Sputum neutrophil counts in healthy subjects: relationship with age

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Shareable abstract (@ERSpublications)
There is an age-related increase in sputum neutrophil % in healthy individuals, with a plateau at age ∼60 years. Sputum neutrophil % increased during follow-up. Age-adjusted thresholds for sputum neutrophilia should be considered. https://bit.ly/3OcOrmO

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Abstract

A threshold of ∼60% has commonly been used in asthma and COPD studies to define the presence of neutrophilic airway inflammation. This threshold is based on relatively young healthy subject datasets. However, age-related increases in sputum neutrophils have been observed previously. We used a healthy cohort, with a comparatively wider age range, to re-evaluate the age-related increase in sputum neutrophils, analysing changes by decade. We also studied the long-term repeatability of sputum neutrophil counts.

Differential sputum cell count data for healthy subjects (n=121) was retrospectively analysed. Subjects with a repeated count (mean interval 4.8 years) were included in longitudinal analysis.

There was a significant positive association between age and sputum neutrophil % (rho=0.24, p<0.01), with 51.2% of subjects having a sputum neutrophil count >60%. Sputum neutrophil counts increased with each decade until ∼60 years where a plateau was observed. The baseline sputum neutrophil % increased significantly at repeated sampling (p=0.02), with excellent long-term repeatability (intraclass correlation coefficient=0.80).

We confirm previous reports of an age-related increase in sputum neutrophil % in healthy individuals and identified a plateau which occurs at age ∼60 years. There was an increase in sputum neutrophil % during longitudinal follow-up, indicating that age-related neutrophilia is a progressive phenomenon. These findings question the use of an unadjusted threshold, in relation to age, to identify the presence of neutrophilic airway inflammation.

Introduction

Neutrophils play a central role in the innate immune response to pathogens, participating in the phagocytosis and killing of pathogens, the secretion of pro-inflammatory mediators and clearance of cellular debris [1]. Increased neutrophil numbers in the lungs have been observed in various diseases including COPD and a subgroup of patients with asthma [2–5]. Furthermore, greater neutrophilic airway inflammation has been associated with more severe disease characteristics in both asthma and COPD [6, 7], implicating neutrophils as a therapeutic target in obstructive lung diseases [8, 9].

Induced sputum is a noninvasive technique for measuring airway inflammation [10]. Sputum cells counts have been used to identify individuals with neutrophilic airway inflammation. Thomas *et al.* [11] reported that sputum neutrophil counts increase with age in healthy individuals (n=66), ranging from a mean of 26.9% in those aged <29 years to 68.5% in those aged ⩾60 years. There are similar reports of increased sputum neutrophil counts with ageing in healthy cohorts with sample sizes from n=51 to n=243 [12–14]. Higher neutrophil counts in relation to age have also been reported in bronchoalveolar lavage [15].

A threshold of 60 or 61% has commonly been used in asthma and COPD studies to define the presence of neutrophilic airway inflammation [16–18]. This threshold is based on the upper limit of healthy control

datasets [11, 19], including that reported by Belda *et al.* [18] (n=96), for which the mean age of the cohort was 36 years but the upper age range was only 60 years, thus omitting older individuals. While previous healthy subject studies have reported mean sputum neutrophil counts ranging from 37.5 to 47.0%, it is notable that the upper limit of the 90% confidence interval ranged from 49 to 93% [20]. Differences between populations may be due to different environmental exposures (*e.g.*, pollution or passive smoke exposure) or differences in the age ranges studied. Nevertheless, a threshold value of ∼60% appears to be within the range observed in healthy subjects, particularly older individuals.

The short-term stability of sputum neutrophils is well described. Studies of healthy subjects from 48 h up to 2 weeks in length [21, 22] have reported intraclass correlation coefficient (ICC) values around 0.75. However, longer-term stability of sputum neutrophil counts in healthy subjects is under-reported and can help elucidate whether higher neutrophil counts in healthy subjects are a transient or stable phenomenon.

A sputum neutrophil cell count threshold of ∼60% is frequently used to define neutrophil airway inflammation, but this value appears to be based on datasets with younger individuals [16, 17, 23, 24]. We have re-examined the relationship between ageing and sputum neutrophil counts in a retrospective analysis of a cohort of healthy subjects who have participated in research studies at our centre. There were two aims: 1) by including a wider age range compared to many previous studies, we analysed the influence of ageing by decade; and 2) we studied the long-term repeatability of sputum neutrophil counts.

