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Goblet cell hyperplasia as a feature of neutrophilic asthma

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Summary

Background: Goblet cell hyperplasia (GCH) is a pathological finding classically reported across asthma severity levels and usually associated with smoking. Multiple biological mechanisms may contribute to excessive mucus production.

Objective: We aimed to decipher the clinical meanings and biological pathways related to GCH in non-smokers with asthma.

Methods: Cough and sputum assessment questionnaire (CASA-Q) responses at entry and 1 year later were compared to clinical and functional outcomes in 59 asthmatic patients. GCH was assessed through periodic-acid shift (PAS) staining on endobronchial biopsies obtained at entry in a subset of 32 patients.

Results: Periodic-acid shift-staining analysis revealed a double wave distribution discriminating patients with (>10% of the epithelial area) or without GCH. CASA-Q scores were mostly driven by overall asthma severity ($P < 0.0001$). CASA-Q scores remained stable at 1 year and were independently associated with BAL eosinophil content irrespective of the presence of GCH. GCH was unrelated to the presence of bronchiectasis at CT, GERD or chronic rhinosinusitis, but correlated well with neutrophilic inflammatory patterns observed upon BAL cellular analysis ($P = 0.002$ at multivariate analysis). BALF bacterial loads were unrelated to GCH or to CASA-Q.

Conclusions and Clinical Relevance: Goblet cell hyperplasia is disconnected from chronic cough and sputum when assessed by a specific questionnaire. GCH is related to neutrophilic asthma whereas symptoms are related to airway eosinophilia. The clinical counterpart of GCH is unlikely assessed by the CASA-Q.

KEYWORDS

asthma, goblet cell hyperplasia, mucus

1 | INTRODUCTION

Chronic bronchitis, defined as recurrent and prolonged cough and sputum production, is commonly associated with COPD, though its clinical significance remained controversial.¹ Interestingly, cough and chronic mucus production are non-specific symptoms also often reported by asthmatic patients. Moreover, autopsy reports

of fatal asthma cases have reported excessively narrowed airways completed by spectacular mucus casts.^{2,3} Increased goblet cells and MUC5AC airway epithelial staining in asthma have been described before and this is now clearly acknowledged as a significant component of asthma pathophysiology.⁴⁻⁶ Interestingly, these outcomes are usually not assessed during asthma care, nor in clinical trials.

New insights into the mechanisms involved in excessive mucus production and goblet cell hyperplasia suggest a predominant role for IL-13 and T2 related inflammation.^{7,8} Unfortunately, most of the IL-13 targeting mAbs failed to improve severe asthma outcomes.^{9,10} In patients with high levels of periostin as a surrogate marker of T2-driven asthma, FEV1 improvements were reported with lebrikizumab—a monoclonal antibody directed against IL13, but no data were provided in terms of prevention or changes in mucus hypersecretion.¹¹ Likewise, no data related to mucus hypersecretion were provided in both tralokinumab or dupilumab recent clinical trials reports.

Symptoms of mucus hypersecretion are mostly subjective and may reflect not only airway goblet cell hyperplasia (GCH) or bronchial gland enlargement but also other mechanisms such as post-nasal drip or GERD, which are likely to be confounding sources.¹² Endoscopists familiar with the assessment of chronic airway diseases often describe discrepancies between reported symptoms and endoscopic findings (high level of complaint but no secretion found in the airways and vice versa).¹³

These symptoms may fluctuate with the level of airway inflammation and whether it should be considered as a phenotypic characteristic remains unknown.¹⁴ Interestingly, the “Cough And Sputum Assessment Questionnaire” (CASA-Q) has been developed in COPD in order to standardize the quantification of these symptoms.¹⁵

In the present study, we investigated the relationships between the potential clinical phenotype of cough and mucus production in asthma with various clinical, pathological and biological parameters in order to assess its potential relevance for pinpointing diagnoses and management.

