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“It is a capital mistake to theorize before one has data. Insensibly one begins to twist facts to suit theories, instead of theories to suit facts.”

— *Sherlock Holmes*, “A Scandal in Bohemia” (Arthur Conan Doyle, 1891)

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1 . Abstract

Background

The aetiology of late-onset eosinophilic asthma is largely unknown

Objectives

(1) To understand the additional effects of oral corticosteroids (OCS) on top of inhaled corticosteroids in type-2 (T2) high asthma to understand why late-onset eosinophilic (LOE) patients are more OCS dependent. (2) To assess IL-5 and solubilised IL-5-receptor- α (sIL-5R α) in the blood and airway of eosinophilic asthmatics to understand if the IL-5/IL-5R α axis may be leveraged to dampen IL-5 basal signalling. (3) To assess the burden of rare, high impact mutations in genes related to IL-5 signalling, and look for clonal haematopoiesis of indeterminate potential (CHIP) in LOE asthma.

Methods

(1) A prospective observational trial was performed comparing adjunctive 30 mg prednisolone daily for 10 days with the effects of initiating 200 mcg beclomethasone twice-daily for 8 weeks in T2-high asthmatics. (2) IL-5 and sIL-5R α were measured in blood and sputum of 50 eosinophilic asthmatics and 17 healthy controls. Flow sorted 1e5 eosinophils from blood and sputum were examined for expression of mIL-5R α

(n=7). (3) 123 patients with LOE asthma and 30 healthy controls were deep-sequenced for high-impact variants in IL-5 signalling genes, and for CHIP mutations, using bespoke panels.

Results

(1) OCS produced greater inhibition of blood eosinophils while ICS produced greater inhibition of FeNO. OCS had a broader suppressive effect on T2-mediators in sputum and serum. (2) There was no relationship between plasma sIL-5R α and IL-5. Levels of sIL-5R α were significantly lower in sputum than plasma for eosinophilic asthmatics ($p < 0.05$). There was marked loss of eosinophil mIL-5R α expression in sputum ($p < 0.0001$). (3) Six missense variants were identified in *IL17RA* and 2 high-impact mutations (one frameshift, one missense) were identified in *THRA* in LOE asthma patients: this did not reach Bonferroni significance.

Conclusion

Further work is required to understand the onset and maintenance of high T2-signalling in LOE asthma patients.

Abstract word count = 300

2 . Abbreviations

5'UTR:	5-prime Untranslated Region
ACQ–5:	Asthma Control Questionnaire–5-item version
AER:	annualised exacerbation rates
AERD:	aspirin exacerbated respiratory disease
ALSPAC:	Avon Longitudinal Study of Parents and Children
AN:	allele number
ATS:	American Thoracic Society
BAL:	bronchoalveolar lavage
BD:	bronchodilator:
BDP:	beclometasone dipropionate
BDR:	bronchodilator responsiveness
CADD:	Combined Annotation Dependent Depletion
CD:	cluster of differentiation
cDC2:	type-2 conventional dendritic cells
CH:	clonal haematopoiesis
CHIP:	clonal haematopoiesis of indeterminate potential
COPD:	chronic obstructive pulmonary disease
DAMP:	damage-associated molecular patterns

DTT:	dithiothreitol
EDTA:	ethylenediaminetetraacetic acid
ELISA:	Enzyme-Linked Immunosorbent Assay
ERS:	European Respiratory Society
FCS:	foetal calf serum
FEF ₂₅₋₇₅ :	forced expiratory flow 25–75%
FeNO:	fraction of exhaled nitric oxide
FEV1:	forced expiratory volume in 1 second
gnomAD:	Genome Aggregation Database
GWAS:	genome-wide association study
HC:	healthy control
HEPES:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSC:	haematopoietic stem cells
HSPC:	haematopoietic stem and progenitor cells
ICS:	inhaled corticosteroids
IgE:	immunoglobulin E
IL-:	interleukin
IL-5R α :	interleukin-5 receptor α
ILC2:	group 2 innate lymphoid cells
iNOS:	inducible nitric oxide synthase

Kb:	kilobases
LABA:	Long-Acting β_2 -Agonist
LLOD:	lower limit of detection
LOE:	late-onset eosinophilic
LTE ₄ :	leukotriene E ₄
mIL-5R α :	membrane bound interleukin-5 receptor α
MRI:	magnetic resonance imaging
NICE:	National Institute for Clinical Excellence
NRAD:	National Review of Asthma Deaths
OCS:	oral corticosteroids
PBS:	phosphate buffered saline
PGD ₂ :	prostaglandin D ₂
SAD:	small airways dysfunction
SARP:	Severe Asthma Research Program
sIL-5R α :	solubilised interleukin-5 receptor α
SNP:	single nucleotide polymorphism
T2:	type-2
TARC:	thymus and activation-regulated chemokine
TNF α :	tumour necrosis factor α
TSLP:	thymic stromal lymphopoietin

vAF: variant allele frequency

VEP: Variant Effect Predictor

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5 . Introduction

Asthma is a chronic inflammatory disorder of the airways affecting over 330 million people worldwide and representing a significant health burden.¹ It is the second leading cause of death among chronic respiratory diseases worldwide.¹ In the United Kingdom (UK) there has been an increase in the incidence of asthma in children and adults over the last 40 years.²⁻⁶ Deaths due to asthma are highest amongst adults aged ≥ 65 years.¹⁷

Asthma is a clinical diagnosis and represents a heterogeneous spectrum of disease. Since 1990 guidelines for the diagnosis of asthma have been based on a combination of symptoms of wheeze, breathlessness, chest tightness and cough alongside variable airflow limitation.⁸ Such syndromic definitions are now refined by objectively measurable phenotypic biomarkers. Biomarkers of eosinophilic airway inflammation, including peripheral blood eosinophil count and fraction of exhaled nitric oxide (FeNO), have been used in the management of severe asthma for the past decade and have recently entered diagnostic guidelines.^{9 10}

Eosinophilic asthma is a phenotype of asthma in which airway inflammation is driven predominantly by eosinophils, an immune cell involved in allergic and type-2 (T2) immune responses. Infiltration of the airway by eosinophils was first described as a

¹ Deaths coded as asthma amongst older age-groups have not been part of previous NRAD reviews due to concern over miscoding of chronic obstructive pulmonary disease in older adults and 'resources not permitting'. Gupta.et.al (2018) Thorax, 73:706-712.

feature of asthma by Gollash more than 130 years ago¹¹, and an association between sputum eosinophilia and treatment response to systemic corticosteroids was recognised by Harry Morrow-Brown in 1958.^{12 13}

Late-onset eosinophilic asthma, synonymous in the literature with adult-onset asthma,¹⁴ is eosinophilic asthma that presents symptomatically in adulthood.

