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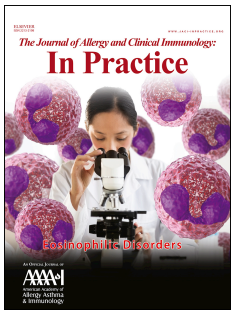

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Longitudinal Impact of Sputum Inflammatory Phenotypes on Small Airway Dysfunction and Disease Outcomes in Asthma

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78

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Abstract

Background: Little is known about the relationship between airway inflammatory phenotypes and some important asthma features such as small airway dysfunction (SAD).

Objective: to describe the longitudinal impact of airway inflammatory phenotypes on SAD and asthma outcomes

Methods:
We measured eosinophil and neutrophil counts in induced sputum at baseline and one year later to stratify 197 adult asthma patients into four inflammatory phenotypes. We conducted a comprehensive assessment of lung function using spirometry, body plethysmography, impulse oscillometry, inert gas single and multiple breath washouts. We compared lung function, asthma severity, exacerbation frequency and symptom control between the phenotypes. We studied the longitudinal impact of persistent sputum inflammatory phenotypes and the change of sputum cell counts on lung function.

Results:
Patients were stratified into eosinophilic (23%, n=45), neutrophilic (33%, n=62), mixed granulocytic (22%, n=43), and paucigranulocytic (24%, n=47) phenotypes. Eosinophilic and mixed granulocytic asthma patients had higher rates of airflow obstruction and severe exacerbation as well as poorer symptom control than paucigranulocytic asthma patients. All SAD measures were worse in eosinophilic and mixed than in paucigranulocytic asthma patients (all p-values <0.05). Eosinophilic asthma also indicated worse distal airflow obstruction, increased ventilation inhomogeneity (all p-values <0.05), and higher tendency for severe exacerbation (p= 0.07) than neutrophilic asthma. Longitudinally, persistent mixed granulocytic asthma was associated with the worst follow-up measures of SAD compared to persistent neutrophilic, persistent paucigranulocytic or non-persistent asthma phenotypes. In patients with stable FEV1, the mean increase in small airway resistance (R5-20) was greater in persistent mixed granulocytic patients (+103%) than in patients with persistent neutrophilic (+26%), p=0.040, or persistent paucigranulocytic asthma (-41%), p=0.028. Multivariate models adjusted for confounders and treatment with inhaled or oral corticosteroids or anti-eosinophilic biologics indicated that the change of sputum eosinophil rather than neutrophil counts is an independent predictor for the longitudinal change in FEV1, FEF_{25-75}, sReff, RV and LCI.

Conclusion:
In asthma, airway eosinophilic inflammation is the main driver of lung function impairment and poor disease outcomes, which might also be aggravated by the coexistence of airway neutrophilia to confer a severe mixed asthma phenotype. Persistent airway eosinophilia might be associated with dynamic SAD even in patients with stable FEV1.

142
143
144 ## Highlights box:

145

146 **What is already known about this topic?**

147 Airway inflammatory patterns indicate differences in clinical asthma features and phenotypes.

148

149 **What does this article add to our knowledge?**

150 • In asthma, eosinophilic airway inflammation is the main driver of SAD and the subsequent poor asthma
151 outcomes.
152 • The coexistence of airway neutrophilia aggravates the impact of eosinophilic airway inflammation on lung
153 function and confers a severe mixed granulocytic asthma phenotype.
154 • Airway eosinophil rather than neutrophil count is the independent predictor of the longitudinal change in
155 all lung function measures, even in patients who are being treated with inhaled or oral corticosteroids or
156 anti-eosinophilic biologics.
157 • Persistent airway eosinophilia was associated with dynamic small airway changes even in patients with
158 stable FEV1.

159

160 **How does this study impact current management guidelines?**

161 In patients with asthma, SAD should prompt the investigation of airway eosinophilia, either directly or via
162 surrogate markers, even in patients who are under eosinophils targeting therapies. In future clinical trials that
163 are investigating eosinophils targeting therapies, the addition of small airway function markers to the routinely
164 used FEV1 might be more appropriate for the evaluation of lung function. Further research elucidating the
165 potential role of eosinophil-neutrophil interaction in the pathophysiology of asthma is warranted.

