# Cross Canada Rounds

## Journal Club

**Eyal Jacobi – Pediatric respiratory Fellow**

**21.03.2109**

# Airway microbiome in adult survivors of extremely preterm birth: the EPICure study

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EUROPEAN RESPIRATORY *journal*

FLAGSHIP SCIENTIFIC JOURNAL OF ERS

European Respiratory Journal 2019 53: 1801225; **DOI:** 10.1183/13993003.01225-2018

# Presentation goals:

1. **Introduction of the field of Microbiome** and how its implemented in the Pediatric respiratory field.

2. **Journal club.**
   - Research question.
   - Methods.
   - Results.
   - Discussion of implementation of the results.

# The Pediatric Microbiome and the Lung

- The term "**microbiome**", originally defined by microbiologist Joshua Lederberg, signifies the "**community of commensal, symbiotic, and pathogenic microorganisms that…share our body space**"*.

- Medical microbiology - focusing on the identification, cultivation, and analysis of specific microbial "pathogens".

- This "**pathogen-oriented**" approach, while enormously helpful and successful for many diseases, often ignored the presence and activities of the numerous other microbes that we now know to inhabit us.

Peterson J, Garges S, Giovanni M, McInnes P, Wang L, Schloss JA, Bonazzi V, McEwen JE, Wetterstrand KA, et al. NIH HMP Working Group. The NIH Human Microbiome Project. Genome Res. 2009; 19:2317–2323. [PubMed: 19819907]

# The Pediatric Microbiome and the Lung

- "**The great plate count anomaly**" - observations made by environmental microbiologists comparing microscopic findings with routine culture of specimens.

- Major discrepancies between **what was observed** using culture (the absence of microbes in airways) and **what was expected** based on other considerations (constant inhalation of microbes).

- "**Cultureindependent**" microbiological techniques that do not rely on microbial growth, but rather detect their molecular signatures (most often their genetic material).

- These newer techniques, often referred to as **microbiome methods**, have provided fascinating new findings that have forced us to revise our notions of how microbes inhabit our bodies in various states of health.

# The Pediatric Microbiome and the Lung - Methods

- **qPCR** - Realtime quantitative polymerase chain reaction remains the most common technique for amplifying and quantifying abundance of specific DNA or RNA sequences.

- **Fluorescent in situ hybridization (FISH)** - fluorescently labeled probes for specific bacterial sequences, followed by fluorescent microscopy to identify their targets and localize them within tissues.

- **16S ribosomal RNA (rRNA) gene** - This gene contains some sequences that are well-conserved among bacteria. Allowing for identification of specific bacterial types, or taxa, often at the phylum, genus or species level.

The Pediatric Microbiom and the lung Michel tracy et al. Curr Opin Pediatr. 2015 June ; 27(3): 348–355.

# The Pediatric Microbiome and the Lung - Methods

- Once the DNA is purified from a sample of interest, PCR is usually used to amplify a large portion of the rRNA gene using primers targeting conserved regions to eventually identify and quantify specific bacteria type\diversity.

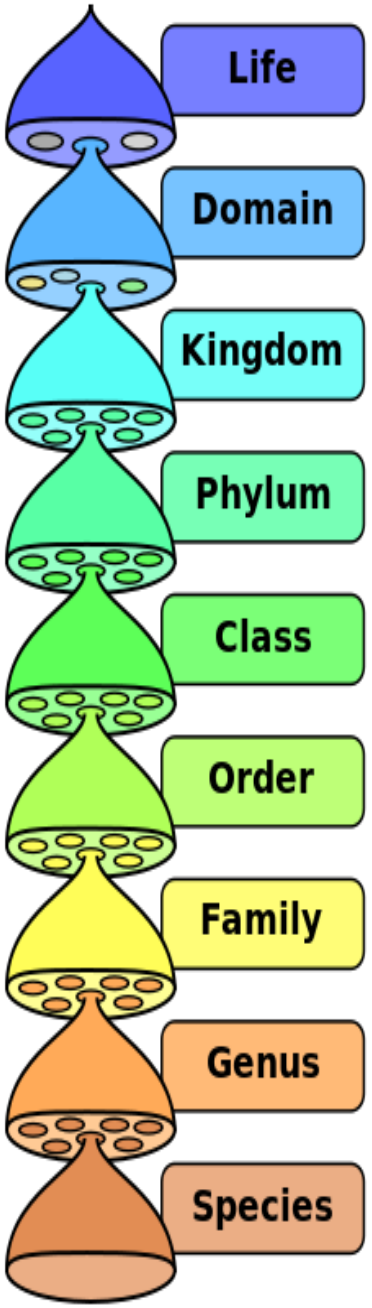
- Sequencing of 16S rRNA gene **amplicons**, followed by computational comparison of the resulting sequence “reads” to taxonomic databases, has yielded high resolution snapshots of microbiomes.

# Microbiome terminology

- **Dysbiosis** – microbial imbalance or maladaptation on or inside the body.

- **Genus** –Taxonomic rank used in the biological classification of living and fossil organisms. In the hierarchy of biological classification, genus comes above species and below family.

