.012	-0.056(-1.40 – 1.29)	0.935	-0.025 (-0.330 – 0.280)	0.875
IL-6	1.10 (-0.076 – 2.28)	0.144 (0.042 – 0.246)	0.010	0.486 (-0.300 – 1.27)	0.236	-0.030 (-0.209 – 0.148)	0.743

Adjusted for *a priori* confounders age (in years), sex and z-BMI. Both bacterial loads and cytokine concentrations are log10 transformed. For all models with *H. influenzae* only children colonized are included thus n = 49, which includes 27 high SES children and 22 low SES children. For all models with *S. pneumonia* only children colonized are included thus n = 36, which includes 15 high SES children and 21 low SES children

IL-1RA and IL-1beta response to *S. aureus* carriage differs between high and low SES

In the low SES, the concentration of IL-1RA seemed to be higher in in *S. aureus* carriers compared to non-carriers, whereas in high SES IL-1RA levels were lower in the carriers compared to non-carriers (**Figure 5A**). A similar pattern was observed for IL-1beta (**Figure 5B**). To quantify these observations, we performed a regression analysis including the *a priori* confounders, showing a significant difference between high and low SES for IL-1RA ($\beta_{interaction}$=-0.700, p=0.007), and a similar trend for IL-1beta ($\beta_{interaction}$=-0.700, p=0.059) (**Table 8**). Since IL-1beta and IL-1RA compete for the same receptor and the activity of IL-1beta is affected by the levels of IL-1RA, the ratio between IL-1RA and IL-1beta was determined (**Figure 5C**). The interaction between *S. aureus* carriage and SES was not seen for the ratio between these cytokines ($\beta_{interaction}$=0.074, p=0.859), but the IL-1RA/IL-1beta ratio tended to be higher in high compared to low SES (β_{SES}=0.465, p=0.078) (**Table 8**). The pattern observed for IL-1beta and IL-1RA did not exist for other IL-1 cytokines, including IL-1alpha (**Figure S1**).

> Description figure 5. All children for which both cytokines and bacterial colonization could be measured were included, thus n = 73 of which 36 low SES and 37 high SES children. The boxes represent the interquartile range and the line within represents the median. The whiskers represent the 1.5 IQ of the upper and lower quartile concentration of the cytokine. SES: Socio-economic status