Authors disagree on how 'late' disease onsets in the late onset phenotype but ≥18 years is often quoted.¹⁵ This phenotype was recognised by Francis Rackemann in 1947¹⁶ as 'intrinsic' by comparison with the 'extrinsic' allergic asthma of childhood which had clear external triggers such as exposure to pollens and animal dander and features of atopy such as eczema. By comparison the 'intrinsically' arising asthma of adulthood lacked these identifiable external allergens and skin prick tests of the period were usually negative. There was also less association with atopic disease. While Rackemann did not appreciate the importance of eosinophilic inflammation to the pathology of the patients with intrinsic asthma he was describing, his terminology is still in use today and he recognised that "the outlook in extrinsic asthma is generally better than in intrinsic asthma"¹⁶ in keeping with limited remission in this asthma phenotype.

5.1 Estimating the prevalence of late-onset eosinophilic asthma in the UK

How common is late-onset eosinophilic asthma? Approximately 7.2 million of the UK population of 69.8 million people have asthma, according to the National Institute for Clinical Excellence (NICE) and Asthma-Lung UK.^{9 17} One estimate of the prevalence

of late-onset eosinophilic asthma can be derived from the Kerkhof et al.¹⁸ who examined the primary care data of 11 million people diagnosed with asthma in UK primary care. Taking a longitudinal perspective, and with data from birth, they reported 69.5% of asthmatics were diagnosed at ≥ 18 years and 56.9% were diagnosed at ≥ 30 years. As peripheral blood eosinophil counts $\geq 0.30 \times 10^9/L$ are a reasonable proxy for the presence of airway eosinophilia¹⁹⁻²¹ they ascribed an eosinophilic phenotype to an asthma patient if they had ever had a measured blood eosinophil count $\geq 0.30 \times 10^9/L$, at least 3 months after any prescribed oral corticosteroid (OCS). Thus, the percentage of all asthmatics with both onset ≥ 18 years *and* deemed to have an eosinophilic phenotype was 34.6% reducing to 28.5% if the threshold for onset is ≥ 30 years.

The Kerkhof et al. estimate of LOE asthma prevalence is broadly corroborated by phenotypic data from the 376,358 individuals with asthma who volunteered to be part of the UK Biobank.²² The criteria of ever having an eosinophil count $\geq 0.30 \times 10^9/L$ likely over-estimates the number with eosinophilic airway inflammation however, based on sputum differential cell counts obtained from the whole population with asthma in a longitudinal cohort of the Bristol ALSPAC² study in Bristol, UK. Sputum is a more sensitive and specific marker for eosinophilic inflammation in asthma and a sputum eosinophil count $\geq 2.5\%$ is an accepted threshold for diagnosis.^{19-21 23 24} Thirty nine percent of the ALSPAC asthma population had a sputum eosinophil count $\geq 2.5\%$. It should be noted that these asthmatics were assessed at 26–27 years of

² Abbreviation; ALSPAC, Avon Longitudinal Study of Parents and Children.

age²⁵, and the relationship between blood and sputum eosinophilia may be different in older age groups. However, in a parallel cohort of young eosinophilic asthmatics from New Zealand with a relatively high median blood eosinophil count of $0.4 \times 10^9/L$ only 50% had a sputum eosinophil count $\geq 2.5\%$.²⁵

Using a cautious estimate of airway eosinophilic inflammation among ~40-50% of the population whose asthma onset ≥ 30 years we arrive at an estimated population prevalence of ~1.6 million to 2.0 million LOE asthmatics in the UK. This is ~ 2.3–2.9% of the UK population and ~23-28% of all asthmatics. This encompasses LOE asthma of *all* severity, the majority of which is mild and treated in primary care.

Severe asthma is defined by the European Respiratory Society (ERS) / American Thoracic Society (ATS) as requiring high dose inhaled corticosteroids (ICS) plus additional controllers and/or OCS or remaining uncontrolled despite optimized treatment.²⁶ Asthma-Lung UK estimate 4%²⁷ of UK asthma patients have severe asthma while estimates in the literature range between 3 and 10%.^{26 28 29} This severe asthma sub-population of 3–10% of account for more than half of the total community costs of asthma.^{30 31 32} There is evidence that LOE asthma is over-represented among severe asthma patients.³³⁻³⁸ A study applying the same approach used for the UK primary care population by Kerkhof et.al. to a secondary care severe asthma population found ~80% had an eosinophilic phenotype while ~51% of the total population had an age of onset ≥ 30 years.³⁹ Estimating therefore, that about half of severe asthma patients in the UK have the LOE asthma phenotype the prevalence of *severe* LOE asthma in the UK is between 1.5 and 5% of all UK asthma cases or, between 1.5 : 1000 and 1 : 200 of the total UK population.

Patients with severe LOE asthma are the focus of the work presented here.

