

DR. MICHAEL FRICKER (Orcid ID : 0000-0002-8587-1774)

Article type : Original Article: Asthma and Lower Airway Disease

Molecular markers of type 2 airway inflammation are similar between eosinophilic severe asthma and eosinophilic COPD

Manuscript Acceptance Date: 14-Dec-2020

Running title: Molecular type 2 airway inflammation in eosinophilic COPD

Authors: Michael Fricker^{1,2,3}, Vanessa M. McDonald^{2,3,4,5}, Natasha A. Winter^{1,2}, Katherine J. Baines^{1,3}, Peter A.B. Wark^{1,3,5}, Jodie L. Simpson^{1,3,5}, Peter G. Gibson^{1,2,3,5}.

Author affiliations:

¹School of Medicine and Public Health, Faculty of Health and Medicine and Priority Research Centre for Healthy Lungs, The University of Newcastle, NSW, Australia

²National Health and Medical Research Council Centre for Excellence in Severe Asthma, Newcastle, NSW, Australia

³Hunter Medical Research Institute, Newcastle, NSW, Australia

⁴School of Nursing and Midwifery, Faculty of Health and Medicine and Priority Research Centre for Healthy Lungs, The University of Newcastle, NSW, Australia

⁵Department of Respiratory and Sleep Medicine, John Hunter Hospital, Newcastle, NSW, Australia

Name and address of corresponding author:

Michael Fricker

Address: Priority Research Centre for Healthy Lungs, Hunter Medical Research Institute, Lot 1 Kookaburra Circuit, New Lambton Heights, NSW, 2305, Australia.

Phone: +61 2 404 20207 Fax: +61 2 404 20046 Email: michael.fricker@newcastle.edu.au

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1111/ALL.14741](https://doi.org/10.1111/ALL.14741)

This article is protected by copyright. All rights reserved

Author contributions: M.F. designed and supervised laboratory studies, designed and performed data analysis, interpreted data and wrote the manuscript. V.M.M. supervised clinical studies, designed data analysis, interpreted data and edited the manuscript. N.A.W. performed laboratory studies, interpreted data and edited the manuscript. K.J.B. supervised laboratory studies and data analysis, edited the manuscript. J.L.S and P.A.W. supervised clinical studies and edited the manuscript. P.G.G. supervised clinical studies, designed data analysis, interpreted data and edited the manuscript.

Word count: Abstract: 249 words; Text: 3442 words; 4 figures; 2 tables

Key words: obstructive airways disease, sputum, cytokine, eosinophil, biomarker, prostaglandin

Abbreviations:

ACQ	Asthma Control Questionnaire
BAL	Bronchoalveolar Lavage
cDNA	Complementary DNA
COPD	Chronic Obstructive Pulmonary Disease
Ct	Cycle threshold
DNA	Deoxyribonucleic Acid
EA	Eosinophilic asthma
ECOPD	Eosinophilic COPD
ELISA	Enzyme Linked Immunosorbent Assay
FEV ₁	Forced Expiratory Volume in 1 second
FVC	Forced Vital Capacity
GOLD	Global Initiative for Chronic Obstructive Lung Disease
HPGDS	Haematopoietic Prostaglandin D Synthase
ICS	Inhaled Corticosteroid
IL	Interleukin
ILC2	Type 2 Innate Lymphoid Cells
mRNA	Messenger RNA
NEA	Non-eosinophilic asthma

NECOPD	Non-eosinophilic COPD
OAD	Obstructive Airways Disease
OCS	Oral Corticosteroid
PBE	Peripheral Blood Eosinophil
PGD ₂	Prostaglandin D ₂
RNA	Ribonucleic acid
qPCR	Quantitative Polymerase Chain Reaction

Abstract:

Background: Airway and systemic eosinophilia are important treatable traits in both severe asthma and COPD. The molecular basis of eosinophilia in COPD is poorly understood but could involve type 2 cytokines (IL5, IL13) and prostaglandin D₂ (PGD₂).

Methods: This study included non-obstructive airways disease (OAD) controls (n=19), a COPD cohort (n=96) and a severe asthma cohort (n=84). Demographics, exacerbation history, disease impact (SGRQ) and spirometry were assessed. Participants were categorized as eosinophilic using either sputum eosinophil proportion ($\geq 3\%$) or blood eosinophil count ($\geq 300/\mu\text{L}$). Sputum type 2 inflammatory measures included PGD₂ by ELISA and gene expression (qPCR) of *IL5*, *IL13* and the haematopoietic PGD₂ synthase (*HPGDS*).

Results: Type 2 markers did not differ across groups except *HPGDS* mRNA which was highest in non-OAD controls and lowest in COPD. *IL5* and *IL13* mRNA and PGD₂ levels were significantly increased in eosinophilic vs non-eosinophilic severe asthma but did not differ between eosinophilic COPD and eosinophilic severe asthma or non-eosinophilic COPD. *HPGDS* expression was higher in eosinophilic severe asthma compared with eosinophilic COPD. Results were similar using sputum or blood eosinophil cut-offs. Sputum *IL5* and *IL13* were highly intercorrelated in severe asthma ($r=0.907, p<0.001$) and COPD ($r=0.824, p<0.001$), were moderately correlated with sputum eosinophils in severe asthma (*IL5* $r=0.440, p<0.001$; *IL13* $r=0.428, p<0.001$) and weakly correlated in COPD (*IL5* $r=0.245, p<0.05$; *IL13* $r=0.317, p<0.05$).

Conclusions: Molecular markers of type 2 airway inflammation do not differ between eosinophilic asthma and eosinophilic COPD, however the relationship between eosinophilia and type 2 airway markers appears weaker in COPD than in severe asthma.

Introduction

Asthma and Chronic Obstructive Pulmonary Disease (COPD) are obstructive airway diseases that feature heterogeneous patterns of airway and systemic inflammation¹. Importantly, markers of airway and systemic inflammation have been shown to relate to variable clinical response to anti-inflammatory therapeutics including inhaled corticosteroids (ICS)². Sputum cell counts allow categorization of asthma and COPD into airway inflammatory phenotypes based on the presence or absence of abnormally elevated levels of eosinophils. Adjustment of ICS dose to suppress sputum eosinophilia reduces exacerbations of asthma and COPD^{3,4}, and use of blood eosinophil

count, a surrogate marker of sputum eosinophilia, is now part of the Global Initiative for Chronic Obstructive Lung Disease (GOLD) strategy document for ICS use to treat COPD⁵. Despite this, eosinophilic airway inflammation is present in a significant proportion of patients with asthma and COPD who are treated with ICS^{1,6}, including those with severe asthma who receive maximal ICS therapy^{7,8}. Sputum eosinophilia has been associated with heightened exacerbation risk in both severe asthma and COPD^{6,9}. Use of add-on monoclonal therapies targeting IL-5 or IL-4/13 signalling, cytokines involved in eosinophilic airway inflammation, reduces exacerbations in severe eosinophilic asthma, however clinical trials of IL-5 targeting monoclonal antibodies in similarly biomarker-enriched COPD populations have produced equivocal results¹⁰. A greater understanding of the molecular features of airway eosinophilia in COPD and their relation to surrogate biomarkers and clinical features of disease such as exacerbation susceptibility is required.

Sputum eosinophilia is associated with increased sputum gene expression of type 2 cytokines IL-4, -5 and -13 in asthma¹¹. Elevated sputum type 2 cytokine gene expression in ICS-treated asthma is associated with increased age, worse lung function, increased sputum and blood eosinophils, and greater frequency of exacerbations in the prior year¹². The relationship of sputum type 2 cytokine gene expression of airway inflammatory and clinical characteristics of COPD is not known. Prostaglandin D₂ (PGD₂) is a lipid signalling molecule that promotes airway eosinophilia and type 2 inflammation, primarily through signalling via the DP₂ (CRTH₂) receptor. PGD₂ promotes type 2 cytokine production by Th₂ lymphocytes and type 2 innate lymphoid cells (ILC₂), and can act as a direct chemoattractant for eosinophils¹³. DP₂ antagonism can reduce airway eosinophilia independently of blood eosinophils in asthma¹⁴, and a post-hoc analysis of a phase 2 trial suggested blockade of DP₂ signalling could reverse established asthma pathology through reduction of airway smooth muscle mass¹⁵. Recent parallel phase 3 trials of DP₂ antagonist fevipiprant failed to demonstrate a significant reduction in asthma exacerbations compared to placebo¹⁶. This is suggestive of an important airway localised contribution of PGD₂ signalling to eosinophilia and pathology in asthma. Haematopoietic prostaglandin D₂ synthase (HPGDS) is expressed in various immune cells and plays an important role in mediating allergic airway inflammation through catalysing the final step in production of PGD₂. Increased soluble PGD₂ in BAL fluid and increased *HPGDS* gene expression in bronchial epithelial brushings have been reported in severe vs mild-moderate asthma¹⁷. Soluble PGD₂ in sputum supernatants was

reported to be increased in eosinophilic and non-eosinophilic severe asthma compared to non-asthma controls¹⁸. Little is known of dysregulation of PGD₂-related markers in COPD clinical airway-derived samples, although increased concentrations of urinary PGD₂ metabolites were detected in COPD vs healthy controls using mass spectrometry¹⁹. Sputum analysis offers potential advantages compared to BAL and brushing analyses due to relatively non-invasive collection procedures, and established standard laboratory methods for gene expression and ELISA-based analyses.

In this study, we aimed to determine if type 2 inflammatory pathways (sputum *IL5*, *IL13*, *HPDGS* gene expression and PGD₂ levels) differed between eosinophilic and non-eosinophilic severe asthma and COPD. A secondary objective was to explore the relationship of these markers to clinical features of COPD and severe asthma. We hypothesized that sputum type 2 inflammatory markers would be increased in eosinophilic vs non-eosinophilic participants irrespective of disease diagnosis. We also hypothesized these measures would relate to important clinical features of COPD and severe asthma.