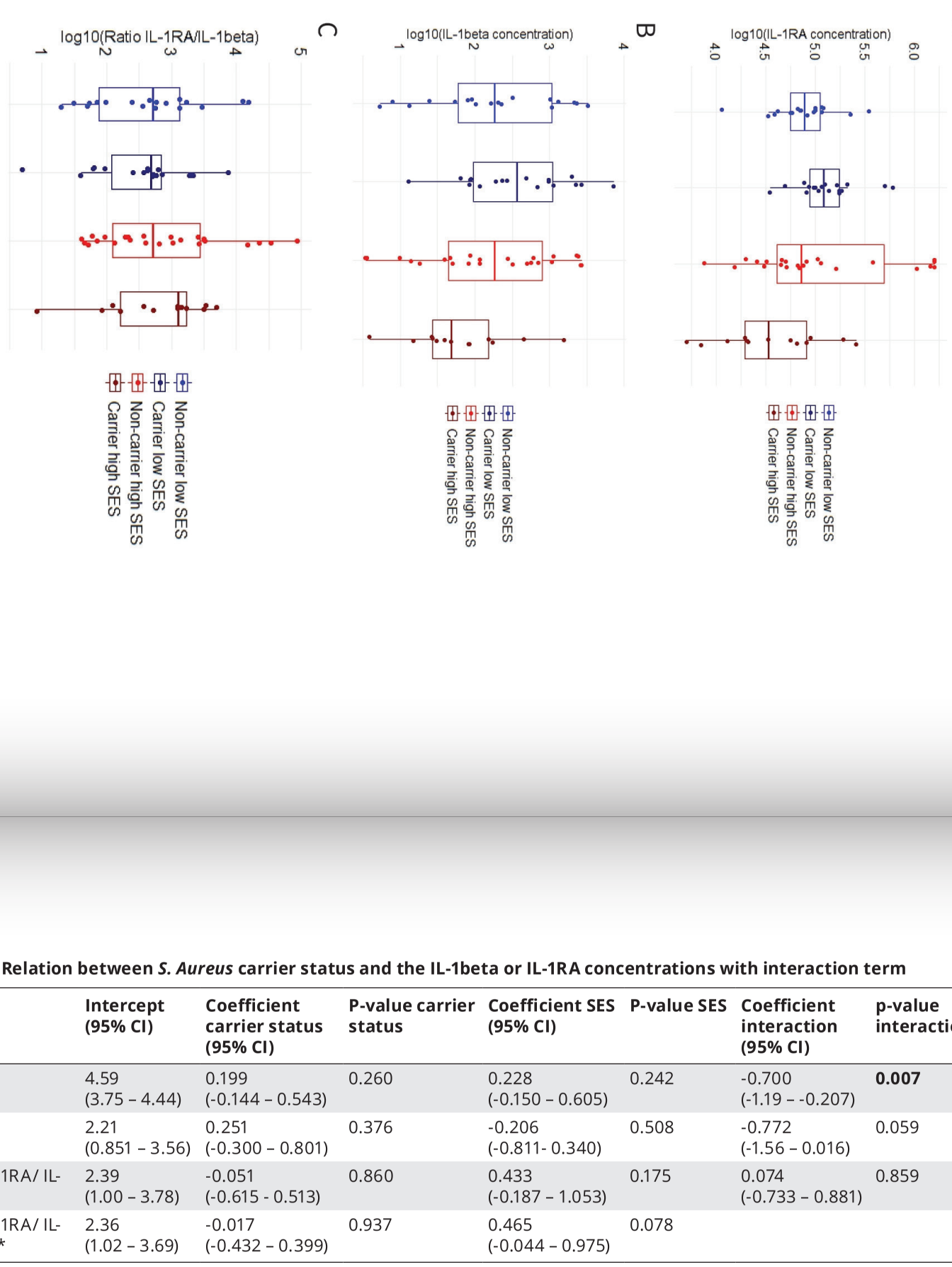

Figure 5. The IL-1RA and IL-1Beta concentrations *in S. Aureus* carrier and non-carrier in high and low SES.

Table 8. Relation between *S. Aureus* carrier status and the IL-1beta or IL-1RA concentrations with interaction term

	Intercept (95% CI)	Coefficient carrier status (95% CI)	P-value carrier status	Coefficient SES (95% CI)	P-value SES	Coefficient interaction (95% CI)	p-value interaction
IL-1RA	4.59 (3.75 – 4.44)	0.199 (-0.144 – 0.543)	0.260	0.228 (-0.150 – 0.605)	0.242	-0.700 (-1.19 – -0.207)	**0.007**
IL-1beta	2.21 (0.851 – 3.56)	0.251 (-0.300 – 0.801)	0.376	-0.206 (-0.811- 0.340)	0.508	-0.772 (-1.56 – 0.016)	0.059
Ratio IL-1RA/ IL-1beta *	2.39 (1.00 – 3.78)	-0.051 (-0.615 - 0.513)	0.860	0.433 (-0.187 – 1.053)	0.175	0.074 (-0.733 – 0.881)	0.859
Ratio IL-1RA/ IL-1beta **	2.36 (1.02 – 3.69)	-0.017 (-0.432 – 0.399)	0.937	0.465 (-0.044 – 0.975)	0.078		

Adjusted for *a priori* confounders age (in years), sex and z-BMI. Cytokine concentrations are log10 transformed. All children for which both cytokines and bacterial colonization could be measured were included, thus n = 73 of which 36 low SES and 37 high SES children. ** log10 transformed and with interaction term, * log10 transformed without interaction term

DISCUSSION

This is to the best of our knowledge the first study evaluating the nasal cytokine response in relation to bacterial colonization in high and low SES children. This study showed increased densities of *H. influenzae* and *S. pneumoniae* in low compared to high SES. The densities of these bacteria were positively associated with IL-1beta and IL-6 levels. After correcting for bacterial density, IL-6 levels were increased in high SES, indicating that IL-6 levels were higher at any given density of *H. influenzae* and *S. pneumoniae* in high compared to low SES.

