Airway microbiome composition correlates with lung function and arterial stiffness in an age-dependent manner

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Abstract

Objective
To investigate age-associated changes in airway microbiome composition and their relationships with lung function and arterial stiffness among genetically matched young and elderly pairs.

Methods
Twenty-four genetically linked family pairs comprised of younger (≤40 years) and older (>60 years) healthy participants were recruited (Total n = 48). Lung function and arterial stiffness (carotid-femoral pulse wave velocity (PWV) and augmentation index (AIx)) were assessed. Sputum samples were collected for targeted 16S rRNA gene amplicon sequencing and correlations between microbiome composition, lung function and arterial stiffness were investigated.

Results
Elderly participants exhibited reductions in lung function (FEV1 (p<0.001), FVC (p<0.001) and percentage FEV1/FVC (p = 0.003)) and a 1.3–3.9-fold increase in arterial stiffness (p<0.001) relative to genetically related younger adults. Elderly adults had a higher relative abundance of Firmicutes (p = 0.035) and lower relative abundance of Proteobacteria (p = 0.014), including specific genera Haemophilus (p = 0.024) and Lautropia (p = 0.020) which were enriched in the younger adults. Alpha diversity was comparable between young and elderly pairs (p>0.05) but was inversely associated with lung function (FEV1%Predicted and FVC %Predicted) in the young (p = 0.006 and p = 0.003) though not the elderly (p = 0.481 and p = 0.696). Conversely, alpha diversity was negatively associated with PWV in the elderly (p = 0.01) but not the young (p = 0.569). Specifically, phylum Firmicutes including the genus Gemella were correlated with lung function (FVC %Predicted) in the young group.
($p = 0.047$ and $p = 0.040$), while Fusobacteria and *Leptotrichia* were associated with arterial stiffness (PWV) in the elderly (both $p = 0.004$).

**Conclusion**

Ageing is associated with increased Firmicutes and decreased Proteobacteria representation in the airway microbiome among a healthy Asian cohort. The diversity and composition of the airway microbiome is independently associated with lung function and arterial stiffness in the young and elderly groups respectively. This suggests differential microbial associations with these phenotypes at specific stages of life with potential prognostic implications.

**Introduction**

Ageing is an identified risk factor for several chronic health conditions, including lung and cardiovascular disease (CVD), that exhibit disproportionate susceptibility, morbidity and mortality in older individuals [1, 2]. During ageing, subclinical lung function decline [3, 4], vascular endothelial dysfunction and arterial stiffening occur in the healthy population [5–7]. In older individuals above age 40 [8], parameters of lung function are inversely associated with arterial stiffness parameters, even after adjustment for cardiovascular risk factors, smoking history and lung disease [9, 10]. Reduced pulmonary function is independently associated with precursors for CVD including arterial stiffness, regardless of age, sex or anthropometry [8–12] and is an independent risk factor for cardiovascular morbidity and mortality [13]. Pulmonary function also predicts the development of atherosclerotic plaques and is a strong risk factor for arterial stiffness, suggesting a causal relationship between the decline in lung function and arterial stiffness [11, 12, 14].

While evidence supports the relationship between declining lung function, arterial stiffness and subsequent onset of CVD [13], the mechanisms underlying these associations are not known. Possible mechanisms include inflammation, as arterial stiffness is markedly increased in patients with chronic obstructive pulmonary disease (COPD), a condition characterized by airflow obstruction and inflammation [15–17]. While systemic inflammation, weight, smoking history, hypercholesterolemia, hypertension and diabetes have all been implicated, they do not fully account for the relationship between pulmonary function and arterial stiffness, suggesting that other factors could be involved [9, 13, 18].

