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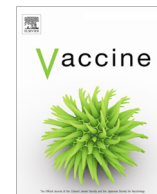
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10-valent pneumococcal conjugate vaccine (PCV10) decreases metabolic activity but not nasopharyngeal carriage of *Streptococcus pneumoniae* and *Haemophilus influenzae*

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ABSTRACT

Background: The effect of pneumococcal vaccination is widely variable when measured by nasopharyngeal carriage of vaccine and non-vaccine targets. The aim of this study was to compare the carriage rates and metabolic activity of *Streptococcus pneumoniae, Staphylococcus aureus, Haemophilus influenzae* and *Moraxella catarrhalis* among children who were or were not vaccinated with PCV10.

Methods: We included children with acute respiratory infection aged 6–23 months from a cross-sectional study (CHIADO-IVAS). Nasopharyngeal aspirates were collected and respiratory pathogens were quantified by nCounter digital transcriptomics (Nanostring) and metagenomic sequencing of 16S ribosomal RNA (Illumina). The metabolic rate was calculated by the ratio between RNA transcripts and 16S DNA reads.

Results: Out of the 80 patients in this study, 53 were vaccinated with PCV10 and 27 were unvaccinated. There was no difference in nasopharyngeal carriage rates of *S. pneumoniae, S. aureus, H. influenzae* or *M. catarrhalis* by either transcriptomic analysis or 16S metagenomics. However, unvaccinated children presented a higher metabolic rate for *S. pneumoniae* compared to PCV10-vaccinated children (Median [25–75th percentiles]: 126 [22.75–218.41] *vs.* 0[0–47.83], p = 0.004). Furthermore, unvaccinated children presented a positive correlation between mRNA counts and 16S DNA reads for *S. pneumoniae* (r = 0.707; p < 0.001) and *H. influenzae* (r = 0.525; p = 0.005), in contrast to vaccinated children. No such effect was observed for *S. aureus* and *M. catarrhalis*.

Conclusions: Vaccination by PCV10 exerts a pathogen-specific effect on pneumococcal metabolic rate. Pathogen RNA/DNA ratio might represent a more sensitive readout for vaccine follow-up, as compared to nasopharyngeal carriage.

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1. Introduction

Nasopharyngeal colonization is a dynamic process in which commensal and potentially pathogenic bacterial agents are constantly acquired and eliminated in the nasopharynx. It represents a fundamental step for invasive disease [1–3], and affects clinical characteristics in children with viral acute respiratory infection (ARI) [4,5]. *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis,* and *Staphylococcus aureus* are commonly carried bacteria in the nasopharynx of children, and present a complex net of interactions among them [6,7]. The use of pneumococcal conjugate vaccines (PCV) alters the dynamics of nasopharyngeal colonization by inducing pathogen-specific immune responses that change carriage rates of the targeted bacteria [8–10] and bystander effects on bacterial pathogens not targeted by the vaccine. However, the effect of pneumococcal vaccination on the metabolic profile of colonizing bacteria has not been established so far.

Transcriptomic profiling of nasopharyngeal samples is a high-throughput method to acquire dynamic phenotypic information

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from colonizing pathogens [11], and represents a tool to assess pathogen metabolic activity through the production of mRNA. It presents high accuracy when assessing pathogen replication or clearance [12,13]. The aim of this study was to compare the carriage rates and metabolic activity of *S. pneumoniae, S. aureus, H. influenzae,* and *M. catarrhalis* among children who were or were not vaccinated with PCV10.

2. Materials and methods

2.1. Patients and samples

We included patients from a prospective cross-sectional study (CHIADO-IVAS) which recruited children aged from 6 to 23 months with ARI seen at the Pediatric Emergency Department of the Federal University of Bahia Hospital, in Salvador, Northeast Brazil, between September 2009 and October 2013 [14]. Inclusion criteria for the CHIADO-IVAS study comprised the presence of either fever, sneeze, running nose, nasal blockage, or cough for up to 7 days. Patients were selected for this study based on the availability of vaccination data and nasopharyngeal aspirate (NPA) for colonization testing. In Salvador, Brazil, the use of PCV10 (Synflorix, GlaxoSmithKline Biologicals, Rixensart, Belgium) was universally introduced in July 2010 for children aged <2 years. It was composed by capsular polysaccharide serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F conjugated to non-typable *H. influenzae* (NTHi) protein D, tetanus toxoid, or diphtheria toxoid. Clinical data was recorded in a standardized form and the vaccine card was checked for PCV application and adherence to the recommended vaccine posology. All included children received the *Haemophilus influenzae* type b (Hib) vaccine. Written informed consent was obtained from legal guardians before recruitment and the study was approved by the Ethics Committee of the Federal University of Bahia.

