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# Rheology predicts sputum eosinophilia in patients with muco-obstructive lung diseases

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## A B S T R A C T

**Background:** Mucus is known to play a pathogenic role in muco-obstructive lung diseases, but little is known about the determinants of mucus rheology. The purpose of this study is to determine which sputum components influence sputum rheology in patients with muco-obstructive lung diseases.

**Methods:** We performed a cross sectional prospective cohort study. Spontaneous sputum was collected from consecutive patients with muco-obstructive lung diseases. Sputum rheology was assessed using the Rheomuco® rheometer (Rheonova, Grenoble); the elastic modulus  $G'$ , viscous modulus  $G''$ , and the critical stress threshold  $\sigma_c$  were recorded. Key quantitative and qualitative biological sputum components were determined by cytology, nucleic acid amplification tests and mass spectrometry.

**Results:** 48 patients were included from January to August 2019. Among them, 10 had asthma, 14 COPD and 24 non-CF bronchiectasis (NCFB). The critical stress threshold  $\sigma_c$  predicted a sputum eosinophilia superior to 1.25% with 89.19% accuracy (AUC = 0.8762).  $G'$  and  $G''$  are positively correlated with MUC5AC protein concentration (( $\rho = 0.361$ ;  $P = .013$ ) and ( $\rho = 0.335$ ;  $P = .021$ ), respectively).  $\sigma_c$  was positively correlated with sputum eosinophilia ( $\rho = 0.394$ ;  $P = .012$ ), MUC5B ( $\rho = 0.552$ ;  $P < .001$ ) and total protein ( $\rho = 0.490$ ;  $P < .001$ ) concentrations.  $G'$  and  $G''$  were significantly higher in asthma patients ( $G' = 14.49[7.18-25.26]$ Pa,  $G'' = 3.0[2.16-5.38]$ Pa) compared to COPD ( $G' = 5.01[2.94-6.48]$ Pa,  $P = .010$ ;  $G'' = 1.45[1.16-1.94]$ Pa,  $P = .006$ ) and to NCFB ( $G' = 4.99[1.49-10.49]$ Pa,  $P = .003$ ;  $G'' = 1.46[0.71-2.47]$ Pa,  $P = .002$ ).

**Conclusion:** In muco-obstructive lung diseases, rheology predicts sputum eosinophilia and is correlated with mucin concentrations, regardless of the underlying disease.

**Clinical trial registration:** (registrar, website, and registration number), where applicable NCT04081740.

### Keywords:

Asthma  
COPD  
Eosinophil  
Mucins  
Non-CF bronchiectasis

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## 1. Introduction

Mucus plugging is known to play a critical, pathogenic role in fatal asthma episodes [1], in the progression of COPD [2–4], and in outcomes in non-cystic fibrosis bronchiectasis (NCFB) [5]. In the clinical setting, chronic bronchitis is a frequent cause for concern for patients and responsible for considerable handicap [6–11]. The pathophysiology of this symptom is probably more complex than

the mere consequence of goblet cell hyperplasia alone, though mucus hyperproduction is a key feature reported in most mucobstructive diseases [12–17].

Mucus covers the apical surface of the airway epithelium and represents the first barrier with the environment. Produced by both epithelial goblet cells and submucosal glands, mucus is composed of 98% water and 2% protein. The latter components are mostly composed of mucins (primarily MUC5AC and MUC5B [18]), which confer a viscoelastic gel behavior [19].

Certain rheological properties of mucus, such as viscosity and elasticity, may actively contribute towards airway plugging. The amount of force required to initiate flow in a viscoelastic gel, i.e. the critical stress threshold, is also a key rheology parameter. Surprisingly, the biological components determining these properties remain largely unclear and debated. Recently, Rubin et al. demonstrated that mucus rheology was a better predictor of antibiotic treatment success in cystic fibrosis exacerbations of bronchiectasis when compared to classic bacteriology and antibiograms from respiratory samples [20]. In addition, Patarin et al. showed that sputum rheology might be a useful marker for discriminating between different types of chronic airway diseases [21], but no relationship with sputum content has been established to explain these observations. The ratio of mucins MUC5AC/MUC5B in mucus seems to be one important factor [22], as well as eosinophils whose survival in mice has been shown to be favored in MUC5B-deficient sputum through lesser interactions with Siglec-F (Sialic acid-binding, immunoglobulin-like lectin) that normally induces eosinophils apoptosis [23].

In the present study, we aimed to assess the contribution of eosinophil and mucin content to mucus rheology from patients with asthma, COPD and NCFB.

## 2. Methods

### 2.1. Patients

All consecutive patients attending the outpatient clinic in the Respiratory Diseases Department of the Montpellier University Hospitals (Montpellier, France) who met eligibility criteria were prospectively enrolled between September and December 2019. Adults aged 18 or more with a confirmed diagnosis of asthma, COPD or NCFB according to international guidelines [24–26] were eligible if they were able to spontaneously produce sputum.

### 2.2. Sputum analysis

A spontaneous unselected sputum sample was collected for each individual patient. All samples were rigorously processed following the same procedure as previously described [21]. Within 2 h of collection, rheology parameters were measured, and then cytology assessments were performed (see following paragraphs). The sputum supernatant was subsequently frozen and stored at  $-80^{\circ}\text{C}$ . In parallel, a part of the sample was sent to routine bacterial direct examination and culture. The sputum flow chart is described in the supplementary appendix (e-Fig. 1).

### 2.3. Rheology

Rheology was assessed on 720  $\mu\text{l}$  of one fresh sputum sample, as previously published by Patarin et al. [21] Briefly, after gentle vortexing at low velocity for 1 min, rheometric analysis was performed using the Rheomuco® rheometer (Rheonova, Grenoble, France) at  $37^{\circ}\text{C}$  with an oscillatory frequency of 1 Hz and an increasing strain.

Measurements of the elastic modulus ( $G'$ ; Pa), the viscous modulus ( $G''$ ; Pa) in the visco-elastic linear domain and the critical stress threshold ( $\sigma_c$ ) were obtained.

### 2.4. Cytology

Preliminary data suggested that total cellular counts or cytological classification were not affected by prior rheological analysis (e-Table 1). Subsequently, the sputum was treated with dithiothreitol (DTT) according to standard procedures [27]. The sample quality was then assessed (as squamous cells are an indicator of saliva content/contamination, a squamous cell count  $<20\%$  of total cell count was required). Sputum samples were classified according to conventionally recognized categories [28]. Briefly, sputum samples with normal neutrophil ( $<61\%$ ) and eosinophil ( $<1.9\%$ ) counts were considered as paucigranulocytic. Sputum samples with normal neutrophil counts and raised eosinophil counts ( $\geq 2\%$ ) were considered as eosinophilic, and sputum samples with raised neutrophil counts ( $\geq 61\%$ ) and normal eosinophil counts were considered as neutrophilic. Finally, those samples with both raised neutrophil and eosinophil counts were considered as mixed granulocytic. Supernatants were then stored at  $-80^{\circ}\text{C}$  for further assessment.