SES has been identified as risk factor for increased nasopharyngeal carriage in a number of studies (4, 11-14). A previous study in Indonesia found increased densities of *S. aureus* in children living in semi-rural compared to urban areas. Furthermore, they identified family income as a risk factor for pneumococcal carriage densities and showed that low maternal education was associated with increased *H. influenzae* densities (14). The results of the current study are in line with these previous findings, however in contrast to the study Fadlyana *et al.* we included children from different SES within one urban center, thereby providing unique insights into the role of SES on nasopharyngeal bacterial carriage and densities in children. Finally, the associations between different microbial species have been described in earlier studies and our results are therefore in line with literature (22, 23).

Without taking bacterial colonization status into account, only IL-1beta was significantly different between high and low SES, while there was a large dynamic range in cytokine levels observed, indicating that local factors are important for driving cytokine levels at the mucosa. In the current study, we examined the association between the cytokine levels and bacterial colonization, a local factor that is thought to play a significant role. Indeed, densities of *H. influenzae* and *S. pneumoniae* were positively associated with IL-1beta and IL-6 levels. The importance of the IL-1 cytokine signaling in the clearance of *S. pneumoniae* has been shown in mice and reduced IL-1 responses have been suggested to be permissive for persistent colonization during infancy (10, 24).Moreover, high levels of IL-6 and IL-1beta were found in response to Non-typeable *H. influenzae* in individuals suffering from chronic suppurative lung disease (25). Other local factors that might be of importance are co-infections. Co-infections with viruses in the nasal cavity are common and are strongly associated with increased SPN nasopharyngeal load and invasive disease in children (23). In addition, co-infection with intestinal helminths have also been shown to increase pneumococcal carriage density and induces the development of invasive

disease(26). Intestinal helminths are known to modulate the immune system and induce a more tolerogenic response in order to favor chronicity of infections, which also affects the response to other pathogens and allergens. In the current study, helminth infections were assessed but only limited number of infections were detected, due to a recent deworming program implemented. However, light infections might have been present as the Kato-Katz method used in this study has limited sensitivity (27).

After adjusting for bacterial densities, increased IL-6 levels were observed in children colonized by *H. influenzae* or *S. pneumoniae* from high compared to low SES, whereas this was not observed for IL-1beta. This might be explained by the role of IL-6 in the IL-1 signaling pathway and its capacity to drive promote the differentiation of Th17-cells. Whereas IL-1beta is primarily initiates the IL-1 signaling, IL-6 production is one of the numerous downstream effects of the IL-1 signaling pathway. Furthermore, high levels of IL-6 are known to promote differentiation of $CD4^+$-cells to form Th17-cells, a cell type that has been shown essential for the clearance *of S. pneumoniae* in mice (28). A study analyzing the Th17-cells and cytokines in adenoidal tissue from *S. pneumoniae*-positive and *S. pneumoniae*-negative children found an increased number of Th17-cells and higher levels of IL-17A and IL-6 in *S. pneumoniae*-negative compared to *S. pneumoniae*-positive children (29). In the current study increased IL-6 levels were observed in the high SES, whereas the density of *H. influenzae* and *S. pneumoniae* was reduced, supporting the role of IL-6 in the control of these bacteria. The IL-17A levels were below the level of detection in most samples collected in this study, however very low levels of IL-17A might be enough to activate local T-cells and therefore be involved in the bacterial clearance.

Since a limited number of children were colonized with *S. aureus* and the presence and density was not associated with that of the other bacteria, responses to colonization by *S. aureus* were not likely to be identified by the canonical correlation analysis. Therefore, the relation between *S. aureus* carriage status and cytokine concentrations was further examined, whereby IL-1 cytokines were of main interest since these cytokines were shown previously to be important for *S. aureus* control (8). In the current study in the low SES increased IL-1beta and IL-1RA levels were observed in *S. aureus* carriers compared to non-carriers, whereas in the high SES decreased levels were found in carriers. The ratio between IL-1RA and IL-1beta was not affected by the carrier status but tended to be decreased in low compared to high SES after adjusting for *S. aureus* carriage. A human nasal inoculation with *S. aureus* showed that IL-1beta was upregulated after inoculation in individuals able to clear the bacteria compared to individuals, but found no