NPA samples were collected from included children using the following protocol: the distance between the entrance of the nostril and the ear lobe was measured as an estimate of the distance from the entrance of the nostril to the nasopharynx; an aseptic plastic sputum catheter was inserted into the nostril until reaching the nasopharynx; negative pressure was applied and approximately 2 mL of nasal secretions were collected and deposited in a sterile tube with 1 mL of Nuclisens Lysis Buffer (Biomerieux, Boxtel, The Netherlands).

2.2. Laboratory procedures

The presence of colonizing bacteria in the nasopharynx of recruited children was quantified using nCounter transcriptomic analysis (which may indicate cellular activity through RNA transcription) and metagenomic sequencing of 16S ribosomal RNA using Illumina Technology.

2.2.1. nCounter analysis

Total RNA (10–50 ng) was extracted using RNEasy (Qiagen, Hilden, Germany) and was subsequently hybridized against probes targeting *S. pneumoniae, H. influenzae, M. catarrhalis,* and *S. aureus,* using the nCounter gene expression system (Nanostring Technologies), which captures and digitally counts individual mRNA transcripts. Probes were chosen based on specificity profiles previously described in the literature [15,16]. The laboratory tests were performed at the Laboratory for Clinical and Epidemiological Virology, Rega Institute for Medical Research, KU, in Leuven, Belgium. Raw data were processed using nSolver 2.0 software (Nanostring Technologies) sequentially correcting three factors: technical variation between batches (positive control RNA), background correction (negative control) and RNA content by adjusting

the counts geometric mean for the 15 housekeeping genes, followed by normalization using logarithmic transformation (base 2).

2.2.2. Metagenomic sequencing

We performed metagenomic sequencing of prokaryotic 16S ribosomal RNA gene (16S rRNA) using the standard Illumina protocol. 16S rRNA is approximately 1500 bp long and contains nine variable regions interspersed between conserved regions, which are frequently used in phylogenetic classification. Herein, we included a dual barcoding strategy to multiplex up to 96 samples per run. The V3 and V4 regions of the rRNA 16S gene were amplified and Illumina sequencing adapters and dual-index barcodes to the amplicon target were added using limited cycle PCR. The amplified DNA was sequenced with MiSeq v3 reagents using paired 300-bp reads, in which ends of each read were overlapped to generate high-quality, full-length reads of the V3 and V4 regions. Data was analyzed using a two-step strategy to maximize identification of clinically relevant bacteria at the species level. First, by using Kraken software and Krona chart visualization (on BaseSpace, Illumina), which allows a rapid and convenient overview of the complete microbiome, as well as unclassified reads as a quality control (see example in Supplementary Fig. 1). Second, after microbiome determination using Greengenes database, bacterial species level assignments (including all clinically relevant) were confirmed by QIIME [17], performing usearch, with chimera checking and quality filtering, plus blast searching in the SILVA 123 release, with 98% similarity and default e-value. Finally, we performed a correlation analysis between both fast (Kraken) and stringent (QIIME) analysis of DNA reads for both *Streptococcus pneumoniae* and *Staphylococcus aureus* (Fig. 1) and closely related species (*Streptococcus equinus* and *Staphylococcus aureus,* respectively), and demonstrated that the two types of analysis were concordant and highly specific.

2.3. Statistical analysis

Categorical variables were compared using chi-square or Fisher's exact test as appropriate and continuous variables were evaluated using Mann-Whitney U as they presented non-parametric distribution. Kruskal-Wallis test was used when the association of a categorical variable with more than 2 levels and a continuous variable was evaluated. Carriage of each bacterium was defined as the detection of 16S DNA in NPA, and bacterial load as the number of reads of 16S rRNA gene. The ratio between mRNA transcripts and 16SDNA reads of each bacterium was calculated to assess metabolic activity of the respective bacterium (*S. pneumoniae, S. aureus, H. influenzae,* or *M. catarrhalis*). Logistic regression was performed to assess the effect of the number of doses of PCV10 on the detection of RNA transcripts or bacterial carriage (16S DNA). Finally, results were clustered according to Spearman's correlation and shown as heatmap to assess the interplay between carried bacteria following pneumococcal vaccination. All statistical tests were two-tailed, (significance level of 0.05) using SPSS software (version 9.0).