166

167 **Key words:**

168 Eosinophilic asthma, mixed granulocytic asthma, airway inflammation, small airway dysfunction

169

170 **Abbreviations:**

171 ACT: asthma control test

172 FEF: forced expiratory flow

173 FeNO: fractional exhaled nitric oxide

174 FEV1: forced expiratory volume in one second

175 LCI: lung clearance index

176 RV: residual lung volume

177 SAD: small airway dysfunction

178 ## Introduction

179

180 Asthma is a heterogeneous disease that comprises variable airway inflammatory phenotypes (1).
181 Identifying these phenotypes is a cornerstone in understanding the pathophysiology of asthma and
182 in the development of targeted asthma therapy (2). In this context, induced sputum cell count allows
183 a viable noninvasive assessment of asthmatics airway inflammation (3). Based on sputum eosinophil
184 cell count, asthma can be broadly classified as eosinophilic or non-eosinophilic (4). When considering
185 the count of sputum neutrophils in this classification, eosinophilic asthma can be further subdivided
186 into eosinophilic or mixed granulocytic, while non-eosinophilic asthma can be subdivided into
187 neutrophilic or paucigranulocytic (4). Although that both eosinophils and neutrophils, separated or
188 combined, have been incriminated in asthmatics airway inflammation (5, 6), they might have
189 different impact on asthma severity, symptom control and lung function impairment (4). For
190 instance, a recent study has shown that a predominant mixed granulocytic asthma indicates worse
191 lung function (FEV1) than the other phenotypes (7). However, studies on this matter were either
192 cross-sectional (4, 8, 9), or have reported the impact of different asthma phenotypes on airflow
193 obstruction only (3, 7), leaving the relationship between asthma inflammatory phenotypes and a
194 wide spectrum of lung function measures, such as measures of small airway dysfunction, largely
195 unexplored. Small airway dysfunction is a highly prevalent feature of asthma that has been linked to
196 disease severity, poor symptom control, frequent exacerbation and physical inactivity (10–12). Small
197 airway dysfunction entails a spectrum of interrelated distal lung function impairments including
198 increased small airway resistance, decreased lung elastance, the subsequent limitation of airflow in
199 the peripheral airways, air trapping, and ventilation inhomogeneity (12). In light of the increasing
200 recognition of the significant role of small airway dysfunction in asthma, it is important to investigate
201 whether different asthma phenotypes confer different associations with markers of small airway
202 dysfunction. Moreover, longitudinal data are required to elucidate the impact of airway
203 inflammation on small airway dysfunction and the ensuing asthma outcomes.

204 Therefore, in this study, we sought to investigate the association between different asthma
205 phenotypes, as defined by sputum cell count, and markers of small airway dysfunction. Moreover,
206 we aimed to explore the longitudinal impact of sputum cell counts on the one-year change in lung
207 function and asthma control.
208

209 ## Methods

210 ***Study design***

211 Eligible subjects were adults with asthma who participated in the observational multicenter All Age
212 Asthma Cohort (ALLIANCE), a longitudinal cohort of pediatric and adult asthma patients, initiated by
213 the German Centre for Lung Research (DZL). The study was approved by the ethics committee at the
214 medical school-Luebeck university (Az.21-215) and is registered at clinicaltrials.gov (adult arm:
215 NCT02419274). Written informed consent was obtained before enrollment. This analysis included
216 adult patients with mild to severe asthma in whom sputum induction was performed at baseline visit
217 and after one-year of follow-up. The participants had to have stable disease without the presence of
218 acute exacerbations or respiratory tract infections within 4 weeks prior to any study visit. Detailed
219 information on recruitment, inclusion and exclusion criteria of the ALLIANCE cohort were described
220 previously (13).
221

222 ***Airway physiology characteristics***

223 We performed body plethysmography, impulse oscillometry (IOS), single and multiple breath
224 washout (SBW, MBW), followed by forced spirometry in accordance with the latest ERS
225 recommendations (14–17). We studied upper airway obstruction using values of forced expiratory
226 volume in the first second (FEV1), its ratio to the forced vital capacity (FVC), and the IOS- defined
227 airway resistance at 20 Hz (R20). Airflow obstruction was defined as FEV1/FVC less than the lower
228 limit of normal (LLN) (18). Regarding the longitudinal change in FEV1, the latest European Respiratory
229 Society/American Thoracic Society recommendations indicate that in long-term (i.e. ≥1 year), a
230 change of 15% or more in the FEV1 is with high confidence clinically meaningful (19).

231 Accordingly, patients who had a relative one-year change of less than 15% were classified as stable
232 FEV1. Markers of small airway dysfunction were: spirometric mean forced expiratory flow at 50% and
233 between 25% and 75% of the forced vital capacity (FEF50%, FEF25–75%), residual lung volume (RV %)
234 and the specific effective airway resistance (sReff %) from body plethysmography, small airway
235 resistance (R5Hz-20Hz, kPa/L/s)) and lung elastance indicated by the resonance frequency from IOS.
236 Further measures of small airway dysfunction were markers of ventilation inhomogeneity i.e. the
237 phase III slope (delta N_2/l) derived from N_2 SBW and the lung clearance index (LCI) measured by N_2
238 MBW test.
239

240 ***Sputum induction and patients' stratification***

241 Sputum induction and processing was done in patients who had a predicted FEV1 of ≥50% following
242 standardized procedures as previously described (20). In summary, the patients inhaled hypertonic
243 saline in ascending concentration (i.e. 3%, 4% and 5%) each for 7 minutes and the induction was
244 discontinued if FEV1 fell by more than 20%. Sputum plugs were collected from all inhalation periods
245 and then pooled, weighed, and treated with four volumes of 0.1% dithiothreitol (DTT, Sputolysin®;
246 Calbiochem, Bad Soden, Germany). Subsequently, total cell counts were determined by
247 haemocytometer and trypan blue staining, (Sigma, Deisenhofen, Germany), and differential cell
248 counts were analyzed on Diff-Quick-stained cytospin preparations (21). Cytospin slide quality was
249 evaluated based on cell morphology, amount of cellular debris and squamous cell contamination and
250 rated using a 5-point scale (low to high: 0, 0.5, 1, 1.5, 2) (22). Samples with slide quality of ≤ 0.5 were
251 excluded from the analysis. Cutoffs of ≥2% and ≥50% were used to define eosinophilic and
252 neutrophilic asthma, respectively (7). Eosinophilic asthma (eosinophils ≥2%) was further subdivided
253 into *eosinophilic* (neutrophils <50%) or *mixed* (neutrophils ≥50%) asthma phenotypes. Likewise, non-
254 eosinophilic asthma (eosinophils <2%) was also subdivided into *neutrophilic* (neutrophils ≥50%) or
255 *paucigranulocytic* (neutrophils <50%) asthma phenotypes. For the longitudinal analysis, patients with
256 eosinophils ≥2% or neutrophils ≥50% at both baseline and follow-up were classified as *persistent*