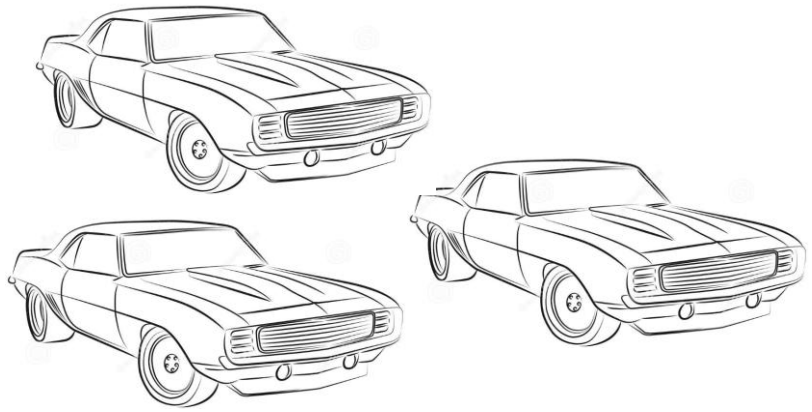
- **Phylum** - is a level of classification or Taxonomic rank below kingdom and above class (eg – Bacteroidetes).

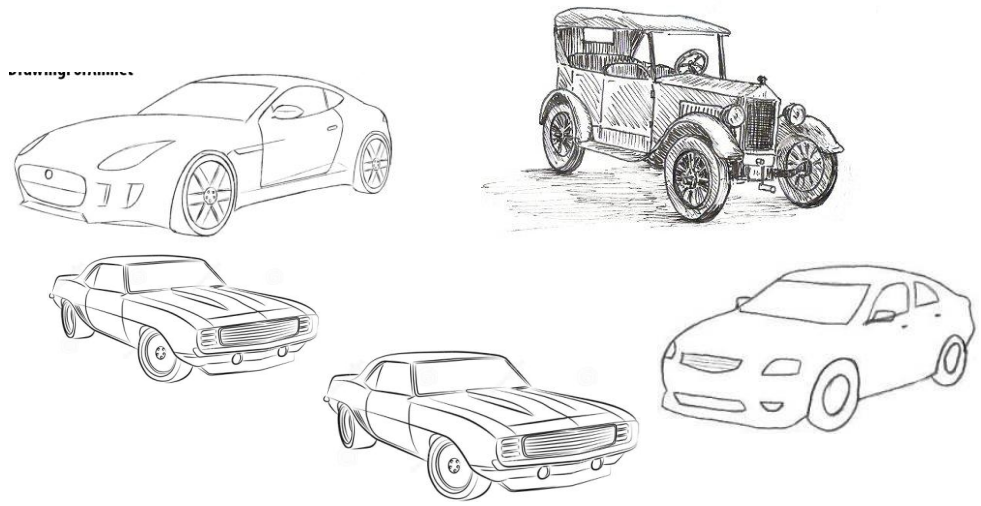


# Microbiome terminology

- **Alpha diversity** - is the mean species diversity in sites or habitates at a local scale.

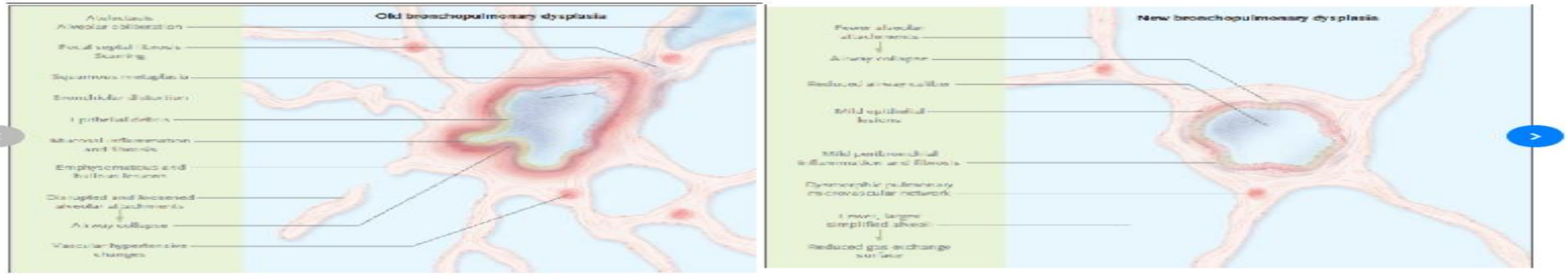
- **Beta diversity** - is the ratio between regional and local species diversity.





# Introduction - BPD and preterm Infants

- Bronchopulmonary dysplasia (BPD) is a major complication of preterm birth that leads to lifelong respiratory morbidity.
- Global and national survival rates of extremely preterm birth have steadily increased over the past decades.
- Recently in more immature infants a “**new**” form of BPD has been defined, characterised by disrupted distal lung development, arrest of alveolarisation and interference with normal vascularisation.




Airway and Parenchymal Damage in Old and New BPD. " Old " and " new " BPD are two different morphologic outcomes of variable combinations of factors capable of injuring lungs of differing maturity. In old BPD, intense inflammation and disruption of normal pulmonary structures lead to a nonhomogeneous airway and parenchymal disease. In contrast, the main feature of new BDP is diffusely reduced alveolar development, which is associated with a clinically significant loss of surface area for gas excange, with airway injury, inflammation, and fibrosis that are usually milder than in old BPD. (From Baraldi E., Filippone M. Chronic Lung Disease after Premature Birth. N Engl J M. 2007, 357(8);1951)

PEDIATRICS
OFFICIAL JOURNAL OF THE AMERICAN ACADEMY OF PEDIATRICS

# The EPICure Study: Outcomes to Discharge From Hospital for Infants Born at the Threshold of Viability

Kate Costeloe, Enid Hennessy, Alan T. Gibson, Neil Marlow, Andrew R. Wilkinson
and for the EPICure Study Group

*Pediatrics* 2000;106;659-671
DOI: 10.1542/peds.106.4.659

# Introduction - The EPICure study

- The EPICure study (Pediatrics, 2000) is a **prospective national longitudinal cohort study** investigating the health outcomes of babies born at <26 weeks of gestation in the United Kingdom and Ireland between March and December 1995.