Methods

Study cohort

Data from healthy nonsmokers who had participated in research studies at the Medicines Evaluation Unit (Manchester University NHS Foundation Trust) were retrospectively analysed. Subjects were aged ⩾18 years old. All participants were not using maintenance antibiotics or oral corticosteroids and had no previous diagnosis of any lung disease. Participants had a ratio of forced expiratory volume in 1 s to forced vital capacity (FEV_1/FVC ratio) of >0.7 and were nonsmokers with a pack-year history of <1. All patients provided written informed consent using protocols approved by local ethics committees (05/Q1402/41, 10/H1016/25 and 16/NW/0836).

Study design

Data for healthy subjects were retrospectively collected from a research database. Sputum samples were obtained during stable state, with no respiratory illness within 4 weeks of sampling. Longitudinal data for samples >6 months apart were analysed.

Sputum induction and processing was performed using a two-step method with Dulbecco's phosphate-buffered saline, then a dithiothreitol step allowing for preparation of cytospins for differential cell counts as previously described [25]. Each sputum sample was objectively assessed for quality; if leukocyte viability was <50% and/or the squamous cell percentage was >30%, the sample was discarded on the basis of poor quality.

Statistical analysis

Comparisons between groups for non-parametric data were assessed using a Wilcoxon matched-pairs signed rank test or Kruskal–Wallis followed by *post hoc* analysis with Dunn's multiple comparisons test, which compared each age group to the 18–29 years group. Associations between non-parametric data were analysed using a Spearman's correlation. Multiple linear regression analyses assessed associations between sputum neutrophils and age while controlling for potentially confounding variables. $p<0.05$ was considered statistically significant. Analyses were performed using GraphPad Prism version 9.00 (GraphPad, San Diego, CA, USA). ICC estimates were calculated with log transformed data using SPSS (25.0, IBM, Armonk, NY, USA) and based on an absolute agreement, two-way mixed effects model [26]. ICC values were interpreted as excellent (>0.75), fair to good (0.40–0.75) or poor (<0.40) [26].

Results

Data from 121 subjects were available for analysis, with 29 subjects having a repeat measurement of sputum neutrophil counts. Subjects had a mean age 46.0 years, FEV_1 % predicted 104.8% and FEV_1/FVC ratio of 78.0% (table 1). The clinical and sputum characteristics for the subjects included in the longitudinal sub-analysis were similar to that of the entire cohort (supplementary table S1).

Baseline sputum cell counts

Median neutrophil cell count % at baseline was 62.0%, with the percentages of other cell types and absolute cell counts shown in table 1. There was a significant positive association between age and sputum

TABLE 1 Baseline demographics for the entire cohort (n=121) [#]

Characteristic	% or mean±SD
Sex, % male	70.3
Age years	46.0±15.7
Smoking status, current %	0.0
BMI kg·m^{-2}	26.7±3.7
FEV_1 L	3.5±1.0
FEV_1 % predicted	104.8±15.7
FEV_1/FVC %	78.0±6.4

Sputum characteristics	Median (range)	Mean±SD	Percentile		95% CI
			10th	90th	
Sputum total cell count ×10^6·g^{-1}	5.64 (0.99–37.00)	8.68±7.99	2.07	21.96	6.66–10.69
Sputum neutrophil %	62.00 (4.00–97.25)	55.20±24.47	16.30	82.50	50.79–59.60
Sputum eosinophil %	0.00 (0.00–3.50)	0.43±0.79	0.00	1.45	0.27–0.60
Sputum lymphocyte %	0.50 (0.00–3.75)	0.85±0.88	0.00	2.00	0.67–1.03
Sputum macrophage %	35.00 (0.25–91.50)	39.37±22.67	12.85	73.40	34.65–44.10
Sputum epithelial cells %	3.25 (0.00–3.75)	6.21±8.46	0.50	15.25	4.45–7.97
Sputum neutrophil cell count ×10^6·g^{-1}	3.11 (0.14–30.53)	5.79±15.40	0.72	15.40	4.16–7.43
Sputum eosinophil cell count ×10^6·g^{-1}	0.00 (0.00–0.75)	0.05±0.13	0.00	0.09	0.02–0.08
Sputum lymphocyte cell count ×10^6·g^{-1}	0.04 (0.00–0.75)	0.07±0.12	0.00	0.17	0.04–0.10
Sputum macrophage cell count ×10^6·g^{-1}	1.72 (0.19–8.25)	2.43±1.87	0.59	5.42	1.95–2.90
Sputum epithelial cell count ×10^6·g^{-1}	0.24 (0.00–1.40)	0.34±0.31	0.04	0.77	0.26–0.42