257 *eosinophilic* or *persistent neutrophilic*, respectively. Patients who had both eosinophils ≥2% and
258 neutrophils ≥50% at baseline and follow-up were classified as *persistent mixed*, while patients who
259 had neither persistent eosinophilia nor persistent neutrophilia or who had persistent
260 paucigranulocytic asthma were classified as *non-persistent/persistent paucigranulocytic* asthma. We
261 compared lung function and asthma outcomes between these phenotypes at baseline and follow-up.
262

263 ***Asthma severity and asthma control***

264 Severe asthma was defined according to European Respiratory Society/American Thoracic Society
265 recommendations (24). Asthma control was assessed based on self-reported symptoms from the
266 asthma control test (ACT) and the frequency of severe exacerbations during the 12 months preceding
267 a study visit, defined as a burst of systemic corticosteroids for at least 3 days (25).
268

269 ***Statistical analysis***

270 We used one-way analysis of variance, Kruskal Wallis or Fisher exact test to determine the
271 significance of differences among clinical variables between the study groups. For pairwise
272 comparisons, post-hoc analyses were done using either Tukey's test or Dunn's test with Bonferroni
273 correction. To test for statistical dependence between two continuous variables, we used Pearson's
274 test and for skewed variables the Spearman's rank test. We used multivariate linear regressions to
275 determine whether the changes in sputum eosinophil or neutrophil counts is an independent
276 predictor for the longitudinal change in lung function even after adjustment for asthma therapy.
277 Statistical analyses were performed using R (version 3.6.2, R Foundation, Vienna, Austria). An alpha
278 error of less than 5% was considered statistically significant.
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283

284 **Results**

285

286 We performed sputum induction in 214 patients. Sputum samples from 17 patients were excluded
287 from the analysis due to poor slide quality. Overall, 197 patients were included at baseline (mean
288 age, 51 ±18 years; 47% male; 50.2% severe asthma; 88.2% inhaled corticosteroids; 22.8% systemic
289 corticosteroids; 13.2% anti-T2 biological therapy). Detailed demographic and baseline clinical
290 characteristics of the subjects stratified according to sputum inflammatory phenotypes are shown in
291 Table 1. Based on predetermined cutoffs of ≥2% for sputum eosinophils and ≥50% for neutrophils,
292 the frequencies of sputum eosinophilia (45%, *n=88*) and sputum neutrophilia (*53%, n=105*) were
293 comparable. Patients were stratified into: eosinophilic (23%, *n=45*), neutrophilic (33%, *n=62),* mixed
294 (22%, *n=43*), and paucigranulocytic (24%, *n=47*) asthma phenotypes. Despite some difference in the
295 percentage of sputum eosinophils between patients with eosinophilic and mixed asthma, they
296 expressed similar absolute counts of eosinophils and varied in neutrophil counts only (Table 1).
297 Additional markers of T2-inflammation (blood eosinophils, FeNO, serum IgE) were significantly higher
298 in eosinophilic and mixed asthma phenotypes than in non-eosinophilic asthma phenotypes,
299 confirming a T2-high inflammation in sputum-based eosinophilic asthma phenotypes (Table 1).
300 Nonetheless, we also observed some increase of T2-markers in patients who were stratified as
301 neutrophilic asthma indicated by the third quartile values of blood eosinophils (230, 10^3/µl), FeNO
302 (28, ppb) and serum IgE (292, ku/l), (Table 1). Patients with both eosinophilic asthma phenotypes
303 (eosinophilic and mixed granulocytic) were older than patients with paucigranulocytic asthma.
304 Otherwise, there were no statistically significant differences between these phenotypes with regard
305 to gender, body mass index or smoking status despite that the neutrophilic phenotype showed a
306 tendency to be associated with overweight and current smoking. Both eosinophilic asthma
307 phenotypes were associated with higher frequencies of severe asthma, higher frequencies of airflow
308 obstruction, worse ACT scores, higher rates of severe exacerbations and higher use and dose of
309 systemic corticosteroids compared with paucigranulocytic asthma (Table 1).

310 Also, when compared to patients with neutrophilic asthma, patients with mixed granulocytic asthma
311 showed a higher tendency to have airflow obstruction (p=0.09) and patients with eosinophilic
312 asthma had a higher rate for acute severe exacerbation (p= 0.042). However, airflow obstruction or
313 rates of acute severe exacerbation were not significantly higher in neutrophilic than in
314 paucigranulocytic asthma patients, p=0.52 and p=0.35, respectively. Moreover, increased sputum
315 eosinophils indicated more severe small airway dysfunction (Figure 1; see Table E1 in this article's
316 Online Repository). Compared to patients with paucigranulocytic asthma, patients with eosinophilic
317 or mixed granulocytic asthma had increased distal airflow obstruction (FEF_{25-75}%, FEF_{50}%), increased
318 small airway resistance (R_{5-20}, sReff %), decreased lung elastance (resonance frequency), increased air
319 trapping (RV %) and ventilation inhomogeneity (LCI, delta N_2/l), (all adj. p-values <0.05, except for R5-
320 20 showed only a tendency, p=0.08). In addition, measures of FEF_{25-75}% and delta N_2/l were
321 significantly worse in eosinophilic than in neutrophilic asthma patients (both adj. p-values <0.05). We
322 also found that only measures of FEF_{50}% and LCI were worse in neutrophilic than in paucigranulocytic
323 asthma patients (both adj. p= 0.014), while none of the small airway dysfunction measures differed
324 significantly between both eosinophilic asthma phenotypes.
325