### 2.5. Total DNA, protein and mucin concentrations, protein identification and $\alpha$ -amylase activity

Frozen supernatants from sputum samples were thawed at room temperature. DNA was extracted (QIAamp DNA Micro Kit) and then quantified with spectrophotometry (NanoDroponeC). Total protein concentration was assessed using colorimetry (RC DC kit (BIO-RAD®)) at 690 nm. Tandem mass spectrometry (Multiple Reaction Monitoring) was performed to quantify both MUC5AC and MUC5B protein concentrations according to previously described techniques, using the LC-MRM method [29]. Peptide sequences were selected according to previous reports [29]. Because *Pseudomonas* sp. can cleave mucins and thus impair their detection [30], we screened and identified peptide sequences at both the N- and C-terminal parts of MUC5B and MUC5AC. Protein identification was performed by high resolution mass spectrometry (HRMS) on Data-Dependent Acquisition. The entire detailed protocol is described in the supplementary material.  $\alpha$ -amylase activity was measured using a colorimetric assay (Abcam® ab102523) at 405 nm every other minute during 1 h (TECAN Infinite® 200 PRO) as a second quality check to assess salivary contamination.

### 2.6. Ethics

This study was approved by the local Institutional Review Board (CHU de Montpellier RECHMPL19\_0430, #2019\_IRB-MTP\_09–10) and registered on [Clinicaltrials.gov](https://clinicaltrials.gov) (NCT04081740). In accordance with French regulations for observational studies, verbal consent was required and patients could participate as long as they were correctly informed and did not oppose the data analysis.

### 2.7. Statistics

Descriptive statistics are provided as medians followed by their interquartile ranges for quantitative variables, and as numbers (%) for qualitative variables. Pairwise comparisons between groups were performed using false discovery rate (FDR)-corrected Conover's tests or Fisher's exact tests. Multivariable, stepwise regression was then performed to explain sputum rheology parameters

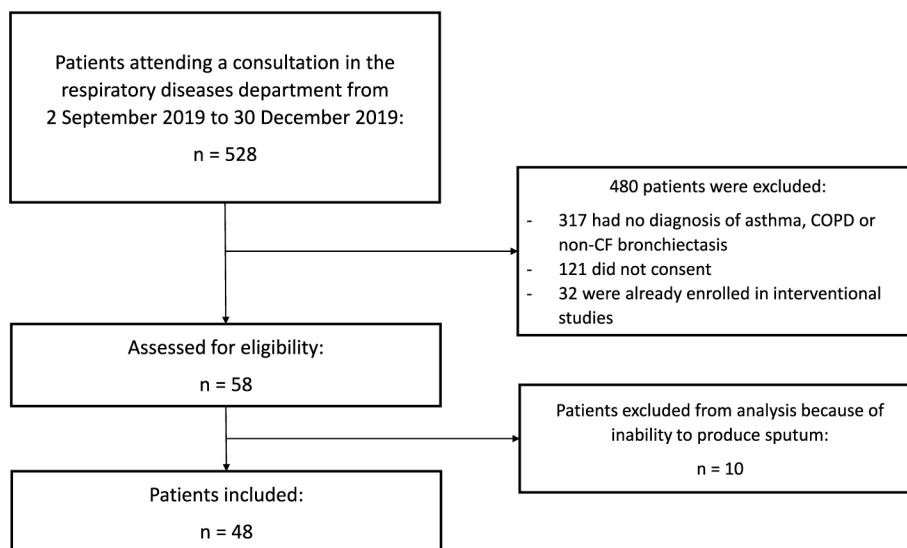


Fig. 1. The study flowchart.

**Table 1** Population characteristics for the total population and each disease group (asthma, chronic obstructive pulmonary disease (COPD) and non-cystic fibrosis bronchiectasis (NCFB). Sample sizes (N) and descriptive statistics (median (inter-quartile) for quantitative variables and number (%) for qualitative variables) are provided. *P* values indicate false discovery rate (FDR) corrected, non-parametric Conover test results for pairwise comparisons (for quantitative variables), or FDR-corrected pairwise Fischer's tests (for qualitative variables). *P*-values <.05 are indicated in bold.

	N	Total (N = 48)	Asthma (N = 10)	COPD (N = 14)	NCFB (N = 24)	<i>P</i> -values		
						<i>Asthma vs COPD</i>	<i>Asthma vs NCFB</i>	<i>COPD vs NCFB</i>
Age (yr)	48	64 (51.25–71)	61 (50.25–64)	67.5 (58.75–69)	64 (34–71)	.440	.750	.440
Sex (% female)	48	25 (52.08%)	8 (80%)	4 (28.57%)	13 (54.17%)	.108	.251	.251
BMI (kg/m <sup>2</sup> )	48	22.22 (20.02–25.62)	24.73 (21.56–28.07)	21.38 (17.89–23.93)	22.22 (20.02–25.05)	.200	.320	.380
Smoking status (% ever smoker)	47	24 (51.06%)	4 (40%)	13 (92.86%)	7 (30.43%)	<b>.013</b>	.692	< <b>.001</b>
Active (%)	24	6 (25%)	2 (50%)	4 (31%)	0 (0%)	.999	.120	<b>.041</b>
Mean pack-years	17	37 (20–50)	20 (9.5–35)	40 (38.5–52.5)	20 (8.75–35)	.120	.860	.100
Steady state vs exacerbation (% exacerbation)	48	25 (52.08%)	5 (50%)	5 (35.71%)	15 (62.5%)	.704	.704	.536
<b>Blood eosinophilia</b>								
cells/mm <sup>3</sup> in the prior week	35	210 (140–260)	230 (100–250)	210 (170–230)	190 (160–310)	.88	.88	.88
≥300 cells/mm <sup>3</sup> in the prior year (%)	41	18 (43.9%)	5 (55.56%)	5 (35.71%)	8 (44.44%)	.532	.532	.999
<b>Lung function</b>								
FEV1 (% predicted)	44	46.5 (33–73.5)	75 (57–78)	28 (22.75–33.25)	55.5 (40–79)	< <b>.001</b>	.140	< <b>.001</b>
FVC (% predicted)	42	75 (57.75–89.75)	89 (79–100)	72 (47–76)	72 (57–95)	<b>.042</b>	.085	.320
FEV1/FVC ratio	42	0.59 (0.4–0.69)	0.58 (0.52–0.75)	0.33 (0.29–0.42)	0.67 (0.59–0.7)	<b>.001</b>	.694	< <b>.001</b>
RV (% predicted)	28	168.5 (131.5–214.47)	–	218.9 (188–301)	138.5 (103.75–153.75)	–	–	< <b>.001</b>
<b>Comorbidities</b>								
Chronic rhinosinusitis (%)	45	25 (55.56%)	9 (90%)	1 (9.09%)	15 (62.5%)	<b>.001</b>	.215	<b>.006</b>
with nasal polyps (%)	45	4 (8.7%)	4 (44.44%)	0 (0%)	0 (0%)	<b>.021</b>	<b>.011</b>	.999
Atopy <sup>a</sup> (%)	47	11 (23.4%)	6 (66.67%)	1 (7.14%)	4 (16.67%)	<b>.015</b>	<b>.016</b>	.633
GERD (treated for) (%)	48	18 (37.5%)	5 (50%)	5 (35.71%)	8 (33.33%)	.999	.999	.999
<b>Exacerbations</b>								
Number in past 12 months	46	2 (1–4)	2 (1–3)	3 (1–4)	2.0 (1.5–3.5)	.900	.900	.900
≥2 in past 12 months (%)	46	32 (69.57%)	6 (66.67%)	9 (64.29%)	17 (73.91%)	.999	.999	.999
Prior hospitalization (%)	46	31 (67.39%)	6 (60%)	10 (76.92%)	15 (65.22%)	.999	.999	.999
Prior ICU (%)	48	14 (29.17%)	3 (30%)	5 (35.71%)	6 (25%)	.999	.999	.999