The lung microbiome encompasses the collective genetic information of all microorganisms that colonize the lung and differs between healthy and diseased individuals [19]. Lower microbiome diversity and the presence of specific microbial taxa are associated with decreased lung function in pathogenic disease states [19, 20]. In healthy individuals, the lung microbiome comprises a community of low density and transiently present microorganisms, whereas pathogenic organisms predominate in disease [21–23]. While the effects of ageing on the lung microbiome remain to be established, the gut microbiome has been found to influence healthy ageing through alteration of inflammatory markers, diet and immune response [24–26]. Furthermore, age-associated alterations in immune function may affect the microbiome (or vice versa), generating low-grade inflammation, a key contributor to arterial stiffness and declining lung function [27]. An emerging question is whether the observed immunopathological associations between gut microbiota and disease also hold true at other anatomical sites such as the
lung, where the microbiome may underpin age-associated changes in lung function with a consequent impact on arterial stiffness.

Here we investigated the effect of age on the airway microbiome in healthy individuals of Asian origin. Age-related changes in airway microbiota, between young and elderly family pairs, were assessed for their association with normative age-associated changes in lung function and arterial stiffness, as potential prognostic indicators of cardiorespiratory health.

### Methods

#### Subjects

Twenty-four genetically linked family pairs (parent and child) were prospectively recruited. Younger participants were 22–39 years old and older participants were 60–71 years old. All participants were non-smokers, not on long-term inhaled medication, had no history of chronic respiratory diseases and resided in Singapore over the 10–12-month period preceding recruitment. None of the female participants were on oral contraceptives or hormone replacement therapy. The participants were recruited through posters, social media and community outreach events. All the participants were briefed on the nature and risks involved in the study and their rights to withdraw their participation without obligation before giving their written informed consent to participate in the study. The procedures in the study were approved by the institutional review board of Nanyang Technological University, Singapore (IRB-2017-12-010).

#### Study design and procedures

Participants abstained from caffeine and dietary supplementation for 24 h and kept to their regular diet and sleep routines, as well as refrained from strenuous physical activity for 48 h prior to their visit to the laboratory. Participants arrived at the laboratory between 0900 h and 1000 h after having consumed a light meal >2 h before the trial. They had their blood pressure (BP) measured and declared that they were well for participation. Nude body weight was measured using an electronic scale (SECA, Hamburg, Germany) and height was measured using a stadiometer (SECA). Body mass index (BMI) was calculated as body weight (kg) divided by height (m) squared. Waist circumference (WC) was recorded using a tape measure (SECA) placed snugly at the waistline, midway between the lowest ribs and iliac crest in a standing position [28], recorded to the nearest 0.1 cm in triplicate and averaged from the measurements.

#### Sputum collection and processing

Participants rinsed their mouth with water, before careful instructions were given on how to produce sputum from a deep cough, using the huff cough method [29]. Participants took three deep maximal breaths, before coughing as hard and as deeply as possible in a standing position. Sputum was collected by spontaneous expectorate from a deep cough (0.5–2 g) into sterile containers and were visually inspected before placing on ice immediately. Sputasol (ThermoFisher Scientific, Massachusetts, United States) was added into the sputum container in equal amounts. The sputum sample was then shaken at 200 rpm at 37 °C for 15 min. RNA-Later solution (ThermoFisher Scientific, Massachusetts, United States) was added and the sample stored in 1 mL aliquots at −80 °C. Saliva samples were assessed in 6 participants (3 matched pairs) to allow comparative assessments between oral and lung sampling. Prior to sputum collection, participants rinsed their mouth with water and saliva samples (5 mL) were collected into a 50 mL sterile tube by allowing saliva to accumulate before expectoration and
the samples were placed on ice immediately [30]. Saliva samples were aliquoted (500 μL) and RNALater solution (1 mL) added and samples stored at –80 °C.

**Spirometry**

Lung function measurement was conducted using a portable spirometer (Spirolab, MIR Medical International Research SRL, Italy), according to international guidelines [31]. Lung function parameters were determined as the forced expiratory volume in 1 s (FEV1), forced vital capacity (FVC) and percentage of FEV1/FVC. The FEV1 and FVC percentage predicted were calculated in the Spirolab software by the ATS/ERS standards, depending on ethnicity group [32].