5.2 What constitutes the late-onset eosinophilic asthma phenotype?

5.2.1 Often refractory to inhaled corticosteroid treatment

That LOE asthma is commonly found amongst severe and/or refractory groups of asthma patients has been highlighted in seminal studies. In the large U.S. severe asthma research program (SARP) five clusters were identified from a large group of severe asthma patients attending secondary care clinics.⁴⁰ Cluster five consisted of patients with adult-onset disease and had the most severe airflow limitation at baseline: this cluster contained the large proportion of LOE asthma patients included in the study. Within the UK, a cluster analysis of asthma phenotypes using data from primary and secondary care performed by Haldar et.al.⁴¹ found an eosinophilic inflammation predominant cluster of patients that was specific to treatment refractory asthma patients attending secondary care (Figure 5-1). This group of patients was typically late-onset and had a slight male preponderance, a finding supported by a nationwide asthma cohort study from Japan.⁴² Other cohort studies have highlighted higher exacerbation rates and higher oral corticosteroid use/dependency in the

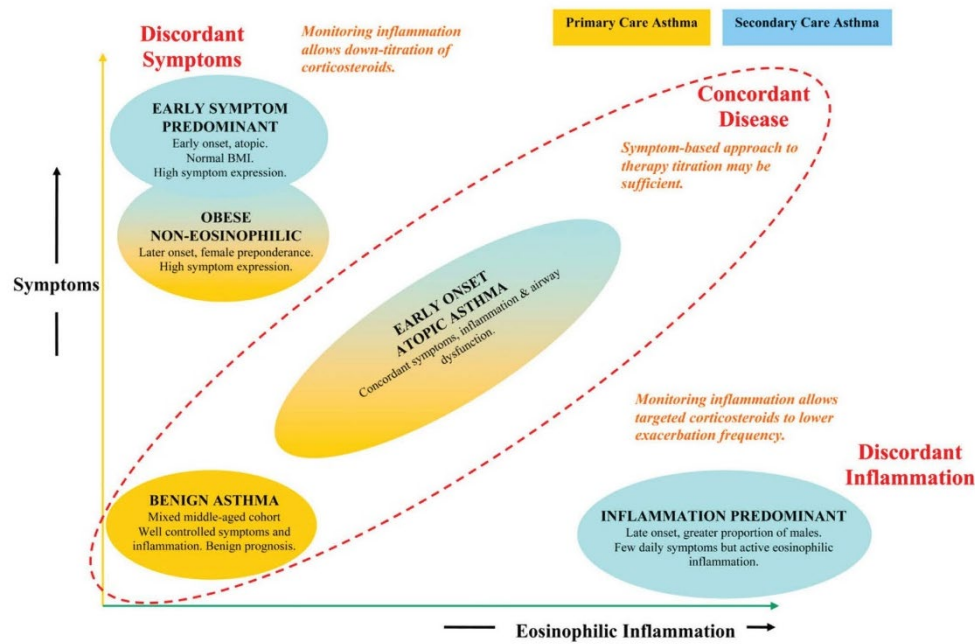


Figure 5-1. Figure from Haldar*, Pavord* et. al. 2008 (Am J Respir Crit Care Med 178: 218–224) “Cluster analysis and clinical asthma phenotypes.” The late-onset eosinophilic asthma cluster (bottom right) was specific to refractory asthma in secondary care.

LOE phenotype of asthma.^{29 36 43} The LOE phenotype is enriched among those severe asthma patients who have the highest levels of eosinophilic inflammation.⁴⁴ The degree to which features of the LOE asthma phenotype are specific to the phenotype and the degree to which they are general features of eosinophilic inflammation-high disease can be difficult to discern.

Key features of the LOE asthma phenotype include the following:

5.2.2 Association with nasal polyposis

One of the features mostly closely associated with this phenotype is nasal polyposis. Nasal polyps share common underlying T2 inflammatory pathophysiology with LOE asthma. Comorbid polyps are characterised by dense eosinophilic tissue infiltration and very high tissue levels of mediators such as IL-5 and periostin that correlate with polyp disease severity,⁴⁵ as levels of these mediators in bronchial tissue correlate with asthma disease severity.⁴⁶⁻⁴⁸ As such, nasal polyps are often understood as manifestations of a single, unified airway disease affecting both the upper and lower airways.^{49 50}

A study of the general asthma population in the Netherlands found LOE asthma was more strongly associated with nasal polyps than any feature, except hay fever.⁵¹ In a select cohort of patients with eosinophilic inflammation from secondary care asthma clinics those patients with late-onset of disease were more than twice as likely to have nasal polyps than childhood-onset eosinophilic patients, with 33.4% of the LOE asthmatic sub-population affected.³⁶ Another study of the LOE asthma phenotype by Amelink.et.al.⁵² found more than half of LOE asthma patients had polyps. Given the prevalence of nasal polyps in the UK population of 1– 6%, and with not all polyps proven eosinophilic at surgical resection,^{53 54} the association between LOE asthma and nasal polyposis appears strong. The LOE asthma phenotype is also more strongly associated with aspirin exacerbated respiratory disease (AERD)^{55 56} and with Samter's triad of asthma, nasal polyposis and aspirin sensitivity.^{57 58}

5.2.3 Elevated IgE without atopy

Patients with LOE asthma typically have lower total serum total immunoglobulin E (IgE) than patients with a childhood-onset allergic phenotype⁵⁹ but a common clinical finding in the work up of LOE patients is a total IgE above the upper limit of normal without signs of specific allergic sensitization.^{36 52} Absence of allergic sensitization in these patients has been challenged by some authors (such as the IDENTIFY project)⁶⁰ who propose that allergens are often identifiable when broader aero-allergen panels are used.^{15 60-62} Others have proposed that an auto-antigen common to the phenotype awaits identification.⁶³ An IgE antibody to components of eosinophilic inflammation itself, such as Charcot-Leyden crystals which have IgE binding properties, has been proposed.⁶⁴ Further, it is argued that higher IgE levels in LOE asthma are a by-product of higher levels of the T2 cytokines IL-4 and IL-13 which promote B cell immunoglobulin class switching to IgE^{65 66} and that group 2 innate lymphoid cells (ILC2s) are key drivers of this effect.⁶⁷ Yet further hypotheses for the raised IgE include production of IgE locally in airway tissue (including bronchial tissue)⁶⁸ and/or as a response to staphylococcal enterotoxins following colonisation of the upper airway due to chronic rhinosinusitis and/or nasal polyp disease: staphylococcal enterotoxins can act as superantigens and drive polyclonal IgE production.⁶⁹⁻⁷¹