BMI = body mass index; CASA-Q = cough and sputum assessment questionnaire; FEV1 = forced expiratory volume in 1 s; FVC = forced vital capacity; GERD = gastroesophageal reflux disease; ICU = intensive care unit; RV = residual volume.

<sup>a</sup> Atopy is defined as positive skin prick tests or specific IgE to one or more inhaled allergens.

(separate models for  $G'$ ,  $G''$  and  $\sigma_c$ ) as a function of sputum components. Data were scaled prior to modeling and the models with

the smallest Akaike Information Criterion from among forward and backward stepwise procedures were retained. To determine the

predictive capacity of rheology to predict sputum eosinophilia, we constructed a Receiver Operating Characteristic curve to discriminate between high ( $\geq 1.25\%$ ) and low ( $< 1.25\%$ ) sputum eosinophilia. The latter threshold (1.25%) was previously published by Hastie et al. and based on 827 patients from the SPIROMICS cohort [31]. The receiver operating curve and associated statistics (Youden's threshold, area under the curve, accuracy, sensitivity and specificity) were computed for  $\sigma_c$  as a predictor of sputum eosinophil percentage  $\geq 1.25\%$  via logistic regression. All computations were performed using the R statistical programming environment [32].

### 3. Results

#### 3.1. Study population

Fifty-eight patients were eligible for the study. Among them, forty-eight patients were able to produce sputum and were included from September to December 2019 (Fig. 1). Ten patients were included in the asthma group, fourteen in the COPD group and twenty-four in the NCFB group, and the associated descriptive statistics are provided in Table 1. The median annual number of exacerbations in the previous year was 2 [interquartile range: 1–4] without significant differences between groups. Median FEV1 (% predicted) was significantly lower in the COPD group (FEV1 = 28% [22.75–33.25]) compared to the asthma group (FEV1 = 75% [57–78]) ( $P < .001$ ) and the NCFB group (FEV1 = 55.5% [40–79]) ( $P < .001$ ). Among patients with data for complete blood counts sampled in the past week ( $n = 35$ ), median blood eosinophil concentration was 210 cells/mm<sup>3</sup> [140–260] and 43.9% of them had  $\geq 300$  eosinophils/mm<sup>3</sup> within the past year. Smoking history was significantly more reported in the COPD (93%) than in the asthma (40%) and the NCFB (30%) groups. All patients with asthma and NCFB reported chronic bronchitis, whereas only 64% of patients with COPD did. Twenty-five patients (52%) were hospitalized for a severe acute exacerbation at inclusion, with no significant differences between groups (Table 1).

#### 3.2. Rheology

The elastic and the viscous moduli were significantly higher in the asthma group ( $G' = 14.49$  [7.18–25.26] Pa,  $G'' = 3$  [2.16–5.38] Pa) versus the COPD group ( $G' = 5.01$  [2.94–6.48];  $G'' = 1.45$  [1.16–1.94] Pa) and versus the NCFB group ( $G' = 4.99$  [1.49–10.49] and  $G'' = 1.46$  [0.71–2.47] Pa) (Fig. 2a et 2b). No significant between-group differences were observed for critical yield stress  $\sigma_c$  (Fig. 2c). Descriptive statistics are also listed in the supplementary material (e-Table 2). No significant differences were found between sputum samples collected at steady state in comparison with sputum collected during an exacerbation (e-Fig. 2).

#### 3.3. Sputum components

Descriptive statistics for sputum components are presented in Table 2. Total cellular count was higher in the NCFB group than in the asthma and COPD groups (respectively 3.66 [1.95–6.92] versus 1.25 [0.82–2.17] ( $P = .025$ ) and 0.42 [0.1–1.05] ( $P < .001$ )), with a predominance of neutrophils in all three groups (median percentage of sputum neutrophils = 89.62% [82.75–93.75]). Sputum eosinophil percentages were higher in the asthma group (1.02 [0–2.62]%) compared to the COPD (0.25 [0–3.12]%) and the NCFB (0 [0–0.25]%) groups, although differences were not significant (Table 2).

Greater concentrations of DNA were found in the NCFB group (916.4 [386.28–1293] ng/ $\mu$ l), with significant differences in comparison to the asthma (104.75 [21.85–419.22];  $P < .001$ ) and COPD

group (30.1 [17.1–161.48] ng/ $\mu$ l;  $P < .001$ ). MUC5B concentrations were significantly higher in both the COPD and asthma groups (respectively 90.55 [57.85–132.82] fmol/ $\mu$ l) and 79.75 [53.28–98.4] fmol/ $\mu$ l) than in the NCFB group (44.6 [26.8–66.7] fmol/ $\mu$ l,  $P = .006$  and  $P = .027$ ). Asthma and COPD patients exhibited higher MUC5AC concentrations (respectively 62.25 [22.95 to 171.7] fmol/ $\mu$ l and 41.3 [40.4 to 91.8] fmol/ $\mu$ l) than patients in the non-CF bronchiectasis group (35.7 [26.85 to 55.45] fmol/ $\mu$ l) (Table 2). Proteomic analysis showed differential patterns among groups: in COPD and asthma samples, MUC5B and MUC5AC proteins were identified in the top 10 of the protein list, whereas in the NCFB samples, MUC5AB and MUC5AC proteins were identified, but in lower amounts (e-Table 3).