3. Results

We included 80 patients with ARI and simultaneous quantification of both RNA transcripts and 16S DNA reads in their NPA. The flow-chart of the included and excluded patients is shown in Fig. 2. The median age was 10.1 months (25–75% percentiles: 8.1–14.7 months) and 31 patients were males (38.8%). Fifty-three patients had received PCV10 (66.3%), out of which 9 (17%) had received 1 dose, 23 (43.4%) had received 2 doses, 14 (26.4%) had received 3 doses and 7 (13.2%) had received 4 doses of PCV10. Overall, carriage of *S. pneumoniae, S. aureus, H. influenzae* and
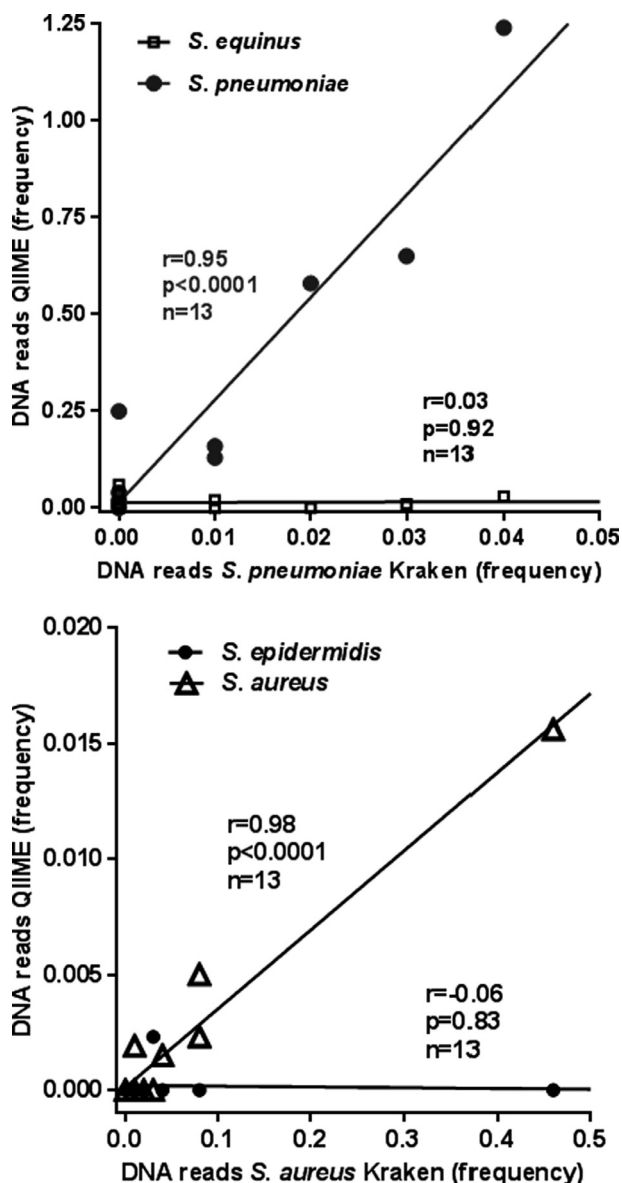
Fig. 1. Specificity of 16S metagenomics reads mapping at species level. A) Frequency of *Streptococcus pneumoniae* reads using either a fast (Kraken) or stringent protocol (QIIME) is highly concordant (Linear regression r = 0.95, p < 0.0001) and specific (no correlation to closely related *Streptococcus equinus*, r = 0.03, p = 0.92) B. Frequency of *Staphylococcus aureus* reads using either a fast (Kraken) or stringent protocol (QIIME) is highly concordant (Linear regression r = 0.98, p < 0.0001) and specific (no correlation to closely related *Staphylococcus epidermidis*, r = −0.06, p = 0.83).