2 | MATERIAL AND METHODS

2.1 | Patients

In 2010-2012, we enrolled 59 patients with asthma referred to our specialized tertiary expert centre for asthma management who consented to participate in the COBRA cohort (IDRCB:2008-A00294-51).¹⁶ Thirty-two of them consented to undergo bronchoscopy examination. The present study was approved by an independent Ethics Committee (*Comité de Protection des Personnes d'Ile de France I*; reference number 09-11962) and written-informed consent was obtained for all participants at inclusion before any procedure. Active or past smoking (with a smoking history of 10 pack-years or more) was exclusion criteria. The flow chart for the present study is detailed in Figure 1.

In addition to all the demographic, clinical and physiologic parameters recorded during routine visits, CASA-Q forms were filled out by the patients before bronchoscopy during the initial visit and again at the second visit (1 year later). CT scans were also routinely performed at entry, and the presence of bronchiectasis (defined as a bronchus to vessel diameters ratio greater or equal to 1.5 and then quoted as present or absence by the expert radiologist blinded to any clinical parameters) was recorded for this specific study.

2.2 | Bronchoscopy

Briefly, a flexible bronchoscope was inserted by the nose under local anaesthesia. A BAL was performed according to routine practice in the right lower lobe by instillation and gentle suction of 50 mL of isotonic saline (twice). Endobronchial biopsies were taken in the left lower lobe as previously described.¹⁷ Two biopsies were immediately stored in formalin for subsequent paraffin-embedding. No safety issues arose during this study.

BAL samples were routinely assessed in order to measure total and differential cells counts and perform routine bacterial cultures. Bacterial cultures were considered positive only when a respiratory pathogen grew at a significant ($\geq 10^3$ cfu/mL) concentration.

2.3 | Staining procedure

Formalin-fixed, paraffin-embedded airway tissue sections (4 μ m) were used for staining. Sections were dewaxed and rehydrated. For morphology, we used Haematoxylin (TM) and Periodic-acid shift (PAS) specific staining to analyse epithelium and RBM thicknesses and mucous cell presence, respectively.

Pictures were obtained using a Nikon Eclipse NiE microscope equipped with a DS-Ri2 camera (Nikon, Tokyo, Japan). Images were captured at room temperature with NIS-Elements software (Nikon; version 4.3) at a resolution of 4908 \times 3264 pixels per manual exposure with fixed shutter time, gain amplification and illumination.

2.4 | Measurement of basal membrane, epithelial thickness and mucus production

Measurements of epithelium and basement membrane thickness were determined and were expressed as the average area/length ratio by using Wilson's method.^{18,19} In brief, a length of 1 mm of basement membrane at least was measured at $\times 40$ magnification by delineating the area and the length of the BM (corresponding more or less to 5 fields, where the orientation was estimated to be correctly perpendicular given the monolayer aspect of the surrounding

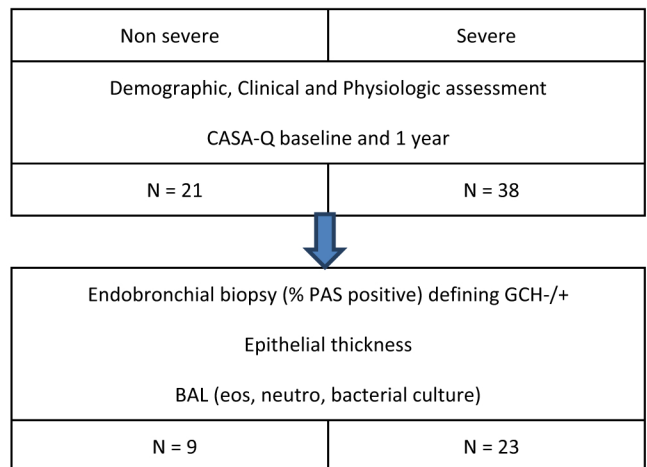


FIGURE 1 Flow chart

epithelium). PAS-staining quantification corresponded to the area of stained bronchial surface epithelium weighted by the bronchial epithelium thickness and was expressed as a percentage of staining intensity per μm using colour segmentation. All measurements were generated with an image analysis program (ImageJ; National Institutes of Health, Bethesda, Md).

2.5 | Statistical analysis

CASA-Q questionnaires were quantified according to the initial report¹⁵ (low scores denoting high levels of symptoms) and variability was assessed using paired *t* tests and linear R^2 regression

coefficients. We also tested the first domain of the CASA-Q score (dedicated to daily sputum production, whereas other domains address the burden of chronic sputum production, the level of cough and its impact).