- A total of 4004 births were recorded, and 811 infants were admitted for intensive care.

- This cohort has been followed-up and assessed at 2.5, 6, 9, 11 and now 19 years of age.

# The EPICure study

Aged 11 years, children who were born extremely preterm compared to their classmates who were born full-term, had significantly more:

1. **Respiratory symptoms.**
2. **Lung function abnormalities with evidence of airway obstruction.**
3. **Ventilation inhomogeneity, Gas trapping and airway hyperresponsiveness.**
4. **Twice the prevalence of asthma.**

Fawke J, Lum S, Kirkby J, et al. Lung function and respiratory symptoms at 11 years in children born extremely preterm: the EPICure study. Am J Respir Crit Care Med 2010; 182: 237–245.

# BPD and Microbiome

- Studies have provided evidence that bacteria may play a role in the development of BPD in preterm infants*.

- Longitudinal study described a characteristic pattern of airway microbial dysbiosis prior to the development of BPD.

- This was characterised by a remarkable **decrease in the richness and α-diversity** with time; in addition to a shift in the bacterial community composition, in contrast to a relatively diverse and stable community in the preterm infants who did not develop BPD.

Lohmann P, Luna RA, Hollister EB, et al. The airway microbiome of intubated premature infants: characteristics and changes that predict the development of bronchopulmonary dysplasia. Pediatr Res 2014; 76: 294–301.

# BPD and Microbiome

- The extent to which microbial succession sustains the dysbiosis of the airway microbiome described in infancy into later life stages has not been investigated.

- How the airway microbiome of BPD survivors differs from the healthy microbiome in adulthood is not known.

- The aim of this study is to characterise the airway microbiome in the EPICure cohort at the age of 19 years and compare it with the healthy microbiome of their counterparts who were born full term.

# Disclosure

- Authors had no Disclosure to be made other then funds that are not related directly to the research.

# Materials and methods

- Induced sputum was collected from 92 participants in the EPICure@19 study during their follow-up visit aged 19 years.

- The average percentage of squamous epithelial cells in one aliquot of each sputum sample was measured by microscopy for quality control.

- Samples were allocated to three groups based on the medical history of participants;

1. Born **extremely preterm without BPD history** (EP no BPD).
2. Born **extremely preterm with neonatal BPD** (EP + BPD).
3. Born **full term-born participants** of the same age, who had been evaluated as classmates in earlier studies (EPICure control group).

# Materials and methods

- Microbiome **investigators were blinded to the group assignment** until analysis was complete.

- Metagenomic DNA was extracted from 500 µL of Sputasol-treated sputum samples using Qiagen Dneasy Blood and Tissue kit (Qiagen, Manchester, UK) as per the manufacturer's protocol.

- The bacterial loads **of H. influenzae, M. catarrhalis and S. pneumoniae** were quantified using multiplex quantitative (q)PCR.

# Materials and methods

- A sequence library was created by amplification of V5–V7 regions of the bacterial 16S rRNA gene from metagenomic DNA using 785 forward primer and 1175 reverse primer.

- The appropriate statistical significance tests were calculated using SPSS (v.23) or QIIME wrapper scripts.

- BPD was defined as receiving supplemental oxygen or respiratory support at 36 weeks postmenstrual age.

# Results

## General characteristics of the participants:

- Induced sputum samples were collected from **92 young-adult participants**;
  51 were born extremely preterm at <26 weeks' gestation.
  41 were born full-term (control group).

- Microbiome analysis was completed on **74 samples with an amplified 16S rRNA gene**:
  36 controls and 38 extremely preterm; 29 of whom had neonatal BPD.

TABLE 1 Demographic and clinical characteristics of all EPICure participants and those with sequenced samples

| | Whole cohort[#] | | | | Sequenced samples[#] | | | |
|---|---|---|---|---|---|---|---|---|
| | EP[#] | | Controls | p-value | EP[#] | | Controls | p-value |
| | BPD | No BPD | | | BPD | No BPD | | |
| **Subjects n** | 37 | 14 | 41 | | 29 | 9 | 36 | |
| **Age years** | 19.02±0.54 | 19.02±0.41 | 19.09±0.52 | 0.803[§] | 18.93±0.66 | 18.82±0.48 | 19.02±0.55 | 0.474[f] |
| **Males** | 35 | 43 | 39 | 0.866[##] | 34 | 56 | 42 | 0.521[##] |
| **Females** | 65 | 57 | 61 | | 66 | 44 | 58 | |
| **Asthma diagnosis** | 57 | 21* | 37 | 0.047[##] | 59 | 22 | 38 | 0.094[##] |
| **Current smoker** | 22 | 29 | 27 | 0.821[##] | 28 | 33 | 33 | 0.873[##] |
| **Passive smoke exposure >30 min·week$^{-1}$** | 22 | 29 | 35 | 0.471[##] | 32 | 22 | 60 | 0.348[##] |
| **Squamous epithelial cell %** | 17.4±17.7 | 17.9±11.9 | 13.8±11.1 | 0.573[f] | 20.9±19.2 | 19.5±14.4 | 14.6±11.3 | 0.566[f] |
| **Prescribed inhalers[¶]** | 60 | 38 | 64 | 0.557[¶¶] | 62 | 17 | 50 | 0.239[¶¶] |
| **Prescribed bronchodilator inhalers[+]** | 53 | 25 | 55 | 0.417[¶¶] | 54 | 17 | 50 | 0.298[¶¶] |
| **Prescribed ICS** | 27 | 13 | 36 | 0.476[¶¶] | 31 | 17 | 33 | 0.883[¶¶] |
| **Antibiotic treatment in past year** | 7 | 13 | 30 | 0.397[¶¶] | 8 | 0 | 27 | 0.395[¶¶] |
| **Treated for respiratory problem in the past year** | 20 | 21 | 25 | 0.393[¶¶] | 22 | 11 | 28 | 0.556[##] |
| **$FEV_1$ L** | 2.66**±0.54 | 3.22±0.76 | 3.57±0.65 | 0.000[§] | 2.63**±0.66 | 3.45±0.76 | 3.55±0.61 | 0.000[§] |
| **$FEV_1$ z-score** | −1.66**±1.09 | −0.911±0.04 | −0.37±0.87 | 0.000[§] | −1.87**±1.17 | −0.75±0.69 | −0.32±0.89 | 0.000[§] |
| **Percentage change in $FEV_1$ with bronchodilator** | 7.93±6.25 | 7.75±7.81 | 5.26±5.60 | 0.077[§] | 9.52±7.77 | 7.19±4.91 | 5.50±5.85 | 0.058[§] |
| **$F_{ENO}$ ppb** | 16.33±12.63 | 18.00±14.72 | 26.47±26.30 | 0.683[f] | 16.50±13.98 | 16.89±15.34 | 25.57±27.71 | 0.830[f] |
| **Eosinophils cells·μL$^{-1}$** | 181±127 | 164±104 | 229±148 | 0.184[f] | 194±141 | 179±128 | 232±156 | 0.399[f] |