#### 3.4. Relating sputum components to rheology

Spearman's correlation tests confirm that  $G'$  and  $G''$  are positively correlated with MUC5AC protein concentration ( $\rho = 0.361$ ;  $P = .013$ ) and ( $\rho = 0.335$ ;  $P = .021$ ), respectively).  $\sigma_c$  was positively correlated with sputum eosinophilia ( $\rho = 0.394$ ;  $P = .012$ ), MUC5B ( $\rho = 0.552$ ;  $P < .001$ ) and Protein ( $\rho = 0.490$ ;  $P < .001$ ) concentrations.

Multiple regression analyses demonstrate that protein content is a highly significant positive determinant of all three rheological parameters, along with MUC5AC (Table 3). MUC5B and the percentage eosinophils also have significant, positive contributions to  $\sigma_c$ . The percentage neutrophils, on the other hand, is significantly associated with decreasing  $G'$  and  $G''$  (and near-significantly so for  $\sigma_c$ ). Similarly, the percentage squamous cells is also negatively correlated with  $\sigma_c$ . Finally, there is a tendency for DNA to be negatively associated with  $G''$  (Table 3).

According to logistic regression,  $\sigma_c$  predicted sputum eosinophilia superior to 1.25% ( $\beta = 0.0218 \pm .0090$ ,  $P = .015$ ; intercept =  $-2.60 \pm 0.7296$ ,  $P < .001$ ); the associated receiver operating curve is presented in Fig. 3 and determined 89.19% accuracy (85.71% sensitivity, 93.33% specificity). The area under the curve (AUC) was 0.8762.

### 4. Discussion

In this study, we provide evidence for the first time that: i) simple sputum rheology at the bedside can be confidently related to sputum cytological and mucin content; ii) the viscoelastic properties of sputum are mostly driven by protein and mucin (MUC5AC in particular) content as compared to other tested components; (iii) the critical stress threshold ( $\sigma_c$ ) at which sputum is forced to flow like a fluid (rather than aggregating more like a solid) is further determined by MUC5B and sputum eosinophilia; (iv)  $\sigma_c$  is predictive of a sputum eosinophilia superior to 1.25% (the latter being a cut-off associated with a more severe COPD phenotype) [31].

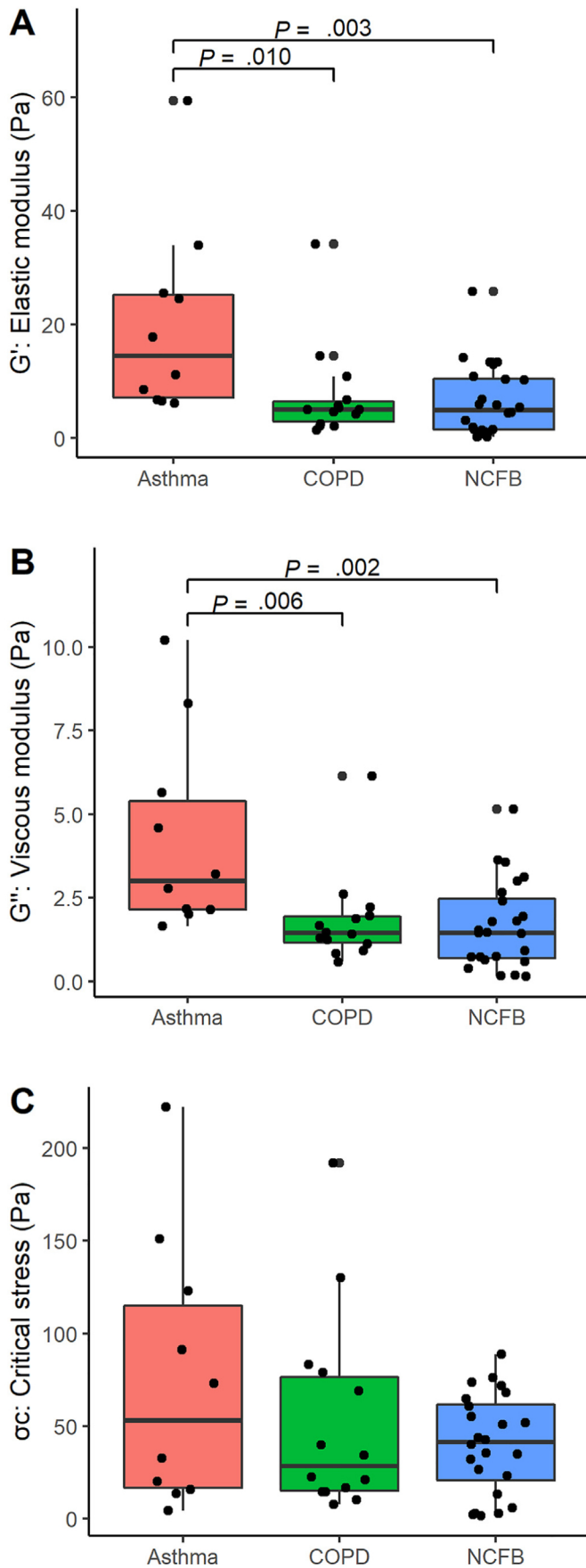
In this study, multivariate models evidenced for the first time what was intuitively known but never previously demonstrated at the bedside: the main determinant of the viscoelastic properties of sputum are the mucins, and in particular, MUC5AC, whose concentrations have been rigorously assessed with mass spectrometry. This may have therapeutic implications in itself, but we feel that future research should also delve deeper into the specificities of each underlying condition. In particular, we demonstrated that the relative “rheological weight” of mucins in NCFB seems less important than in other conditions, likely due to the involvement of inflammatory cells and in particular eosinophils. In contrast to what has been previously published [21], patients with asthma have the highest elastic and viscous moduli here, compared to patients with COPD. This could be explained by the higher severity of asthmatic subjects in our study in comparison with the ones in the article by

As concerns rheology-targeting treatments, one should keep in mind that MUC5AC and MUC5B are highly complex glycoproteins and the promoter of their related encoding genes is a carrier of a very high number and variety of regulation sites [33]. Targeting MUC5AC for treatment is complex and most previous attempts have failed to achieve this aim [34]. Perhaps the EXPEDITION trial assessing the effect of Dupilumab on airway inflammation in patients with asthma could give us some answers (NCT02573233). In vivo and in vitro, very few molecules are able to reduce the viscoelastic behavior of sputum [35,36]. However, other sputum components may be targeted. For example, DNase was successfully marketed in cystic fibrosis [37]. Similarly targeting eosinophils in sputum should be achievable via monoclonal antibodies (mAbs) such as anti-Interleukine 5/5R or Anti-Interleukine 4R/13R, though the impacts of the latter on sputum content are currently under-reported. From our observations, we may also reasonably hypothesize that the presence of eosinophil-derived elements such as Charcot-Leyden crystals or Eosinophil Extra-cellular Traps (EETs) [38,39] could be involved. As a consequence, targeting Charcot-Leyden Crystals with Galectin 10 antibodies could be an option [40]. Another option would be to change cell fate during epithelial differentiation in order to prevent goblet cell hyperplasia; this could be successfully achieved by Notch pathway interference via anti-Jagged antibodies [41–43].