Methods

Study population and clinical measures

This study involved three groups (total n=199): COPD (n=96), severe asthma (n=84) and non-obstructive airway disease (OAD) controls (n=19). Participants were recruited via the respiratory ambulatory care clinics and research database of the Department of Respiratory Medicine at John Hunter Hospital (NSW, Australia), and/or by media advertisement. Studies were approved by institutional ethics committees (08/08/20/3.10, 12/12/12/3.06). All participants provided written informed consent. Detailed diagnostic criteria, inclusion and exclusion criteria are provided in the online supplement. Participants with OAD had stable disease (see online supplement) and no participants had overt respiratory infection at time of sample collection. Eosinophilic status of participants was determined on the basis of sputum $\geq 3\%$ eosinophils²⁰, or in separate analyses on the basis of $\geq 300/\mu\text{L}$ blood eosinophils.

Clinical procedures

Participant demographics were assessed and 12-month exacerbation history (emergency presentations, hospital admissions and unscheduled oral corticosteroid courses) was recorded by questionnaire. Disease-related health status was assessed by questionnaire (St. George Respiratory Questionnaire [SGRQ] and asthma control determined (Asthma Control Questionnaire [ACQ]). Spirometry pre- and post-bronchodilator (Forced Expiratory Volume in 1 second [FEV₁], Forced Vital Capacity [FVC], FEV₁/FVC) were measured and sputum induction performed as previously described²⁰. Blood was collected, full blood count was performed and serum prepared and stored at -80°C.

Sputum processing and airway inflammatory phenotyping

Sputum portions were separated from saliva, and selected portions were processed to cell suspension or stored at -80°C in buffer RLT (*Qiagen*) for subsequent gene expression analyses. Cytospins of sputum cell suspension were prepared and sputum supernatant stored as previously described (see online supplement). Counts of 400 non-squamous sputum cells on May-Grunwald Giemsa-stained cytospins were performed.

Gene expression analysis

Whole sputum RNA isolation, cDNA synthesis and qPCR to measure *IL5*, *IL13* and *HPGDS* gene expression in whole sputum was performed as previously described (see online supplement)^{21,22}. For reporting gene expression, change in cycle threshold (ΔC_t) between the target gene and the housekeeper gene β -actin was log transformed ($2^{-\Delta C_t}$), giving relative abundance of target compared to housekeeper.

Sputum PGD₂ measurement

Soluble PGD₂ was measured in sputum supernatant as previously published¹⁸ using a commercially available kit following manufacturer's instructions (*Cayman Chemicals Cat#512011*) (see online supplement).

Statistical analysis

Statistical analysis was conducted using GraphPad Prism (version 7). Where data were non-normally distributed, Mann-Whitney was used for two-way comparisons, Kruskal-Wallis with Dunn's multiple comparisons adjustment was used for comparisons between 3-4 groups, and Spearman correlation applied. For normally distributed data, Student's t-test or ANOVA with Bonferroni multiple comparisons adjustment were used for comparisons. Non-normally distributed data are reported as median (Q1, Q3), normally distributed data are reported as mean (SD), n.

Results

Clinical characteristics

Participant characteristics are summarized in supplemental table 1. A greater proportion of participants with severe asthma were female. Participants with COPD were significantly older, and were taking a lower mean dose of ICS than severe asthma participants. Pre and post-bronchodilator spirometry measures were lowest in the COPD group and highest in the non-OAD controls, as expected. Participants with COPD had a significantly higher SGRQ score compared to severe asthma. Both COPD and severe asthma participants had higher sputum eosinophil proportion than non-OAD controls. Sputum neutrophil proportion was highest in COPD.

Sputum eosinophilia is associated with increased type 2 cytokine expression in severe asthma but not COPD

We compared sputum gene expression of *IL5* and *IL13* between disease groups. A trend towards increased expression of *IL5* in COPD and severe asthma compared to non-OAD controls was observed, although this did not reach statistical significance (Figure 1A). *IL13* expression did not differ between disease groups (Figure 1B). Overall *IL5* and *IL13* expression in COPD and severe asthma were similar.

To examine relationship of the markers to airway eosinophilic status, participants with COPD or severe asthma were categorized as eosinophilic or non-eosinophilic based on sputum eosinophils $\geq 3\%$ or $< 3\%$ (Table 1). *IL5* and *IL13* gene expression were significantly increased in eosinophilic (EA) vs non-eosinophilic (NEA) severe asthma (Figure 1C, D). In contrast, neither *IL5* nor *IL13* gene expression were significantly altered in eosinophilic (ECOPD) vs non-eosinophilic COPD (NECOPD). Neither *IL5* nor *IL13* gene expression differed significantly between EA and ECOPD. We compared the extent of sputum eosinophilia in ECOPD and EA, and found no significant difference between the two groups (Figure 1E, Supplemental table 1). Sputum neutrophil proportion was higher in ECOPD than EA (Supplemental table 1). Approximately a third of EA and NEA participants were taking maintenance OCS for their asthma (Table 1). Sputum *IL5* and *IL13* expression did not differ between participants who were or were not receiving maintenance OCS treatment in either EA or NEA (Supplemental figure 1).

Sputum PGD₂ pathway components are increased in eosinophilic severe asthma

Sputum supernatant PGD₂ did not differ between disease groups (Figure 2A). No significant difference was observed in sputum PGD₂ between ECOPD and NECOPD (Figure 2B). In contrast sputum PGD₂ was significantly increased in EA vs NEA, although levels were similar in EA vs NECOPD. We observed significant differences in sputum *HPGDS* expression between disease groups, with highest relative expression in non-OAD controls, followed by severe asthma, with expression lowest in participants with COPD (Figure 2C). *HPGDS* expression did not significantly differ between ECOPD and NECOPD, or EA and NEA, although trends towards increased expression in eosinophilic phenotypes were observed. *HPGDS* expression was significantly higher in EA vs ECOPD (Figure 2D).

Interrelationship of sputum type 2 inflammatory and PGD₂ pathway measures

Sputum *IL5* and *IL13* expression were strongly correlated with each other in both COPD and severe asthma (Table 2). Sputum *IL5* and *IL13* expression showed moderate correlations with sputum eosinophil proportion in severe asthma, and weak but statistically significant correlations with sputum eosinophil proportion in COPD. Sputum PGD₂ was weakly correlated with sputum eosinophils in severe asthma, but not in COPD, but was unrelated to *IL5*, *IL13* and *HPGDS* gene expression. *HPGDS* expression was weakly but significantly correlated with sputum eosinophils in both COPD and severe asthma.

Elevated peripheral blood eosinophil count is associated with increased sputum type 2 cytokine and PGD₂ in severe asthma but not COPD

Participants were categorized as peripheral blood eosinophil (PBE) high (≥ 300 eosinophils per μL) or low (< 300 per μL) and sputum measures compared (Supplemental table 2). Sputum *IL5* and *IL13* expression were increased in PBE high vs PBE low severe asthma (Figure 3A, B). In contrast, *IL5* and *IL13* expression did not differ between PBE high and PBE low COPD. *IL5* and *IL13* expression did not significantly differ between PBE high severe asthma and PBE high COPD. Sputum eosinophils were elevated in both PBE high severe asthma and COPD compared to PBE low groups (Figure 3C). Sputum eosinophils did not significantly differ between PBE high severe asthma and PBE high COPD. Sputum PGD₂ was increased in PBE high vs low severe asthma, but did not differ between PBE high and low COPD (Figure 4A). No differences between PBE high and low groups were observed for *HPGDS* expression in severe asthma or COPD,

although *HPGDS* expression was greater in PBE high severe asthma than PBE high COPD (Figure 4B).

Clinical associations of sputum type 2 cytokine and PGD₂ pathway measures

Markers showed no difference between gender in disease groups (Supplemental figure 2). No difference in any marker measured (including sputum eosinophil proportion) was observed in COPD or severe asthma participants categorized as frequent (≥ 2) or infrequent (0-1) exacerbators defined by the use of unscheduled OCS courses in the prior 12 months (Supplemental figure 3). Increased *IL13* gene expression was weakly but significantly correlated with increased pre- and post-bronchodilator FVC in participants with COPD only ($r=0.232$ and $r=0.234$ respectively; Supplemental table 3). *IL5* and *IL13* mRNA expression and PGD₂ levels did not correlate with any other clinical variable tested in either COPD or severe asthma participants. *HPGDS* gene expression was weakly positively correlated with pre- and post-bronchodilator spirometry measures in both COPD and severe asthma, and was negatively correlated with SGRQ in severe asthma, suggesting increased gene expression was related to lesser health-status impairment (Supplemental table 3).

Discussion

This study aimed to determine whether airway and systemic eosinophilia were associated with increased presence of molecular markers of type 2 airway inflammation in COPD and severe asthma. Comparison of eosinophilic with non-eosinophilic participants with severe asthma or COPD demonstrated increased sputum gene expression of key type 2 cytokines IL-5 and IL-13 in EA vs NEA but not in ECOPD vs NECOPD. However, a direct relationship between eosinophil measures and *IL5* and *IL13* expression was observed in COPD, albeit weaker than that observed in severe asthma, and no significant differences in *IL5* and *IL13* expression were observed between EA and ECOPD. Sputum PGD₂ was increased in EA vs NEA defined using either sputum or blood eosinophil cut-offs, however did not differ with ECOPD. Gene expression of the key PGD₂ synthetic enzyme HPGDS was increased in EA vs ECOPD using both sputum and blood eosinophil cut-offs. In sum, a relationship exists between molecular type 2 markers and eosinophilia in severe asthma and COPD, although use of clinically-relevant sputum or blood eosinophil cut-offs was less effective in enriching for a type 2 high subpopulation in COPD.