Data are presented as mean±SD or %, unless otherwise stated. EP: extremely preterm birth; BPD: bronchopulmonary dysplasia; ICS: inhaled corticosteroids; $FEV_1$: forced expiratory volume in 1 s; $F_{ENO}$: exhaled nitric oxide fraction. [#]: whole cohort n=92, of which EP n=51, sequenced samples n=74, of which EP n=38; [¶]: $\beta_2$-adrenoreceptor agonists: salbutamol (Ventolin), terbutaline (Bricanyl), salmeterol (Serevent, Seretide); muscarinic receptor antagonist: ipratropium (Atrovent); leukotriene receptor antagonist: montelukast (Singulair); ICS: beclomethasone (Becotide), budesonide (Pulmicort), fluticasone (Flixotide, Seretide); [+]: $\beta_2$-agonists and muscarinic receptor antagonists; [§]: calculated by ANOVA; [f]: p-value calculated by Kruskal–Wallis test; [##]: p-value calculated by Chi-squared test; [¶¶]: p-value calculated by Fisher's exact test. *: p<0.05; **: p<0.01.

# Results

- Within the sequenced cohort, **forced expiratory volume in 1 s (FEV1) was significantly lower in the EP + BPD group compared to controls** (mean difference −0.91 L, 95% CI −1.24–−0.59 L) **and the EP no BPD** group (mean difference −0.81 L, 95% CI −1.31–−0.32 L).

- After adjustment for age, sex and body size using z-scores, **the mean FEV1 z-score of the EP + BPD group was significantly lower compared to the control group and the EP no BPD group;** mean differences (95% CI) were −1.55 (−2.05–−1.05) and −1.13 (−1.88–−0.37), respectively.

# Results

- The prevalence of **self-reported asthma was relatively higher in the EP + BPD group** (59%) **and controls** (38%), compared to the EP no BPD group (22%), although this did not reach statistical significance.

- Authors mention that it is likely that there is significant overdiagnosis of “asthma” here, but we did not consider it is ethical to stop asthma treatment for the purposes of research.

- At the time of sample collection, the mean±SD exhaled nitric oxide fraction (FeNO) concentration was 16.59±14.10 ppb and 25.57±27.71 ppb in the preterm and control groups, respectively ( $p>0.05$).

# Results

- The mean±SD **eosinophil counts in blood** were 190±136 cells·µL−1 and 232±156 cells·µL−1 in the preterm group and controls, respectively ( p>0.05).

- 47% of the EP group and 50% of the control group were **prescribed inhalers**. **No statistically significant differences were found** across the three groups with respect to the number of patients who were prescribed bronchodilator inhalers, inhaled corticosteroids or those who had been **treated with antibiotics** for respiratory problems in the year prior to sample collection.

- The numbers of **males and females, smokers** and those who were **exposed to passive smoking** (>30 min per week) were similar across the three groups.

# Microbial community composition

1. The bacterial communities **were significantly less diverse and less rich** in **the sputum samples from the whole EP group compared to controls**.

2. The mean±SEM difference in Chao 1 and Fisher α indices between the whole EP group and the controls were −39±13 ($p<0.05$)

3. Other richness and α-diversity indices including the number of observed OTUs and PD whole tree also **showed significantly less diverse and less rich microbiota in the EP group.**

# Microbial community composition

4. **Within the EP group**, the trend observed in all previously mentioned α-diversity indices suggests that **those with neonatal BPD had the least diverse and rich microbial communities, while controls had the highest values** and was statistically significant only in Chao 1 when tested by ANOVA ( $p<0.05$).

5. In principal coordinate analysis of weighted Unifrac β-diversity index, **the samples from EP participants clustered significantly**, **regardless of neonatal BPD status**; whereas the samples from controls were scattered ( $p<0.01$ by ANOSIM and $p<0.05$ by PERMANOVA).