**Arterial stiffness measurement**

An indirect and non-invasive measure of arterial stiffness was determined by the SphygmoCor XCEL device (AtCor Medical Pvt, Ltd, Sydney, NSW, Australia). The arterial stiffness parameters included carotid-femoral arterial pulse wave velocity (PWV) and augmentation index (AIx). The participants rested quietly in a supine position at room temperature for 15 min before measurements of arterial pulse pressure waveforms, by an inflated cuff at the brachial artery. SphygmoCor system calculates the central aortic augmentation pressure (AP) by subtracting the pressure at the first systole resulting from the return of the reflected wave from the systolic pressure. The AIx was calculated as the ratio of AP to pulse pressure [33]. PWV was measured simultaneously with pressure transducers, by acquiring a carotid pulse by applanation tonometry and a femoral pulse by volumetric displacement, within a cuff around the upper thigh (femoral artery) [34, 35]. The pulse waves were captured electronically on a computer using the SphygmoCor system and accepted by the system after consistent high-quality waveforms were measured. The average of approximately 3–5 measurements were taken.

**DNA extraction and 16s rRNA gene sequencing**

Sputum and saliva samples were thawed on ice and homogenised using glass beads (1mm, Sigma-Aldrich) using a bead mill homogeniser (VWR). DNA was purified using the Roche High-pure PCR Template Preparation Kit (Roche) as previously described [36]. Blank extractions from sterile PBS were also performed and served as negative extraction controls. All extracted samples were quantitated using the Qubit dsDNA High Sensitivity (HS) Assay Kit (Invitrogen) and visually assessed for integrity by electrophoresis on a 0.8% agarose gel.

**Sequence data processing and taxonomic assignment**

Using extracted sputum DNA samples, libraries for targeted amplicon sequencing were prepared following the “16S Metagenomic Sequencing Library Preparation” guide (Part# 15044223 rev. B, Illumina) [37]. This 300-bp paired-end sequencing protocol was performed on a MiSeq sequencing platform (Illumina) at the Lee Kong Chian School of Medicine (LKCMedicine), Singapore. Targeted amplicon sequences were analysed using the 16S metagenomics tool (version 1.0.1; Illumina), using as a taxonomic database the Illumina-curated version of the May 2013 Greengenes Consortium Database (greengenes.secondgenome.com) release and the Ribosomal Database Project (RDP) Classifier as the classification algorithm. Control samples from negative PCR and blank DNA extractions were also sequencing and assessed to detect potential contaminants using the decontam statistical package [38].
Statistical analysis

All statistical analyses were performed using Statistical Package for Social Sciences, version 23 (SPSS, Inc., Chicago, IL) and R version 3.3.3 (R Foundation for statistical computing, Vienna, Austria). Numerical variables are presented as mean (standard deviation, SD) in text and figures unless otherwise stated. The participant characteristics were analysed using paired t-test to assess potential differences between younger and older groups. Between group differences for relative abundance of both saliva and sputum microbiome phyla and genera, as well as alpha diversity (Shannon, Simpson and inverse Simpson index) were also assessed using paired t-test. Due to the skewed distribution of data, a square root transformation was performed for relative abundances of Firmicutes, Proteobacteria, Haemophilus and Lautropia, and a logarithmic transformation was performed for PWV to achieve a normal distribution for paired t-test. Pearson’s and Spearman’s correlation was used to evaluate associations between two variables that were either normally distributed or not normally distributed respectively, including any sub-group analyses. Principal coordinate analysis (PCoA) was used to assess the beta diversity and overall lung microbiome composition with age. PCoA plots for were generated using the first two principal coordinates according to age categories (young vs. elderly), as well as according to sample type (saliva vs. sputum). The ‘vegan’ R package (version 2.4–5) was used to calculate alpha diversity and implement ‘adonis’, which uses permutational multivariate analysis of variance, to test for statistical significance of association of overall beta diversity of lung microbiome composition with age. Bray Curtis distances between paired and unpaired young-elderly comparisons were assessed using the ”dist_groups” function from the R package ’udist’. All the results in the tables and figures are presented using the non-transformed data. A value of p < 0.05 was considered statistically significant.