M. catarrhalis was found in 38 (47.5%), 62 (77.5%), 80 (100%) and 80 (100%) patients, respectively, as defined by 16Smetagenomic analysis. All children presented carriage of bacteria from the *Streptococcus* genus. RNA transcripts of *S. pneumoniae*, *S. aureus*, *H. influenzae* and *M. catarrhalis* were detected in 27 (33.8%), 80 (100%), 77 (96.3%), 71 (88.8%) of patients, respectively.

We found no difference in detection rates of RNA transcripts or bacterial carriage (16S DNA) between children who had or had not received PCV10 (Table 1). There was also no difference in the median RNA counts or bacterial load (16SDNA reads) from *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* between vaccinated and unvaccinated patients (Table 2). Children who received PCV10 had higher *S. aureus* RNA counts compared to unvaccinated children, but there was no difference in the number of 16S DNA reads.

Moreover, there was no difference in bacterial load of the complete *Streptococcus* genus (Median [25–75th percentiles]: 14.75 [4.21–39.54] *vs.* 17.21 [2.58–39.34], p: 0.895).

The comparison of the ratio of RNA transcripts and 16S DNA reads between PCV10-vaccinated and unvaccinated children are shown in Table 3. Unvaccinated children had higher RNA/DNA ratio from *S. pneumoniae* compared to vaccinated children, reflecting a higher metabolic rate. There was no difference on the RNA/DNA ratio between vaccinated and unvaccinated children for the remaining bacteria.

Finally, we found that unvaccinated children presented a positive correlation between mRNA counts and 16S DNA reads for *S. pneumoniae* and *H. influenzae*, as shown in Fig. 3. Vaccinated children presented no correlation between mRNA transcripts and 16S DNA reads for any of the studied bacteria.

When the effect of the number of doses of PCV10 on the RNA counts or 16S DNA reads was assessed, we found differences in the RNA counts from *M. catarrhalis*(Median [25–75th percentiles]: 4.88 [1.16–5.87] *vs.* 5.75 [4.65–6.9] *vs.* 5.94 [4.87–7.38] *vs.* 3.8 [0–5.52] for children who received 1, 2 3, or 4 doses of PCV10, respectively; p = 0.039). There was no significant difference in RNA counts, 16S DNA reads or in the ratio between RNA counts and 16S DNA reads according to dosage of PCV10 for any of the remaining pathogens (data not shown). Also, there was no effect of the number of doses of PCV10 on the rates of detection of RNA transcripts or bacterial carriage (16S DNA) (data not shown).

Finally, clustering of pathogen mRNA counts and 16S DNA reads and PCV10 vaccination status (according to Spearman's correlation) revealed three independent clusters, as shown in Fig. 4. A first cluster was formed by *S. pneumoniae* 16S DNA reads, highly correlated to total *Streptococcus* genus DNA frequency. The second cluster displayed strongly correlated 16S DNA reads and mRNA counts of both *H. influenzae* and *S. aureus*. The third cluster shows strong correlation between *S. pneumoniae* replication (mRNA counts), vaccination status and *M.* RNA counts and 16S DNA reads, in agreement with the effects observed in our univariate results. Thus, PCV10 vaccination strongly influences the dynamics of both vaccine (*S pneumoniae*) and non-vaccine targets (the entire *Streptococcus* genus and *M. catarrhalis*).

4. Discussion

We found that PCV10 vaccination did not alter carriage rates by *S. pneumoniae*, *H. influenzae*, *M. catarrhalis*, and *S. aureus*. Nevertheless, colonizing pneumococci in unvaccinated children had a higher metabolic rate than in vaccinated children. Moreover, a significant correlation between RNA transcripts and 16S rRNA DNA reads was found only for *S. pneumoniae* and *H. influenzae* in unvaccinated children. Altogether, these findings indicate that pneumococcal conjugate vaccination affects the biological activity of bacterial respiratory pathogens in the nasopharynx of children.