# Microbial community composition

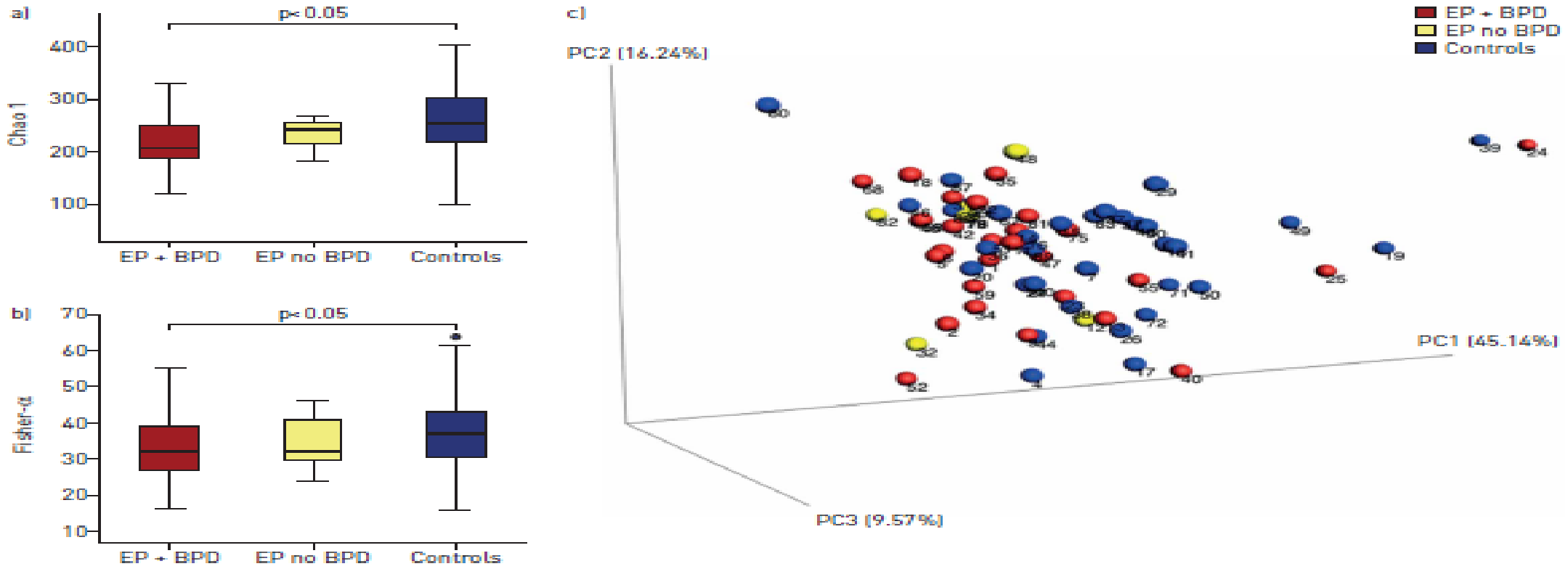



FIGURE 1 Comparison of the richness and α-diversity of microbial communities in sputum between the extreme preterm birth survivors (EP), with and without neonatal bronchopulmonary dysplasia (BPD), and controls. Richness and α-diversity measured by a) Chao 1 ($p<0.05$ by ANOVA); b) Fisher α-diversity index ($p=0.07$ by ANOVA); nevertheless, Fisher-α was significantly lower in the whole EP group compared to controls ($p<0.05$ by t-test). c) Principal coordinate analysis of weighted UniFrac β-diversity index ($p<0.01$ by ANOSIM and $p<0.05$ by PERMANOVA comparing the whole EP group (n=37) and controls (n=33); $p>0.05$ by ANOSIM and PERMANOVA comparing the three groups: EP + BPD (n=28), EP no BPD (n=9), controls (n=33)).

# Microbial community composition – phylum level

- The bacterial community at phylum level was dominated by Firmicutes, followed by Bacteriodetes, then Proteobacteria and Actinobacteria.

- The samples from both EP groups, with and without BPD, had a **significantly lower relative abundance (RA) of the phylum Bacteriodetes** compared to the control group ($p<0.05$ by Kruskal–Wallis test).

- Differences were compensated by a nonsignificant increase in the relative abundance of Firmicutes.

# Microbial community composition – genus level

Looking at the composition of the microbial communities at **genus level**

- the relative abundance of **Prevotella was significantly lower** in both EP groups, with and without BPD, in comparison to the control group ($p<0.05$ by Kruskal–Wallis test).

- This was compensated for by a nonsignificant and inconsistent increase in relative abundance of other genera such as Streptococcus, Veillonella, Rothia and Neisseria, which are **normal microbiota in airways.**

