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SUPPORTING INFORMATION

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Mepolizumab does not alter the blood basophil count in severe asthma

To the Editor:

Mepolizumab (anti-IL-5) depletes blood and airway eosinophils, and, clinically, allows down-titration of oral corticosteroid and a reduction in the frequency of eosinophil-dependent exacerbations.¹ Basophils also express IL-5R α , participate in T2-mediated inflammatory pathways² and have been associated with exacerbation frequency.³ Whilst basophil progenitors are unlikely to depend on IL-5 for development,⁴ blood basophil counts measured in routine clinical laboratories suggest they decrease following mepolizumab treatment.⁵⁻⁸

Our primary objective was to determine whether anti-IL-5 monoclonal antibody treatment reduces blood basophil levels as an additional potential efficacy mechanism. To achieve this, we measured blood basophils, eosinophils and other type 2 inflammatory cells, before and after 16 weeks of mepolizumab ("Nucala," GlaxoSmithKline) by flow cytometry. Patient eligibility criteria are in the online supplement and the study schedule in Figure S1.

Blood samples were obtained from 26 severe asthma subjects, attending a difficult asthma clinic at a single UK centre, at baseline and following a median (IQR) of 16 (16-17) weeks of mepolizumab,

administered as a 100 mg subcutaneous injection every 4 weeks. In 2 cases, it was not possible to obtain post-treatment samples (n = 1, withdrew consent; n = 1, discontinued), totalling 24 (Figure S1 and Table S1). We also recruited 15 nonasthmatic healthy controls (Table S1) to obtain samples at parallel time points but without an intervention (Figure S1). Flow cytometric measurements were compared with data derived through the routine pathology service, which utilizes an ADVIA 2120/2120i analyser (Siemens, UK). A detailed description of the methodology for both approaches is described in the online supplement, and for flow cytometry, the gating strategy is shown in Figure S2. Our criteria for identifying cell subsets were as follows: eosinophils (CD45⁺CD3⁻CD193⁺CD294⁺SSC^{hi}CD123^{-/+}), basophils (CD45⁺CD3⁻CD193⁺CD294⁺SSC^{lo}CD123⁺), cTH2 and peTH2 (both CD4⁺CD294⁺ but CD161⁻ or ⁺, respectively), cTC2 (CD8⁺CD294⁺) and ILC2s (Lineage⁻CD294⁺CD161⁺).

For methodological comparisons, data from asthma and healthy subjects (n = 39) were pooled. A good correlation was observed between flow cytometry and the ADVIA 2120i for total cells ($r^2 = 0.24$, $P = 0.0014$) and eosinophils ($r^2 = 0.75$, $P < 0.0001$), but not basophils ($r^2 = 0.06$, $P = 0.13$ Figure S3). In addition, the change in cell concentration between baseline and follow-up showed a good correlation

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FIGURE 1 Eosinophil and basophils were measured per 100 μL blood using flow cytometry (A and B, left) and on an ADVIA 2120/2120i analyser (A and B, right). In the asthma group, the eosinophil and basophil concentration was measured before and after 16 wks of mepolizumab. In the healthy group, the eosinophil and basophil concentration was measured at baseline and 16 wks later. Within-group comparisons were made with a Wilcoxon matched-pairs test or a paired *t* test (healthy group: A, right and B, left). Dashed line indicates ADVIA 2120/2120i upper reference range