5.2.4 Unlikely to remit spontaneously

Clinically, late-onset asthma is regarded as a phenotype in which spontaneous remission is rare.^{44 72 73} A general asthma population study in Italy reported the probability of remission was strongly and inversely related to the age at onset (62.8% and 15.0% in the <10- and ≥ 20 -years age-at-onset groups, respectively) (Figure 5-2).⁷⁴ A Finnish 12-year secondary-care follow-up study of 203 patients with late-onset asthma – not specifically eosinophilic patients but with a median blood eosinophil count of $0.28 \times 10^9/L$ – reported a remission rate of 6 (3%).⁷⁵ It is possible that rates of spontaneous remission in late-onset severe eosinophilic asthma are lower than this. Reports of spontaneous remission in LOE asthma are conspicuously absent from the literature.

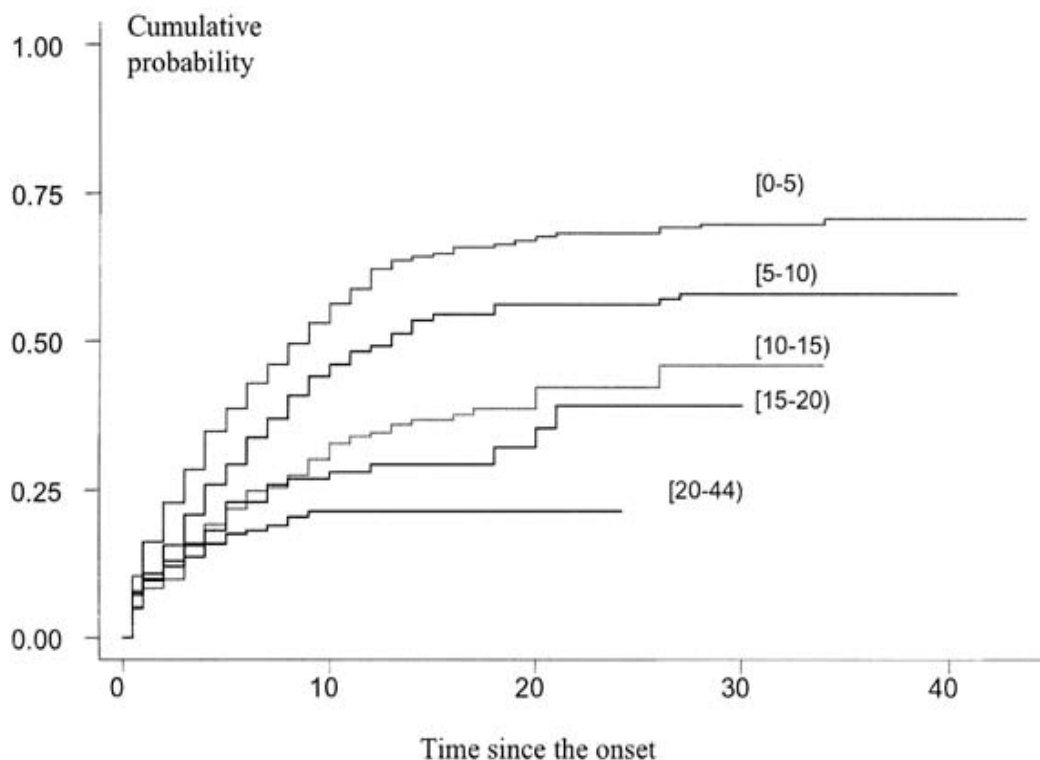


Figure 5-2. Figure from de Marco et al. 2002 (Journal of Allergy and Clinical Immunology, 110:2 228-235) “Incidence and remission in asthma: A retrospective study on the natural history of asthma in Italy.” Kaplan-Meier cumulative probability curves of remission in different age of onset asthma groups (i.e., 0-5, 5-10 years etc). Note, patients were not assayed for eosinophilic inflammation and there is no data for patients with age of onset ≥ 44 years.

By contrast spontaneous remission in childhood onset asthma is well described with ~15 %–65 % of children entering spontaneous remission by early adulthood in longitudinal studies, depending on the definition of remission used.^{76 77 78}

5.2.5 Association with rapid loss of lung function

It is frequently reported that subjects with LOE asthma have lower lung function despite shorter duration of disease when compared with subjects with early-onset asthma.^{59 79} Lung function loss has been closely correlated with eosinophilic inflammation and exacerbations⁸⁰⁻⁸⁴ – two key features of the LOE asthma phenotype. Exacerbations, which are a key feature of the phenotype, also drive lung function loss: linear modelling of lung function data from the DREAM and MENSA phase III trials of the anti-IL-5 antibody mepolizumab demonstrated each eosinophilic exacerbation produced a 50 ml decrease of FEV1.⁸⁵ It is unclear if there is an independent association between *later-onset* of eosinophilic asthma specifically and lung function decline independent of the recognised strong positive correlation between eosinophilic asthma and lung function decline (e.g., Figure 5-3). Similarly,

there is an association between LOE asthma and mucous plugging / small airways pruning⁸⁶ although whether this relationship exists independent of the relationship frequently described between eosinophilic inflammation and mucous plugging / small airways loss⁸⁷⁻⁸⁹ is unclear.