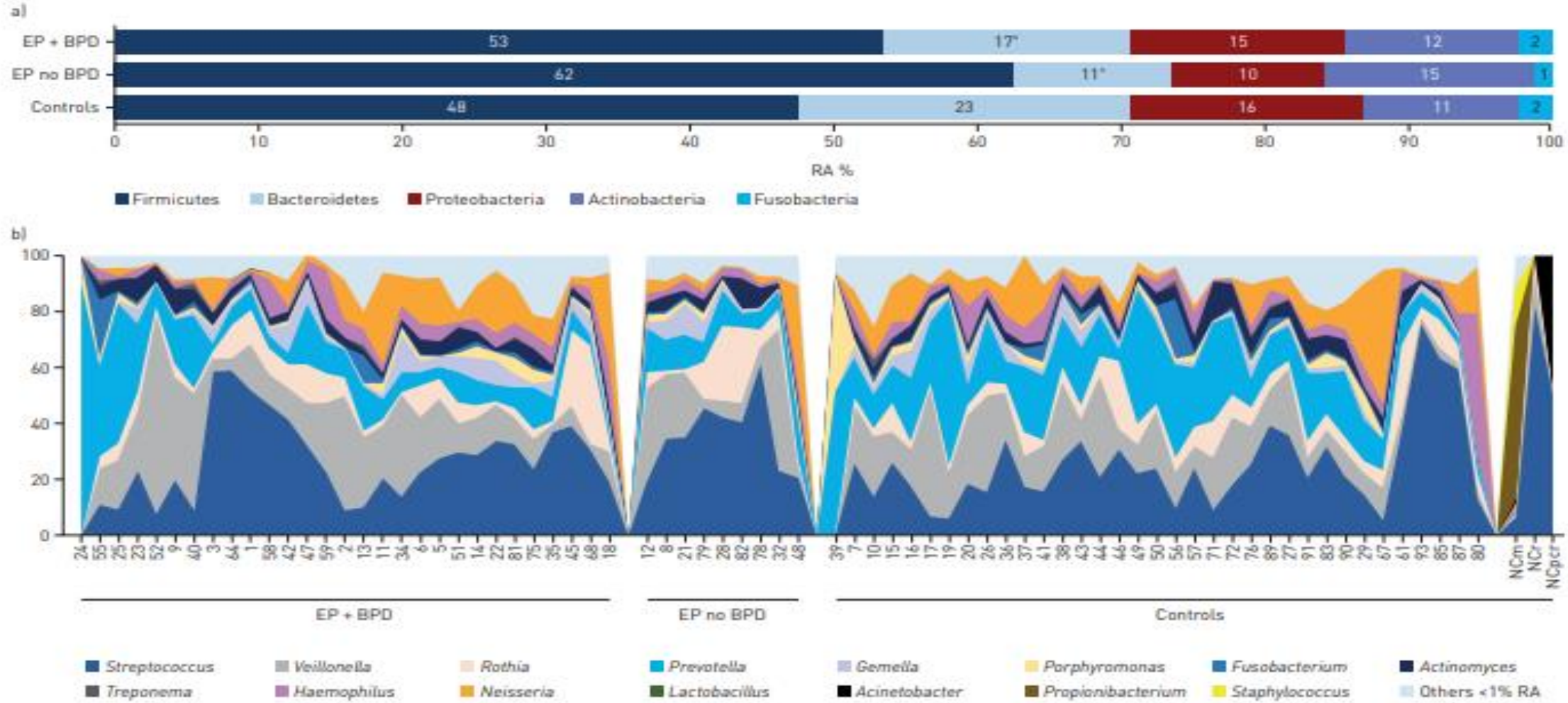
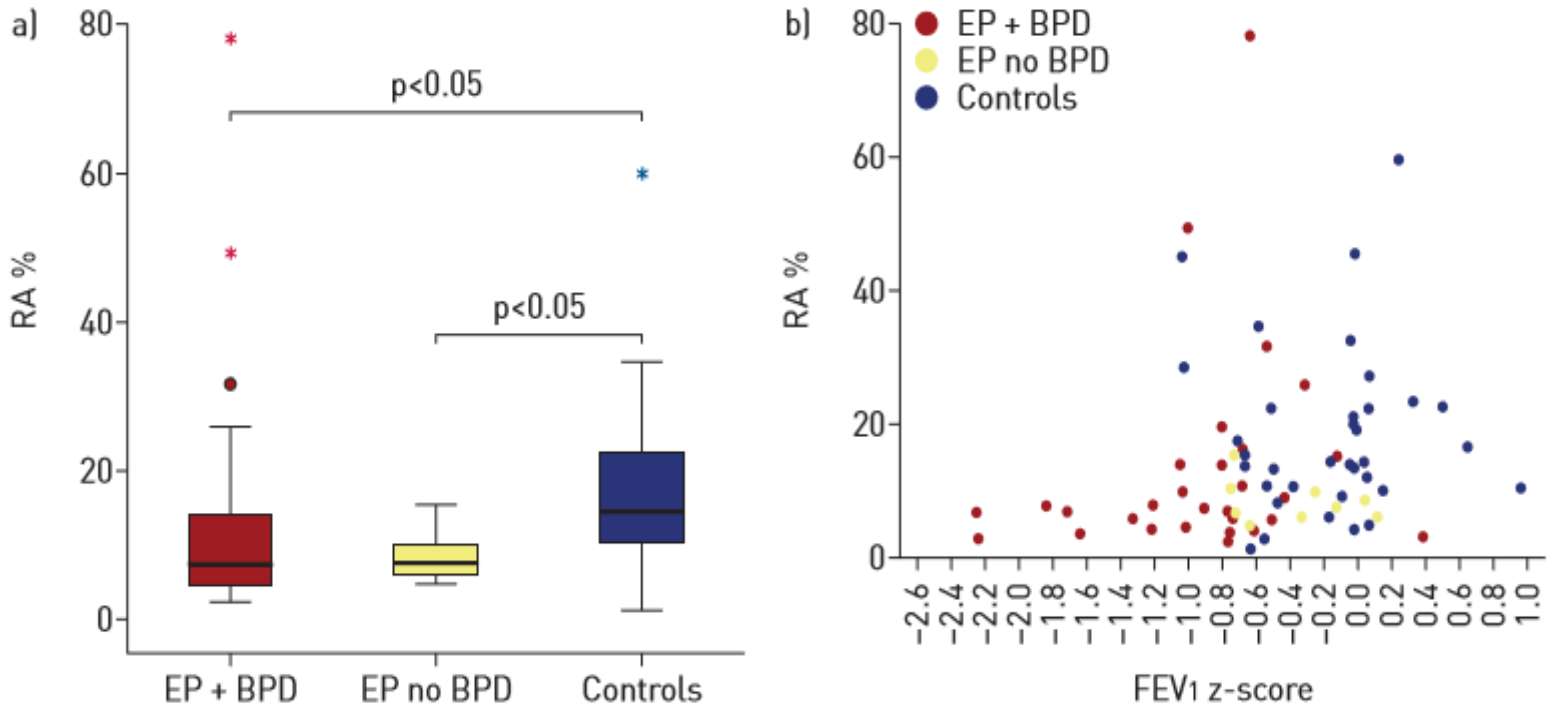