Upcoming trials include the “COPD CARhE” study (Chronic Airway Disease, Mucus Rheology and Exacerbations: a Randomized Controlled Trial of COPD Patients, NCT04339270), which will juxtapose symptom-based versus rheology-based azithromycin strategies. Indeed, azithromycin has been shown to reduce exacerbations in asthma, COPD, and NCFB [44–46]. Our hypothesis is that this treatment success could be due to the mucus modifying properties of this macrolide [47,48]. Rheology, which is correlated to the mucin content of mucus, could thus objectively guide the prescription of this antibiotic.

One of our main findings is the accuracy of the critical stress  $\sigma_c$  for predicting sputum eosinophilia superior to 1.25%. This biomarker might be a promising non-invasive decision-making tool in the care pathway. Indeed, sputum eosinophilia has been shown to be closely related to COPD severity in terms of future exacerbations [31], to be a reliable biomarker of reduction of exacerbations correlated with CT mucus scores in patients with moderate to severe asthma [49] and may identify a subgroup of patients in NCFB who could benefit from a biologic “anti-T2” therapy [50,51]. As a reflection of airway eosinophilia, rheology could thus help the physician to rapidly phenotype their patients, appreciate their adherence to anti-inflammatory therapies or identify patients refractory to their latest biologic treatment.

Due to its pragmatic design, the main limitation of this study is that a significant proportion of patients (10 over 58) were not able to produce spontaneous sputum and were excluded. Although this might represent a lack of power for pairwise group comparisons, the good predictive value of sputum eosinophilia by rheology would not have been affected by this issue. The quality criteria for sputum samples could be another limitation and an obstacle to



**Fig. 2.** Boxplots demonstrating the distributions of (A) the elastic modulus ( $G'$ ; Pa), (B) the viscous modulus ( $G''$ ; Pa) and (C) the critical stress threshold ( $\sigma_c$ ; Pa) for three disease groups (asthma in red, chronic obstructive pulmonary disease (COPD) in green and non-cystic fibrosis bronchiectasis (NCFB) in blue). Medians, interquartile intervals, and ranges are indicated by horizontal lines, boxes and whiskers, respectively.  $P$  values indicate non-parametric Conover tests for pairwise comparisons that remain significant after a false discovery rate correction. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

**Table 2**

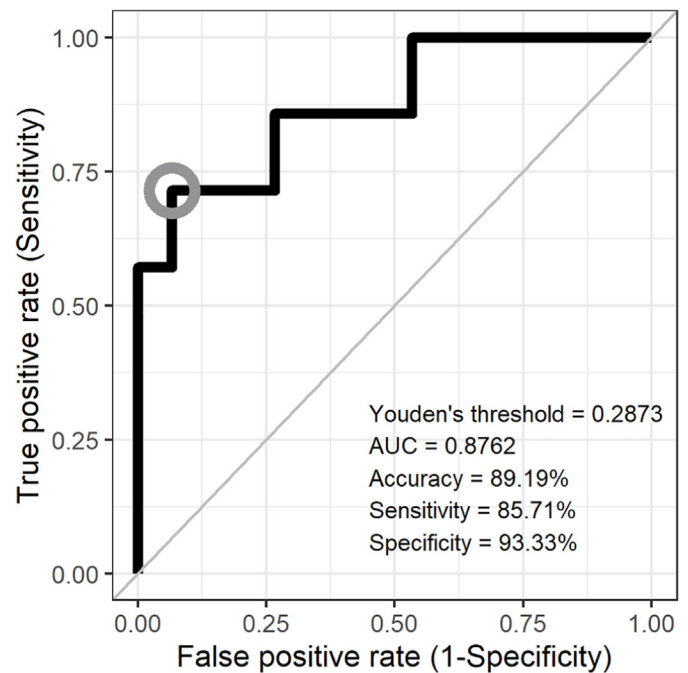
Sputum components for the total population and each disease group (asthma, chronic obstructive pulmonary disease (COPD) and non-cystic fibrosis bronchiectasis (NCFB). Sample sizes (N) and descriptive statistics (median (inter-quartile) for quantitative variables and number (%) for qualitative variables) are provided. *P* values indicate false discovery rate (FDR) corrected, non-parametric Conover test results for pairwise comparisons (for quantitative variables), or FDR-corrected pairwise Fischer's tests (for qualitative variables). *P*-values <.05 are indicated in bold.

	Total (n = 48)	Asthma (n = 10)	COPD (n = 14)	NCFB (n = 24)	<i>P</i> -values		
					Asthma vs COPD	Asthma vs NCFB	COPD vs NCFB
<b>Cell counts</b>							
Total cellular count ( × 10 <sup>6</sup> /g)	1.56 (0.71–4.01)	1.25 (0.82–2.17)	0.42 (0.1–1.05)	3.66 (1.95–6.92)	<b>.034</b>	<b>.025</b>	<b>&lt; .001</b>
Neutrophil count ( × 10 <sup>6</sup> /g)	1.93 (0.85–4.05)	1.14 (0.54–1.86)	0.84 (0.59–0.97)	3.20.99 (1.77–6.75)	.522	<b>.016</b>	<b>.004</b>
Eosinophil count ( × 10 <sup>6</sup> /g)	0.06 (0–2.61)	0.99 (0–2.21)	0.32 (0–3.26)	0 (0–1.78)	.920	.790	.790
<b>Cell percentages</b>							
Sputum neutrophilia (%)	89.62 (82.75–93.75)	85 (77.94–88.74)	81.5 (73.12–92.38)	92 (89.06–96.1)	.945	<b>.043</b>	<b>.043</b>
Sputum eosinophilia (%)	0.12 (0–0.56)	1.02 (0–2.62)	0.25 (0–3.12)	0 (0–0.25)	.750	.400	.400
Squamous cells (%)	1.25 (0.25–7.88)	1.25 (0.75–2.75)	50.38 (2.25–90)	0.38 (0–2.12)	.100	.174	<b>&lt; .001</b>
<b>Classifications</b>							
Neutrophilic (%)	29 (63.04%)	4 (44.44%)	4 (30.77%)	21 (87.5%)	.662	<b>.030</b>	<b>.002</b>
Eosinophilic (%)	0(0%)	0(0%)	0(0%)	0(0%)			
Mixed granulocytic (%)	6 (13.04%)	2 (22.22%)	3 (23.08)	1 (4.17%)	.999	.261	.261
Paucigranulocytic (%)	2 (4.35%)	1 (11.11%)	0(0%)	1 (4.17%)	.716	.716	.999
Squamous (%)	9 (19.57%)	2 (22.22%)	6 (46.15)	1 (4.17%)	.380	.261	<b>.013</b>
<b>DNA and proteins</b>							
DNA (ng/μL)	317.95 (31.62–836.8)	104.75 (21.85–419.22)	30.1 (17.1–161.48)	916.4 (386.28–1293)	.249	<b>&lt; .001</b>	<b>&lt; .001</b>
Proteins (mg/mL)	20.36 (11.96–30.71)	18.04 (14.6–30.22)	20.71 (10.62–23.75)	23.57 (11.96–35.72)	.660	.660	.660
MUC5AC (fmol/μL)	40.75 (26.3–82.1)	62.25 (22.95–171.7)	41.3 (40.4–91.8)	35.7 (26.85–55.45)	.958	<b>&lt; .001</b>	<b>&lt; .001</b>
MUC5B (fmol/μL)	64(32.75–89.05)	79.75 (53.28–98.4)	90.55 (57.85–132.82)	44.6 (26.8–66.7)	.677	<b>.027</b>	<b>.006</b>
α-amylase activity (mU/mL)	842.96 (462.8–2601.27)	1904.76 (685.12–2639.77)	804.59 (290.96–3794.99)	810.46 (524.12–1845.5)	.770	.770	.770