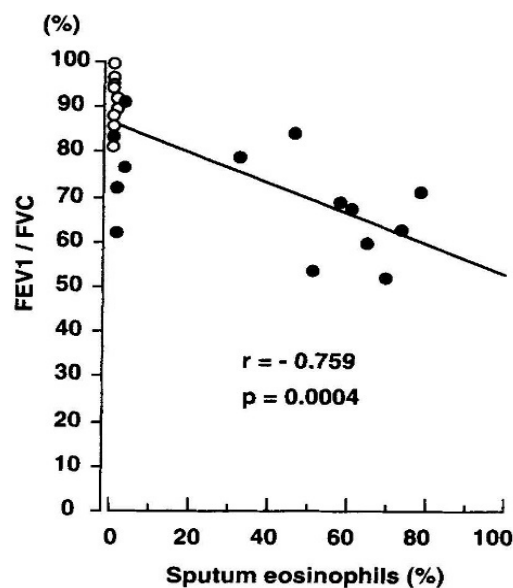


Figure 5-3. Figure from Shoji et.al. 1998 (Journal of Asthma, 35(3) 243–249) “Clinical Implications of Protein Levels of IL-5 in Induced Sputum in Asthmatic Patients.” A correlation between sputum eosinophil counts and airflow obstruction is presented for a cohort of Japanese asthma patients maintained on oral theophylline and inhaled beta-agonist as required (presumed prior to routine clinical use of inhaled corticosteroids). Open circles represent normal controls; closed circles represent asthmatics.

5.2.6 Response to anti-IL-5/anti-IL-5R α and anti-IL-4R α /IL-13 biologics

The trait of eosinophilic inflammation has been successfully targeted in randomized controlled trials of the monoclonal antibodies directed against IL-5 (mepolizumab and reslizumab),^{90 91} against IL-5R α (benralizumab)^{92 93} and against anti-IL-4R α /IL-13 (dupilumab)^{94 95} which reduce exacerbations of asthma and reduce maintenance oral corticosteroid requirements.⁹⁶⁻⁹⁸

Mepolizumab, benralizumab and dupilumab have greater efficacy in patients with higher eosinophil counts.^{99 100 101} For dupilumab, there is additionally a positive relationship between FeNO levels and clinical response.¹⁰¹ Given the high levels of eosinophilic inflammation associated with LOE asthma, and the high predictive value of positive response to corticosteroids, it is unsurprising that LOE asthma patients are recognised among those patients most likely to benefit from anti-IL-5/IL-5R α and anti-IL-4R α /IL-13 treatment. One of the few *post hoc* analyses of the Phase III trials to be stratified by age of onset, Chipps et al.¹⁰² supports this clinical supposition. There was greater response to mepolizumab, benralizumab and dupilumab in patients ≥ 40 years, despite the common entry requirements for blood eosinophil counts and exacerbations treated with OCS. Conversely, in the same analysis, trials of the anti-IgE monoclonal antibody treatment omalizumab showed greater benefit in those patients with age of onset ≤ 18 years. The REALITI-A prospective observational study of oral corticosteroid weaning on mepolizumab treatment¹⁰³ also noted trends towards a greater reduction of clinically significant exacerbations with older age at onset. This conclusion was not reached however, by the *post hoc* review of the MENSA/MUSCA mepolizumab trials which did not report major

differences in efficacy by age of onset.¹⁰⁴ Furthermore, it is also noted that *post hoc* reviews of mepolizumab and benralizumab did not find baseline levels of IgE (which are typically higher in early onset allergic eosinophilic patients) to be associated with exacerbation rates or magnitude of beneficial effects.^{104 105} Again, it is hard to dissociate a specific signal for LOE asthma patient response from the beneficial effects to patients with high levels of eosinophilic inflammation.

5.2.7 Other features of the late-onset eosinophilic phenotype

Several other factors have been proposed to characterise the LOE asthma phenotype including smoke exposure and stressful life events.⁴³ We must also be mindful of the possibility of recall bias when patients recount age of onset of symptoms.¹⁰⁶ Formal studies in this area however, suggest that the majority of patients provide an accurate age of symptom onset.⁴³

5.3 Extrinsic (allergic) asthma vs intrinsic (non-allergic) eosinophilic asthma

The distinctions drawn by Francis Rackemann between ‘extrinsic’ and ‘intrinsic’ causes of asthma are now considered an oversimplification, with different clinical phenotypes exhibiting overlapping mechanisms. However, Rackemann’s initial distinction continues to frame discussion of the underlying pathobiology and will be used here for orientation.

Briefly, extrinsic allergic asthma is driven by adaptive immune responses to environmental allergens resulting in IgE mediated T2 inflammation. Inhaled allergens e.g., house dust mite and animal dander, are taken up by antigen presenting cells which promote the Th2 differentiation of CD4⁺ T-cells via IL-4 signalling. Th2 cells stimulate B-cell class switching to produce allergen specific IgE. IgE binds FcεRI receptors on mast cells and basophils.¹⁰⁷ On re-exposure to an allergen the cross-linking of IgE on mast cells and basophils triggers degranulation and the release of histamine, leukotrienes and prostaglandins.^{108 109} An initial 'early-phase' response is characterised by broncho-constriction followed by a 'late-phase' response driven by the production of T2 cytokines which result in eosinophil recruitment to the airway, airway hyper-responsiveness, goblet hyperplasia and other features of airway remodelling.¹¹⁰⁻¹¹² This pathobiology is associated with infant eczema, atopy in general, sensitization to house dust mite specifically, and childhood onset of asthma (this pattern of progression is often termed 'the atopic march'¹¹³). It is also associated with high levels of specific and total IgE.¹¹⁴⁻¹¹⁶

Intrinsic 'non-allergic' asthma is characterized by non-IgE mediated inflammation. There is a greater role for the innate immune system and epithelial dysfunction. In this model of asthma airway epithelial cells respond abnormally and release alarmins such as thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 which in turn activate and expand innate lymphoid cells (ILC2s)¹⁰³ and activate dendritic cells.¹¹⁷ Dendritic cells drive Th2 differentiation of T cells and production of cytokines while ILC2s substantially upregulate production of cytokines – especially IL-13, but also IL-4 and IL-5.¹¹⁸⁻¹²⁰ ILC2s further drive Th2 differentiation of CD4⁺ T-cells and recruitment of eosinophils to the airway and airway remodelling. This process does not require

antigen specific T-cell priming and murine models of asthma lacking T and B cells (Rag^{-/-} mice) still develop airway eosinophilia and features of remodelling.^{121 122} Consequently, this pathobiology is often considered 'upstream' of extrinsic asthma.¹²³ A schema of updated and contemporary versions of the extrinsic and intrinsic pathways presented by Lambrecht et.al¹²⁴ is reproduced in Figure 5-4.