Trials of therapeutic blockade of type 2 cytokines and the PGD₂ pathway have aimed to prevent the occurrence of exacerbations in severe asthma and COPD. Targeted treatment of eosinophilic severe asthma with monoclonal antibody therapies targeting type 2 cytokine signalling reduces exacerbations²³. In contrast, eosinophilic COPD remains poorly understood, the molecular features of this phenotype are largely unknown, and trials of IL-5 blocking antibody therapies have produced equivocal results¹⁰. In this study we observed that sputum *IL5* and *IL13* gene expression was increased in severe EA vs NEA defined using a sputum cut-off. This finding is in agreement with prior reports, supporting the notion that molecular type 2 airway inflammation is corticosteroid refractory in severe asthma¹². The majority (94%) of the COPD population studied here were also treated with ICS. In contrast to severe asthma, we observed no significant increase in *IL5* or *IL13* sputum gene expression in ECOPD vs NECOPD. Correlation analyses indicated that a relationship did exist between sputum eosinophil proportion and type 2 cytokine expression in COPD, however these correlations were weaker than those observed in severe asthma. Cytokine expression did not differ between EA and ECOPD. We have previously reported that a sputum gene expression signature of 6 biomarkers (6-gene signature [6GS], including Charcot-Leyden crystal galectin [*CLC*]; carboxypeptidase 3 [*CPA3*]; deoxyribonuclease 1-like 3 [*DNASE1L3*]; alkaline phosphatase, liver/bone/kidney [*ALPL*]; *CXCR2*; and *IL1B*) provides excellent airway

inflammatory phenotyping and exacerbation prediction in both asthma^{22,24} and COPD²⁵, suggesting that our data are not explained by an unconsidered confounding factor associated with sputum gene expression studies in COPD. Neutrophil proportion was higher in ECOPD vs EA participants although eosinophil proportions did not differ. Increased sputum neutrophils in ECOPD participants could reflect their increased age compared to EA, or might reflect slightly different ongoing inflammatory processes in the airways. It is possible that alternative type 2-related inflammatory processes, unmeasured in this study, could play a relatively more prominent role in promoting eosinophilia in COPD. George and colleagues recently demonstrated that the bronchial epithelial transcriptome of COPD associated with peripheral blood eosinophilia was not defined by a clear type 2 inflammatory molecular signature in contrast to severe asthma, supporting the hypothesis that alternative mechanisms may promote airway and systemic eosinophilia in COPD²⁶. Potential alternative mechanisms proposed include airway-localised eosinophilopoiesis triggered by epithelial alarmins²⁷, or localised production of eosinophil chemokines including eotaxins CCL11 and CCL24²⁸. However, data from smaller trials of mepolizumab and benralizumab demonstrate depletion of both sputum and blood eosinophils in eCOPD, supporting a role of IL-5 in promoting eosinophilia. Thus, eosinophils may perhaps not play a prominent direct role in promoting exacerbations in ICS-treated COPD, but may be elevated in conjunction with other unknown disease-promoting processes^{29,30}.

In this study we also report that eosinophilic COPD defined by blood eosinophil cut-off does not feature elevated airway type 2 molecular markers, in contrast to eosinophilic severe asthma. Blood eosinophil count is a critical biomarker for selection of individuals with severe asthma who will benefit clinically from treatment with biologicals targeting type 2 cytokines²³. Trials of the IL5 blocker mepolizumab and the IL-5 receptor blocker benralizumab in COPD populations employed similar participant selection criteria to successful severe asthma trials, including confirmation of elevated blood eosinophil count and exacerbation history at screening. Despite this, parallel trials in COPD produced equivocal results for mepolizumab³¹, and failed to significantly reduce exacerbations with benralizumab³². Post-hoc analyses of the mepolizumab COPD trials demonstrated an association between increasing blood eosinophil count and greater reduction in exacerbation rate, and further post-hoc analyses of the benralizumab trials demonstrated potential utility of elevated blood eosinophils (≥220/uL) in combination with stringent clinical selection criteria to identify a responsive subpopulation in COPD³³. Our data suggest that use of a blood

eosinophil cut-off (300 cells/uL) associated with type 2 biologic clinical response in severe asthma does not enrich for a population with significantly increased type 2 molecular airway inflammation in COPD, despite the presence of elevated sputum eosinophils in this population.

Our observation of increased presence of sputum PGD₂ on a per cell basis in eosinophilic severe asthma is consistent with the potential role of this molecule in promoting airway eosinophilia. Sputum PGD₂ was similar in ECOPD vs EA but was not elevated in ECOPD vs NECOPD. PGD₂ promotes airway eosinophilia in asthma via the receptor DP2, acting as a direct chemoattractant for eosinophils, or indirectly via enhancement of type 2 cytokines in Th2 lymphocytes and ILC2. Our analyses indicated no relationship between sputum PGD₂ levels and sputum *IL5* or *IL13* expression in either asthma or COPD, suggesting it may not be a critical factor in promoting type 2 cytokine expression in the airway lumen. In contrast the reported significant albeit weak relationship with sputum eosinophilia supports a potential role as a local eosinophil chemoattractant, and this is consistent with asthma clinical trial data where the DP2 antagonist fevipiprant reduced sputum eosinophil proportion independently of blood eosinophils¹⁴. *HPGDS* encodes one of two PGD₂ synthases, and it is expressed in a number of immune cells. Perhaps surprisingly, sputum *HPGDS* expression was highest in non-OAD controls, and no relationship between *HPGDS* and supernatant PGD₂ levels was observed. A prior study in bronchial brushings and BAL reported increased brushing *HPGDS* expression in severe asthma vs mild to moderate and non-asthma controls, however similar to our study no association between brushing *HPGDS* expression and BAL fluid PGD₂ levels was observed¹⁷.

This study has several limitations. The study was cross-sectional in nature, and requirements for adequate sputum to prepare both cell suspension and reserve raw sputum for gene expression studies may have introduced some participant inclusion bias. Longitudinal studies of the stability of type 2 markers and airway eosinophilia in COPD are lacking and are merited in the future. Although COPD and severe asthma participants had similar overall ICS use and dose, we can't exclude that ICS treatment affected type 2 markers differentially in the two groups. Participants with COPD were older and more likely to be male compared to those with severe asthma. These demographic differences were expected but remain a potential limitation of the study. Whilst the non-OAD and COPD cohorts excluded present smokers, this was not the case in the severe asthma cohort which included 7 current smokers. We did not assess atopic status of participants in this

study and therefore could not address the relationship to type 2 markers measured. The study was conducted at a single research centre and confirmation in independent cohorts in the future would be desirable, although the consistency of clinical and molecular analyses conducted is a strength when considering the comparisons between disease groups. Almost one third of the participants with severe asthma were treated with maintenance OCS which could have affected results, although our analysis indicated OCS treatment did not alter sputum *IL5* and *IL13* expression. Only four participants were treated with biologics in this study, all of whom had severe asthma and were treated with Xolair, thus treatment with biologics was unlikely to have affected the results.

In sum, although molecular markers of type 2 airway inflammation were similar in ECOPD and severe EA, the relationship of type 2 markers to eosinophilia was weaker in COPD. In contrast to severe asthma, ECOPD defined by clinically validated sputum or blood eosinophil cut-offs did not feature increased molecular markers of type 2 airway inflammation compared to NECOPD. These data support the hypothesis that airway and systemic eosinophilic inflammation in COPD is promoted, at least in part, by mechanisms distinct to the canonical type 2 inflammatory pathway that is prominent in asthma. We posit that the lack of enrichment of a molecular type 2-high subpopulation in COPD using eosinophil-based cut-offs demonstrated in this study may explain the relatively limited clinical benefit observed in recent clinical trials of IL-5 blockers in ECOPD.

Acknowledgments: This study was supported by the National Health and Medical Research Council (NHMRC) Centre of Research Excellence in Severe Asthma (project identifier 1078579) and an NHMRC project grant (project identifier 1200185). M.F. was supported by a Thoracic Society of Australia and New Zealand AstraZeneca Respiratory Research Fellowship. P.G.G. was supported by an NHMRC Practitioner Fellowship. The authors thank Lily Williams, Bilal Malik, Heather MacDonald, Lakshitha Gunawardhana, Bridgette Donati, Kellie Fakes, Naomi Fibbens, Michelle Gleeson, Gabrielle LeBrocq, Amber Smith, Kelly Steel, Paola Urroz and Catherine Delahunty for technical assistance.

Conflict of interest statement: Dr. Fricker reports grants from Thoracic Society of Australia and New Zealand, during the conduct of the study; Dr. McDonald reports grants from NHMRC, grants from Ramaciotti Foundation, grants from Lung Foundation of Australia, grants from Hunter Medical Research Institute, grants from John Hunter Hospital, from The University of Newcastle, during the conduct of the study; grants and personal fees from GSK, grants and personal fees from AstraZeneca, outside the submitted work; Dr. Winter has nothing to disclose; Dr. Baines reports grants from NHMRC, grants from Australian Lung Foundation, grants from John Hunter Charitable Trust, outside the submitted work; In addition, Dr. Baines has a patent 6 gene signature for inflammatory phenotype of copd PCT/AU2018/050644, International application filed on 26/06/18. Australia: AU2018293553; Europe: EP18823411.6; China: CN201880052278.X; The United States: US16/624,995 pending; Dr. Wark has nothing to disclose; Dr. Simpson has nothing to disclose; Dr. Gibson reports personal fees from AstraZeneca, GlaxoSmithKline, Novartis, grants from AstraZeneca, GlaxoSmithKline, outside the submitted work.