difference in IL-1RA levels. Furthermore, they showed that IL-1RA/IL-1beta ratio was significantly decreased in individuals with persistent *S. aureus* and proposed this ratio as metric for the clearance of *S. aureus* in the nasal mucosa rather than the expression of individual cytokines (8). In the current study, the IL-1RA/IL-1beta ratio tended to be increased in high compared to low SES, indicating that the response to *S. aureus* in low SES children might be reduced. No significant differences in the IL-1RA/IL-1beta ratio between carriers and non-carriers were found, however, it should be kept in mind that the duration of the colonization was not measured in the current study and some of the carriers would be able to clear *S. aureus* over time. To elucidate the relation between SES and *S. aureus* persistence, longitudinal studies are thus needed.

A factor that can affect the bacterial colonization and cytokine responses and which can be impacted by SES is the vaccination status against pneumococcal and *H. influenzae* type B (Hib) diseases. Unfortunately, we were not able to obtain reliable information regarding the vaccination status for the children in this study. The children in our study were not vaccinated through their national childhood vaccination program. However, in a private clinic on payment additional vaccines can be administered, which predominantly involves the Hib vaccine. Although vaccination status might differ between SES its effect on the bacterial load detected here is probably limited, since the (sero)types prevalent in Indonesia only partially match with the available vaccines. A cross-sectional study amongst 302 young children in Indonesia in 2016, thus after Hib vaccine was incorporated in the national program, but before the pneumococcal conjugate vaccine (PCV13) was introduced, showed a carriage rate of 27.5% of *H. influenzae*, but none of these isolates was type B (30). Moreover, before the introduction of Hib vaccination it was shown that Hib vaccination would not have an effect on the pneumonia incidence in Indonesia whereas it in Africa and South America significantly did, indicating that in Indonesia other respiratory pathogens are responsible for LRTIs (31). Moreover, a study by Dunne *et al*. showed that only 46% of the pneumococcal isolates obtained from Indonesian children are covered by the PCV13 (30). While location-specific serotype circulation is of course possible, one can expect that the isolates found in our study are also (sero)types that will only partly be covered by the vaccine, which limits the expected potential effect of differences in vaccination status between high and low SES on the results.

One of the limitations of the current study is that only four pathogenic bacteria were measured in a cross-sectional manner, whereas it is known that viral coinfections and microbiota in general are also important in

shaping responses. Moreover, future studies should assess changes and responses longitudinally. In this study sampling took place in a single season, dry season. There are indications that season can impact the bacterial colonization(32). Although in the current study sampling for both high and low SES took place in the same season and city, and children from both groups were sampled at the same time, the effect of season on the main outcome can not be ruled out. Furthermore, the small sample size for some analyses limits our analysis. In addition, while school-going children are important drivers of transmission within communities, most childhood pneumonia occurs before the age of 5, therefore it would also be of interest to study mucosal immune responses in infants in future studies.

To conclude, the results in the current study indicate that the local immune response to nasopharyngeal bacterial colonization is altered by SES. Since nasopharyngeal carriage of these bacteria precedes disease and local immune responses are important in controlling potential pathogenic bacteria elucidating the relation between the bacterial colonization and local cytokine response is an important first step in understanding the bacteria-host relationship. Insights in this relationship are important for the development of vaccine strategies and treatment options and will eventually lead to reduced LRTIs worldwide.

Acknowledgements

The authors thank Koen Stam and Mikhael Manurung for their advice on the statistical analysis and all participants involved in this study as well as all students of the LUMC and Hasanuddin University for their efforts on sample collection in participating primary schools in Makassar. We thank the Hasanuddin University Medical Research Center (HUM-RC) for laboratory facilities during sample collections and Hasanuddin University for their support.