Patients were divided by the intensity of the PAS staining as a GCH- and GCH+ group since a bimodal distribution clearly identified a clear cut-off value of 10% of the epithelial area stained by PAS.

Between-group comparisons were assessed using a Mann Whitney U test. Rho's Spearman correlation coefficients were computed when required. Data were presented as mean and standard deviation when normally distributed and as median and interquartiles otherwise.

TABLE 1 Patient characteristics

	Non-severe	Severe	P Value
N	21	38	
Age	53 (27)	51 (16)	0.52
Gender (% F)	57	65	0.71
Median BMI (interquartiles)	27 (24-31)	27 (24-29)	0.84
Disease duration (y)	33 (30)	26 (15)	0.12
Median age at onset (interquartile) (y)	36 (16-46)	27 (20-34)	0.16
Formerly smokers (%yes)	24	21	1
Smoking history (P/Y)	5.6 (2.5)	2 (1)	0.58
Exacerbations in the last 12 mos	0.5 (1)	2.9 (2)	<0.01
Bronchiectasis at CT (% yes)	43	50	0.80
GERD (% yes)	80	75	0.75
Rhinosinusitis (% yes)	86	92	0.66
Allergy (% with at least one positive skin prick test)	48	58	0.63
Positive bacterial BAL culture (%)	1 (5%)	3 (8%)	0.99
Blood eosinophils (cell/mm ³)	206 (145)	450 (590)	<0.01
Inhaled steroids (% with daily dose > 1000 μg eq beclo)	33	97	<0.01
Maintenance OCS (% yes)	0	50	<0.01
Median OCS daily dose mg/d (interquartile)	0	10 (20)	<0.01
LAMA n (% yes)	2 (9)	7 (18)	<0.01
SAMA n (% yes)	0 (0)	23 (61)	<0.01
LTRA n (% yes)	2 (9)	6 (16)	
Macrolides n (% yes)	0	3 (8)	0.61
FEV1 (% predicted value)	92 (30)	70 (25)	<0.01
FVC (% predicted value)	98 (24)	80 (22)	<0.01
VR (% predicted value)	118 (32)	141 (40)	<0.01
Reversibility (%)	5 (12)	12 (8)	<0.01
BAL Cellularity (1000 cell/mL)	107 (50.5)	153 (103)	<0.01
Eosinophils (%)	1.5 (2.25)	3.1 (4)	0.13
Neutrophils (%)	10 (14.7)	18 (19)	0.05
Lymphocytes (%)	6 (7.5)	8 (6)	0.12
Macrophages (%)	74 (15.2)	64 (27)	0.03
CASA-Q score at entry	62.2 (3.5)	33.9 (13)	<0.01
CASA-Q score at 1 y	62.8 (7)	33 (12)	<0.01

Data are presented as median with (interquartiles ranges) or percentages.