326 ***Longitudinal impact of persistent sputum inflammatory phenotypes on lung function***

327 We induced and analyzed sputum samples of 141 patients at one-year follow-up. Samples of six
328 patients with poor slide quality were excluded from the analysis. Missing follow-up samples were
329 due to drop outs, patients' refusal for a second sputum induction or due to follow-up FEV1 of < 50%
330 predicted. Based on the longitudinal sputum cell counts, 135 patients were stratified as persistent
331 eosinophilic (*n=29*), persistent neutrophilic (*n=43*) and persistent mixed (*n=16*) asthma phenotypes.
332 For the rest of the patients (*n=47*), they had neither persistent sputum eosinophilia nor neutrophilia
333 or had persistent paucigranulocytic asthma (Table 2). This longitudinal stratification revealed that the
334 persistent mixed asthma phenotype was associated with the worst follow-up measures of small
335 airway dysfunction. Consequently, all follow-up small airway dysfunction markers were significantly

336 worse in patients with persistent mixed asthma than in patients with non-persistent/persistent
337 paucigranulocytic asthma phenotype, (all adj. p-values < 0.05). We also noted that patients with
338 persistent mixed asthma had worse follow-up asthma control than non-persistent/persistent
339 paucigranulocytic asthma patients indicated by lower ACT score (17.0 ±4.4 vs. 20.9±3.8, adj. p=
340 0.019) and higher annualized rate of acute severe exacerbation (2.4±3.0 vs. 0.8±2.2, adj. p=0.034).
341 Further, measures of sReff% and delta N2/l were worse in persistent mixed than in persistent
342 neutrophilic asthma patients, (p=0.019 and 0.020), respectively (Table 2). In addition, persistent
343 eosinophilic asthma indicated worse follow-up measures of FEF25-75%, FEF50%, sReff % and RV%
344 than patients with non-persistent/persistent paucigranulocytic asthma, (all adj. p-values < 0.05).
345 None of small airway dysfunction markers or measures of asthma control differed significantly in
346 persistent eosinophilic versus persistent neutrophilic asthma patients.
347

348 ***Longitudinal impact of the change of sputum cell counts on lung function***

349 In a further step, we correlated changes in sputum cell counts with the one-year change in lung
350 function measures. Univariate regressions indicated that the change in sputum eosinophil counts
351 correlates better with the changes in all lung function measures than the change in sputum
352 neutrophil counts (see Table E2 in this article's Online Repository). We also found that the increase in
353 sputum eosinophils confer a stronger impact on both airflow obstruction and small airway
354 dysfunction than the increase in sputum neutrophils, (Table 3). Multivariate regressions adjusted for
355 cofounders and for the change in the dose of inhaled and oral corticosteroids and also for the
356 presence of anti-T2 biological therapy, showed that the change in sputum eosinophils is a stronger
357 predictor for the longitudinal change in lung function than the change in sputum neutrophils in well
358 fit models (multiple R^2 up to 0.74) as the increase in sputum eosinophils remained an independent
359 predictor for the change in FEV1, FEF_{25-27}, sReff and RV after adjustment for asthma therapy (Table
360 3).

361 ***Longitudinal association of sputum cells counts with small airway dysfunction in patients***
362 ***with stable FEV1***

363 Based on a minimal clinically important difference of 15% for the one-year change in FEV1, we
364 classified the patients into: improved (n=18), declined (n=9) or stable (n=105) FEV1. This longitudinal
365 classification showed increased sputum eosinophils (+2.7% [1.4, 3.7]) in patients who had declined
366 FEV1 versus sputum eosinophils reduction (-15.4% [- 40.0, - 0.18]) in patients who had their FEV1
367 improved, (p<0.01). We also found that patients with stable FEV1 had a relatively small change in
368 their sputum eosinophil count (0.15% (-0.6 - 1.4]), and that the change in sputum neutrophils count
369 was similar between these patients with improved, declined, or stable FEV1, (p=0.29), (Table E3
370 available in this article's Online Repository). While the one-year changes in small airway dysfunction
371 markers were all in concordance with the one-year change in FEV1 (Table E3), the longitudinal
372 change in FEV1 might not accurately reflect the magnitude of change in small airway dysfunction (see
373 Table E4 in the Online Repository). Also in this respect, in a subgroup of patients with stable FEV1, in
374 whom the FEV1 change was less than 15%, we identified the presence of dynamic small airways'
375 changes, indicated by the changes in the frequency dependence of resistance (R5-20). The one year
376 change for R5-20 (median and, KPa/L per s) was (+103%, 0.05 [-0.02, 0.11]), (+82%, 0.02 [-0,01,
377 0.05]), (+26%, 0.0 [-0.04, 0.03]) and (-41%, -0.015 [-0.05,0.02]) in patients with persistent mixed
378 granulocytic, persistent eosinophilic, persistent neutrophilic and non-persistent/persistent
379 paucigranulocytic asthma, respectively, (p=0.038). A pairwise post-hoc comparison indicated that the
380 one year change in R5-20 in persistent mixed granulocytic patients differed significantly from non-
381 persistent/persistent paucigranulocytic, (p= 0.028) as well as from persistent neutrophilic asthma
382 patients, (p=0.040). In contrast, the one-year changes in FEV1 was similar between these persistent
383 sputum phenotypes (p=0.64). Moreover, nearly half of the patients (n=44/98) had a relative R5-20
384 change of at least 50% from baseline. An increase of at least 50% in R5-20 was mainly observed in
385 45% of patients with persistent mixed granulocytic and in 42% of patients with persistent