The degree to which these broad pathways lead to clinical endophenotypes are the subject of ongoing experimental investigation.¹²⁵ It is proposed that important clinical outcomes can be traced to the relative contributions from the two pathways to the phenotype. This might include the better outcomes of childhood onset asthmatic cohorts (presumed IgE mediated) with the anti-IgE medication omalizumab¹⁰², and the relatively lesser effect of the anti-IL-5 mepolizumab on annualised exacerbation rates (AER) in exacerbation-prone children in the MUPPITS-2 trial¹²⁶ when compared to the effects of mepolizumab on AER in adults.³ There is limited impact of anti-IL-5/IL-5R α treatment on the trait of airway hyper-responsiveness / bronchoconstriction that is characteristic of allergic asthma early phase responses. Failure to improve bronchoconstriction through IL-5 blockade (unlike IL-4 and IL-13) has also been demonstrated *in vitro*.^{127 128} Hypothetically, this may be a reason for reduced efficacy in preventing exacerbations among allergic eosinophilic asthmatics.^{129 130}

³ The inclusion threshold of a blood eosinophil count of ≥ 150 cells/ μ L may have been the reason or a bigger contributing factor

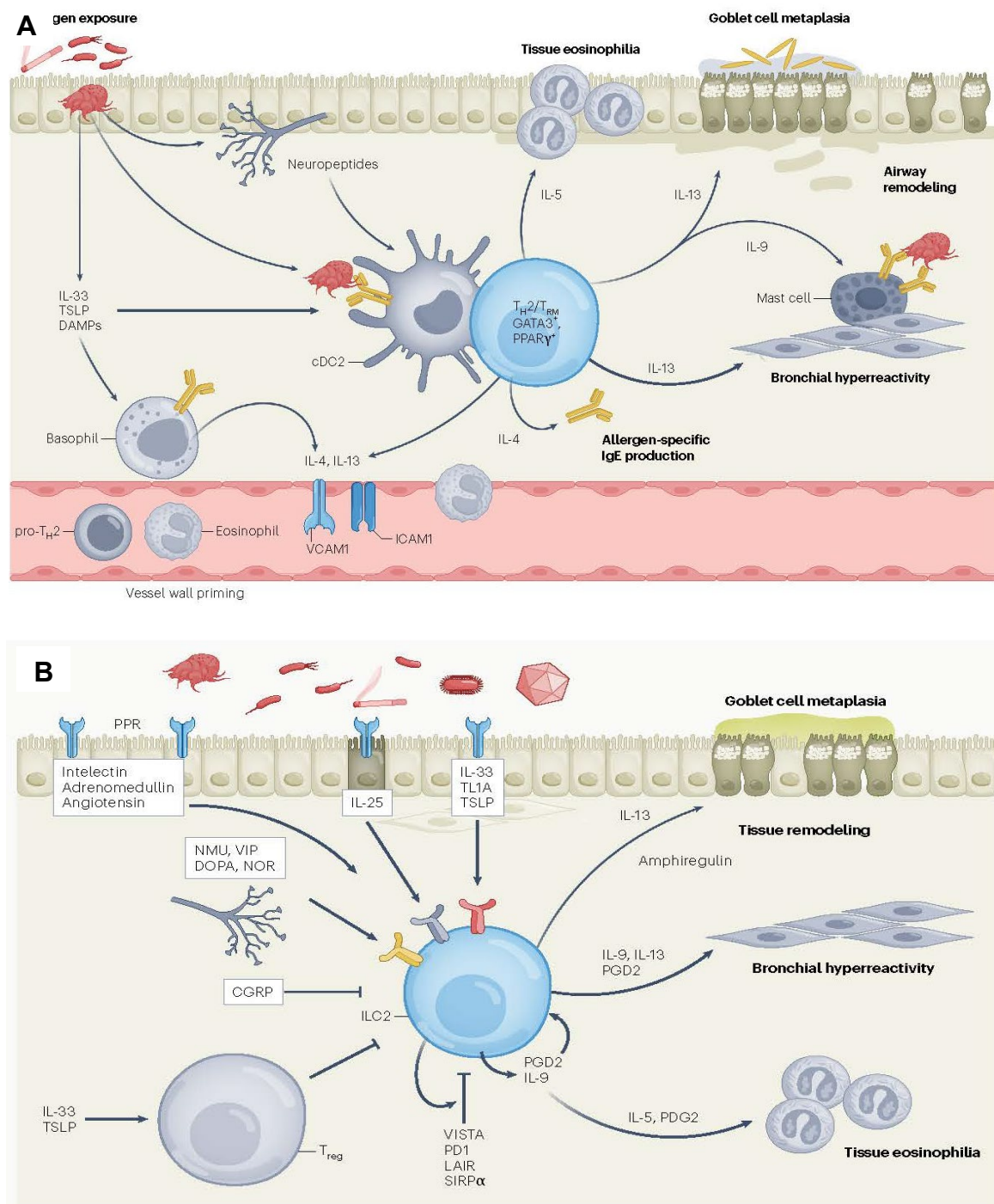


Figure 5-4. Figures from Lambrecht et.al. 2025 (Nature Immunology, 26:1233–1245) “The immunology of asthma.” A contemporary, updated schema of the extrinsic and intrinsic pathways in T2-high asthma. Briefly, (A) Allergic “extrinsic” asthma. Allergen exposure triggers release of epithelial alarmins e.g., IL-33, TSLP, DAMPs and neuropeptides triggering cDC2 activation. cDC2s detect and present allergens to T cells which differentiate to Th2 cells producing T2 cytokines. Basophils also produce cytokines that