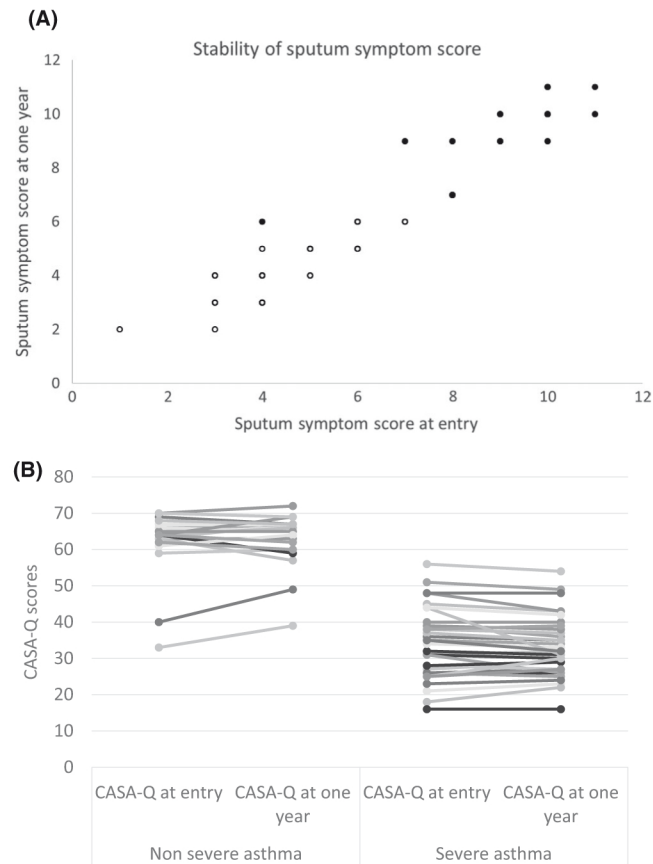


FIGURE 2 A, Linear regression between sputum symptom score at entry and at 1 y in severe (open circles) and non-severe (black circles) asthma patients. B, Stability of CASA-Q total score at entry and 1 y later among non-severe (left panel) and severe (right panel) asthma patients. (colour changes are just intending to improve clarity)

3 | RESULTS

Patient characteristics are presented in Table 1. Fifty-nine patients were enrolled and had complete follow-up at 1 year. Thirty-eight (64.5%) patients presented severe asthma as defined by the ERS/ATS task force: these patients were poorly controlled, with two exacerbations or more in the last 12 months while receiving a GINA step 4/5 treatment (half required a maintenance regimen with daily oral steroids). All others had non-severe asthma controlled with treatment below step 4 of the GINA scale (Table 1). CASA-Q values at entry and 1 year later showed high stability. Higher CASA-Q and “sputum domain” scores were recorded in severe versus non-severe asthmatics (Figures 2A,B). BAL culture was found positive for a respiratory pathogen in three severe and one non-severe asthma patients. No fungi were found.

Periodic-acid shift-staining analysis (Figure 3A) demonstrated a bimodal distribution distinguishing one group with stained areas below 10% (called GCH-) and one group with stained areas >10% (called GCH+). CASA-Q scores (both at entry and 1 year later) were not significantly different between the two groups (Figure 3B).

Patients with GCH had higher BAL neutrophil content (expressed as a percentage of total BAL leucocytes count), and thicker airway epithelia ($P < 0.001$). Absolute leucocytes counts differed between groups: BAL eosinophils 79.5 (79.9) in GCH+ vs 14.1 (17.8) in GCH- and BAL neutrophils 5.6 (3.8) vs 9.6 (12.5). Results were expressed as median and interquartile values.

Univariate and multivariate analysis were conducted to identify factors related to¹ CASA-Q at entry and 1 year later, and² GCH (Table 2). Interestingly, only BAL eosinophil content (expressed as a percentage of total BAL leucocytes count) was significantly and independently related to CASA-Q at entry, such that BAL eosinophilia was protective vis-à-vis cough and sputum symptoms. This was confirmed 1 year later, where asthma severity was found as an additional predictor. Age at onset did not affect any of these outcomes. In contrast, GCH based on PAS staining of the endobronchial biopsy was found to be independently associated with BAL neutrophilia (expressed as a percentage of total BAL leucocytes count). When patients were categorized as neutrophilic or eosinophilic according to classically reported thresholds (neutrophilic when >61% and eosinophilic when >1.9%), exact Fisher's tests failed to detect any relationship between GCH and the so-called inflammatory phenotype. Nonetheless, BAL neutrophilia significantly differed between groups ($28.0\% \pm 14.6$ vs $8.6\% \pm 6.2$, $P < 0.001$). Of note, BMI was not associated with the neutrophilic trait (26 ± 5 kg/m² in eosinophilic vs 29 ± 7 kg/m² in neutrophilic asthma patients, $P = 0.27$). Noteworthy, positive BAL bacterial culture, bronchiectasis at CT, GERD and rhinosinusitis were not significantly associated with GCH.