Data presented as mean±SD or median (range) unless specified otherwise. BMI: body mass index; FEV_1: forced expiratory volume in 1 s; FVC: forced vital capacity. [#]: 30 subjects did not have full differential cell counts data available aside from neutrophil %; 58 subjects did not have absolute sputum counts available.

neutrophil % (rho=0.24, p<0.01, figure 1a), remaining significant when adjusted for sex, FEV_1 and FEV_1/FVC ratio (supplementary table S2). No association was observed between age and absolute neutrophil counts (p=0.94), or other cell types (supplementary table S3).

When subjects were split into age groups by decade, the median sputum neutrophil % increased numerically up to 60 years of age, after which no further increase was observed. Median sputum neutrophil counts were 41.3, 53.1, 69.8, 72.0, 64.5 and 56.5% for subjects aged 18–29, 30–39, 40–49, 50–59, 60–69 and 70+ respectively. Subjects aged 40–49 and 50–59 had a significantly higher sputum neutrophil % compared to those aged 18–29 years (ANOVA p=0.04, *post hoc* p=0.01 and 0.03 respectively, figure 1b).

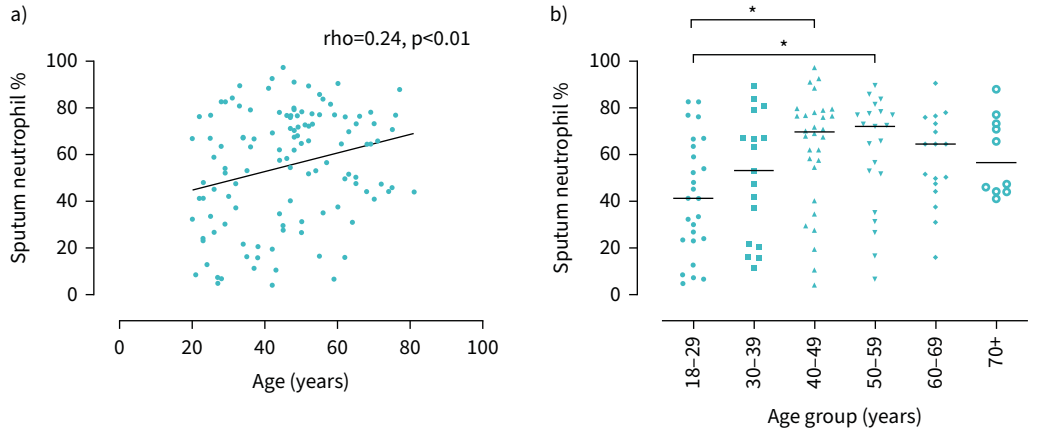

FIGURE 1 Association between a) sputum neutrophil % and age, and b) sputum neutrophil % for different age groups in healthy non-smoking subjects. a) n=121 and b) n=26, 17, 30, 21, 17 and 10 for age groups 18–29, 30–39, 40–49, 50–59, 60–69 and 70+ respectively. *: p<0.05.

Overall, 51.2% of subjects had a sputum neutrophil count >60%. The proportion of subjects with a sputum neutrophil % >60 in each age group was 26.9, 47.1, 66.7, 61.9, 52.9 and 50% for those aged 18–29, 30–39, 40–49, 50–59, 60–69 and 70+ years respectively.

Longitudinal measurements

29 subjects had a repeated sputum sample, with a mean interval of 4.8 years (range: 2.7–8.0). The baseline sputum neutrophil % was 53.3, increasing significantly to 65.8 at repeated sampling (p=0.02; figure 2a). There were increases in neutrophil % for 19 subjects. The ICC for sputum neutrophil % was 0.80. Bland–Altman analysis shows a positive bias of 10.2%, compatible with an increase in neutrophil % over time (figure 2b). Differences between measurements appeared to be related to the mean measurement, with smaller differences observed at lower mean measurements. The limits of agreement (−39.86–60.25) indicate that relatively large increases between visits may occur in some individuals.