References

1. Collaborators GBDCoD. Global, regional, and national age-sex specific mortality for 264 causes of death, 1980-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet. 2017;390:1151-1210.
2. Collaborators GBDLRI. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of lower respiratory infections in 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet Infect Dis. 2018;18:1191-1210.
3. Simell B, Auranen K, Kayhty H, et al. The fundamental link between pneumococcal carriage and disease. Expert Rev Vaccines. 2012;11:841-855.
4. Mackenzie GA, Leach AJ, Carapetis JR, Fisher J, Morris PS. Epidemiology of nasopharyngeal carriage of respiratory bacterial pathogens in children and adults: cross-sectional surveys in a population with high rates of pneumococcal disease. Bmc Infect Dis. 2010;10.
5. von Eiff C, Becker K, Machka K, Stammer H, Peters G, Grp S. Nasal carriage as a source of Staphylococcus aureus bacteremia. New Engl J Med. 2001;344:11-16.
6. Chochua S, D'Acremont V, Hanke C, et al. Increased Nasopharyngeal Density and Concurrent Carriage of Streptococcus pneumoniae, Haemophilus influenzae, and Moraxella catarrhalis Are Associated with Pneumonia in Febrile Children. PLoS One. 2016;11:e0167725.
7. Park DE, Baggett HC, Howie SRC, et al. Colonization Density of the Upper Respiratory Tract as a Predictor of Pneumonia-Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus, and Pneumocystis jirovecii. Clin Infect Dis. 2017;64:S328-S336.
8. Cole AL, Muthukrishnan G, Chong C, et al. Host innate inflammatory factors and staphylococcal protein A influence the duration of human Staphylococcus aureus nasal carriage. Mucosal Immunol. 2016;9:1537-1548.
9. Liao SL, Hsu SY, Lai SH, et al. Infant anemia is associated with reduced TLR-stimulated cytokine responses and increased nasopharyngeal colonization with Moxarella catarrhalis. Sci Rep-Uk. 2018;8.
10. Kuipers K, Lokken KL, Zangari T, Boyer MA, Shin S, Weiser JN. Age-related differences in IL-1 signaling and capsule serotype affect persistence of Streptococcus pneumoniae colonization. Plos Pathog. 2018;14.
11. Adegbola RA, DeAntonio R, Hill PC, et al. Carriage of Streptococcus pneumoniae and Other Respiratory Bacterial Pathogens in Low and Lower-Middle Income Countries: A Systematic Review and Meta-Analysis. Plos One. 2014;9.
12. Hanieh S, Hamaluba M, Kelly DF, et al. Streptococcus pneumoniae Carriage Prevalence in Nepal: Evaluation of a Method for Delayed Transport of Samples from Remote Regions and Implications for Vaccine Implementation. Plos One. 2014;9.
13. Dagan R, Juergens C, Trammel J, et al. Modeling pneumococcal nasopharyngeal acquisition as a function of anticapsular serum antibody concentrations after pneumococcal conjugate vaccine administration. Vaccine. 2016;34:4313-4320.
14. Fadlyana E, Dunne EM, Rusmil K, et al. Risk factors associated with nasopharyngeal carriage and density of Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, and Staphylococcus aureus in young children living in Indonesia. Pneumonia. 2018;10.