**Table 3**

Multivariable regression results for explaining sputum rheology parameters (*G'*, *G''* and  $\sigma_c$ ) as a function of sputum components. Only the best models minimizing the Akaike Information Criterion are presented (from among forward and backward stepwise procedures). Standardized coefficients and associated standard errors are presented so as to facilitate coefficient comparisons. *P*-values in bold indicate variables with significant contributions to a model.

Variable	Standardized		<i>P</i> -value
	Coefficient	Standard error	
<b>Model 1: Variables predicting the elastic modulus (<i>G'</i> in Pa) of sputum samples</b> Adjusted <i>r</i> <sup>2</sup> = .8791; <i>P</i> < .001			
Proteins (mg/mL)	.3829	.0587	<b>&lt; .001</b>
MUC5AC (fmol/μL)	.6439	.0886	<b>&lt; .001</b>
Sputum neutrophilia (%)	-.2504	.0958	<b>.014</b>
Sputum eosinophilia (%)	.0939	.0604	.130
Squamous cells (%)	-.1450	.0715	.051
<b>Model 2: Variables predicting the viscous modulus (<i>G''</i> in Pa) of sputum samples</b> Adjusted <i>r</i> <sup>2</sup> = .7513; <i>P</i> < .001			
Proteins (mg/mL)	.6561	.1108	<b>&lt; .001</b>
DNA (ng/μL)	-.2375	.1197	.056
MUC5AC (fmol/μL)	.3768	.1279	<b>.006</b>
Sputum neutrophilia (%)	-.3242	.1372	<b>.024</b>
Squamous cells (%)	-.2047	.1047	.059
<b>Model 3: Variables predicting the critical stress threshold (<math>\sigma_c</math> in Pa) of sputum samples</b> Adjusted <i>r</i> <sup>2</sup> = .8058; <i>P</i> < .001			
Proteins (mg/mL)	.3611	.0754	<b>&lt; .001</b>
MUC5AC (fmol/μL)	.2555	.1125	<b>.030</b>
MUC5B (fmol/μL)	.2517	.0937	<b>.011</b>
Sputum neutrophilia (%)	-.2281	.1251	.078
Sputum eosinophilia (%)	.3484	.0862	<b>.000</b>
Squamous cells (%)	-.2339	.0907	<b>.015</b>



**Fig. 3.** The receiver operation characteristic (ROC) curve for the prediction of high versus low sputum eosinophilia using the critical stress threshold ( $\sigma_c$  in Pa) as a predictor in logistic regression. The grey circle indicates Youden's best threshold.

routine use. In our study, we used a classic quality standard for sputum (a squamous cell count <20% of total cell count) and added

a measure of  $\alpha$ -amylase activity to exclude salivary contamination as previously published [29]. Contamination was observed, was modest, and importantly, was equally distributed amongst the strata, indicating it did not bias our results, which is consistent with

previous reports [21].

At last, one could object to the absence of a control group. Patarin et al. have previously reported data about the performance of rheology to discriminate between patients with mucro-obstructive lung diseases and healthy subjects [21]. As a consequence, it would not have been ethical to perform further procedures in this category of patients. Moreover, the aim of our study was to assess the performance of rheology to predict sputum content.

#### 4.1. Interpretation

Sputum rheology is driven primarily by mucins and eosinophils in asthma, COPD and NCFB, and accurately predicts sputum eosinophilia. Given the recent technological advances rendering on-site rheology quite feasible in the clinic, rheology-based diagnosis and therapeutic strategies can now be tested.

#### Ethics approval and consent to participate

This study was approved by the local Institutional Review Board (CHU de Montpellier RECHMPL19\_0430, #2019\_IRB-MTP\_09–10) and registered on [Clinicaltrials.gov](https://clinicaltrials.gov) (NCT04081740). In accordance with French regulations for observational studies, verbal consent was required and patients could participate as long as they were correctly informed and did not oppose the data analysis.

#### Consent for publication

All the authors give their consent for publication in *Respiratory Research*.

#### Availability of data and material

The data that support the findings of this study are available from the corresponding author, JC, upon reasonable request.

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#### Authors' contributions

Mathilde Volpato, Jeremy Charriot and Aurelie Petit performed the cytology, protein assays and rheological analysis of the samples. Arnaud Bourdin, Isabelle Vachier and Jeremy Charriot participated in the design of the study. Jerome Vialaret and Christophe Hirtz performed the mass spectrometry measurements. Nicolas Molinari, Eric Matzner-Lober and Carey Suehs performed the statistical analyses. Jeremy Patarin gave counsel and expertise about the rheological analysis. All the authors equally contributed to the writing of the manuscript.

#### Declaration of competing interest

Dr. Suehs reports grants from Astra Zeneca, outside the submitted work.

Dr Patarin is a full-time employee of Rheonova. Rheonova designed and built the rheometer used in this study.

Pr. Molinari reports personal fees from Astra Zeneca, grants from

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The remaining authors (MV, JV, CH, AP, EML, IV, JC) have no conflicts of interest to disclose.