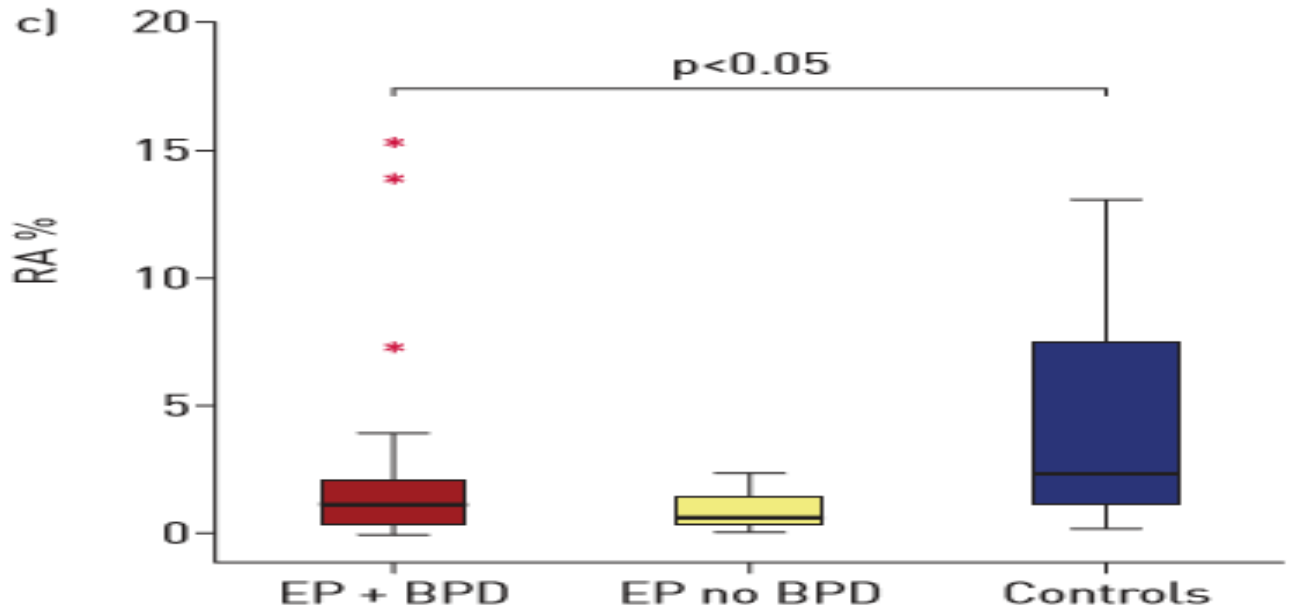

FIGURE 2 Comparison of the airway microbiome profile in the EPICure groups at a) phylum level and b) genus level. a) At phylum level, the Bacteroidetes relative abundance (RA) was significantly lower in both extremely preterm-born (EP) groups compared to controls ($p<0.05$ by Kruskal–Wallis test); b) at the genus level, the *Prevotella* relative abundance was significantly lower in EP groups compared to controls ($p<0.05$ by Kruskal–Wallis test). NCm: extraction negative control of the saline matrix used for sputum induction; NCr: extraction negative control of the diluted Sputasol and reagents; NCpcr: PCR negative control. Sample size: EP + BPD n=29, EP no BPD n=9 and controls n=36. *: $p<0.05$ by Mann–Whitney test.

# Microbial community composition

- Prevotella was completely absent in two negative controls and present at 0.4% RA in the extraction negative control of the sputum induction matrix, This gives confidence that the impact of environmental contamination was minimal.




- **Prevotella did correlate significantly with the FEV1 z-score** (p=0.02), but not with a self-reported diagnosis of asthma or ther clinical parameters such as smoking status, exposure to passive smoking, self-reported diagnosis of asthma, FeNO, blood eosinophil count or use of inhalers.




- Operational taxonomic unit (OTU) 4458304 contributed most to the observed difference in genus Prevotella RA across the study groups ($p<0.05$ by Kruskal–Wallis test).

- Phylogenetic tree of OTU 4458304 was 100% identical to **Prevotella melaninogenica** strains as obtained by basic local alignment search tool (BLAST) analysis .

# Load and prevalence of airway bacteria using multiplex qPCR

- The loads and prevalence of the three bacteria S. pneumoniae, H. influenzae and M. catarrhalis **were similar within the three study groups.**

- None of the differences in prevalence and load of the three organisms across the study groups were statistically significant.

- M. catarrhalis was the least prevalent and the least populous organism.

# Discussion

- First study to investigate the airway microbiome in adult survivors of preterm birth in comparison to matched full term born controls.

- The airway microbiome profile in the three study groups was consistent with previous studies*. It was dominated by Firmicutes, Bacteriodetes, Proteobacteria and Actinobacteria.

- The extremely preterm group had significantly less diverse and less rich microbial communities in comparison with the control group.