help prime the vessel wall to upregulate integrins required for recruitment of eosinophils. Mast cells use allergen-specific IgE to detect allergens and contribute to bronchial hyperreactivity. Mast cells, basophils and Th2 cells also react to epithelial alarmins and neuropeptides directly. (B) Non-allergic “intrinsic” asthma. ILC2 cells are activated in response to epithelial alarmins, such as IL-33 and TSLP derived from ciliated cells and IL-25 from tuft cells. ILC2 cells are potent producers of cytokines, particularly IL-13. Neuropeptides either stimulate or suppress ILC2s. IL-9 and prostaglandin D2 (PGD2) are important amplifiers and effectors of ILC2 function. Abbreviations: T2, type-2; TSLP, thymic stromal lymphopoietin; DAMPs, damage-associated molecular patterns; cDC2, type-2 conventional dendritic cells; IgE, immunoglobulin E.

5.4 The bone marrow compartment in eosinophilic asthma

In asthma, eosinophils identified in sputum and airway tissue are largely derived from progenitor cells in the bone marrow. These cells are terminally differentiated, originating from haematopoietic CD34⁺ stem cells, and do not appear to proliferate once they have left the bone marrow.¹³¹ As such, the bone marrow plays a central role in pathobiology of eosinophilic asthma through increased eosinophil lineage commitment from haematopoietic stem cells. Patients with eosinophilic asthma demonstrate elevated CD34⁺ IL-5Rα⁺ eosinophilic progenitors, not only within the bone marrow but also in the peripheral circulation and airways.^{132 133} eosinophil development in the bone marrow is tightly regulated by the co-ordinated expression of key transcription factors, including GATA-binding protein 1 (GATA-1), CCAAT/enhancer-binding protein-α (C/EBPα) PU box-binding protein (PU.1), Friend of GATA-1 (FOG-1) and interferon consensus sequence-binding protein (ICSBP).¹³¹ Among these, GATA-1 is critical for eosinophil differentiation. Mice lacking a high-affinity palindromic site in the GATA1 promotor (ΔdblGATA mice) exhibit a selective absence of eosinophils.¹³⁴ Such eosinophil-deficient models have been instrumental in elucidating the role of eosinophils in allergic disease.¹³⁵

5.5 IL-5 in eosinophilopoiesis and eosinophilic inflammation

The differentiation of eosinophils within the bone marrow is strongly regulated by IL-3, GM-CSF and IL-5.¹³² Starting with the discovery of IL-5 in 1987¹³⁶ the crucial role of this pleiotropic cytokine for the differentiation, maturation, and survival of eosinophils became evident. IL-5 acts at multiple levels throughout the eosinophil lineage, regulating eosinophil production, differentiation, activation, migration and survival.¹³⁷ In murine models, upon IL-5 stimulation via direct injection or using IL-5 transgenic or reporter mice, the bone marrow can produce several orders of magnitude more eosinophils.¹³⁸⁻¹⁴¹ IL-5 knockout mice are ostensibly eosinophil deficient, but still develop small numbers of blood and tissue eosinophils through baseline homeostatic transcriptional mechanisms.^{142 143} They cannot mount blood or tissue eosinophilia in response to infection with helminths or sensitization and challenge with allergens, because these responses are IL-5 dependent.^{142 143} IL-5 supports local eosinophilopoiesis in the airway, acting on eosinophil progenitors recruited to inflamed lung tissue.¹⁴⁴⁻¹⁴⁶ Although a cytokine, IL-5 also has selective chemoattractant effects on eosinophils.^{147 148}

Bronchoscopy sub-studies of the anti-IL-5 monoclonal antibody treatment mepolizumab in humans have shown a significant reduction in bronchial eosinophils with treatment.¹⁴⁹ These include one study performed specifically in LOE patients with fixed airflow obstruction.¹⁵⁰ Another study demonstrated attenuated eosinophil recruitment to the lung in patients taking mepolizumab following a segmental allergen challenge.¹⁵¹ Both mepolizumab and the intravenous anti-IL-5 treatment reslizumab reduce sputum and blood eosinophilia.¹⁵²⁻¹⁵⁵

IL-5 has effects beyond eosinophils including priming the activation and survival of basophils, which express functional IL-5 receptor α (IL-5R α) and respond to IL-5.¹⁵⁶ IL-5 has indirect effects on airway structural cells too: airway remodelling is suppressed in IL-5 knockout mouse models.^{157 158} The results of these animal studies have some corroboration in bronchial biopsies taken from asthmatic patients treated with mepolizumab. Anti-IL-5 treatment reduced the reticular basement membrane by reducing deposition of extracellular matrix proteins such as pro-collagen, tenascin and lumican.¹⁴⁹ How much these effects might be attributed to IL-5 effects independent of eosinophil depletion remains to be determined. The anti-eosinophil small molecule therapy dexamipexole has clinical benefit in asthma but no effect on chronic rhinosinusitis with nasal polyposis (CRSwNP), unlike the anti-IL-5 mepolizumab.^{159 160} IL-5 does have a broader range of effects on other cells which express IL-5R α and may contribute to disease through alternate or additional mechanisms.¹⁶⁰ *In vitro* studies of epithelial cells show downregulation of genes related to epithelial barrier function following exposure to IL-5. *Ex vivo* analyses of mast cells show that IL-5 can prime them for activation,¹⁶¹ and co-culture of fibroblasts with IL-5 can induce their proliferation.¹⁶²

The production of IL-5 has been attributed to cells of both the innate and adaptive immune systems in the airways, as discussed. These include mast cells which are the main source of IL-5 in the bronchial tissue of healthy people, according to the single cell RNA sequencing of the Human Lung Cell Atlas project.¹⁶³ It also includes ILC2s, and activated Th2 lymphocytes.^{48 164} The processes underlying the basal regulation of sustained IL-5 signalling in LOE asthma however, remains unknown. What is clear is that tissue and luminal airway levels of IL-5 are higher than systemic

levels of IL-5 in eosinophilic asthma,^{165 166} that IL-5 levels are significantly higher in the airways of eosinophilic asthmatics than healthy controls,¹⁶⁷ and that systemic levels of IL-5 in severe eosinophilic asthma are usually higher than systemic levels of IL-5 in healthy counterparts.¹⁶⁸