References:

1. Hiles SA, Gibson PG, McDonald VM. Disease burden of eosinophilic airway disease: Comparing severe asthma, COPD and asthma-COPD overlap. *Respirology*. 2020.
2. Garudadri S, Woodruff PG. Targeting Chronic Obstructive Pulmonary Disease Phenotypes, Endotypes, and Biomarkers. *Ann Am Thorac Soc*. 2018;15(Suppl 4):S234-S238.
3. Green RH, Brightling CE, McKenna S, et al. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet*. 2002;360(9347):1715-1721.
4. Siva R, Green RH, Brightling CE, et al. Eosinophilic airway inflammation and exacerbations of COPD: a randomised controlled trial. *Eur Respir J*. 2007;29(5):906-913.
5. Singh D, Agusti A, Anzueto A, et al. Global Strategy for the Diagnosis, Management, and Prevention of Chronic Obstructive Lung Disease: the GOLD science committee report 2019. *Eur Respir J*. 2019;53(5).
6. Hastie AT, Martinez FJ, Curtis JL, et al. Association of sputum and blood eosinophil concentrations with clinical measures of COPD severity: an analysis of the SPIROMICS cohort. *Lancet Respir Med*. 2017;5(12):956-967.
7. Hastie AT, Mauger DT, Denlinger LC, et al. Baseline sputum eosinophil + neutrophil subgroups' clinical characteristics and longitudinal trajectories for NHLBI Severe Asthma Research Program (SARP 3) cohort. *J Allergy Clin Immunol*. 2020;146(1):222-226.
8. McDonald VM, Clark VL, Cordova-Rivera L, Wark PAB, Baines KJ, Gibson PG. Targeting treatable traits in severe asthma: a randomised controlled trial. *Eur Respir J*. 2020;55(3).
9. Denlinger LC, Phillips BR, Ramratnam S, et al. Inflammatory and Comorbid Features of Patients with Severe Asthma and Frequent Exacerbations. *Am J Respir Crit Care Med*. 2017;195(3):302-313.
10. Yousuf A, Ibrahim W, Greening NJ, Brightling CE. T2 Biologics for Chronic Obstructive Pulmonary Disease. *J Allergy Clin Immunol Pract*. 2019;7(5):1405-1416.
11. Peters MC, Mekonnen ZK, Yuan S, Bhakta NR, Woodruff PG, Fahy JV. Measures of gene expression in sputum cells can identify TH2-high and TH2-low subtypes of asthma. *J Allergy Clin Immunol*. 2014;133(2):388-394.

12. Peters MC, Kerr S, Dunican EM, et al. Refractory airway type 2 inflammation in a large subgroup of asthmatic patients treated with inhaled corticosteroids. *J Allergy Clin Immunol*. 2019;143(1):104-113 e114.
13. Brusselle GG, Maes T, Bracke KR. Eosinophils in the spotlight: Eosinophilic airway inflammation in nonallergic asthma. *Nat Med*. 2013;19(8):977-979.
14. Gonem S, Berair R, Singapuri A, et al. Fevipiprant, a prostaglandin D2 receptor 2 antagonist, in patients with persistent eosinophilic asthma: a single-centre, randomised, double-blind, parallel-group, placebo-controlled trial. *Lancet Respir Med*. 2016;4(9):699-707.
15. Saunders R, Kaul H, Berair R, et al. DP2 antagonism reduces airway smooth muscle mass in asthma by decreasing eosinophilia and myofibroblast recruitment. *Sci Transl Med*. 2019;11(479).
16. Brightling CE, Gaga M, Inoue H, et al. Effectiveness of fevipiprant in reducing exacerbations in patients with severe asthma (LUSTER-1 and LUSTER-2): two phase 3 randomised controlled trials. *Lancet Respir Med*. 2020.
17. Fajt ML, Gelhaus SL, Freeman B, et al. Prostaglandin D(2) pathway upregulation: relation to asthma severity, control, and TH2 inflammation. *J Allergy Clin Immunol*. 2013;131(6):1504-1512.
18. Hilvering B, Hinks TSC, Stoger L, et al. Synergistic activation of pro-inflammatory type-2 CD8(+) T lymphocytes by lipid mediators in severe eosinophilic asthma. *Mucosal Immunol*. 2018;11(5):1408-1419.
19. Zhang Y, Zhang G, Clarke PA, et al. Simultaneous and high-throughput quantitation of urinary tetranor PGDM and tetranor PGEM by online SPE-LC-MS/MS as inflammatory biomarkers. *J Mass Spectrom*. 2011;46(7):705-711.
20. Simpson JL, Scott R, Boyle MJ, Gibson PG. Inflammatory subtypes in asthma: assessment and identification using induced sputum. *Respirology*. 2006;11(1):54-61.
21. Baines KJ, Simpson JL, Wood LG, Scott RJ, Gibson PG. Transcriptional phenotypes of asthma defined by gene expression profiling of induced sputum samples. *J Allergy Clin Immunol*. 2011;127(1):153-160, 160 e151-159.
22. Fricker M, Gibson PG, Powell H, et al. A sputum 6-gene signature predicts future exacerbations of poorly controlled asthma. *J Allergy Clin Immunol*. 2019;144(1):51-60 e11.

23. Fricker M, Heaney LG, Upham JW. Can biomarkers help us hit targets in difficult-to-treat asthma? *Respirology*. 2017;22(3):430-442.
24. Baines KJ, Simpson JL, Wood LG, et al. Sputum gene expression signature of 6 biomarkers discriminates asthma inflammatory phenotypes. *J Allergy Clin Immunol*. 2014;133(4):997-1007.
25. Baines KJ, Negewo NA, Gibson PG, et al. A Sputum 6 Gene Expression Signature Predicts Inflammatory Phenotypes and Future Exacerbations of COPD. *Int J Chron Obstruct Pulmon Dis*. 2020;15:1577-1590.
26. George L, Taylor AR, Esteve-Codina A, et al. Blood eosinophil count and airway epithelial transcriptome relationships in COPD versus asthma. *Allergy*. 2020;75(2):370-380.
27. Bhalla A, Mukherjee M, Nair P. Airway Eosinophilopoietic and Autoimmune Mechanisms of Eosinophilia in Severe Asthma. *Immunol Allergy Clin North Am*. 2018;38(4):639-654.
28. Jogdand P, Siddhuraj P, Mori M, et al. Eosinophils, basophils and type 2 immune microenvironments in COPD-affected lung tissue. *Eur Respir J*. 2020;55(5).
29. Brightling CE, Bleecker ER, Panettieri RA, Jr., et al. Benralizumab for chronic obstructive pulmonary disease and sputum eosinophilia: a randomised, double-blind, placebo-controlled, phase 2a study. *Lancet Respir Med*. 2014;2(11):891-901.
30. Dasgupta A, Kjarsgaard M, Capaldi D, et al. A pilot randomised clinical trial of mepolizumab in COPD with eosinophilic bronchitis. *Eur Respir J*. 2017;49(3).
31. Pavord ID, Chanez P, Criner GJ, et al. Mepolizumab for Eosinophilic Chronic Obstructive Pulmonary Disease. *N Engl J Med*. 2017;377(17):1613-1629.
32. Criner GJ, Celli BR, Brightling CE, et al. Benralizumab for the Prevention of COPD Exacerbations. *N Engl J Med*. 2019;381(11):1023-1034.
33. Criner GJ, Celli BR, Singh D, et al. Predicting response to benralizumab in chronic obstructive pulmonary disease: analyses of GALATHEA and TERRANOVA studies. *Lancet Respir Med*. 2020;8(2):158-170.

Tables:

Table 1. Clinical characteristics and sputum differential cell data for participants categorized by sputum eosinophilia

	Severe non-eosinophilic asthma (n=34)	Severe eosinophilic asthma (n=50)	Non-eosinophilic COPD (n=52)	Eosinophilic COPD (n=44)
Female (%)	47	62	40	32
Age	59.9 (15.4) ^{†,‡}	55.9 (12.9) ^{†,‡}	70.5 (6.3)	69.7 (8.4)
BMI	32.7 (8.2)	31.5 (7.4)	30.63 (7.9)	31.92 (8.1)
Ex-smoker (n)	19	20	49	39
Current smoker (n)	3	4	0	0
ICS yes/no	34/0	50/0	48/4	40/4
ICS dose	2000 (2000,2000) ^{†,‡}	2000 (2000,2000) ^{†,‡}	2000 (1000,2000)	1500 (1000,2000)
Maintenance OCS yes/no	11/23	15/35	0/52	1/43
LABA yes/no	34/0	49/0	47/5	39/5
LTRA yes/no	2/32	4/45	1/51	0/44
Preβ2 FEV1 % pred	72.46 (22.6) ^{†,‡}	69.06 (20.9) ^{†,‡}	47.12 (16.2)	51.9 (15.1)
Preβ2 FVC % pred	81.62 (16.8)	84.78 (17.5) ^{†,‡}	75.08 (15.9)	73.78 (15.8)
Preβ2 FEV1/FVC	0.67 (0.12) ^{†,‡}	0.63 (0.14) ^{†,‡}	0.48 (0.17)	0.52 (0.14)
Postβ2 FEV1 % pred	73.77 (22.1) ^{†,‡}	70.47 (20.4) ^{†,‡}	49.86 (17.0)	54.35 (15.9)
Postβ2 FVC % pred	82.49 (16.0)	85.16 (16.9) [‡]	77.57 (16.1)	75.27 (15.7)
Postβ2 FEV1/FVC	0.67 (0.12) ^{†,‡}	0.64 (0.14) ^{†,‡}	0.49 (0.17)	0.54 (0.14)
St Georges Respiratory Questionnaire	45.8 (20.6) [†]	50.7 (18.0)	56.4 (16.4)	54.7 (18.7)
Asthma Control Questionnaire	2.0 (1.3)	2.4 (1.1)	-	-
OCS courses (past 12 months)	1 (1, 4.25)	2 (1, 5.5)	2 (1,3)	2 (0.25, 3)