Pneumococcal vaccination promotes a decrease in vaccine-covered *S. pneumoniae* serotypes associated with an increase in non-vaccine-covered serotypes [8,18], which has been reported to result in an unaltered overall frequency of pneumococcal carriage [19]. Also, recent evidence suggests that PCV10 does not reduce the carriage rates by *H. influenzae* despite the presence of NTHi protein D in its composition [8,18]. No difference in carriage rates of *M. catarrhalis* or *S. aureus* have been reported following pneumococcal vaccination in a clinical trial evaluating the effectiveness of PCV10 in nasopharyngeal colonization of pathogenic bacteria [8]. Accordingly, we found no difference on the carriage rates of either *S. pneumoniae*, *H. influenzae*, *M. catarrhalis* or *S. aureus* between PCV10 vaccinated or unvaccinated children. However, PCV use also induces changes in the metabolic profile of colonizing
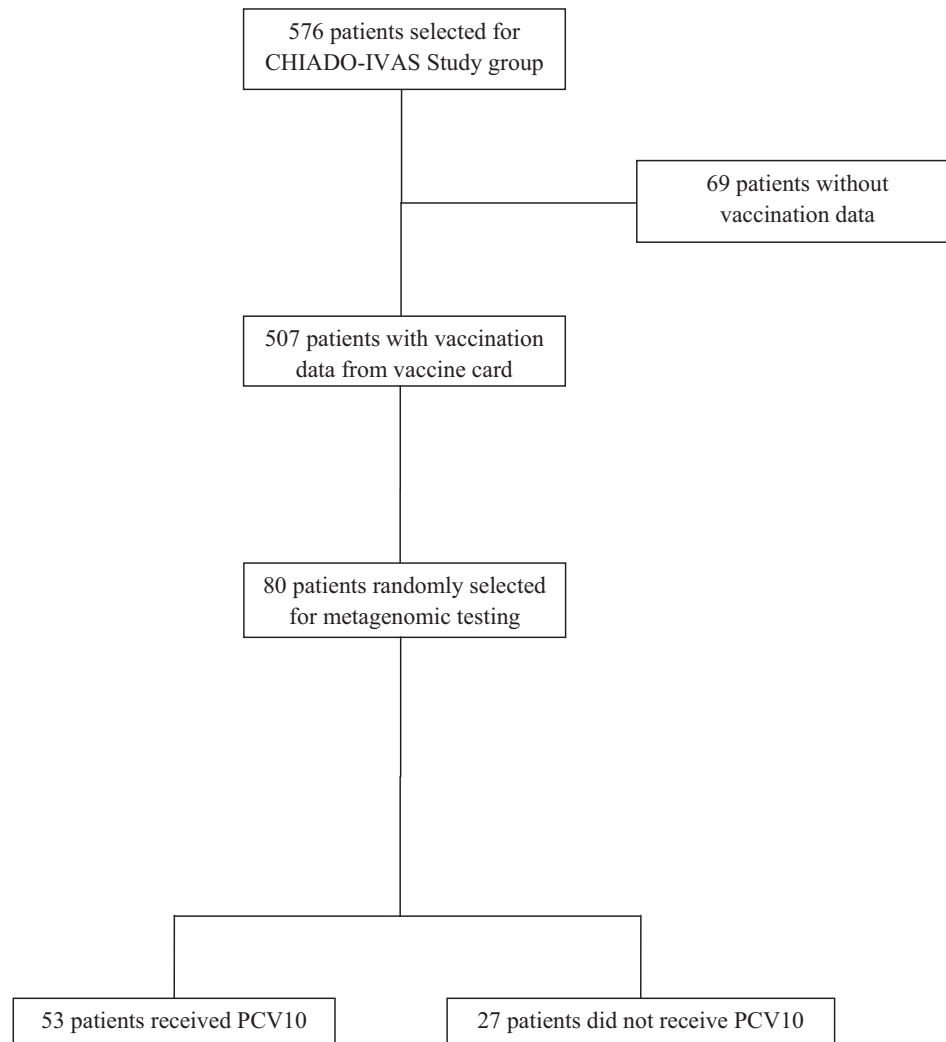
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      │ 53 patients received PCV10 │    │ 27 patients did not receive PCV10 │
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Fig. 2. Flow-chart of the included and excluded cases in this study.

Table 1
Comparison of clinical characteristics, rates of detection of RNA transcripts and 16S DNA between PCV10-vaccinated and unvaccinated patients.