Results

Age-associated changes in physiology of study cohort

Anthropometry and blood pressure. The demographics of healthy participants in our study are presented in Table 1. Despite no difference in weight (p = 0.734), older adults were shorter in stature (p < 0.001) and had higher BMI (p = 0.03) than the younger adults. Waist circumference was also greater in older than younger adults (p = 0.03). Compared with younger adults, older adults also had higher systolic BP (p = 0.002), although diastolic BP was similar between groups (p = 0.213) (Table 1).

Lung function and arterial stiffness. Lung function parameters including FEV1 (p < 0.001), FVC (p < 0.001) and FEV1/FVC (p = 0.003) were all reduced in older adults compared with their younger family pairs (Table 1). Compared with younger adults, older adults had 41% or 1.4 L lower FEV1, 33% or 1.3 L lower FVC and 7% lower percentage of FEV1/FVC. However, adjusted FEV1 (%Predicted) and FVC (%Predicted) were comparable between younger and older adults (p = 0.063 and p = 0.361) suggesting lower lung function values were reflective of natural lung function decline in our healthy elderly cohort. The percentage of predicted value for FEV1/FVC percentage in younger and older family pairs was comparable (p = 0.157), and the values were all within healthy ranges [39]. Compared with younger adults, older adults had increased arterial stiffness, indicated by PWV and AIx (all p < 0.001). In both younger and older adults, arterial stiffness indicated by PWV and AIx were also within healthy ranges [35], and were 1.3-fold and 3.9-fold higher in older than in younger adults respectively (Table 1). Lung function was inversely associated with arterial stiffness. However, when age was adjusted for, in a partial correlation model, the associations between FEV1, FVC and
Age-associated changes in lung microbiome composition

Composition of lung microbiome from sputum samples in healthy individuals. The main phyla and genera identified in the airway microbiome of younger and older adults are shown in Fig 1. Sputum samples from healthy individuals, both young and elderly, consisted mainly of microbes from phylum Firmicutes (46%), Proteobacteria (16%), Bacteroidetes (16%), Actinobacteria (14%) and Fusobacteria (6%). The main genera detected were *Streptococcus* (24%), *Prevotella* (12%), *Neisseria* (10%), *Rothia* (8%), *Veillonella* (7%), *Granulicatella* (4%), *Fusobacterium* (4%), *Actinomyces* (3%), *Gemella* (2%), *Haemophilus* (2%), *Porphyromonas* (2%), *Leptotrichia* (2%), *Campylobacter* (1%) and *Lautropia* (1%) (Fig 1A). Comparison of saliva samples (total n = 6) suggested a high degree of overlap between the microbiota of healthy sputum and saliva with no differences in alpha- (Shannon diversity index (SDI) p = 0.304) or beta-diversity (p = 0.582) (S1 Fig). However, sputum samples, which are commonly used to represent the lung microbiome, exhibited subtle differences in specific taxa including higher relative abundance of *Granulicatella* (4.3% vs. 3.0%, p = 0.031), lower relative abundance of *Leptotrichia* (0.8% vs. 1.4%, p = 0.013) and lower relative abundance of *Corynebacterium* (0.7% vs. 0.1%, p = 0.031) (S1 Fig).

Comparison of lung microbiota present in genetically related young and elderly family pairs. In order to allow some degree of control for genetic confounders, we assessed the airway microbiota in related young-elderly family pairs. PCoA analysis revealed that the sputum microbiome of younger and older adults was more similar when analysed as matched family pairs. Here, the measured Bray Curtis distance of matched young-elderly pairs was lower (equating to more similar microbiome profiles) when compared to the average distance.
between all other possible un-matched young-elderly comparisons ($p = 0.036$) (Fig 1C and 1D). Paired analysis showed that older adults generally had higher relative abundance of Firmicutes (47.4% vs 43.9%, $p = 0.035$) and lower Proteobacteria (13.9% vs 19.0%, $p = 0.014$) with lower average relative abundance of Proteobacteria including *Haemophilus* (2.0% vs 2.8%, $p = 0.024$) and *Lautropia* (0.5% vs 1.1%, $p = 0.020$) (Fig 2). No significant differences in alpha diversity measures were observed between young and elderly groups (S2 Fig). Beta-diversity analysis also showed no difference between age groups in overall lung microbiome composition ($p = 0.420$, Fig 1B).