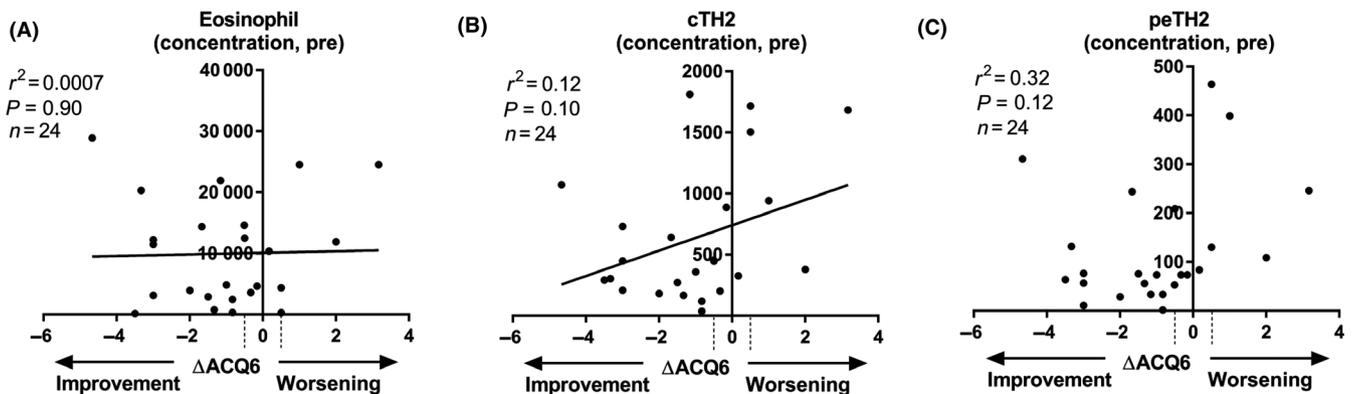
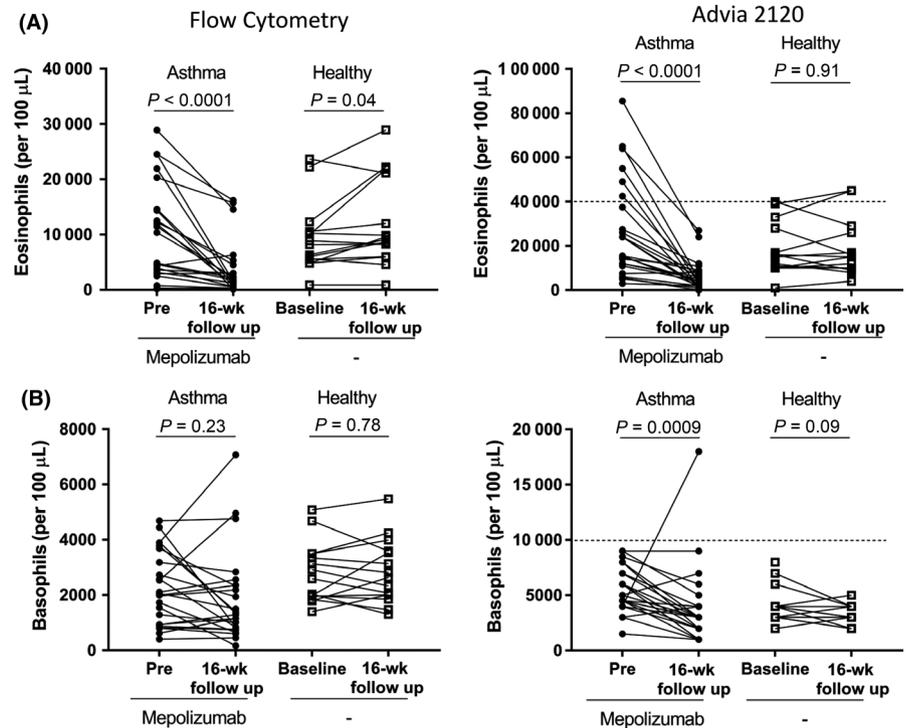


FIGURE 2 (A) Eosinophil, (B) cTH2 and (C) peTH2 concentration pre-mepolizumab (y-axis) plotted with the absolute change in ACQ6 score between baseline and 16 wks post-mepolizumab (ΔACQ6 , x-axis). Linear regression (A, B) or Spearman (C) analysis was performed. Dashed lines on x-axis represent the minimal clinically important difference (± 0.5)

between the two analytical methods for eosinophils ($r^2 = 0.72$, $P < 0.0001$, $n = 38$) but not basophils ($r^2 = 0.02$, $P = 0.39$, $n = 38$).