4 | DISCUSSION

Cough and sputum are troublesome symptoms often reported by asthmatic patients. In order to address the specificity of this phenotype, we queried chronic mucus production in lifelong non-smoking asthmatic patients. Interestingly, we found that the clinical phenotype assessed by measuring the cough and sputum questionnaire CASA-Q was very stable over 1 year, mostly associated with asthma severity. Moreover, BAL eosinophilia was found to be protective. Surprisingly, CASA-Q scores were not related to Goblet Cell Hyperplasia (GCH) assessed by the percentage of positive staining of goblet cells on endobronchial biopsies. Nor was it related to the ability of reconstituted ALI cultures to release mucin in vitro. Moreover, rhinosinusitis, GERD, bronchiectasis or the presence of bacteria in the BAL fluid were not related to this phenotype. Surprisingly, GCH was related to BAL neutrophilia.

Chronic mucus hypersecretion is classically seen as a feature of COPD and might be seen as an overlapping criterion between asthma and COPD,²⁰ whereas key studies demonstrated that GCH is a pathophysiological feature of asthma.⁴⁻⁶ Asthmatic patients often complain about these symptoms, and we found that it was associated with severe asthma. The clear discrimination between severe

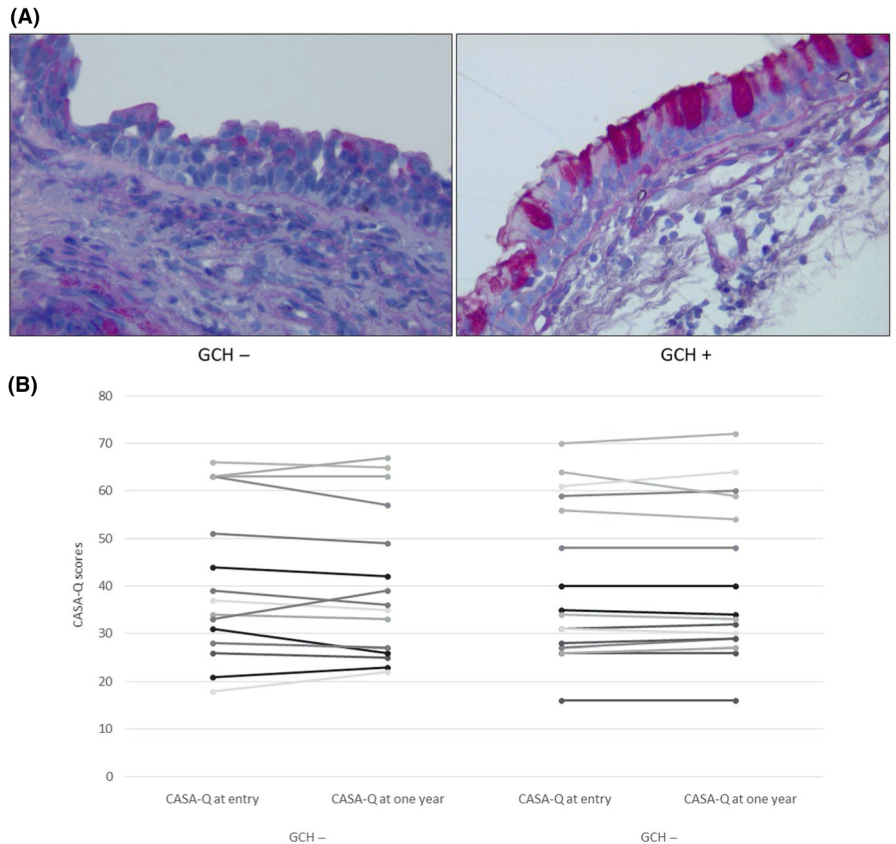


FIGURE 3 A, PAS staining in GCH+ and GCH- (representative pictures). B, Stability of CASA-Q total score at entry and 1 y later among patients without (left panel) and with (right panel) goblet cell hyperplasia (GCH). (colour changes are just intending to improve clarity)

asthma and COPD is not established according to symptoms or imaging parameters.²¹ For example, bronchiectasis is described on HRCT-scan images both in COPD and asthma.²²

Whereas T2 inflammation mostly through IL13 signalling is known to affect mucus hypersecretion in asthma, other known pathways such as EGFR appear more relevant to COPD, but here again overlap may exist.^{1,23,24} Indeed, in the SPIROMICS analysis, no relationships between T2 biomarkers and mucin concentration assessed in the induced sputum were found.