**Association of lung microbiome with lung function and arterial stiffness**

**Association of lung microbiome alpha diversity with lung function and arterial stiffness.** Alpha diversity of the lung microbiome was associated with lung function in an age-dependent manner. While SDI was associated with both FEV1 (%Predicted) and FVC (%Predicted) in the cohort as a whole (all $r = -0.314$, $p = 0.032$), the association was driven by the younger group (Fig 3A and 3B). In the younger group, FEV1 (%Predicted) and FVC (%Predicted) were both associated with SDI ($r = -0.546$, $p = 0.006$ and $r = -0.573$, $p = 0.003$).
However, in the older group, the association between FEV1 (%Predicted) and FVC (%Predicted) with SDI were not significant ($r = -0.155, p = 0.481$ and $r = -0.086, p = 0.696$) (Fig 3A and 3B). Conversely, alpha diversity of the lung microbiome was correlated with arterial stiffness (PWV) in the older but not the younger.
group ($r = -0.516$, $p = 0.01$ vs $r = 0.132$, $p = 0.569$) (Fig 3C). Correlations with inverse Simpson index showed similar associations as SDI (Table 2).

**Age-dependent association of lung microbiome composition and lung function.** We found that within the younger cohort, the relative abundance of microbes from the Firmicutes phylum ($r = 0.409$, $p = 0.047$) and specifically the Gemella genus ($r = -0.422$, $p = 0.04$) was associated with FVC (%Predicted) (Fig 4A and 4B). However, similar to correlations with SDI, these associations between FVC (%Predicted) were seen in the younger but not the elderly cohort ($r = -0.200$, $p = 0.360$ and $r = 0.422$, $p = 0.710$) (Fig 4A and 4B). In younger subjects, the relative abundance of Actinomyces genus was also inversely associated with absolute FEV1 but this association was not observed among older individuals ($r = -0.408$, $p = 0.048$ vs. $r = 0.230$, $p = 0.290$) (Table 2). Within the older group, the only association with lung function parameters was the relative abundance of phylum Actinobacteria, which was positively associated with absolute FVC ($r = 0.447$, $p = 0.033$), an association that was not observed in the younger group ($r = -0.070$, $p = 0.747$) (Fig 4C).

**Age-dependent association of lung microbiome composition and arterial stiffness.** In contrast to the associations between lung microbiome and lung function, which were more prevalent in the younger group, the associations between lung microbiome and arterial stiffness were more pronounced in the older group. Within the older group, the arterial stiffness parameter PWV exhibited a strong inverse association with relative abundances of the Fusobacteria ($r = -0.570$, $p = 0.004$), specifically the genus *Leptotrichia* ($r = -0.569$, $p = 0.004$) (Fig 4D and 4E). These associations of PWV with both Fusobacteria and *Leptotrichia* relative
Table 2. Correlation matrix comparing lung microbiome composition with lung function and arterial stiffness parameters among young and elderly subjects.