Discussion

In a cohort of healthy subjects, we observed that sputum neutrophil counts were age dependent. This age-related increase in sputum neutrophil counts appeared to reach a plateau at age ∼50 to 60 years. There was a wide range of neutrophil counts, with younger subjects displaying neutrophil counts up to ∼80%, and a considerable number of subjects had neutrophil counts >60% (51.2%). These findings question the commonly used threshold of ∼60% to define neutrophilic airway inflammation in healthy subjects. The longitudinal follow-up showed an increase in sputum neutrophil % over time, compatible with ageing processes.

Previous studies have observed a significant positive correlation between age and sputum neutrophil levels [11, 12, 14], with rho values of 0.58, 0.50 and 0.40 reported. The strength of the association in the present study (rho=0.24) was lower compared to these previous reports. However, we included a comparatively large proportion of older subjects aged >60 years, above which we observed a plateau in sputum neutrophil % count, which is also evident on close inspection of the cohort described by Thomas *et al.* [11]. This plateau clearly can influence the rho value when evaluating the association between age and neutrophil counts.

The longitudinal increase in the median sputum neutrophil % at a mean interval of ∼5 years can be attributed to increasing age-related airway neutrophilia, compatible with the cross-sectional analysis reported here. We are not aware of any previous studies of the long-term repeatability of sputum neutrophil counts in healthy subjects. These data indicate that higher neutrophil counts in healthy subjects are not a transient phenomenon, and that increases are commonly observed over time. The long-term repeatability, determined using ICC, was excellent (0.80). The ICC provides an estimate of the overall concordance between baseline and follow-up by considering between-subject variation expressed as a proportion of the total variance, which includes between-subject differences and within-subject variability during repeated measurements [27], thus indicating that 80% of the total variability observed here was due to between-subject variability. Bland–Altman analysis provides different information, focusing on within subject variability at an individual level. This revealed greater potential for variation at higher neutrophil counts, with the limits of agreement (−39.86–60.25%) indicating that relatively large changes can be

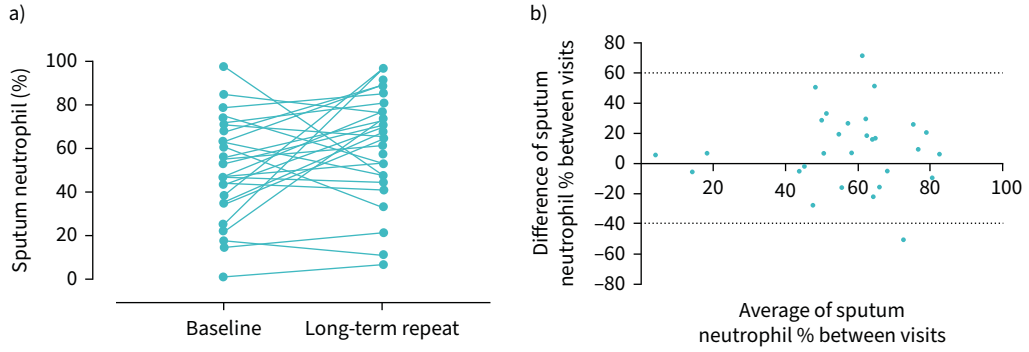

FIGURE 2 a) Sputum neutrophil % at baseline and long-term follow-up visits, and b) Bland–Altman plot of the difference *versus* the mean of two repeat measurements of sputum neutrophil % over time for healthy non-smoking subjects. n=29. Long-term defined as >6 months from baseline.

expected in some individuals. Our overall interpretation of these various analyses of repeated measurements is that the group mean sputum neutrophil % increases over time due to ageing, with the Bland–Altman analysis showing that some changes at an individual level can be relatively large, while the ICC analysis demonstrates that this within-subject variability is small compared to between-subject variability.

In the cross-sectional (baseline) analysis, 51.2% of individuals had a sputum neutrophil % >60%. Similarly, it has been reported that the upper 95th percentile of sputum neutrophil counts within a healthy cohort (mean±$_{SE}$ age 36.98±2.03 years, n=41) was 73.6%, with a median count of 53.75%, indicating again that many healthy subjects can have sputum neutrophil counts >60% [28]. Our results, in conjunction with previous cohorts [11–14], support a different approach to defining neutrophilic airway disease using sputum samples, with the effects of ageing taken into account.

Our overall interpretation of the cross-sectional analysis is not based only on p-values, but we also considered the overall pattern of the median values, and the smaller sample size of subgroups which reduced statistical power. However, our main inferences are based on the significant p-values showing an increase in sputum neutrophil % from age 40 to 59 years.