The CASA-Q questionnaire was highly stable over 1 year. This finding was expected according to clinical experience and previous reports. Indeed, patients were included and interviewed at steady state in order to limit the risk that an exacerbation could bias the responses. Although the four domains of the CASA-Q explore different items, the domain specifically related to sputum production was highly correlated to the total score, and so both analyses were found to provide exactly the same results. It seems that coughing is highly related to sputum complaints, even in asthma (at least in this population). Mucus biophysical properties including viscosity, elasticity and tenacity are likely more closely related to Patient Reported Outcomes and clearly deserves more focused studies using clinically approved rheometers.^{25,26}

Interestingly, BAL eosinophil counts expressed in percentages were unrelated with sputum production symptoms, whereas it is now considered as a surrogate T2 marker. GCH is in the predictive T2 model thought to occur especially as a consequence of excess of IL13.⁸ This is surprising given that IL-13, IL-5 and eotaxin are

released by T2 cells (both ILC2 and Th2) and are involved in the recruitment and activation of eosinophils. Previous studies^{6,27,28} have reported an association between sputum eosinophils and airway mucus in stable asthma. Charcot Leyden crystals have the potential to explain the link between T2 airway inflammation and mucus rheological properties.²⁹ Unfortunately, chronic mucus hypersecretion remained unaddressed in all recent trials that tested biologics directed towards T2 cytokines, including the anti IL-13 monoclonal antibodies lebrikizumab, tralokinumab and less specifically dupilumab. Indeed, most of the current literature associates mucus hypersecretion with viral and/or bacterial infection,^{30,31} bronchiectasis or with neutrophilic asthma.³² Noteworthy, neutrophilic asthma is frequently presented as a manifestation of asthma-COPD overlap, or associated with smoking asthma. LAMA or macrolides are potentially interfering with chronic mucus secretion, but the present study could not address this interesting issue; it clearly deserves additional studies. Macrolides were thought to interfere more with neutrophilic asthma,³³ but finally a significant reduction in the exacerbation rates were observed irrespective of the presence of T2-surrogate biomarkers.³⁴

As mentioned above, although BAL eosinophils appeared unrelated with high sputum symptom scores, BAL neutrophilia was associated with GCH.

Whether these are coincident findings, or inter-twined variables, or consequences of a common physiopathological pathway, remains unknown. Of note, targeting neutrophils in asthma was shown to improve certain asthma outcomes.³⁵

TABLE 2 Multivariate analyses identifying factors independently related to CASA-Q at entry and at 1 y, then to the presence of goblet cell hyperplasia (GCH) and mucin production in air-liquid interface (ALI) cultures

	Estimate	Std. Error	P-value
CASA-Q at entry			
(Intercept)	60.317	7.492	<0.001
Age (y)	0.063	0.098	0.522
Bronchiectasis	1.74	2.733	0.527
Severe vs non-severe	-30.681	3.653	<0.001
Duration of asthma	-0.017	0.102	0.871
Age at onset (y)	3.455	0.011	0.244
Exacerbations last 12 mos	0.84	1.004	0.407
GERD	-3.851	2.99	0.204
Rhinosinusitis	-1.566	4.247	0.714
Pack-years	0.2	0.169	0.242
BAL eosinophils (% leucocytes)	1.007	0.411	0.018
BAL neutrophils (% leucocytes)	-0.073	0.123	0.555
Positive bacterial BAL culture	3.98	6.617	0.550
CASA-Q at 1 y			
(Intercept)	60.24	6.196	<0.001
Age (y)	0.092	0.081	0.263
Bronchiectasis	1.056	2.261	0.643
Severe vs non-severe	-31.368	3.021	<0.001
Age at onset (y)	0.964	0.01	0.488
Duration of asthma (y)	-0.028	0.084	0.741
Exacerbations in the last 12 mos	0.716	0.831	0.393
GERD	-3.137	2.473	0.211
Rhinosinusitis	-1.676	3.513	0.635
Pack-years	0.15	0.14	0.288
BAL eosinophils (% leucocytes)	0.948	0.34	0.008
BAL neutrophils (% leucocytes)	-0.111	0.102	0.283
Positive bacterial BAL culture	5.082	5.473	0.358
Presence of GCH			
(Intercept)	-9.353	10.678	0.392
Age (y)	0.007	0.097	0.945
Bronchiectasis	0.637	2.443	0.797
Severe vs non-severe	7.855	5.267	0.152
Duration of disease (y)	-0.058	0.095	0.550
Age at onset (y)	6.022	1.022	0.322
Exacerbations in the last 12 mos	-1.572	0.83	0.073
GERD	2.291	2.823	0.427
Rhinosinusitis	0.196	3.621	0.957
Pack-years	-0.025	0.184	0.894
CASA-Q at 1 y	0.168	0.142	0.250
BAL eosinophils (% leucocytes)	0.002	0.404	0.997
BAL neutrophils (% leucocytes)	0.407	0.115	0.002
Positive bacterial BAL culture	-10.365	6.339	0.118