	Vaccinated patients (n = 53)	Unvaccinated patients (n = 27)	P
RNA transcripts			
S. pneumoniae	15 (28.3%)	12 (44.4%)	0.149
S. aureus	53 (100%)	27 (100%)	–
H. influenzae	51 (96.2%)	26 (96.3%)	1
M. catarrhalis	47 (88.7%)	24 (88.9%)	1
16SrRNA DNA			
S. pneumoniae	25 (47.2%)	13 (48.1%)	0.934
S. aureus	41 (77.4%)	21 (77.8%)	0.966
H. influenzae	53 (100%)	27 (100%)	–
M. catarrhalis	53 (100%)	27 (100%)	–
Clinical characteristics			
Sex Male	20 (37.7%)	11 (40.7%)	0.794
Age in months, median (p25th-p75th)	9.2 (7.9–13.9)	10.8 (8.2–16.8)	0.228
History of fever[a]	41 (78.8%)	22 (81.5%)	0.782
History of running nose	47 (88.7%)	25 (92.6%)	0.710
History of hoarseness	17 (32.1%)	8 (29.6%)	0.8231
History of otalgia	3 (5.7%)	1 (3.7%)	1
History of wheezing	20 (37.7%)	13 (48.1%)	0.371
History of dyspnea	16 (30.2%)	5 (18.5%)	0.262
History of chest retraction	5 (9.4%)	0 (0%)	0.161

[a] N: 79 patients due to missing data.

Table 2
Comparison of median (interquartile interval) RNA transcripts counts and 16S rRNA DNA reads between PCV10-vaccinated and unvaccinated patients.

	Vaccinated patients (n = 53)	Unvaccinated patients (n = 27)	P
RNA transcripts			
S. pneumoniae	0 (0–1.49)	0 (0–3.43)	0.112
S. aureus	8 (6.25–9.42)	5.62 (4.58–7.08)	0.001
H. influenzae	11.47 (6.53–19.04)	13.98 (6.3–17.14)	0.695
M. catarrhalis	5.52 (4.07–6.46)	5.58 (4.26–6.26)	0.819
16S DNA			
S. pneumoniae	0 (0–0.02)	0 (0–0.03)	0.982
S. aureus	0.01 (0.01–0.05)	0.01 (0.01–0.2)	0.508
H. influenzae	0.45 (0.24–14.82)	1.24 (0.23–11.51)	0.927
M. catarrhalis	13.44 (0.82–53.24)	37.5 (11.05–54.49)	0.191

pneumococci, as suggested by previous mathematical modelling [20]. *Streptococcus pneumoniae* have sensor-kinase signal systems that detect environmental parameters that allow changes in their genetic programs in response [21]. Therefore, rather than solely affecting carriage rates, pneumococcal vaccination may promote changes in the biological behavior of colonizing pathogenic bacteria in the nasopharynx by changing environmental characteristics.

Herein, we found that carried pneumococci in PCV10-vaccinated children have a lower metabolic rate compared to those

Table 3

Comparison of the ratio between RNA transcripts counts and 16S rRNA DNA reads between PCV10-vaccinated and unvaccinated patients who had carriage of each bacterium detected by metagenomic analysis.

	Vaccinated patients	Unvaccinated patients	p
S. pneumoniae (n = 38)	0 (0–47.83)	126 (22.75–218.41)	0.004
S. aureus (n = 62)	292.87 (82.74–733.47)	253.99 (140.35–594.58)	0.982
H. influenzae (n = 80)	18.85 (0.55–47.21)	7.5 (0.66–36.83)	0.590
M. catarrhalis (n = 80)	0.21 (0.07–7.39)	0.12 (0.07–0.52)	0.359

in unvaccinated children (Table 3). This finding corroborates the lower invasive potential of colonizing pneumococci following PCV use [19]. The reduction in pneumococcal activity in vaccinated children may represent an adaptive response from this bacterium in a hostile environment created by the activated immune system. Also, unvaccinated children had a positive correlation between bacterial load (16S rRNA DNA reads) and mRNA counts, indicating that carried bacteria were in an active replicating state. Vaccinated children, however, had a poor correlation, which might represent inhibition of replication or even bactericidal activity of the immune system.

Vaccinated children also presented a poor correlation between bacterial load (16S rRNA DNA reads) and mRNA counts from *H. influenzae*. The effect of PCV10 on nasopharyngeal carriage of *H influenzae* has not been elucidated so far. Prymula et al. (2009) reported a decrease in the rates of carriage after the booster dose of a NTHi Protein D conjugated pneumococcal vaccine, whereas Vesikari et al. (2016) found no difference in the carriage rates between PCV10-vaccinated and unvaccinated children [8,22]. In Brazil, higher rates of colonization by *H influenzae* were detected

in PCV10 vaccinated children [18]. In our study, the carriage rates by this bacterium were extremely high both in vaccinated and unvaccinated children, so no differences were found in carriage rates or bacterial load. However, we hypothesize that PCV10 yields an antigen-specific response that suppresses the bacterial metabolic activity or leads to bacterial killing, thereby creating the poor correlation between carriage and transcription for *H. influenzae* in vaccinated children.