386 eosinophilic asthma versus only in 11% and 23% of patients with non-persistent/persistent
387 paucigranulocytic or patients with persistent neutrophilic asthma, respectively, (p=0.046).
388

389 Discussion

390 Small airway dysfunction is a frequent feature of asthma that has been linked to disease severity,
391 poor symptom control and severe exacerbation. Unfortunately, there are limited data evaluating
392 relationships between small airway dysfunction and asthmatics airway inflammation. In this cohort
393 study, the use of sputum induction to typify airway inflammation, combined with the comprehensive
394 assessment of lung function and patients' clinical characteristics was designated to enhance our
395 understanding of different asthma phenotypes and their association with small airway dysfunction.
396 We demonstrated the impact of eosinophilic versus non-eosinophilic asthma on lung function and
397 disease outcomes, while also addressing the presence of mixed granulocytic airway inflammation,
398 which appeared to confer a severe asthma phenotype with the greatest lung function impairment.
399 Overall, airway eosinophilic inflammation was associated with more severe small airway dysfunction,
400 poorer asthma control and more frequent severe exacerbation. These findings were confirmed
401 longitudinally as persistent eosinophilic inflammation indicated sustained small airway dysfunction
402 and poorer asthma control, particularly, in patients with persistently elevated eosinophils and
403 neutrophils i.e. mixed asthma phenotype. Moreover, the change in sputum eosinophils rather than
404 sputum neutrophils was an independent predictor for the longitudinal change of lung function.
405 Furthermore, our data indicates that the paucigranulocytic phenotype predicts a mild asthma
406 phenotype with preserved lung function and better asthma outcomes.
407 In our study, where nearly half of the patients were severe asthmatics, most of them had increased
408 sputum granulocytes, mainly sputum neutrophilia (53%). Despite that the vast majority of the
409 patients had inhaled or oral corticosteroids therapy, a notable proportion of them had sputum
410 eosinophilia (45%). The higher frequency of neutrophilic asthma is consistent with the findings of
411 some previous studies (26, 27) but also in the contrary to others where higher frequencies of

412 eosinophilic or paucigranulocytic asthma were reported (28). In fact, there is a considerable
413 heterogeneity in the reported frequencies of asthma phenotypes which can be attributed to some
414 variations in the granulocyte counts used to define each phenotype. In addition, there are also
415 discrepancies between these studies' cohorts concerning other factors such as asthma severity, the
416 presence of acute exacerbation during sputum induction, and the use of inhaled and oral
417 corticosteroid therapy (9, 29). We also found that airway eosinophilic inflammation was associated
418 with multiple clinical indicators of asthma severity. Airway eosinophilic inflammation, as identified by
419 sputum cell count, has frequently been linked to poor symptom control and severe exacerbations
420 (30, 31). Our data also suggest that in asthma, airway eosinophilic inflammation is closely associated
421 with small airway dysfunction. This important association confirms the pathological findings of
422 previous studies on asthma patients, which demonstrated that the small airways were
423 predominantly infiltrated with activated eosinophils compared to the large airways (32, 33). This
424 finding also emphasizes that targeting eosinophilic airway inflammation should be a mainstream
425 treating strategy of small airway dysfunction in asthma as we observed that anti-T2 biological
426 therapy substantially improves small airway dysfunction (34).
427 Persistent airway eosinophilic inflammation might present in a subgroup of severe asthma patients
428 who are treated with inhaled or even oral systemic corticosteroids (35, 36). Accordingly, our data
429 indicate that persistent airway eosinophilic inflammation confers persistent severe small airway
430 dysfunction and poor asthma control; this was particularly observed in patients with persistent mixed
431 asthma phenotype. The longitudinal analysis revealed that patients with persistent mixed asthma
432 had the worst follow-up measures of small airway dysfunction, poorer symptom control and more
433 frequent exacerbation, when compared to non-persistent eosinophilic or persistent non-eosinophilic
434 airway inflammation. These findings also support the notion that the interplay between airway
435 eosinophils and neutrophils might have a critical role in the pathogenesis of asthma (37, 38). The
436 longitudinal analysis also indicated that the change of sputum eosinophil counts rather than
437 neutrophil count is an independent predictor for the longitudinal change of small airway dysfunction