As expected, following 16 weeks of mepolizumab, we observed a significant reduction in blood eosinophil concentration (flow cytometry [mean \pm SD] -6442 ± 6852 , ADVIA $-20\,688 \pm 19\,355$ cells/100 μL , Figure 1A) and frequency (flow cytometry [mean \pm SD] $-1.2 \pm 1.2\%$ of total leukocytes, Figure S4A) compared with baseline levels for both methods. This decrease was not observed in our control group (mean \pm SD 2475 ± 4148 , 285 ± 6977 cells/100 μL and $0.70 \pm 1.38\%$, respectively) (Figure 1A). Notably, the reduction in blood eosinophil levels following mepolizumab was related to baseline eosinophil levels ($r^2 = 0.69$, $P < 0.0001$, Figure S5).

In contrast to eosinophils, basophil concentration and frequency at baseline were similar to that following 16 weeks of

mepolizumab, when measured by flow cytometry (pre vs post [mean \pm SD] 2232 ± 1309 vs 1873 ± 1647 cells/100 μL , $P = 0.23$, Figure 1B and 0.40 ± 0.19 vs $0.37 \pm 0.27\%$ of total leukocytes, $P = 0.076$, Figure S4B). Surprisingly, measurements obtained on the ADVIA 2120i suggested a statistically significant reduction in the basophil concentration following 16 weeks of mepolizumab (pre vs post [mean \pm SD] 5521 ± 2003 vs 3792 ± 3623 cells/100 μL , $P = 0.0009$, Figure 1B). The mean \pm SD reduction in basophil concentration of -1667 ± 3988 cells/100 μL in asthma was significantly greater compared with the change observed in the control group (vs -571 ± 1222 , $P = 0.011$, Mann-Whitney). In the healthy group, the concentration and frequency of basophils were similar when comparing baseline and follow-up samples, regardless of analytical method (Figures 1B and S4).

These data suggest that basophil concentration and frequency, alongside other T2 inflammatory cells (Figure S6), are likely to be IL-5/mepolizumab-independent in severe asthma. However, our real-world study was not sufficiently powered to detect small differences in relation to basophil concentration or frequency. A strength of our flow cytometric approach is that we have measured cell concentration as well as frequency, and also reported recently identified T2 cell subsets (eg peTH2 cells). Our data suggest there were also no indirect effects of mepolizumab on type-2 polarised T cell or group 2 ILC concentration over this 16-week time frame as indicated by others.⁵

Clinically, we observed a significant change in ACQ6 symptom score from a baseline of 2.9 ± 1.6 to 1.9 ± 1.3 at 16 weeks post-treatment, which is a reduction of -0.92 (97.73% CI of -2 to -0.16 , Wilcoxon matched pairs, $P = 0.0085$), corresponding to an improvement in symptoms above the minimal clinically important difference (MCID) threshold of -0.5 . Since baseline eosinophil concentration was associated with basophils ($r = 0.53$, $P = 0.0073$), cTH2 ($r = 0.59$, $P = 0.0023$), peTH2 ($r = 0.45$, $P = 0.026$) and TC2 ($r = 0.45$, $P = 0.026$) cells but not total cells ($r = 0.2$, $P = 0.35$) or ILC2s ($r = 0.2$, $P = 0.35$), we examined their relationship to ACQ6 improvement (Δ ACQ6). The baseline cellular parameters described above were not associated with Δ ACQ6 (Figure 2, shown for eosinophils, cTH2 and peTH2 cells only). Δ ACQ6 was also not associated with the Δ change in eosinophil ($r^2 = 0.04$, $P = 0.36$) or basophil ($r^2 = 0.09$, $P = 0.15$) levels post-treatment, limiting the utility of these measurements as symptom response biomarkers.

Of further interest was the effect of mepolizumab on eosinophil and basophil cell surface expression of the IL-3 receptor α (CD123), and their relationship to Δ ACQ6. IL-3 is upregulated in the serum of poorly controlled asthmatic patients,⁹ and it can potentiate eosinophil chemotactic and degranulation responses. Recently, mepolizumab treatment, in the context of allergen challenge,⁸ was associated with reduced levels of IL-3R α mRNA and protein on circulating blood but not lung eosinophils.