Charlson ES, Bittinger K, Haas AR, et al. Topographical continuity of bacterial populations in the healthy human respiratory tract. Am J Respir Crit Care Med 2011; 184: 957–963.

# Discussion

- Research did not find significant differences between the airway microbiome profiles of the extremely preterm groups with and without neonatal BPD.

- A trend was observed in which the α-diversity and richness of the microbial communities in the BPD group was slightly, but not significantly lower than the group without BPD, and significantly lower than the control group.

- This trend may be important, but our study did not have sufficient statistical power to be able to confirm a difference in all microbial diversity indices.

# Discussion

- This Research has demonstrated a significant shift away from Bacteriodetes, driven particularly by reduction in the relative abundance of genus Prevotella in both extremely preterm groups, with and without BPD, relative to the control group.
- Prevotella relative abundance correlated significantly with FEV1 z-score, but had no association with other clinical parameters such as smoking status, exposure to passive smoking, self-reported diagnosis of asthma, FeNO, blood eosinophil count or use of inhalers.
- This research has found that P. melaninogenica was the species that contributed most to the observed reduction in total Prevotella abundance in the preterm groups in comparison to the control group.

# Discussion

- Observational studies** that have investigated the lung microbiome have commonly detected Prevotella in lung tissues and bronchoalveolar lavage of **healthy subjects**.
  It is suggested that Bacteriodetes relative abundance is linked with healthy lung microbiome.

- A shift in community composition away from Bacteriodetes towards Proteobacteria has been observed in people with COPD and those with asthma.

Pragman AA, Lyu T, Baller JA, et al. The lung tissue microbiota of mild and moderate chronic obstructive pulmonary disease. Microbiome 2018; 6: 7.
Erb-Downward JR, Thompson DL, Han MK, et al. Analysis of the lung microbiome in the “healthy” smoker and in COPD. PLoS One 2011; 6: e16384.

# Discussion

- A similar trend in which there is a shift from Bacteriodetes to Firmicutes, mainly streptococci, has been reported by ZHANG et al. [2016] in severe asthma.

- HILTY et al. [2010] reported a shift in the community membership away from Bacteriodetes (mainly Prevotella), towards Proteobacteria (mainly Haemophilus), as well as Firmicutes in children with asthma.

# Discussion

- Prevotella species are obligate anaerobes that have been regarded as opportunistic members of the oral microbiota as well as other body sites and have been isolated with other bacteria in mixed anaerobic infections and lower respiratory tract infections.

Kedzia A et al. Incidence of anaerobic bacteria in respiratory tract infections]. Pneumonol Alergol Pol 2003; 71: 68–73.

# Discussion

- It is not clear what role Prevotella plays in lower respiratory microbial homeostasis.

- Studies that compared the lung microbiome in health and disease suggest that Prevotella is associated with health, and is quickly replaced by members of Proteobacteria or Firmicutes in various chronic lung conditions.

Dickson RP, Erb-Downward JR, Martinez FJ, et al. The microbiome and the respiratory tract. Annu Rev Physiol 2016; 78: 481–504
Einarsson GG, Comer DM, McIlreavey L, et al. Community dynamics and the lower airway microbiota in stable chronic obstructive pulmonary disease, smokers and healthy non-smokers. Thorax 2016; 71: 795–803.

# Discussion

- The role of Prevotella species in pathogenesis has received little attention, possibly because Prevotella is difficult to culture and is not usually isolated from specimens in routine microbiology laboratories.

- Further research is required to understand the interactions of Prevotella species with the host immune system and with other microbes within the lung microbial communities, and to characterize the strains that may be beneficial to respiratory health.

# Discussion

Conventionally, S. pneumoniae, H. influenzae and M. catarrhalis have been classified as typical airway pathogens. These organisms normally reside harmlessly within the human nasopharynx .

Studies have demonstrated the potential consequences of these organisms in COPD and asthma.

Research did not find significant differences in the bacterial load nor the prevalence of these pathogens between the three study groups.

Garcha et al. Changes in prevalence and load of airway bacteria using quantitative PCR in stable and exacerbated COPD. Thorax 2012; 67: 1075–1080

# Conclusions

- Extremely preterm birth is associated with a significant dysbiosis in airway microbiome at 19 years of age regardless of neonatal BPD status.

- This is characterized by a shift in the community composition away from Bacteriodetes as manifested in a significant drop in Prevotella relative abundance.

# Limitations of study

1. The main limitations of this study relate to the **small sample** size and an **inability to induce sputum in all subjects**, which may not be random, as it is more challenging to induce sputum in healthy subjects compared to those with respiratory pathology.

2. Inclusion criteria – BPD not related to severity. Preterm according to GA only not taking into consideration other factors such as weight, infection, exposure to antibiotics, other comorbidities such as PDA etc.

# Limitations of study

3. Lower hypertonic saline strength was used for sputum induction in those who were labelled as asthmatics which might have accounted for some of the variations between the groups.

4. Dx of Asthma - asthma prevalence was self-reported; no objective testing to confirm or refute this; and many subjects were prescribed asthma inhalers.

5. EPICure and other preterm cohort studies have previously reported that BPD survivors often have airflow obstruction later in life which can be mislabelled as asthma.

# Limitations of study

6. Other spirometry variables were not measured or compared (example: FEV1\FVC ratio) could assist to better clinical correlate microbiome to measured variables.

# Plans for the future

- Prospective cohort studies for the extreme Preterm population to be conducted following microbial characteristics and potential PFTs and different intervals while implementing them on clinical symptoms.

# Thank you very much