15. Wahyuni S, van Dorst M, Amaruddin AI, et al. The relationship between malnutrition and TH 2 immune markers: a study in school-aged children of different socio-economic backgrounds in Makassar, Indonesia. Trop Med Int Health. 2021;26:195-203.
16. Thwaites RS, Jarvis HC, Singh N, et al. Absorption of Nasal and Bronchial Fluids: Precision Sampling of the Human Respiratory Mucosa and Laboratory Processing of Samples. J Vis Exp. 2018.
17. Nikolaou E, Blizard A, Pojar S, et al. Minimally Invasive Nasal Sampling in Children Offers Accurate Pneumococcal Colonization Detection. Pediatric Infectious Disease Journal. 2019;38:1147-1149.
18. Olwagen CP, Adrian PV, Nunes MC, Madhi SA. Evaluation of the association of pneumococcal conjugate vaccine immunization and density of nasopharyngeal bacterial colonization using a multiplex quantitative polymerase chain reaction assay. Vaccine. 2018;36:3278-3285.
19. Bogaert D, Keijser B, Huse S, et al. Variability and Diversity of Nasopharyngeal Microbiota in Children: A Metagenomic Analysis. Plos One. 2011;6.
20. de Onis M, Onyango AW, Borghi E, Siyam A, Nishida C, Siekmann J. Development of a WHO growth reference for school-aged children and adolescents. B World Health Organ. 2007;85:660-667.
21. Gonzalez I, Dejean S, Martin PGP, Baccini A. CCA: An R package to extend canonical correlation analysis. J Stat Softw. 2008;23:1-14.
22. Margolis E, Yates A, Levin BR. The ecology of nasal colonization of Streptococcus pneumoniae, Haemophilus influenzae and Staphylococcus aureus: the role of competition and interactions with host's immune response. BMC Microbiol. 2010;10:59.
23. Chien YW, Vidal JE, Grijalva CG, et al. Density interactions among Streptococcus pneumoniae, Haemophilus influenzae and Staphylococcus aureus in the nasopharynx of young Peruvian children. Pediatr Infect Dis J. 2013;32:72-77.
24. Lemon JK, Miller MR, Weiser JN. Sensing of Interleukin-1 Cytokines during Streptococcus pneumoniae Colonization Contributes to Macrophage Recruitment and Bacterial Clearance. Infect Immun. 2015;83:3204-3212.
25. Pizzutto SJ, Upham JW, Yerkovich ST, Chang AB. High Pulmonary Levels of IL-6 and IL-1 beta in Children with Chronic Suppurative Lung Disease Are Associated with Low Systemic IFN-gamma Production in Response to Non-Typeable Haemophilus influenzae. Plos One. 2015;10.
26. Law AE, Shears RK, Lopez Rodas AA, et al. Intestinal helminth co-infection is an unrecognised risk factor for increased pneumococcal carriage density and invasive disease. Sci Rep. 2021;11:6984.
27. Tarafder MR, Carabin H, Joseph L, Balolong E, Jr., Olveda R, McGarvey ST. Estimating the sensitivity and specificity of Kato-Katz stool examination technique for detection of hookworms, Ascaris lumbricoides and Trichuris trichiura infections in humans in the absence of a 'gold standard'. Int J Parasitol. 2010;40:399-404.
28. Zhang Z, Clarke TB, Weiser JN. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. J Clin Invest. 2009;119:1899-1909.
29. Jiang XL, Zhang GL, Yang T, et al. Association of Pneumococcal Carriage and Expression of Foxp3+Regulatory T Cells and Th17 Cells in the Adenoids of Children. Respiration. 2015;90:25-32.

30. Dunne EM, Murad C, Sudigdoadi S, et al. Carriage of Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, and Staphylococcus aureus in Indonesian children: A cross-sectional study. PLoS One. 2018;13:e0195098.
31. Gessner BD, Sutanto A, Linehan M, et al. Incidences of vaccine-preventable Haemophilus influenzae type b pneumonia and meningitis in Indonesian children: hamlet-randomised vaccine-probe trial. Lancet. 2005;365:43-52.
32. Carrion D, Kaali S, Kinney PL, et al. Examining the relationship between household air pollution and infant microbial nasal carriage in a Ghanaian cohort. Environ Int. 2019;133.

Supplementary materials:

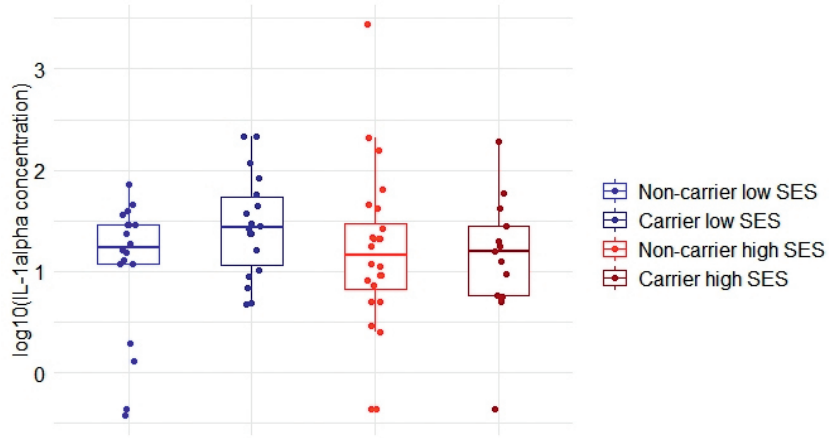

Figure S1: IL-1α concentration in *S. aureus* carrier and non-carrier in high and low SES.
All children for which both cytokines and bacterial colonization could be measured were included, thus n = 73 of which 36 low SES and 37 high SES children. SES: Socio-economic status.