Consistent with Kelly et al, we observed a decrease in eosinophil IL-3R α cell surface expression in response to mepolizumab in asthma (pre [mean \pm SD] 415 ± 306 vs post 204 ± 192 GMFI, $P < 0.0001$, $n = 21$) and not in our healthy control group (baseline [mean \pm SD] 587 ± 626 vs post 558 ± 535 GMFI, $P = 0.32$, $n = 15$) (example in Figure S2 and cumulative in S7). This represents a % decrease of $-54 \pm 25\%$ in asthma compared with $+9 \pm 55\%$ in the healthy group (mean \pm SD, $P < 0.0001$, Mann-Whitney test). We also noted that eosinophil expression of CRTH2 (CD294) was increased following mepolizumab (Figure S7); however, there was no relationship between the reduction in eosinophil IL-3R α expression and CRTH2 expression. Importantly, there was no relationship between changes in eosinophil IL-3R α or CRTH2 expression with Δ ACQ6 in these patients. Furthermore, there were no mepolizumab-dependent effects on eosinophil/basophil Siglec-8, CD69 or IL-5R α expression (not shown), consistent with others⁸ or basophil IL-3R α (Figure S7); and thus, these parameters were not examined for a relationship with Δ ACQ6.

In summary, our flow cytometric data do not support a direct inhibitory effect of mepolizumab on basophil levels, and therefore, clinical benefit is likely to be independent of basophils. Our data suggest that the specificity and sensitivity of basophil detection on routine clinical analysers should be validated prior to reporting/interpreting basophil data in the context of an intervention. Our data do support others⁸ that mepolizumab reduces eosinophil, but not basophil, IL-3R α expression and, importantly, extends the applicability of this phenomenon to the "real-world" scenario. However, neither changes in eosinophil levels nor changes in IL-3R α expression were associated with clinical efficacy determined by change in asthma control in this study, and thus, biological correlates of response to treatment require further study.

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Circulating miRNAs as diagnostic tool for discrimination of respiratory disease: Asthma, asthma-chronic obstructive pulmonary disease (COPD) overlap and COPD

To the Editor,

Asthma is a respiratory condition characterized by reversible airflow obstruction and airway inflammation usually provoked by eosinophils,¹ which is usually associated with atopy and early onset. Although asthma normally presents itself alone, it does not protect against chronic obstructive pulmonary disease (COPD), which is another lung pathology that differs from asthma in the non-reversible airflow obstruction, adulthood onset and the triggering by noxious substances.² This condition of having concomitant diagnosis of asthma and COPD has been identified as asthma-COPD overlap (ACO), and it is characterized by the features of both diseases.³

Nowadays, only spirometry and clinical manifestations can differentiate between both pathologies. Their differentiation is important because their treatment is different. The use of inhaled corticosteroids, the cornerstone of asthma management, could be harmful in COPD, since it has been associated with the occurrence of

pneumonia. For this reason, they are not indicated in COPD, unless some clinical characteristics are present, the presence of high blood eosinophil counts being the most important, which defines ACO. Thus, the use of disease biomarkers might help the clinical practice. MicroRNAs (miRNAs) are small noncoding RNAs associated with disease mechanisms and have been described as good biomarkers. We have previously described that differential miRNAs expression obtained from eosinophils of asthmatics compared with healthy individuals was able to cluster asthmatics and healthy subjects.⁴ Five of these miRNAs (miR-1246, miR-144-5p, miR-320a, miR-185-5p and miR-21-5p) measured in serum were able to differentiate asthmatics from healthy subjects by statistical models.⁴ We hypothesized that some of these miRNAs could be specific to asthma, and they may differentiate asthma from other respiratory diseases. Therefore, the aim of the study was to compare these miRNAs between asthmatics, COPD and ACO and to analyse whether they could be used as asthma biomarkers.

Abbreviations: ACO, asthma-COPD overlap; AMPK, AMP-activated protein kinase; AUC, area under the curve; COPD, chronic obstructive pulmonary disease; FEV1, forced expiratory volume in the first second; Fox, forkhead box; FVC, forced vital capacity; IL, interleukin; MAPK, mitogen-activated protein kinases; PI3K-AKT, phosphoinositide-3-kinase-protein kinase B; RNA, ribonucleic acid; ROC, receiver operating characteristic; sRAGE, soluble form of receptor for advanced glycation end products; TGF- β , transforming growth factor beta; YKL-40, Chitinase 3-like 1.

Rodrigo-Muñoz and Rial equally contributed to this publication.