438 and airflow obstruction. Multivariate regressions adjusted for the dose of inhaled and oral
439 corticosteroids and for an adjuvant anti-T2 biological therapy demonstrated that the increase of
440 sputum eosinophil count has a significant negative impact on lung function.
441 A further finding was the high concordance between the longitudinal changes in small airway
442 function markers and the change in the FEV1, which has confirmed the previously described direct
443 association between small airway dysfunction and upper airflow obstruction (12). Moreover, the
444 relative change in FEV1 after one year, and the consistent changes in small airway function markers,
445 were also associated with consistent and significant changes in sputum eosinophils count.
446 Interestingly, in patients with relatively stable FEV1, we observed dynamic changes in small airways,
447 indicated by increased frequency dependence of resistance (R5-20), mainly, in patients with
448 persistent mixed granulocytic asthma. Additionally, the one-year change of at least 50% in R5-20 was
449 observed in nearly half of the patients who had stable FEV1. Nevertheless, it is important to notice,
450 that there are no generally accepted normal reference ranges or MCID for the measurements of
451 impulse oscillometry (39). However, the applied cut-off value of 50% for the change in R5-20 was
452 beyond its' recently reported mean coefficient of variation of 33.1% (95% CI:19.5 – 46.7) (39) and are
453 also in line with the cut-off changes that are applied for the diagnosis of airway hyperresponsiveness,
454 using forced oscillation technique (16). The presence of dynamic small airway changes despite a
455 relatively stable FEV1 suggests that changes in small airway dysfunction are potential treatment
456 outcome to investigate in future clinical trials, particularly, in those investigating anti-eosinophilic
457 asthma therapies.
458 Airway neutrophilic inflammation is linked to asthma severity and frequent exacerbation (40, 41). To
459 our knowledge, no previous study has reported a direct association between airway neutrophilic
460 inflammation and small airway dysfunction in asthma. In our cohort, while all measures of small
461 airway dysfunction were numerically worse in neutrophilic than in paucigranulocytic asthma patients
462 only, differences in measures of LCI and FEF_{2575} reached statistical significance. In addition, a
463 coexistent airway neutrophilia in patients with airway eosinophilic inflammation in persistent mixed

464 asthma was associated with the worst measures of small airway dysfunction. Based on these
465 findings, it might be reasonable to speculate that airway neutrophilic inflammation plays a role in the
466 pathogenesis of small airway dysfunction. However, a caveat with this assumption is that some
467 patients who were stratified as neutrophilic had an anti-T2 biological therapy and some of them had
468 elevations in T2-markers (blood eosinophils, FeNO and serum IgE), suggesting that some of the
469 neutrophilic asthma patients had primarily eosinophilic or T2-high asthma. In addition, airway
470 neutrophilic inflammation might contribute indirectly to small airway dysfunction. We recently
471 reported that increased extracellular DNA production in asthmatic airway, a collateral mechanism of
472 airway neutrophilic inflammation, indicates a broad lung function impairment including small airway
473 dysfunction (6). So far, therapies targeting neutrophilic airway inflammation in asthma failed to
474 improve asthma outcomes (42), leaving the complicated role of airway neutrophilic inflammation in
475 asthma and small airway dysfunction uncertain.
476 In this study, nearly 24% of the patients had paucigranulocytic asthma. As we found, this phenotype
477 was associated with the best lung function measures and asthma outcomes. In spite of this, 36% of
478 the patients with paucigranulocytic asthma had airflow obstruction and 38% experienced at least one
479 severe exacerbation in the previous year. The observed uncoupling of asthma activity from airway
480 inflammation in the paucigranulocytic asthma phenotype brings to question whether asthma activity
481 might present independent form active airway inflammation (43) or is it rather a state of repressed
482 airway inflammation that underestimate the disease progression in a subgroup of the patients.
483 Considering the high variability of sputum cell counts (44), two sputum samplings might be a
484 shortcoming of our study and multiple sputum sampling could have demonstrated the relationship
485 between fluctuations in lung function and sputum cell variability more clearly. Another limitation of
486 our study was the number of dropouts at follow-up. Nevertheless, to the best of our knowledge, this
487 is the first longitudinal study that correlated a broad spectrum lung function assessment and
488 patients' clinical characteristics with sputum inflammatory phenotypes.
489

490 In summary, in patients with asthma, airway eosinophilic inflammation is the main driver of lung
491 function impairment and poor disease outcomes, which might also be aggravated by the coexistence
492 of airway neutrophilia to confer a severe mixed asthma phenotype. The presence of SAD in patients
493 with asthma should prompt the investigation of airway eosinophilia, either directly or via surrogate
494 markers, even in patients who are being treated with inhaled or oral corticosteroids or even with
495 anti-eosinophilic biologics. The observed dynamic changes in the small airways in patients with
496 relatively stable FEV1 emphasizes the significance of evaluating small airway dysfunction in
497 eosinophilic asthma.
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509 Conception and design of the study: MA, HW, KFR, FT; statistical analysis MA and FT; sputum
510 processing and analysis: FP; acquisition, and interpretation: MA, TB, FT, HW; drafting the manuscript:
511 MA, HW; critically revises the manuscript: KFR, AMK, EvM, MVK, VV,HB and GH. All authors revised
512 the manuscript for intellectual content and approved it for publication. All authors read and
513 approved the final manuscript.
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518 # Figure legend:

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520 ## Figure 1: Small airway dysfunction in different asthma phenotypes:

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522 Post hoc Dunn's test indicated statistically significant differences in all small airway dysfunction measures between
523 eosinophilic and paucigranulocytic asthma patients as well as between mixed granulocytic and paucigranulocytic asthma
524 patients except for the measure of R5-20; the statistical differences showed a high tendency (p-value =0.08). Measures of
525 FEF25-75% and delta N2/l were significantly worse in eosinophilic than in neutrophilic asthma patients (both adj. p-values
526 <0.05). Measures of FEF50% and LCI were worse in neutrophilic than in paucigranulocytic asthma patients (both adj. p=
527 0.014). None of the small airway dysfunction measures differed significantly between both eosinophilic asthma phenotypes
528 (all p-values >0.05). FEV1: forced expiratory volume in first second, FVC: forced vital capacity, FEF50% and FEF25–75: mean
529 forced expiratory flow at 50% and between 25% and 75% of the forced vital capacity, R5-20: small airway resistance (total
530 lung resistance – large airway resistance), X5: lung reactance at 5 Hz, RV: residual volume, sReff: specific effective airway
531 resistance, LCI: lung clearance index from multiple breath washout, delta N2: the slope of phase III nitrogen single-breath
532 washout. Phenotypes*: eosinophilic*: patients with sputum eosinophils ≥2% and neutrophils <50%, *mixed*: mixed granulocytic
533 sputum; eosinophils ≥2% and neutrophils ≥ 50%, *neutrophilic:* sputum eosinophils < 2% and neutrophils ≥50%, *pauci:*
534 paucigranulocytic; eosinophils <2% and neutrophils < 50%.