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#### Abbreviations

BMI	=	body mass index
CASA-Q	=	cough and sputum assessment questionnaire;
COPD	=	chronic obstructive pulmonary disease
FDR	=	false discovery rate
FEV1	=	forced expiratory volume in 1 s
FVC	=	forced vital capacity
GERD	=	gastroesophageal reflux disease
ICU	=	intensive care unit
MUC5AC	=	Mucin 5AC
MUC5B	=	Mucin 5B
NCFB	=	non-cystic fibrosis bronchiectasis
RV	=	residual volume

#### References

- [1] E.M. Dunican, D.C. Watchorn, J.V. Fahy, Autopsy and imaging studies of mucus in asthma. Lessons learned about disease mechanisms and the role of mucus in airflow obstruction, *Annals ATS* 15 Supplement\_3 (2018) S184–S191.
- [2] T. Aikawa, S. Shimura, H. Sasaki, M. Ebina, T. Takishima, Marked goblet cell hyperplasia with mucus accumulation in the airways of patients who died of severe acute asthma attack, *Chest* 101 (1992) 916–921.
- [3] J.C. Hogg, F.S.F. Chu, W.C. Tan, D.D. Sin, S.A. Patel, P.D. Pare, et al., Survival after lung volume reduction in chronic obstructive pulmonary disease: insights from small airway pathology, *Am. J. Respir. Crit. Care Med.* 176 (2007) 454–459.
- [4] J.C. Hogg, F. Chu, S. Utokaparch, R. Woods, W.M. Elliott, L. Buzatu, et al., The nature of small-airway obstruction in chronic obstructive pulmonary disease, *N. Engl. J. Med.* 350 (2004) 2645–2653.
- [5] K. Ramsey, G. Radicioni, D. Hill, C. Ehre, B. Button, N.E. Alexis, et al., Airways mucus pathogenesis in patients with non-cystic fibrosis bronchiectasis, *Eur. Respir. J.* 52 (suppl 62) (2018).
- [6] J. Vestbo, E. Prescott, P. Lange, Association of chronic mucus hypersecretion with FEV1 decline and chronic obstructive pulmonary disease morbidity. Copenhagen City Heart Study Group, *Am. J. Respir. Crit. Care Med.* 153 (1996) 1530–1535.
- [7] P.-R. Burgel, P. Nesme-Meyer, P. Chanez, D. Caillaud, P. Carré, T. Perez, et al., Cough and sputum production are associated with frequent exacerbations and hospitalizations in COPD subjects, *Chest* 135 (2009) 975–982.
- [8] S. Accordini, A.G. Corsico, L. Calciano, R. Bono, I. Cerveri, A. Fois, et al., The impact of asthma, chronic bronchitis and allergic rhinitis on all-cause hospitalizations and limitations in daily activities: a population-based observational study, *BMC Pulm. Med.* 15 (2015) 10.
- [9] P.M. Meek, H. Petersen, G.R. Washko, A.A. Diaz, V. Klm, A. Sood, et al., Chronic bronchitis is associated with worse symptoms and quality of life than chronic airflow obstruction, *Chest* 148 (2015) 408–416.
- [10] M.M. de Oca, R.J. Halbert, M.V. Lopez, R. Perez-Padilla, C. Tálamo, D. Moreno, et al., The chronic bronchitis phenotype in subjects with and without COPD: the