|          | FEV1   | FVC    | FEV1 (%pred) | FVC (%pred) | AIX    | PWV    |
|----------|--------|--------|-------------|-------------|--------|--------|
| **Young** |        |        |             |             |        |        |
| SDI      | −0.362 | −0.411 | −0.546      | −0.573      | −0.068 | 0.132  |
|          | (0.082)| (0.031)| (0.006)**  | (0.003)**   | (0.769)| (0.569)|
| ISI      | −0.396 | −0.460 | −0.440      | −0.482      | 0.009  | 0.169  |
|          | (0.055)| (0.024)| (0.032)     | (0.017)*    | (0.967)| (0.465)|
| Firmicutes | 0.318  | 0.390  | 0.363       | 0.409       | 0.159  | 0.160  |
|          | (0.130)| (0.060)| (0.081)     | (0.047)*    | (0.492)| (0.489)|
| Gemella  | 0.082  | 0.017  | −0.348      | −0.422      | −0.072 | −0.080 |
|          | (0.704)| (0.937)| (0.095)     | (0.040)*    | (0.756)| (0.732)|
| Actinobacteria | −0.055 | −0.070 | 0.064       | 0.023       | 0.019  | −0.121 |
|          | (0.799)| (0.747)| (0.767)     | (0.916)     | (0.935)| (0.603)|
| Actinomyces | −0.408 | −0.391 | −0.198      | −0.142      | −0.119 | 0.205  |
|          | (0.048)| (0.057)| (0.354)     | (0.508)     | (0.608)| (0.372)|
| Fusobacteria | −0.151 | −0.168 | −0.395      | −0.364      | 0.014  | 0.146  |
|          | (0.480)| (0.432)| (0.056)     | (0.080)     | (0.953)| (0.528)|
| Leptotrichia | −0.132 | −0.112 | −0.353      | −0.216      | 0.012  | 0.170  |
|          | (0.538)| (0.603)| (0.090)     | (0.310)     | (0.960)| (0.461)|
| Lautropia | 0.043  | −0.124 | 0.015       | −0.209      | −0.492 | −0.023 |
|          | (0.843)| (0.563)| (0.944)     | (0.327)     | (0.003)| (0.058)|
| **Elderly** |        |        |             |             |        |        |
| SDI      | −0.151 | −0.157 | −0.155      | −0.086      | 0.035  | −0.516 |
|          | (0.493)| (0.475)| (0.481)     | (0.696)     | (0.870)| (0.001)|
| ISI      | −0.216 | −0.206 | −0.112      | −0.017      | 0.055  | −0.588 |
|          | (0.323)| (0.346)| (0.611)     | (0.939)     | (0.789)| (0.004)|
| Firmicutes | 0.190  | 0.099  | −0.031      | −0.200      | 0.193  | 0.118  |
|          | (0.386)| (0.653)| (0.888)     | (0.360)     | (0.366)| (0.582)|
| Gemella  | −0.259 | −0.215 | −0.032      | 0.082       | 0.124  | 0.280  |
|          | (0.232)| (0.324)| (0.887)     | (0.710)     | (0.564)| (0.186)|
| Actinobacteria | 0.347  | 0.447  | 0.382       | 0.322       | 0.137  | 0.097  |
|          | (0.105)| (0.033)| (0.072)     | (0.134)     | (0.522)| (0.651)|
| Actinomyces | 0.230  | 0.238  | 0.124       | 0.073       | 0.023  | −0.350 |
|          | (0.290)| (0.274)| (0.572)     | (0.742)     | (0.915)| (0.094)|
| Fusobacteria | −0.361 | −0.344 | −0.286      | −0.114      | 0.083  | −0.570 |
|          | (0.091)| (0.108)| (0.187)     | (0.605)     | (0.701)| (0.004)|
| Leptotrichia | −0.284 | −0.221 | −0.318      | −0.157      | 0.207  | −0.569 |
|          | (0.189)| (0.310)| (0.139)     | (0.473)     | (0.332)| (0.004)|
| Lautropia | −0.088 | −0.112 | 0.254       | 0.080       | 0.034  | −0.093 |
|          | (0.690)| (0.612)| (0.242)     | (0.716)     | (0.873)| (0.665)|

Microbiome indices of alpha diversity including the Shannon diversity index (SDI) and inverse Simpson index (ISI) as well as specific phyla and genera showing significant correlation with lung function (FEV1, FVC, FEV1%predicted, FVC %predicted) and arterial stiffness (augmentation index (AIX) and pulse wave velocity (PWV)) are displayed for younger and elderly sub-groups. p values are shown in brackets. Significantly correlation scores are indicated by bold typeface.

*p < 0.05.

**p < 0.01.

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abundances were not significant in the younger group (r = 0.146, p = 0.528 and r = 0.170, p = 0.461) (Fig 4D and 4E). The only genus exhibiting correlation with arterial stiffness in the young was Lautropia, which was associated with AIX an observation that was not evident in the older cohort (r = −0.492, p = 0.023 vs r = 0.034, p = 0.873) (Fig 4F). These age-specific associations between airway microbiome composition, lung function and arterial stiffness are summarised in Fig 5.
While a growing number of studies detail the ageing gut microbiome and its consequence for health and disease, comparable analyses of the respiratory microbiome are lacking [19, 40, 41]. Although several studies have investigated the airway microbiome in the context of respiratory disease, the present study is the first to explore age-associated differences in lung microbiome.