Total sputum cell count (x10 ⁶ /mL)	4.46 (3.15,10.8)	4.10 (2.79, 9.09)	5.76 (3.04, 12.42)	4.73 (2.81, 8.33)
Sputum cell viability (%)	80.2 (66.9, 91.4)	64.41 (47.2, 80.2) [†]	86.7 (67.7 94.5)	79.15 (63.6, 87.9)
Sputum macrophages (%)	45.63 (26.6, 61.4) [†]	40.5 (27.3, 58.4) [†]	24.9 (10.3, 43.5)	32.9 (20.5, 43.4)
Sputum neutrophils (%)	53.8 (31.3, 71.2) ^{†, §}	39 (17.4, 53.4) ^{†, ‡}	69.13 (46.3, 85.7) [‡]	53.15 (39.6, 63.8)
Sputum eosinophils (%)	0.5 (0.25, 1) ^{‡, §}	8.75 (4.2, 17.4)	1.13 (0.57, 1.75) ^{‡, §}	5.625 (3.75, 15.1)
Sputum lymphocytes (%)	0.75 (0, 1.56)	0.75 (0, 1.78)	0.75 (0.25, 1.94)	1.25 (0.5, 2.19)
Sputum columnar epithelial cells (%)	1.5 (0.19, 4.56)	2.5 (0.75, 5.31)	1.25 (0, 5.19)	2.25 (1, 4.25)

[†] = p<0.05 vs non-eosinophilic COPD, [‡] = p< 0.05 vs eosinophilic COPD, [§] = p<0.05 vs eosinophilic severe asthma

Table 2. Correlations of sputum type 2 inflammatory measures

		<i>IL13</i> mRNA	<i>HPGDS</i> mRNA	PGD ₂ pg/10 ⁶ viable cells	Sputum Eosinophils %
Severe Asthma	<i>IL5</i> mRNA	0.907 *** (80)	0.297 ** (80)	0.136 (78)	0.440 *** (80)
	<i>IL13</i> mRNA		0.240 * (80)	0.057 (78)	0.428 *** (80)
	<i>HPGDS</i> mRNA			0.006 (82)	0.236 * (84)
	PGD ₂ pg/10 ⁶ viable cells				0.283 ** (82)
COPD	<i>IL5</i> mRNA	0.824 *** (90)	0.242 * (90)	0.084 (88)	0.245 * (90)
	<i>IL13</i> mRNA		0.244 * (90)	0.095 (88)	0.317 * (90)

<i>HPGDS</i> mRNA			-0.060 (91)	0.271^{**} (93)
PGD ₂ pg/10 ⁶ viable cells				0.204 (94)

Spearman R values reported (n for analyses in brackets). * = p<0.05, ** = p<0.01, *** = p<0.001.

COPD – Chronic Obstructive Pulmonary Disease; *IL5* – interleukin 5; *IL13* – interleukin 13;

HPGDS – Haematopoietic Prostaglandin D2 Synthase; PGD₂ – Prostaglandin D2.

Figure legends:

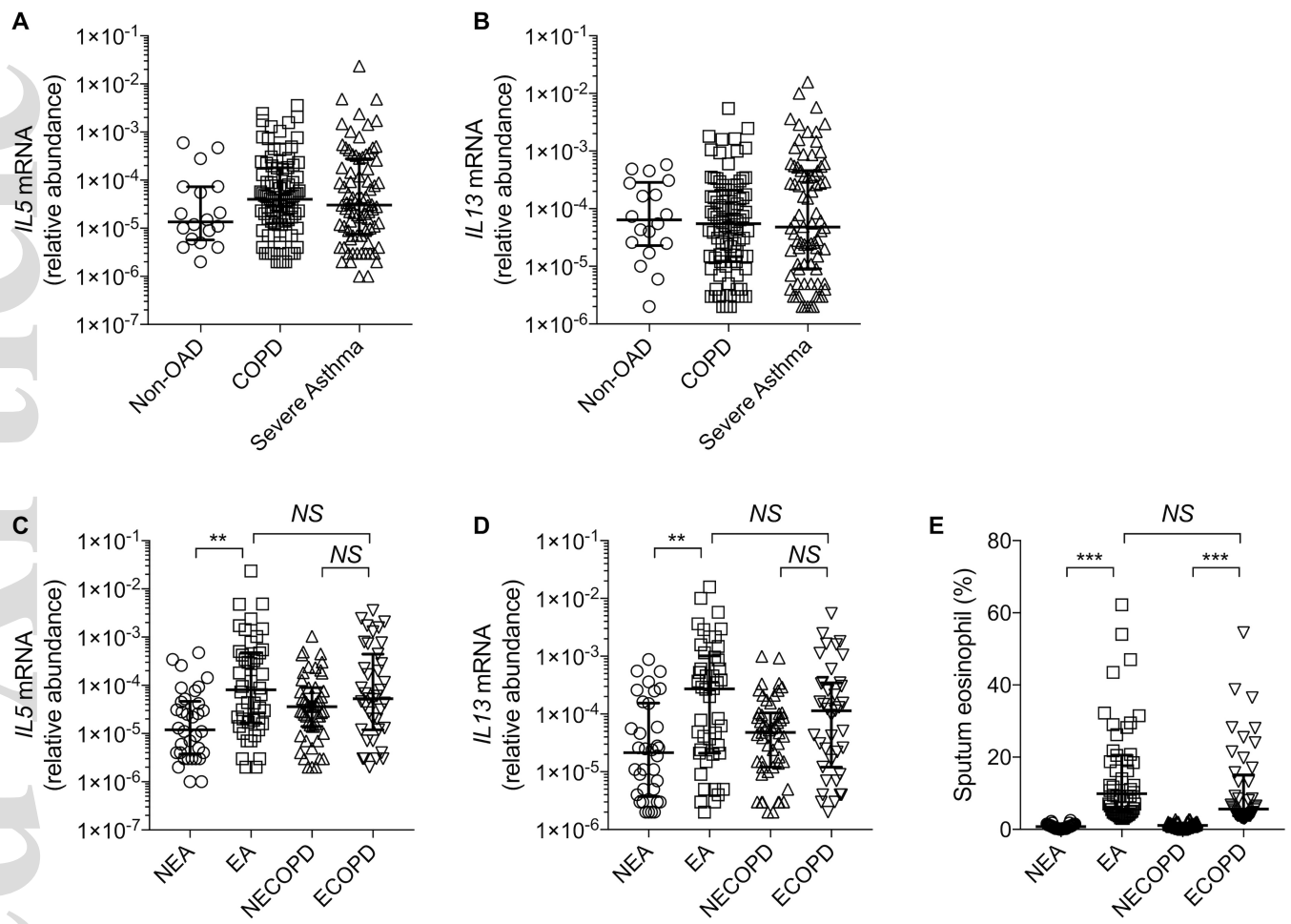
Figure 1. Sputum type 2 cytokine gene expression between disease groups and sputum eosinophil subgroups. Sputum *IL5* mRNA (A) and *IL13* mRNA (B) were quantified in non-OAD controls (n=18), COPD (n=90) and severe asthma (n=80). Quantity of target mRNA is expressed as relative abundance to the housekeeper gene *BACTIN*. Sputum *IL5* mRNA (C), *IL13* mRNA (D) and eosinophil proportion (E) in non-eosinophilic (NEA) and eosinophilic (EA) severe asthma, non-eosinophilic (NECOPD) and eosinophilic (ECOPD) COPD. Dunn's multiple comparison results indicated where Kruskal-Wallis test reached significance. ** = $p < 0.01$, *** = $p < 0.001$.

Figure 2. Sputum PGD₂ levels and *HPGDS* gene expression between disease groups and sputum eosinophil subgroups. Sputum PGD₂ levels were quantified in non-OAD controls (n=18), COPD (n=94) and severe asthma (n=83) (A). Sputum *HPGDS* mRNA was quantified in non-OAD controls (n=19), COPD (n=93) and severe asthma (n=84) (C). Sputum PGD₂ (B) and *HPGDS* mRNA (D) in non-eosinophilic (NEA) and eosinophilic (EA) severe asthma, non-eosinophilic (NECOPD) and eosinophilic (ECOPD) COPD. PGD₂ expressed as pg per 10⁶ viable sputum cells. Quantity of target mRNA is expressed as relative abundance to the housekeeper gene *BACTIN*. Dunn's multiple comparison results indicated where Kruskal-Wallis test reached significance. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

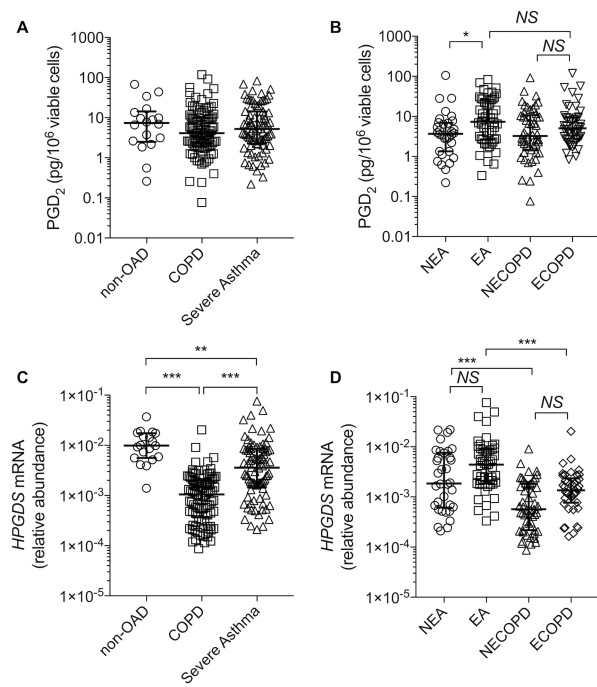
Figure 3. Sputum type 2 cytokine gene expression between peripheral blood eosinophil high or low severe asthma and COPD. Sputum *IL5* mRNA (A), *IL13* mRNA (B) and eosinophil proportion (C) in severe asthma and COPD participants categorized according to high (≥ 300 eosinophils per μL) or low (< 300 eosinophils per μL) peripheral blood eosinophil count. Quantity of target mRNA is expressed as relative abundance to the housekeeper gene *BACTIN*. Kruskal-Wallis test with Dunn's multiple comparisons indicated. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Figure 4. Sputum PGD₂ levels and *HPGDS* gene expression between peripheral blood eosinophil high or low severe asthma and COPD. Sputum PGD₂ levels (A) and *HPGDS* mRNA (B) in severe asthma and COPD participants categorized according to high (≥ 300 eosinophils per μL) or low (< 300 eosinophils per μL) peripheral blood eosinophil count. PGD₂ expressed as pg per 10⁶ viable sputum cells. Quantity of target mRNA is expressed as relative abundance to the