- PLATINO study, *Eur. Respir. J.* 40 (2012) 28–36.
- [11] L. Lahousse, L.J.M. Seys, G.F. Joos, O.H. Franco, B.H. Stricker, G.G. Brusselle, Epidemiology and impact of chronic bronchitis in chronic obstructive pulmonary disease, *Eur. Respir. J.* 50 (2017).
- [12] C.L. Ordoñez, R. Khashayar, H.H. Wong, R. Ferrando, R. Wu, D.M. Hyde, et al., Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression, *Am. J. Respir. Crit. Care Med.* 163 (2001) 517–523.
- [13] A.L. Innes, P.G. Woodruff, R.E. Ferrando, S. Donnelly, G.M. Dolganov, S.C. Lazarus, et al., Epithelial mucin stores are increased in the large airways of smokers with airflow obstruction, *Chest* 130 (2006) 1102–1108.
- [14] D.A. Groneberg, P.R. Eynott, T. Oates, S. Lim, R. Wu, I. Carlstedt, et al., Expression of MUC5AC and MUC5B mucins in normal and cystic fibrosis lung, *Respir. Med.* 96 (2002) 81–86.
- [15] P.-R. Burgel, D. Montani, C. Danel, D.J. Dusser, J.A. Nadel, A morphometric study of mucins and small airway plugging in cystic fibrosis, *Thorax* 62 (2007) 153–161.
- [16] Z.-G. Chen, Y.-Y. Li, Z.-N. Wang, M. Li, H.-F. Lim, Y.-Q. Zhou, et al., Aberrant epithelial remodeling with impairment of cilia architecture in non-cystic fibrosis bronchiectasis, *J. Thorac. Dis.* 10 (2018) 1753–1764.
- [17] K.A. Ramsey, A.C.H. Chen, G. Radicioni, R. Lourie, M. Martin, A. Broomfield, et al., Airway mucus hyperconcentration in non-cystic fibrosis bronchiectasis, *Am. J. Respir. Crit. Care Med.* 201 (2020) 661–670.
- [18] K. Okuda, G. Chen, D.B. Subramani, M. Wolf, R.C. Gilmore, T. Kato, et al., Localization of secretory mucins MUC5AC and MUC5B in normal/healthy human airways, *Am. J. Respir. Crit. Care Med.* 199 (2019) 715–727.
- [19] R.C. Boucher, Muco-obstructive lung diseases, *N. Engl. J. Med.* 380 (2019) 1941–1953.
- [20] J.T. Ma, C. Tang, L. Kang, J.A. Voynow, B.K. Rubin, Cystic fibrosis sputum rheology correlates with both acute and longitudinal changes in lung function, *Chest* 154 (2018) 370–377.
- [21] J. Patarin, É. Ghiringhelli, G. Darsy, M. Obamba, P. Bochu, B. Camara, et al., Rheological analysis of sputum from patients with chronic bronchial diseases, *Sci. Rep.* 10 (2020), 15685.
- [22] M.E. Lachowicz-Scroggins, S. Yuan, S.C. Kerr, E.M. Dunican, M. Yu, S.D. Carrington, et al., Abnormalities in MUC5AC and MUC5B protein in airway mucus in asthma, *Am. J. Respir. Crit. Care Med.* 194 (2016) 1296–1299.
- [23] Endogenous airway mucins carry glycans that bind Siglec-F and induce eosinophil apoptosis, PubMed, <https://pubmed.ncbi.nlm.nih.gov/25497369/>. (Accessed 22 October 2021).
- [24] 2020 GINA Main Report. Global Initiative for Asthma - GINA. <https://ginasthma.org/gina-reports/>. Accessed 25 May 2020.
- [25] Gold reports for personal use. Global initiative for chronic obstructive lung disease - gold. <https://goldcopd.org/gold-reports/>. (Accessed 25 May 2020).
- [26] E. Polverino, P.C. Goeminne, M.J. McDonnell, S. Aliberti, S.E. Marshall, M.R. Loebinger, et al., European Respiratory Society guidelines for the management of adult bronchiectasis, *Eur. Respir. J.* 50 (2017).
- [27] Leader of the Working Group, A. Efthimiadis, A. Spanevello, , Members of the Working Group, Q. Hamid, M.M. Kelly, M. Linden, R. Louis, et al., Methods of sputum processing for cell counts, immunocytochemistry and in situ hybridisation, *Eur. Respir. J.* 20 (Supplement 37) (2002) 19S–23S.
- [28] P. Haldar, I.D. Pavord, Noneosinophilic asthma: a distinct clinical and pathological phenotype, *J. Allergy Clin. Immunol.* 119 (2007) 1043–1052.
- [29] M. Kesimer, A.A. Ford, A. Ceppe, G. Radicioni, R. Cao, C.W. Davis, et al., Airway mucin concentration as a marker of chronic bronchitis, *N. Engl. J. Med.* 377 (2017) 911–922.
- [30] A.G. Henderson, C. Ehre, B. Button, L.H. Abdullah, L.-H. Cai, M.W. Leigh, et al., Cystic fibrosis airway secretions exhibit mucin hyperconcentration and increased osmotic pressure, *J. Clin. Invest.* 124 (2014) 3047–3060.
- [31] A.T. Hastie, F.J. Martinez, J.L. Curtis, C.M. Doerschuk, N.N. Hansel, S. Christenson, et al., Association of sputum and blood eosinophil concentrations with clinical measures of COPD severity: an analysis of the SPIROMICS cohort, *Lancet Respir. Med.* 5 (2017) 956–967.
- [32] R. Core Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, Vienna, Austria, 2021.
- [33] P. Thai, A. Loukoianov, S. Wachi, R. Wu, Regulation of airway mucin gene expression, *Annu. Rev. Physiol.* 70 (2008) 405–429.
- [34] E.V.S. Ha, D.F. Rogers, Novel therapies to inhibit mucus synthesis and secretion in airway hypersecretory diseases, *PHA* 97 (2016) 84–100.
- [35] Rheology of cystic fibrosis sputum after in vitro treatment with hypertonic saline alone and in combination with recombinant human deoxyribonuclease I, *Am. J. Respir. Crit. Care Med.* (2020). <https://www.atsjournals.org/doi/full/10.1164/ajrccm.156.1.9512074>. (Accessed 26 May 2020).
- [36] J. Tamaoki, K. Takeyama, E. Tagaya, K. Konno, Effect of clarithromycin on sputum production and its rheological properties in chronic respiratory tract infections, *Antimicrob. Agents Chemother.* 39 (1995) 1688–1690.
- [37] S. Shak, D.J. Capon, R. Hellmiss, S.A. Marsters, C.L. Baker, Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum, *Proc. Natl. Acad. Sci. USA* 87 (1990) 9188–9192.
- [38] R. Dworski, H.-U. Simon, A. Hoskins, S. Yousefi, Eosinophil and neutrophil extracellular DNA traps in human allergic asthmatic airways, *J. Allergy Clin. Immunol.* 127 (2011) 1260–1266.
- [39] C.S. Hwang, S.C. Park, H.-J. Cho, D.-J. Park, J.-H. Yoon, C.-H. Kim, Eosinophil extracellular trap formation is closely associated with disease severity in chronic rhinosinusitis regardless of nasal polyp status, *Sci. Rep.* 9 (2019) 1–8.
- [40] E.K. Persson, K. Verstraete, I. Heyndrickx, E. Gevaert, H. Aegerter, J.-M. Percier, et al., Protein crystallization promotes type 2 immunity and is reversible by antibody treatment, *Science* 364 (2019).
- [41] J.e. Phillips, S. Davis, J. Werner, T. Huber, L. Feng, B.d. Bennett, et al., Anti-Jagged 1 antibody AMG 430 reduces airway mucus in mouse models of obstructive pulmonary disease, in: B63. ANIMAL MODELS OF COPD, American Thoracic Society, 2019. A3839–A3839.
- [42] N.R. Gough, Converting mucus-making cells into mucus-clearing cells, *Sci. Signal.* 8 (2015) ec362–ec362.
- [43] D. Lafkas, A. Shelton, C. Chiu, G. de Leon Boenig, Y. Chen, S.S. Stawicki, et al., Therapeutic antibodies reveal Notch control of transdifferentiation in the adult lung, *Nature* 528 (2015) 127–131.
- [44] R.K. Albert, J. Connett, W.C. Bailey, R. Casaburi, J.A.D. Cooper, G.J. Criner, et al., Azithromycin for prevention of exacerbations of COPD, *N. Engl. J. Med.* 365 (2011) 689–698.
- [45] G.G. Brusselle, C. Vanderstichele, P. Jordens, R. Deman, H. Slabbynck, V. Ringoet, et al., Azithromycin for prevention of exacerbations in severe asthma (AZISAST): a multicentre randomised double-blind placebo-controlled trial, *Thorax* 68 (2013) 322–329.
- [46] C. Wong, L. Jayaram, N. Karalus, T. Eaton, C. Tong, H. Hockey, et al., Azithromycin for prevention of exacerbations in non-cystic fibrosis bronchiectasis (EMBRACE): a randomised, double-blind, placebo-controlled trial, *Lancet* 380 (2012) 660–667.
- [47] T. Tanabe, S. Kanoh, K. Tsushima, Y. Yamazaki, K. Kubo, B.K. Rubin, Clarithromycin inhibits interleukin-13-induced goblet cell hyperplasia in human airway cells, *Am. J. Respir. Cell Mol. Biol.* 45 (2011) 1075–1083.
- [48] S. Lu, H. Liu, J.M. Farley, Macrolide antibiotics inhibit mucus secretion and calcium entry in SWine airway submucosal mucous gland cells, *J. Pharmacol. Exp. Therapeut.* 336 (2011) 178–187.
- [49] R.H. Green, C.E. Brightling, S. McKenna, B. Hargadon, D. Parker, P. Bradding, et al., Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial, *Lancet* 360 (2002) 1715–1721.
- [50] J. Davison, G. Johnson, K. Jiwa, A. Cranson, R. Wake, B. Hood, et al., P112 Deeper phenotyping of non CF bronchiectasis through sputum differential counts, *Thorax* 68 (Suppl 3) (2013). A125–A125.
- [51] E.M. Dunican, B.M. Elicker, D.S. Gierada, S.K. Nagle, M.L. Schiebler, J.D. Newell, et al., Mucin plugs in patients with asthma linked to eosinophilia and airflow obstruction, *J. Clin. Invest.* 128 (2018) 997–1009.