Airway mucus is a complex assembly of mucin, water, salts, inflammatory cells, desquamated epithelial cells, bacteria and exogenous particles, and the relative contribution of each of these components to symptoms is probably highly subject to variations among patients and diseases.

We further explored the symptoms reported by the patients and found that despite their stability, they were unrelated to any specific comorbid condition, such as GERD or rhinosinusitis.

The obvious limitation of the present report is the subjectivity of the symptoms reporting, even though a validated questionnaire was used. Other limitations include that GERD was rated according to patient declaration and CT scans were not quantitatively assessed.

Airway inflammation was presently assessed in the BAL, whereas classically asthma endotypes are determined from induced sputum samples³⁶ and it is rather unknown how the two overlap. We should also recall that patterns of airway inflammation are variable spontaneously or with corticosteroids,^{37,38} whereas the present study was based on a unique sample. Whether sputum neutrophil percentages or absolute numbers are more biologically relevant is worth a debate for the present study but also in general. It can be argued that mediator concentration in sputum would depend more on absolute rather than relative numbers³⁹ but this could not be confirmed in the present study.

The other potential explanation for our negative findings is that we could not address the bronchial gland contribution to symptoms. Finally, one may hypothesize that a little bit of each of these potential contributors may participate in sputum symptoms (ie, a little from the airway epithelium, a little from the nose and a little from the gut, for example), and in the end we believe that mucus hypersecretion should be seen as a component of all traits to be addressed in a future precision medicine strategy. Given our results, the CASA-Q is not adapted for achieving this goal.²¹

In conclusion, chronic mucus hypersecretion complaints among lifelong non-smoking (or very mild smoking) asthmatics are frequently seen in severe patients. Although very stable, this feature was unrelated to biopsy GCH phenotype. Interestingly, *in our study the neutrophilic phenotype was associated* with GCH, suggesting that this specific *phenotype* needs to be specifically targeted in clinical trials. Better understanding the mechanisms related to GCH phenotypes may offer new therapeutic avenues.

AUTHOR CONTRIBUTION

KA included patients, performed samples, assessed data, prepared and reviewed the MS. AB supervised and analysed data, participated in the study design, reviewed statistical analysis and wrote the MS. CV, CG, CT, JC and IV performed assays and reviewed the MS. PC and DG designed the study, assessed the data and reviewed the MS.

CONFLICT OF INTEREST

Pr. Chanez reports grant support and personal fees from Boehringer Ingelheim, GlaxoSmithKline, AstraZeneca, Novartis, Teva, Chiesi,

Boston Scientific and ALK, personal fees from Johnson & Johnson, Merck Sharp & Dohme, Sanofi, SNCF, Centocor and Almirall, grant support from Roche outside the submitted work. Pr. Bourdin reports personal fees, non-financial support and other support from Astra Zeneca, grant support, personal fees, non-financial support and other support from GlaxoSmithKlein, grant support, personal fees, non-financial support and other support from Boehringer Ingelheim, personal fees, non-financial support and other support from Novartis, personal fees and other support from Teva, personal fees and other support from Regeneron, personal fees, non-financial support and other support from Chiesi Pharmaceuticals, personal fees, non-financial support and other support from Actelion, other support from Gilead, and personal fees and non-financial support from Roche outside the submitted work. Mrs Vernisse, Mrs Garulli, Dr Alagha, Dr Tummino, Dr Charriot, Dr Vachier, Dr Gras report having nothing to disclose.

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