**Fig 4. Physiological correlates of the microbiome at phylum and genus level.** Sub-group correlations between specific lung microbiota, lung function and arterial stiffness parameters are indicated. Lung function—microbiome correlations were observed in the young but not the elderly group, between FVC (% Predicted) and relative abundance of (A) phylum Firmicutes and (B) genus Gemella. Correlations observed in the elderly but not the young group included (C) FVC and relative abundance of the genus Actinobacteria. Arterial stiffness and lung microbiome correlations were observed in the elderly group, between pulse wave velocity and relative abundance of (D) phylum Fusobacteria and (E) genus Leptotrichia while correlation between (F) augmentation index and the relative abundance of Lautropia was observed in young subjects. Young and elderly measures are indicated by unfilled and filled circles respectively. The line of best fit is indicated with a broken line for the young population and with a continuous line for the elderly. Correlation coefficients and associated p values are indicated for young sub-group ($r_{\text{young}}$) and elderly sub-groups ($r_{\text{elderly}}$).

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**Discussion**

While a growing number of studies detail the ageing gut microbiome and its consequence for health and disease, comparable analyses of the respiratory microbiome are lacking [19, 40, 41]. Although several studies have investigated the airway microbiome in the context of respiratory disease, the present study is the first to explore age-associated differences in lung microbiome.
composition, with specific focus on healthy individuals of Asian origin. Our findings demonstrate remarkable consistency between young and elderly healthy microbiome profiles while identifying subtle taxonomic differences related to lung function and arterial stiffness in an age-dependent manner. These include associations between the lung microbiome and lung function (FEV1%Predicted and FVC %Predicted) in the younger adults, and arterial stiffness (PWV) in the elderly, suggesting that these relationships have greater relevance at different stages of life.

Given the paucity of evidence, the role of the airway microbiome in ageing is still unknown though long-speculated to be of clinical significance [42, 43]. Here, we have explored the effects of ageing on microbial constituents of the healthy airway microbiome by employing a paired genetic matching strategy, which controls the confounding influence of host genetics. Among patients with chronic respiratory disease such as COPD, overrepresentation of the Proteobacteria and reduction of Firmicute abundance is observed [44–47]. Enrichment of specific Proteobacteria including Haemophilus in the sputum microbiome of patients with COPD [48], and Lautropia in cystic fibrosis patients represent examples of disease-associated dysbiosis [46, 49]. In our healthy participants, however, the overall representation of phylum Proteobacteria (16%) was much lower than in disease states (~44%) [44]. Likewise, the average Firmicutes representation in our study (46%) was higher compared to diseased states (~16%) [44]. It is plausible that ‘healthy’ ageing of the lungs promotes enrichment of the Firmicutes
and reduced relative abundance of Proteobacteria such as *Haemophilus* and *Lautropia*, which contrasted with the dysbiosis associated with chronic respiratory diseases.

We observed correlation between microbiome composition and lung function among younger subjects, noting an inverse relationship between lung microbiome diversity (SDI) and lung function (FEV1 % predicted and FVC % predicted). Microbial diversity thus negatively correlated with lung function in our study, which contrasted with respiratory disease states where positive correlation is generally observed [50, 51]. At the phylum level, overall Firmicutes abundance positively associated with lung function (FVC %Predicted) in the younger adults in this study. This suggests enrichment of the Firmicutes has a beneficial effect on lung function and might explain the negative correlation between lung function and SDI. The increased Firmicute abundance, or that of other beneficial microbiome, could drive a corresponding reduction in SDI. Observed association between *Gemella* (a Firmicute linked to pulmonary exacerbation in cystic fibrosis patients) is also associated with reduced lung function in our young cohort, further suggesting its potentially negative implications for respiratory health [20, 52]. The positive influence of Firmicute abundance on lung function might be contingent on the presence and abundance of specific genera within this phylum, such as *Gemella*. The Actinobacteria (in the elderly) the genus *Actinomyces* (in the young) were associated with increased absolute FVC and decreased FEV1 respectively (Fig 5). However, absolute values of FEV1 and FVC are less robust lung function parameters that do not take into consideration normative physiological lung function decline with age. The association between lung microbiome and lung function parameters (FEV1 and FVC %Predicted) may thus be more relevant and pronounced in the younger group (Fig 5).