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Table 1: Demographic and clinical characteristics for patients stratified based on sputum cell count

Characteristic	Eosinophilic	Mixed granulocytic	Neutrophilic	Paucigranulocytic	P -value
N baseline	45	43	62	47	-
Age, years	53 (49-59)	57 (50-65)	52 (41-63)	45 (33-55)	0.002
Sex, male%	47	47	47	49	0.99
Body mass index, kg/m²	27.3 (24.6-29.6)	25.8 (23.0-29.3)	28.2 (24.3-32.0)	26.0 (23.5-29.0)	0.16
Severe asthma,%	67	65	50	21	<0.001
Current smoker, %	4	2	14	6	0.12
Maintenance OCS use,%	27	35	26	4	0.001
OCS dose, mg	10.0 (6.8-13.1)	10.0 (5.0-10.0)	11.5 (9.4-20)	3.5 (2.7-4.3)	0.027
Controller ICS use, %	96	93	87	77	0.033
ICS dose, µg	500 (400-1000)	500 (400-1000)	500 (318-1000)	500 (250-500)	0.035
Biological therapy,%	15	19	14	4	0.14
Airflow obstruction, %	63	74	50	36	0.002
FEV1, %	71 (61-87)	78 (62-93)	83 (67-102)	96 (85-105)	<0.001
FVC%	101 (90-112)	106 (98-115)	105 (93-104)	110 (100-108)	0.12
FEV1/FVC, %	59 (53-70)	58 (52-67)	68 (55-75)	71 (66-79)	<0.001†
ACT score	17.5 (11-22)	17.0 (14-21)	18.5 (13-22)	22 (10-24)	<0.001
Severe exacerbation,%	78	58	53	38	0.002*
Number of severe exacerbations	2 (1-4)	1 (0-4.5)	1 (0-3)	0 (0-1)	<0.001
Sputum cell count					
Eosinophils, %	16 (7-42)	7 (4-16)	0.3 (0-1.0)	0.2 (0-0.8)	<0.001†*
Eosinophils, 10^6/ml	0.22 (0.08-1.8)	0.23 (0.12-0.59)	0.01 (0.0-0.04)	0 (0-0.1)	<0.001†*
Neutrophils, %	30 (24-38)	68 (58-77)	74 (61-86)	30 (17-41)	<0.001*
Neutrophils, 10^6/ml	0.55 (0.30.1.4)	2.37 (1.1-4.1)	2.74 (1.4-5.2)	0.41 (0.19-0.71)	<0.001*
Macrophages, %	37 (18-54)	15 (9-24)	19 (10-31)	60 (50-72)	<0.001*
Macrophages, 10^6/ml	0.89 (0.44-1.6)	0.46 (0.25-0.88)	0.65 (0.38-1.3)	0.84 (0.58-1.4)	0.12*
Blood eosinophils, 10^3/µl	410 (290-790)	430 (250-570)	130 (80-230)	180 (140-320)	<0.001†*
Total serum IgE ku/l	158 (69-477)	165 (46-452)	82 (37-292)	93 (43-230)	0.068
FeNO, ppb	40 (22-77)	42 (25-56)	17.5 (11-28)	20 (14-32)	<0.001†*

Values are presented as median and interquartile range. OCS: oral corticosteroids, OCS dose: prednisolone equivalent dose, ICS: inhaled corticosteroids, ICS dose: fluticasone equivalent dose, FEV1: forced expiratory volume in first second, FVC: forced vital capacity, ACT: asthma control test, FeNO: fractional exhaled nitric oxide. Airflow obstruction is defined as FEV1/FVC < LLN. Severe exacerbation: one or more severe exacerbations within 12 months before study visit.
P-values are from ANOVA, Fisher-exact or Kruskal-Wallis-Tests to indicate the statistical significance of the differences in measured parameters between the groups. The post-hoc analysis indicates significant differences (p<0.05) in the pairwise comparison as follows: † Neutrophilic vs. mixed granulocytic, * neutrophilic vs. eosinophilic.

Table 2: Lung function characteristics in asthma patients stratified longitudinally by sputum cell counts