We found higher mRNA counts from *S. aureus* in children who received PCV10, indicating that although the carriage rates were similar in vaccinated and unvaccinated children, colonizing strains in vaccinated children had a higher metabolic profile. As we found lower metabolic activity of *S. pneumoniae* in vaccinated children, this finding corroborates the negative association between nasopharyngeal colonization by *S. pneumoniae* and *S. aureus* [5,6]. Regarding nasopharyngeal carriage of *M. catarrhalis,* we found no difference on the carriage rates or metabolic activity of this bacterium between PCV10 vaccinated and unvaccinated children. Differences in mRNA counts among children who received different number of doses of PCV10 may have been caused by changes in nasopharyngeal bacterial interactions due to vaccination, as dynamic interactions between different colonizers have been reported [23]. Indeed, clustering was observed between mRNA counts from *S. pneumoniae* and 16S DNA reads and mRNA counts from *M. catarrhalis* (Fig. 4).

The limitations of this study should be noted. The group of vaccinated children was not homogenous, as children received different number of doses of PCV10. However, we found no effect of the number of doses of PCV10 on the rates of detection of RNA transcripts or bacterial carriage (16S DNA), as assessed by logistic regression. Similarly, there was no effect of the number of doses of PCV10 on the bacterial load or on the metabolic rate for any of the studied bacteria. Therefore, the differences in the vaccination schemes did not significantly affect the outcome variables from
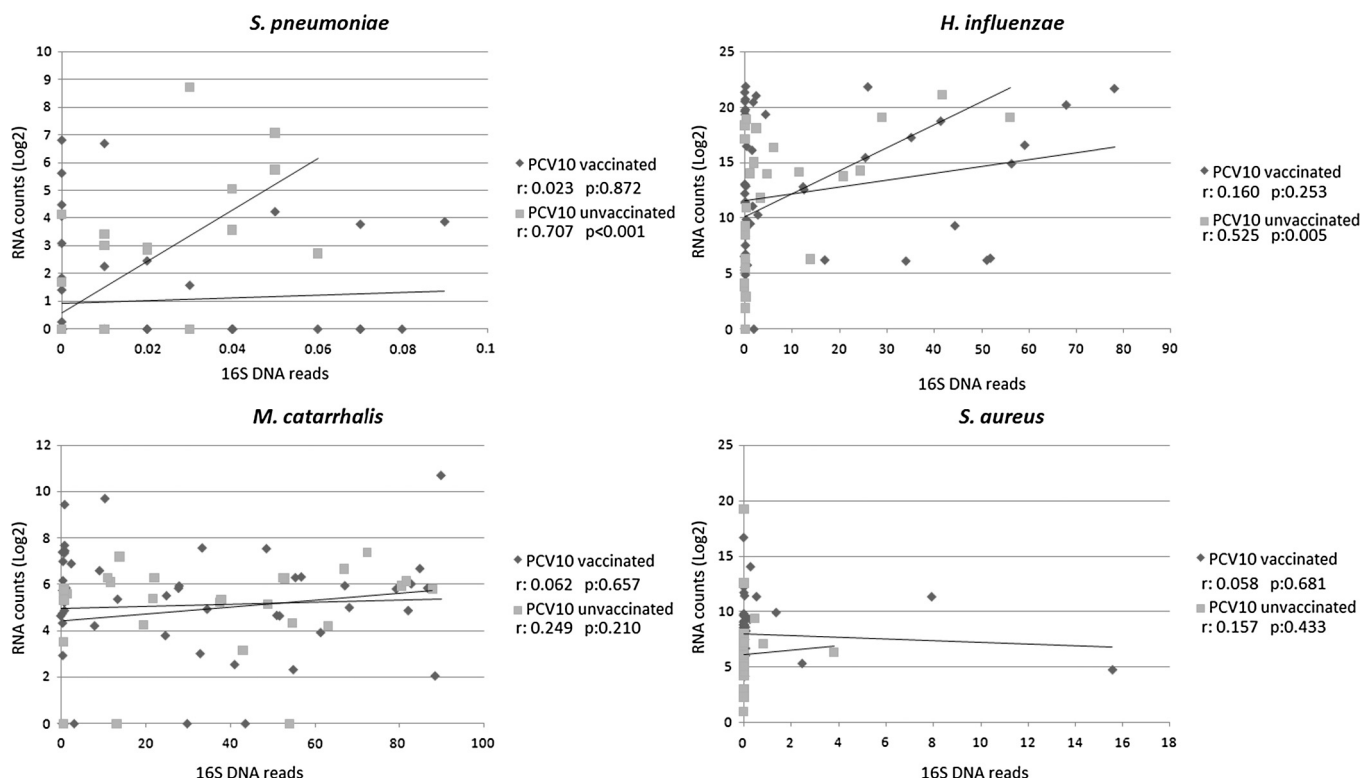