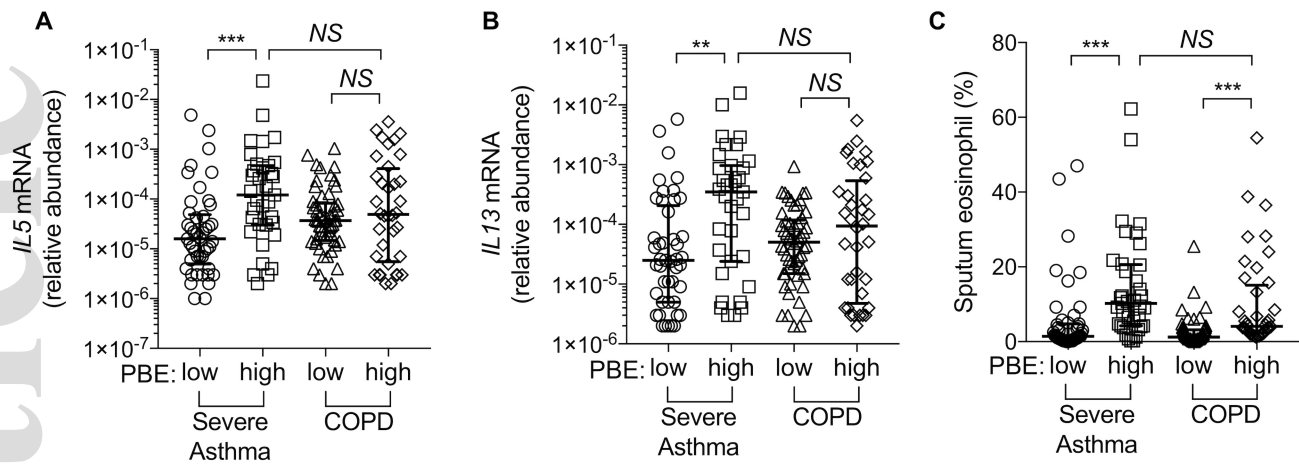
housekeeper gene *BACTIN*. Kruskal-Wallis test with Dunn's multiple comparisons indicated. * = $p < 0.05$, *** = $p < 0.001$.



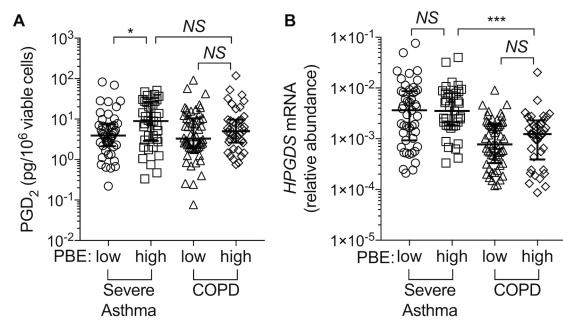
all_14741_f1.jpg



all_14741_f2.jpg



all_14741_f3.jpg



all_14741_f4.jpg

Molecular markers of type 2 airway inflammation are similar between eosinophilic severe asthma and eosinophilic COPD

Authors: Michael Fricker, Vanessa M. McDonald, Natasha A. Winter, Katherine J. Baines, Peter A.B. Wark, Jodie L. Simpson, Peter G. Gibson.

ONLINE SUPPLEMENT

Supplemental methods

Study participants

Participants with OAD met the following diagnostic criteria. Participants with COPD had a physician confirmed diagnosis of COPD and documented evidence of incompletely reversible airflow limitation with a post bronchodilator FEV_1 % predicted $<80\%$ AND $FEV_1/FVC <0.70$ OR physician diagnosis and objective confirmation from CT chest or PFT. Participants with severe asthma had a physician-confirmed diagnosis and met the following criteria: documented evidence of bronchodilator reversibility of $\geq 12\%$ OR AHR OR peak flow diary (diurnal variation $\geq 15\%$ or $>50\text{mL}$); taking maximal ICS/LABA therapy (GINA step 4 treatment – described as 1000 mcg ICS plus LABA) AND/OR maintenance prednisone; post-bronchodilator FEV_1 % predicted $<80\%$ and $FER <0.7$ OR ACQ score ≥ 1.5 OR experiencing a severe exacerbation in the previous 12 months requiring unscheduled OCS use, emergency room presentation or hospitalization.

Participants had stable disease at study visit, defined as an absence of recent respiratory infection, acute exacerbation or change in maintenance therapy in the four weeks prior to recruitment. Participants were excluded if pregnant, breastfeeding or had a primary diagnosis of another respiratory disease (bronchiectasis, pulmonary fibrosis, active tuberculosis or lung cancer). Participants with COPD and non-OAD controls did not include current smokers, while current smokers were not excluded from the severe asthma cohort. Non-OAD control participants had no diagnosis of significant respiratory disease (e.g. asthma, COPD, bronchiectasis), and did not present with hypertonic saline-induced AHR during spirometry and sputum induction.

Sputum induction and analysis

Spirometry (Medgraphics, CPFS/DTM usb Spirometer, Breezesuite v7.1, Saint Paul, USA) was performed pre- and post-bronchodilator (400 µg Salbutamol or equivalent). Sputum induction was performed with hypertonic saline (4.5%) as previously described, with the exception of participants with FEV₁ <1L, where 0.9% saline was used. For inflammatory cell counts, selected sputum portions were dispersed using dithiothreitol, and total cell count and viability determined. Cytospins were prepared, stained with May-Grunwald–Giemsa and a differential cell count obtained from 400 non-squamous cells. For gene expression analyses, 100 µL of selected sputum was added to Buffer RLT (Qiagen, Hilden, Germany) and stored at -80°C until RNA extraction.

Gene expression analysis

Sputum gene expression of *IL5*, *IL13* and *HPGDS* was performed as previously described. Sputum RNA was extracted using Qiagen RNeasy Mini Kits and a QIAcube following manufacturer's instructions. RNA was reverse-transcribed to produce cDNA using a high capacity cDNA reverse transcriptase kit (*Thermofisher*, #4374966) following manufacturer's instructions. Gene expression measurements of *IL5*, *IL13*, *HPGDS* and the housekeepers *BACTIN* and *18S* was performed using standard Taqman real-time qPCR methods and validated primer sets (*Applied Biosystems*, *Thermofisher*). For *HPGDS* expression, cDNA was diluted up to 1:10, and samples were excluded if cycle threshold (Ct) values for housekeepers exceeded 20 (*18S*) or 30 (*BACTIN*) as previously described¹. Due to relatively low levels of expression, *IL5* and *IL13* were measured in undiluted cDNA samples. Gene expression data were expressed as relative abundance of target genes to the housekeeper *BACTIN* using the formula $2^{-\Delta Ct}$, where $\Delta Ct = \text{target gene Ct} - \text{BACTIN Ct}$. Where target genes were undetectable,

a ΔCt value within 0.5 of the largest detected ΔCt (ie lowest detectable abundance for that gene) for that target gene was generated using a random number generator. *IL5* and *IL13* were undetectable in between 26-33% of COPD and severe asthma sputum samples. *HPGDS* mRNA was undetectable in 22% of COPD samples and in 1% of severe asthma sputum samples.

Sputum supernatant PGD₂ measurement

Concentration of Prostaglandin D₂ (PGD₂) in sputum supernatant samples was determined using the PGD₂-MOX (methoxime) ELISA Kit (Catalogue #512011; Cayman Chemical, Michigan, US). An initial quality control experiment determined that a 1:20 dilution of DTT should be present with standards to negate matrix effects of the dithiothreitol (DTT) dispersion (Sputolysin® reagent; Catalogue #560000; Calbiochem®) reagent present in assayed sputum supernatants. Sputum supernatant samples were combined in a 1: 1 ratio with the supplied methyl oximating (MOX) reagent and heated at 60°C for 30 minutes. These methoximated samples were then spun at 550×g for 5 minutes and diluted 1:2 using the provided ELISA buffer for assay. The remainder of the assay was performed according to manufacturer's instructions. Standards, samples and control wells were prepared in duplicate according to the manufacturer's instructions and incubated overnight (~18 hours) at 4°C. Following overnight incubation, 200 µL of Ellman's Reagent was added per well and incubated at room temperature on a plate shaker at 400rpm for 90 minutes in the dark. Absorbance was measured at a wavelength of 412nm using the CLARIOstar® microplate reader (BMG LABTECH, Germany). Analysis of the assay was performed according to the manufacturer's instructions, using the Cayman Chemical duplicate workbook for Prostaglandin D₂-MOX ELISA Kit (<https://www.caymanchem.com/analysisTools/elisa>).

Supplementary references

1. Baines KJ, Simpson JL, Wood LG, et al. Sputum gene expression signature of 6 biomarkers discriminates asthma inflammatory phenotypes. *J Allergy Clin Immunol.* 2014;133(4):997-1007.