“Inflamm-ageing” describes the increase, associated with age, of pro-inflammatory cytokines and subsequent persistent low-grade inflammation. Inflamm-ageing can occur in the absence of infection and is thought to drive the age-dependent deterioration in immunological competence (immunosenescence) [29]. It is known that serum interleukin (IL)-6 levels increase in an age-dependent manner [30]. The age-associated increase in sputum neutrophil % was less evident in subjects aged >60 years, with a numerical decrease (that was not statistically significant) in age groups >60 years compared to 40–59 years. A plausible explanation for this phenomenon is that changes in inflammation and chemotactic gradients caused by inflamm-ageing eventually reach a maximum level, as it has been shown that temporal gradients limit neutrophil accumulation *in vitro* [31]. Furthermore, the inhibition of cell death in the presence of inflammatory mediators such as granulocyte–macrophage colony-stimulating factor and IL-2, also lipopolysaccharide, has shown to be reduced in elderly individuals, and therefore apoptosis of neutrophils may become more common with age [32].

There are inconsistent reports of associations between age and peripheral blood neutrophil counts [12, 33, 34]. Granulopoiesis appears to be unaffected by age [35], suggesting that any increase in sputum neutrophils observed here is not simply due to increased bone marrow production.

Neutrophils exhibit a short lifespan in the circulation with prolonged survival in tissue, promoted by a pro-inflammatory milieu [36]. Therefore, neutrophil sequestration within the lung may be enhanced by inflamm-ageing. Neutrophil function itself demonstrates immunosenescence through various mechanisms including impaired chemotactic accuracy (resulting in delayed pathogen killing and collateral damage from proteinase release) [37], defective phagocytosis and neutrophil extracellular trap (NET) formation [38], increased spontaneous reactive oxygen species (ROS) release [39] and impaired bactericidal activity [38]. Furthermore, the phagocytic ability of macrophages decreases with age and therefore limits clearance of apoptotic neutrophils [40]. In addition, neutrophils may facilitate the induction of senescence of other cells *via* ROS production and subsequent telomere dysfunction [41]. Age-related, airway-centric changes have been reported for innate immune receptor (Toll-like receptor 2) and mediator (matrix metalloproteinase-9) expression and function, which were associated with neutrophilic inflammation in healthy individuals [13]. Collectively, these findings demonstrate multiple immunosenescence mechanisms contributing to age-related airway neutrophilia.

The present analysis is limited by the relatively small sample size used to investigate longitudinal follow-up and variation in study period for longitudinal analyses due to the nature of the data collation. Furthermore, it was not feasible to investigate the cause for the age-related increase in sputum neutrophil counts, such as neutrophil chemokines or biomarkers of inflamm-ageing, due to the study design being a retrospective analysis.

Conclusion

The present analysis confirms previous reports of an age-related increase in sputum neutrophil % in healthy individuals and identifies a plateau which appears to occur at age ∼60 years. There was an increase in sputum neutrophil % during longitudinal follow-up. In our healthy cohort, >50% of individuals had sputum neutrophil counts >60%. This questions the use of this threshold to identify the presence of excessive neutrophilic airway inflammation in patients with obstructive lung diseases.

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Provenance: Submitted article, peer reviewed.

Statement of ethics: The study was conducted according to the guidelines of the Declaration of Helsinki, and the study protocol was reviewed and approved by local Research Ethics Committees (05/Q1402/41, 10/H1016/25 and 16/NW/0836). Written informed consent was obtained from all subjects involved in the study.

Data availability statement: The datasets generated and/or analysed during the current study and additional related documents are not publicly available.

Author contributions: AB and DS were responsible for the concept and design of study, data acquisition, data interpretation and drafting the manuscript. AB analysed the data and DS oversaw all analyses. Both authors have approved the final version to be published and are jointly accountable for all aspects of the work.

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Conflict of interest: D. Singh has received sponsorship to attend and speak at international meetings, honoraria for lecturing or attending advisory boards from the following companies: Aerogen, AstraZeneca, Boehringer Ingelheim, Chiesi, Cipla, CSL Behring, Epiendo, Genentech, GlaxoSmithKline, Glenmark, Gossamerbio, Kinaset, Menarini, Novartis, Pulmatrix, Sanofi, Teva, Theravance and Verona. A. Beech has no conflicts of interest to declare.

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