Fig. 3. Correlation between mRNA counts and 16S rRNA DNA reads from *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis* and *Staphylococcus aureus* among PCV10 vaccinated and unvaccinated children.
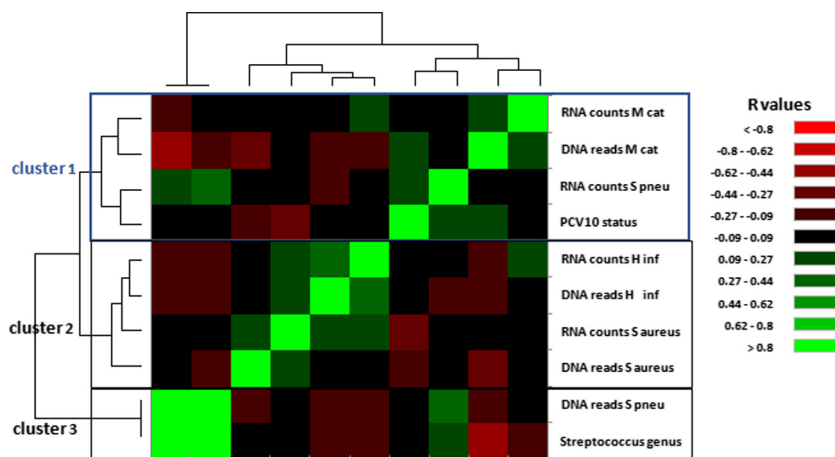
Fig. 4. Dynamic interplay between pathogenic genera and species at both RNA and DNA level following PCV10 vaccination. RNA and DNA levels of respiratory pathogens were quantified by nCounter digital transcriptomics and 16S metagenomics, respectively. Results were clustered according to Spearman's correlation and shown as heatmap (red = negative, green = positive). The blue cluster (1) indicates vaccination status is strongly correlated to *S. pneumoniae* RNA levels and *M. catarrhalis* DNA levels, whereas *H. influenzae* and *S. aureus* cluster together at both RNA and DNA level. Cluster 3 confirms *S. pneumoniae* DNA levels (carriage) are independent from its RNA levels, but are strongly correlated to the DNA frequency of the entire Streptococcus genus. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

this study. Finally, although carriage rates described herein were extremely high for some pathogenic bacteria, high carriage rates for the studied bacteria have been described elsewhere [23–25]. Furthermore, we applied a highly sensitive method (and still specific, as shown in Fig. 1), so higher carriage rates were expected when compared to previous reports.

In conclusion, vaccination with PCV10 did not affect carriage rates or the bacterial load of colonizing *S. pneumoniae, H. influenzae, M. catarrhalis,* and *S. aureus.* However, we found lower metabolic activity of *S. pneumoniae* in vaccinated children, paralleled by higher RNA counts for *S. aureus.* Furthermore, only unvaccinated children presented a positive correlation between bacterial load and mRNA transcripts for *S. pneumoniae* and *H. influenzae,* indicating an immune-mediated suppressive or bactericidal effect in vaccinated children. Altogether, our data demonstrates that pneumococcal conjugate vaccination alters pathogen RNA/DNA ratio in the nasopharyngeal microbiome, which might represent a more sensitive readout for vaccine follow-up, as compared to nasopharyngeal carriage.

Conflicts of interest

None to declare.

Acknowledgements

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.06.048.

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