Supplemental tables

Supplemental table 1. Participant characteristics

	Non-OAD controls (n=19)	COPD (n=96)	Severe Asthma (n=84)
Female (%)	32%	36.5%	56.0%
Age	48.4 (17.9)	70.1 (7.3) ^{*, #}	57.5 (14.0)
BMI	27.6 (4.6)	31.2 (8.0)	32.0 (7.7)
Ex-smoker (n)	7	88	39
Current smoker (n)	0	0	7
ICS yes/no	-	88/8	84/0
ICS dose	-	2000 (1000,2000) [#]	2000 (2000,2000)
Maintenance OCS yes/no	-	1/95	26/58
LABA yes/no	-	86/10	83/0
LTRA yes/no	-	1/95	6/77
Preβ2 FEV1 % pred	93.2 (12.6)	49.0 (15.9) ^{*, #}	70.5 (21.5) ^{&}
Preβ2 FVC % pred	93.8 (12.1)	74.4 (15.7) ^{*, #}	83.5 (17.2) ^{&}
Preβ2 FEV1/FVC	0.78 (0.08)	0.49 (0.16) ^{*, #}	0.65 (0.13) ^{&}
Postβ2 FEV1 % pred	96.8 (12.4)	51.7 (16.6) ^{*, #}	71.8 (21.1) ^{&}
Postβ2 FVC % pred	93.9 (12.2)	76.5 (15.8) ^{*, #}	84.1 (16.5) ^{&}
Postβ2 FEV1/FVC	0.81 (0.08)	0.51 (0.16) ^{*, #}	0.65 (0.13) ^{&}
St Georges Respiratory Questionnaire	-	55.7 (17.4) [#]	48.7 (19.2)
Asthma Control Questionnaire	-	-	2.2 (1.2)
OCS courses (past 12 months)	-	2 (0.5, 3)	2 (1, 5)
Total sputum cell count (x10 ⁶ /mL)	3.38 (2.12, 6.55)	5.13 (2.88, 10.17)	4.19 (2.88, 9.59)
Sputum cell viability (%)	72.8 (54.2, 81.9)	80.6 (67.2, 92.8) [#]	74.1 (51.6, 87.2)
Sputum macrophages (%)	63.5 (35.4, 74.1)	29.6 (15.6, 43.5) ^{*, #}	41.1 (27.8, 58.8)
Sputum neutrophils (%)	30.1 (14.1, 43.4)	60.6 (42.1, 80.9) ^{*, #}	43.6 (22.2, 62.9)
Sputum eosinophils (%)	0.6 (0.2, 1.3)	1.9 (0.8, 4.1) [*]	3.5 (0.8, 11.6) ^{&}
Sputum lymphocytes (%)	2.5 (1.8, 5.3)	1 (0.3, 2) [*]	0.8 (0, 1.7) ^{&}
Sputum columnar epithelial cells (%)	2.5 (0.4, 5.6)	2 (0.5, 4.6)	2.1 (0.5, 4.9)

Mean (SD) or median (Q1,Q3). * = p<0.05 COPD vs non-OAD controls. # = p<0.05 COPD vs Severe Asthma. & = p<0.05 Severe asthma vs controls. OAD – Obstructive Airway Disease; COPD – Chronic Obstructive Pulmonary Disease; BMI – Body Mass Index; ICS – Inhaled Corticosteroid; OCS – Oral Corticosteroid; Preβ2 – Pre beta-2 agonist; Postβ2 – Post beta-2 agonist; FEV1 – Forced Expiratory Volume in 1 second; FVC – Forced Vital Capacity

Supplemental table 2. Clinical characteristics and sputum differential cell data for participants categorized by peripheral blood eosinophil count threshold.

	Severe non-eosinophilic asthma (n=47)	Severe eosinophilic asthma (n=37)	Non-eosinophilic COPD (n=56)	Eosinophilic COPD (n=40)
Female (%)	47	68	36	38
Age	58.4 (14.4) ^{†,‡}	56.3 (13.6) ^{†,‡}	71.4 (7.0)	68.3 (7.5)
BMI	33.3 (8.3)	30.2 (6.6)	30.3 (7.6)	32.5 (8.5)
Ex-smoker (n)	26	18	53	35
Current smoker (n)	6	1	0	0
ICS yes/no	47/0	37/0	51/5	37/3
ICS dose	2000 (2000,2000) [‡]	2000 (2000,2000) [‡]	1500 (500,2000)	2000 (1000,2000)
Maintenance OCS yes/no	16/31	10/27	0/56	1/39
Preβ2 FEV1 % pred	70.44 (22.0) ^{†,‡}	70.5 (21.1) ^{†,‡}	50.7 (16.1)	47.3 (15.3)
Preβ2 FVC % pred	82.8 (17.8) [‡]	84.4 (16.5) [‡]	76.7 (15.0)	71.3 (16.6)
Preβ2 FEV1/FVC	0.65 (0.13) ^{†,‡}	0.64 (0.14) ^{†,‡}	0.49 (0.16)	0.50 (0.15)
Postβ2 FEV1 % pred	71.32 (21.6) ^{†,‡}	72.47 (20.6) ^{†,‡}	53.21 (16.8)	50.09 (16.3)
Postβ2 FVC % pred	83.51 (17.5) [‡]	84.78 (15.3) [‡]	78.91 (15.4)	73.29 (16.2)
Postβ2 FEV1/FVC	0.65 (0.12) ^{†,‡}	0.66 (0.15) ^{†,‡}	0.51 (0.16)	0.52 (0.16)
St Georges Respiratory Questionnaire	47.3 (19.9) [†]	50.5 (18.3)	53.9 (15.8)	58.0 (19.4)
Asthma Control Questionnaire	2.2 (1.2)	2.2 (1.1)	-	-
OCS courses (past 12 months)	1 (1, 5)	2 (1, 4.75)	1 (0,3)	2 (1, 3.75)
Total sputum cell count (x10 ⁶ /mL)	3.96 (2.88,9)	4.59 (2.84, 10.67)	5.22 (2.7, 10.17)	5.09 (3.06, 11.25)
Sputum cell viability (%)	77.8 (65.8, 88.3)	63.6 (46.0, 80.8) ^{†,‡}	80.8 (63.6, 92)	80.6 (71.2, 94.2)
Sputum macrophages (%)	44.5 (29.8, 58) ^{†,‡}	32 (19.6, 59.6)	33.1 (15.8, 46.6)	28.5 (15.6, 41.6)
Sputum neutrophils (%)	44.9 (27, 63.3) [†]	41 (18.1, 62.5) ^{†,‡}	62.3 (42.2, 80.5)	58.1 (41.3, 80.9)
Sputum eosinophils (%)	1 (0.25, 4) ^{‡,§}	9.25 (3.9, 18)	1.3 (0.75, 3.2) ^{‡,§}	4.1 (2.4, 15.1)
Sputum lymphocytes (%)	1 (0, 2)	0.5 (0, 1.25)	1.25 (0.25, 2.2)	0.75 (0.1, 2)
Sputum columnar epithelial cells (%)	2 (0.25, 5)	2.3 (0.75, 4.75)	1.75 (0.25, 5.25)	2 (0.63, 4.1)

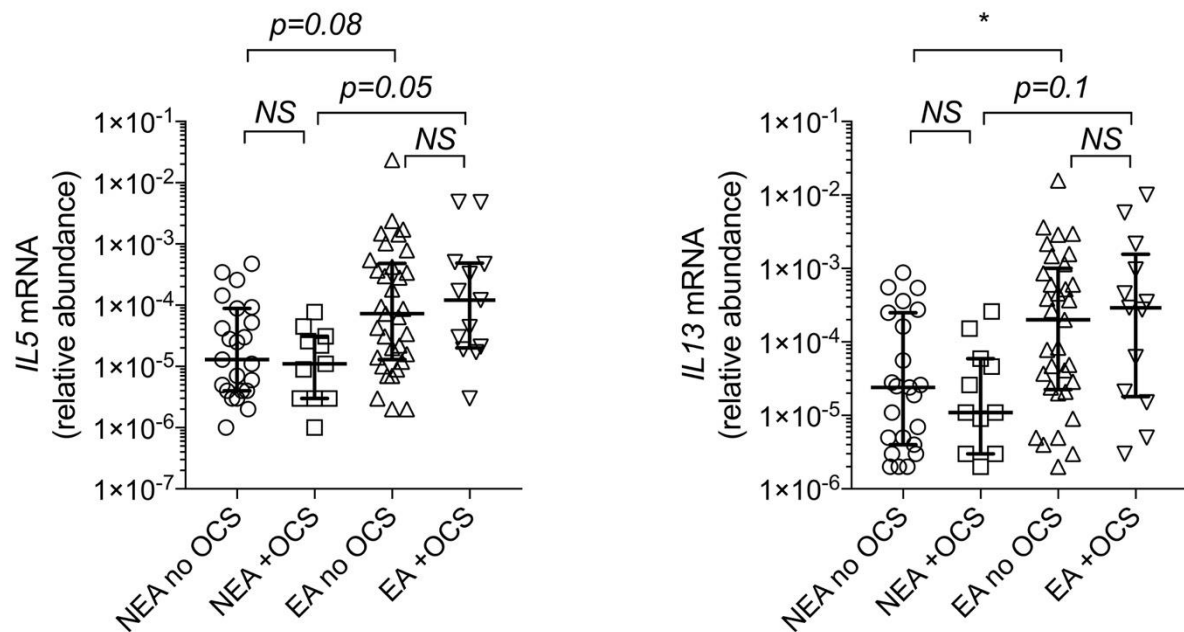
Participants categorized as eosinophilic (peripheral blood eosinophil count $\geq 300/\mu\text{L}$) or non-eosinophilic (peripheral blood eosinophil count $< 300/\mu\text{L}$). [†] = p<0.05 vs non-eosinophilic COPD, [‡] = p<0.05 vs eosinophilic COPD. [§] = p<0.05 vs eosinophilic severe asthma.