In contrast to lung function, we found that associations between airway microbiome composition and arterial stiffness were largely confined to older adults. Here, lung microbiome diversity was inversely associated with arterial stiffness parameter PWV (a negative clinical indicator), in the older adults. Reduced microbial diversity in lower airways is linked to inflammatory phenotypes of the airways [53–55], which could increase both systemic inflammation and arterial stiffness that occur during ageing [23, 48]. Our findings highlight the relative abundance of Fusobacteria phylum and its genus *Leptotrichia* in inverse association with arterial stiffness in older adults. The relevance of the Fusobacteria in lung respiratory disease has been noted, where they are associated with beneficial effects [56–58]. While associations between particular organisms and lung function occur mainly in the younger group, distinct organisms in the lung are linked to arterial stiffness in the elderly. These results highlight the complexity of the microbial interactions that may underpin age-dependent relationships between lung function and arterial stiffness across different stages of life (Fig 5).

Our study has several limitations. Although associations can be drawn from the study results, the cross-sectional design does not conclusively prove causality. This study is also unable to directly assess temporal dynamics of the lung microbiome during ageing given the lack of a longitudinal component. While the younger and older participants in this study are family pairs, which minimize genetic influences, we did not control for other confounders such as environmental factors, lifestyle or living conditions, that could have influenced the lung microbiome and represent significant confounders. As targeted bacterial 16s rRNA gene amplicon sequencing was employed, the resolution does not support species-level characterization of the microbiome, which could be important in understanding the ecological and functional interaction with ageing. Though our study was explorative in nature, it provides a first insight into age-dependent variability in the airway microbiome and its potential implications for respiratory and cardiovascular health. We have identified several potential microbial taxa whose clinical relevance should be further explicated in larger studies incorporating longitudinal experimental design to fully delineate the core healthy airway microbiome and the
temporal dynamics of beneficial and deleterious taxa that may serve as early prognostic indicators of lung health status.

Supporting information

**S1 Fig. Analysis of microbiome profiles of saliva and sputum.** (A) Comparison of lung microbiome composition as detected by 16s rRNA gene profiling in DNA derived from saliva and sputum. Average relative abundance (RA) for the most representative taxa (present at >1%) is illustrated by circle size. Colour denotes phylum level membership. The saliva (V) and sputum (S) of three young (Y01-Y03) and three elderly matched family pairs (S01-S03) were analysed. (B) Principle co-ordinate analysis (PCoA) of Bray-Curtis distance between microbiome profiles observed in saliva samples (red open triangles) and sputum samples (black open circles) with indicated centroids (filled circles). Assessment of (C) Shannon diversity index and differences in relative abundance of (D) genus Granulicatella, (E) genus Leptotrichia and (F) genus Corynebacterium in saliva vs sputum samples among genetically paired young and elderly subjects. * = p < 0.05.

(TIF)

**S2 Fig. Alpha diversity indexes are shown between genetically paired young and elderly subjects.** No significant differences are observed in (A) Shannon diversity index (B) Simpson index and (C) Inverse Simpson index between young (white bars) and elderly (grey bars) paired groups. Black connecting lines indicate young-elderly pairs. ns = not significant.

(TIF)

**S1 Table. Associations between lung function and arterial stiffness parameters, with and without adjustment for age in a partial correlation model.** Correlation coefficients and corresponding p values are shown in brackets. *p < 0.05, **p < 0.01, ***p < 0.001. Significant correlation scores are indicated by bold typeface. AIx = Augmentation index, PWV = Pulse wave velocity.

(DOCX)

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