Small airway dysfunction markers	Persistent eosinophilic (*n=29*)	Persistent Mixed (*n=16*)	Persistent Neutrophilic (*n=43*)	Non-persistent/ persistent pauci phenotypes (*n=47*)	P
FEV, l					
Baseline	2.48 (2.0-2.98)	2.14 (1.56-2.41)	2.58 (2.14-3.14)	2.98 (2.44-3.52)	<0.01
FU	2.26 (1.88-2.96)	2.17 (1.75-2.39)	2.56 (1.97-3.15)	2.97 (2.44-3.53)	<0.01
FEV1, %					
Baseline	83 (67-91)	76 (65-84)	82 (68-97)	92 (75-101	0.025
FU	81 (66-88)	80 (72-86)	83 (69-95)	95 (85-103)	<0.01
FEF_{25-75}, l					
Baseline	1.29 (0.86-178)	0.94 (0.65-1.32)	1.54 (0.82-2.16)	1.99 (1.34-2.78)	<0.01
FU	1.22 (0.75-195)	0.92 (0.69-1.07)	1.29 (0.91-2.15)	1.90 (1.47-2.83)	<0.01
FEF_{25-75}, %					
Baseline	37 (27-60)	39 (25-49)	49 (28-72)	58 (49-84)	<0.01
FU	41 (25-54)	35 (29-45)	47 (33-67)	67 (47-83)	<0.01
FEF_{50}, %					
Baseline	39 (29-66)	34 (25-41)	46 (28-72)	58 (40-80)	<0.01
FU	42 (24-55)	35 (23-43)	48 (31-63)	61 (47-87)	<0.01
R5-20, KPa/L per s					
Baseline	0.07 (0.03-0.18)	0.15 (0.09-0.23)	0.09 (0.05-0.18)	0.10 (0.07-0.14)	0.12
FU	0.08 (0.03-0.19)	0.19 (0.09-0.21)	0.10 (0.07-0.15)	0.08 (0.05-0.12)	0.012
Resonance frequency					
Baseline	14 (11-21)	20 (16-22)	15 (10-22)	16 (11-19)	0.22
FU	14 (10-20)	21 (17-23)	16 (12-19)	13 (10-16)	0.01
RV, %					
Baseline	126 (103-139)	126 (120-167)	125 (109-150)	110 (100-128)	<0.01
FU	129 (117-152)	144 (132-219)	125 (110-141)	114 (100-132)	<0.01
sReff, %					
Baseline	122 (94-154)	153 (109-216)	103(75-160)	94 (72-118)	<0.01
FU	118 (88-195)	160 (112-219)	100 (84-154)	88 (70-114)	<0.01†
LCI					
Baseline	6.30 (5.85-6.90)	7.63 (6.35-8.42)	6.40 (5.70-7.30)	5.88 (5.44-6.50)	<0.01
FU	6.81 (5.74-7.23)	7.20 (6.16-8.39)	6.57 (6.13-6.88)	6.0 (5.67-6.65)	0.015

Delta N2 /L					
Baseline	2.6 (1.9-3.3)	4.0 (2.7-6.0)	2.5 (1.3-4.5)	1.7 (1.3-2.5)	<0.01
FU	2.3 (1.6-2.8)	3.3 (2.3-5.3)	2.1 (1.4-3.1)	1.8 (1.3-2.9)	0.019†
Sputum cell count					
Eosinophils-BL, %	15.9 (7.6-33.2)	9.9 (3.8-17.1)	0.5(0.00-1.6)	0.4 (0.0-2.1)	<0.001
Eosinophils-FU, %	7.4 (3.9 -21.2)	6.6 (4.6-17.1)	0.9 (0.00-2.4)	0.5 (0.0-1.4)	<0.001
Neutrophils-BL, %	37.0 (24.2-42.1)	69.0 (60.0-77.0)	74.0(61.0-83.0)	43.0 (21.0-47.0)	<0.001
Neutrophils-FU, %	48.5 (33.8-64.9)	64.0 (60.0-69.0)	77.0 (65.0 -85.0)	42.0 (29.0-54.0)	<0.001

Values are presented as median and interquartile range. FU: follow-up. FEF50% and FEF25–75: mean forced expiratory flow at 50% and between 25% and 75% of the forced vital capacity, R5-20: small airway resistance (total lung resistance – large airway resistance), X5: lung reactance at 5 Hz, RV: residual volume, sReff: specific effective airway resistance, LCI: lung clearance index from multiple breath washout, delta N2: the slope of phase III nitrogen single-breath washout.
P-values are from ANOVA, Fisher-exact or Kruskal-Wallis-Tests to indicate the statistical significance of the differences in measured parameters between the groups. The post-hoc analysis indicates significant differences ($p<0.05$) in the pairwise comparison as follows: †: persistent neutrophilic vs. persistent mixed granulocytic, * persistent neutrophilic vs. persistent eosinophilic.

Table 3: Multivariate regressions of the change in sputum granulocytes as predictor of the change in lung function adjusted for asthma therapy

One-year Change in lung function measures	Change in sputum eosinophils		Change in sputum neutrophils		Model Multiple R^2
	Standardized coefficient	P-value	Standardized coefficient	P-value	
ΔFEV1%	-0.588	<0.01	-0.480	0.091	0.72
ΔFEF_{25-75}%	-0.439	<0.01	-0.264	0.21	0.74
ΔLCI%	0.264	0.23	-0.152	0.54	0.59
Δ Delta N2 %	0.081	0.63	0.034	0.90	0.54
RV%	0.414	0.045	0.410	0.17	0.61
sReff%	0.356	0.032	0.395	0.11	0.68
R5-20, KPa/L per s	0.255	0.384	0.159	0.74	0.43

FEV1: forced expiratory volume in first second, FEF25–75: mean forced expiratory flow between 25% and 75% of the forced vital capacity, R5-20: small airway resistance (total lung resistance – large airway resistance), X5: lung reactance at 5 Hz, RV: residual volume, sReff: specific effective airway resistance, LCI: lung clearance index from multiple breath washout, delta N2: the slope of phase III nitrogen single-breath washout.
Multivariate models were adjusted to age, gender, change ICS, change in OCS dose and to presence of anti T2- biological therapy