Supplemental table 3. Correlation of type 2 inflammatory markers with clinical characteristics.

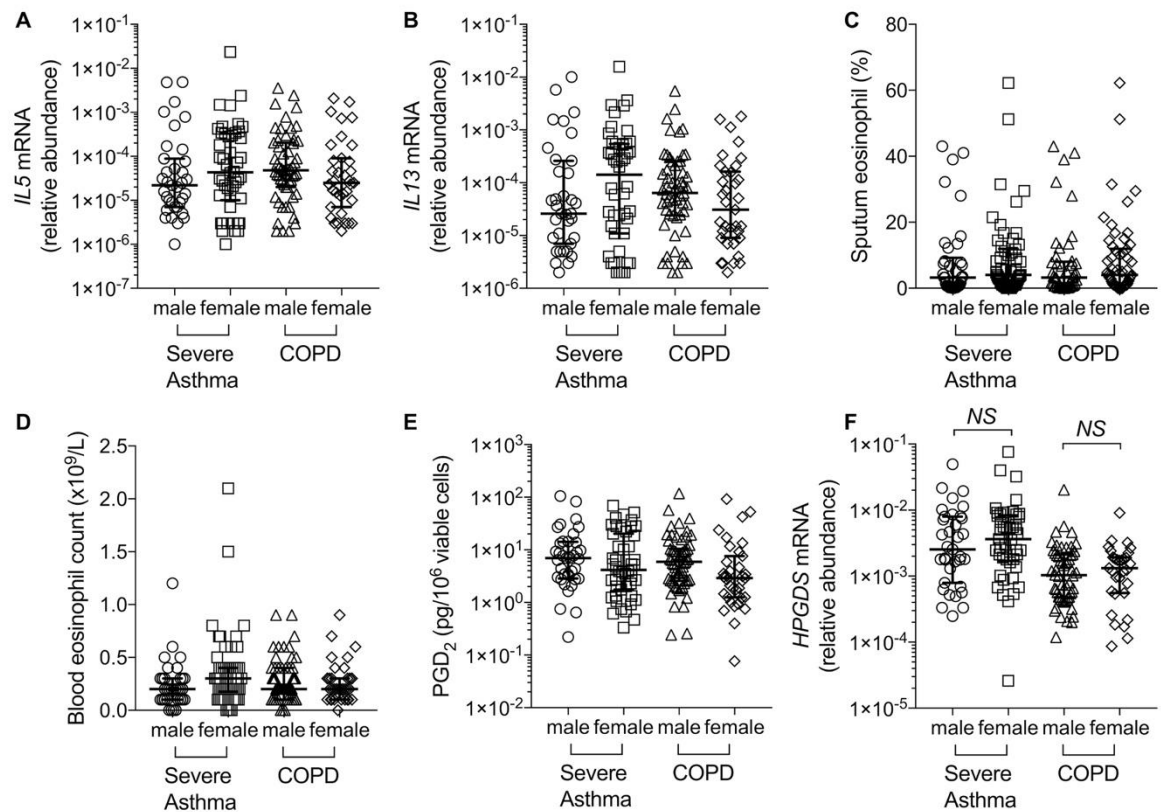
	Severe Asthma				COPD			
	<i>IL5</i> mRNA	<i>IL13</i> mRNA	<i>HPGDS</i> mRNA	PGD2 pg /10 ⁶ viable cells	<i>IL5</i> mRNA	<i>IL13</i> mRNA	<i>HPGDS</i> mRNA	PGD2 pg /10 ⁶ viable cells
Age	0.087	0.018	0.067	-0.144	-0.202	-0.188	-0.071	0.128
BMI	0.019	-0.059	0.023	-0.081	-0.159	-0.164	0.151	0.029
SGRQ	-0.051	-0.053	-0.289*	-0.015	0.006	-0.092	-0.170	-0.036
Preβ2 FEV1 % pred	-0.001	-0.050	0.338**	-0.032	-0.103	0.024	0.234*	-0.061
Preβ2 FVC % pred	0.005	0.018	0.266*	-0.113	0.151	0.232*	0.048	-0.140
Preβ2 FEV1/FVC	0.001	-0.063	0.248*	0.025	-0.193	-0.109	0.237*	-0.011
Postβ2 FEV1 % pred	0.017	-0.031	0.353**	-0.045	-0.096	0.044	0.238*	-0.052
Postβ2 FVC % pred	-0.041	-0.025	0.258*	-0.136	0.164	0.234*	-0.013	-0.201
Postβ2 FEV1/FVC	0.065	-0.004	0.276*	0.070	-0.160	-0.073	0.261*	0.032

Spearman r value (n) displayed. COPD – Chronic Obstructive Pulmonary Disease; BMI – Body Mass Index; Preβ2 – Pre beta-2 agonist; Postβ2 – Post beta-2 agonist; FEV1 – Forced Expiratory Volume in 1 second; FVC – Forced Vital Capacity. SGRQ – St Georges Respiratory Questionnaire. * = p<0.05; ** = p<0.01

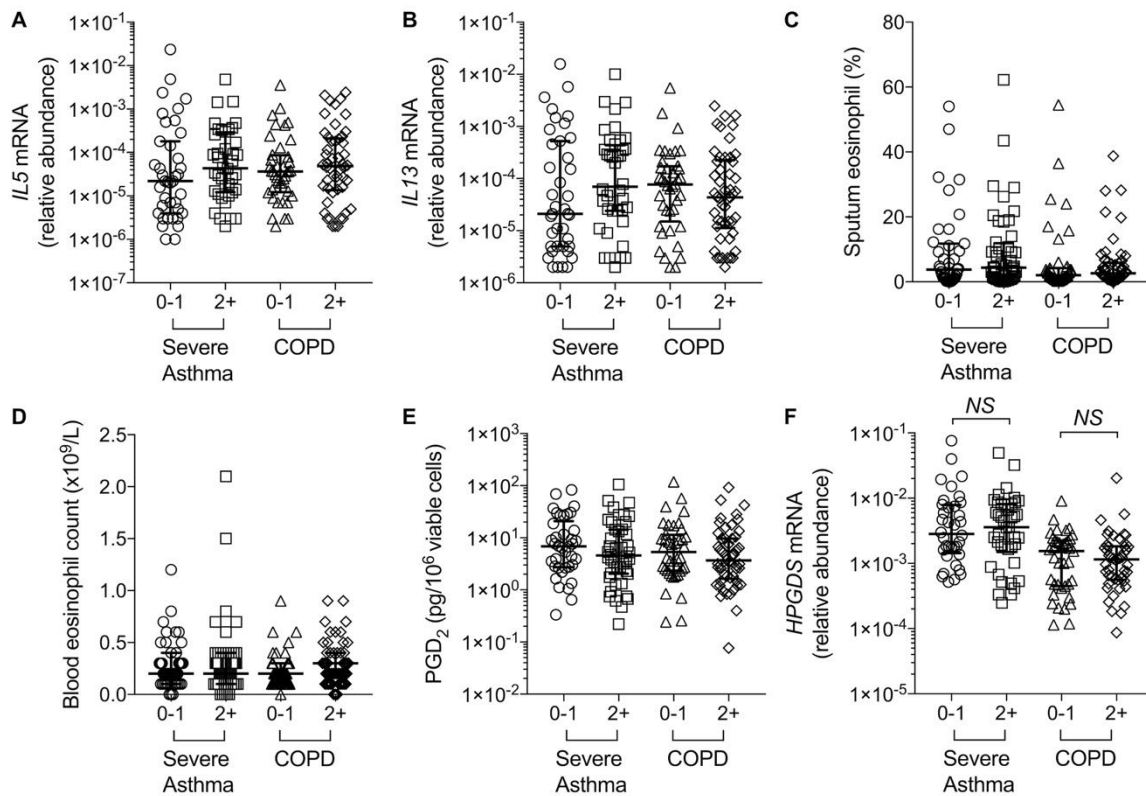
Supplemental figures



Supplemental figure 1. Sputum *IL5* and *IL13* expression in NEA and EA participants categorized by maintenance OCS treatment use. Sputum *IL5* mRNA (A) and *IL13* mRNA (B) in participants with severe asthma categorized into four groups based on presence or absence of sputum eosinophilia and whether or not they were treated with maintenance OCS for their asthma. Dunn's multiple comparison results indicated where Kruskal-Wallis test reached significance. *NS* = non-significant, * = $p < 0.05$.



Supplemental figure 2. Sputum and blood molecular type 2 airway inflammatory markers and eosinophils by gender in severe asthma and COPD. Sputum *IL5* mRNA (A) and *IL13* mRNA (B), sputum eosinophil proportion (C), peripheral blood eosinophil count (D), sputum *HPGDS* mRNA (E) and sputum supernatant PGD_2 were quantified in severe asthma and COPD populations, and are shown by gender. Quantity of target mRNA is expressed as relative abundance to the housekeeper gene *BACTIN*. Kruskal-Wallis test was performed, no significant differences detected.



Supplemental figure 3. Sputum and blood molecular type 2 airway inflammatory markers and eosinophils by exacerbator status in severe asthma and COPD cohorts. Sputum *IL5* mRNA (**A**) and *IL13* mRNA (**B**), sputum eosinophil proportion (**C**), peripheral blood eosinophil count (**D**), sputum *HPGDS* mRNA (**E**) and sputum supernatant PGD_2 were quantified in severe asthma and COPD populations. Participants within each disease group were categorized as infrequent (0-1) or frequent exacerbators on the basis of exacerbations requiring unscheduled courses of OCS in the 12 months prior to sample collection. Quantity of target mRNA is expressed as relative abundance to the housekeeper gene *BACTIN*. Dunn's multiple comparison results indicated where Kruskal-Wallis test reached significance. NS = non-significant.