Many aspects of IL-5 signalling in eosinophilic asthma remain unanswered. The precise molecular mechanisms governing IL-5 transcription in humans, beyond the transcription factors *GATA3* and *STAT6*, are largely unknown.¹⁶⁹ In the case of ILC2 cells triggering amplified production of IL-5 for example, what determines the triggering threshold on exposure to the alarmins IL-25 and IL-33, what effects intermittent or chronic exposure to these alarmins have, and how basal IL-5 signalling is maintained (particularly important to the intrinsic model of asthma), are yet unknown.¹⁷⁰ How IL-5 expression is down or up-regulated in steroid resistant asthma is also an important research question.¹⁷¹

5.6 Plan

In this work I will explore the mechanism of IL-5 signalling in 3 separate novel studies in each of 3 chapters:

- (i) 'Differential effects of inhaled and oral corticosteroids on T2 inflammation'

In the first chapter I look at the response to the initiation of low-dose inhaled corticosteroids (ICS) in corticosteroid naïve patients with T2-high asthma and compare this to the addition of oral corticosteroids (OCS) in

patients who have T2-high asthma despite medium-high dose ICS. The additional benefits of OCS to patients established on ICS are not understood, despite OCS being an established adjunctive therapy for ICS refractory disease. The aim of this work is to understand why patients with late-onset eosinophilic asthma are enriched among the most OCS-dependent severe asthma patients. The effects of ICS and OCS treatments on systemic and airway IL-5 levels are weighed along with the effects on other T2 mediators.

- (ii) 'What is the role of soluble IL5 receptor α (sIL5R α) in eosinophilic asthma?'

In the second chapter I measure the solubilised IL-5R α receptor in the plasma and airway of eosinophilic asthmatics for the first time and look at eosinophil expression of mIL-5R α in the blood and sputum of eosinophilic asthmatics. The aim of this study is to assess whether the regulatory effects of sIL-5R α on IL-5 seen *in vitro* may be applicable to eosinophilic asthma *in vivo*.

- (iii) 'Searching for rare, high impact germline mutations and somatic mutations in late-onset eosinophilic asthma'

In the third chapter I look for rare, high-impact genetic mutations in genes that could influence basal signalling of IL-5 and T2 inflammation (e.g., *IL5RA*). I do this by deep sequencing a cohort of highly phenotyped LOE asthma patients using a targeted gene panel. The gene burden of mutations is assessed with reference to a large publicly available dataset of sequenced healthy controls (gnomAD). The aim of this approach is to highlight important mechanistic pathways in eosinophilic asthma: it has not been used in asthma before. I also consider whether somatic mutations in these genes, or mutations that drive the development of clonal haematopoiesis of indeterminate potential (CHIP), could have a role in LOE asthma. I assess the prevalence of CHIP with reference to a pool of healthy controls sequenced with the LOE asthmatics. I also sequence a small group of young healthy controls (a 'panel of normals') to help distinguish small clones from sequencing artefact. There have been no studies of clonal haematopoiesis in asthma to date. The aim of this analysis is to determine if there are elevated rates of CHIP in LOE asthma.

In the Summary Discussion section, I bring together the findings from these chapters and discuss how they might inform the future research of myself and/or others in LOE asthma.

6 . Differential effects of inhaled and oral corticosteroids on T2 inflammation

In this chapter I perform a prospective observational study, comparing the initiation of low-dose inhaled corticosteroids (ICS) in corticosteroid naïve patients with T2-high asthma with the addition of oral corticosteroids (OCS) in patients with T2-high asthma despite medium-high dose ICS. The additional benefits of OCS to patients established on ICS are not understood despite OCS being an established adjunctive therapy for ICS refractory disease. One of the aims of this work is to understand why patients with late-onset eosinophilic asthma are enriched among the most OCS-dependent severe asthma patients.

6.1 Introduction

6.1.1 Late-onset eosinophilic asthma and benefit from oral corticosteroids

Around a quarter of asthma cases in the UK are the late-onset eosinophilic asthma phenotype yet the phenotype constitutes around half of treatment refractory patients referred to severe asthma clinics.⁴ Cohort studies of LOE asthma patients have consistently documented that this group often require high doses of anti-inflammatory medications.^{36 52 172} It is recognised that the LOE asthma phenotype is prominent among patients with persistent T2 inflammation despite inhaled corticosteroid (ICS) treatment. These ICS refractory patients may benefit from the addition of oral corticosteroid (OCS) treatment.^{24 29 35 173 174} One illustration of this comes from the longitudinal 2-week placebo controlled BIOAIR trial of prednisolone treatment in a group of 85 severe asthma participants where change in lung function was the outcome measure (Figure 6-1).³⁵ The median daily beclomethasone equivalent (BDP) inhaled corticosteroid dose among the recruited patients was 1600 mcg (mean 2,044 mcg, SD $\pm$ 912). Despite the high daily dose of ICS, these severe asthma participants had demonstrably high baseline biomarkers of eosinophilic inflammation: mean sputum eosinophil count was 17.9%, mean peripheral blood eosinophil count was $0.38 \times 10^9/L$ and mean FeNO 47 ppb. These patients were also inadequately controlled with mean ACQ-5 score 2.03. The main predictors of

⁴ See Introduction for estimates

improvement in FEV1 $\geq 12\%$ when compared to any improvement in FEV1 were, *older age of onset*, higher baseline FeNO, with higher baseline sputum eosinophil counts trending to significance.

	Asthma group	Non-responders $\Delta\text{FEV}_1 < 0\%$	Responders $\Delta\text{FEV}_1 \geq 0\%$	p Value (responders $\Delta\text{FEV}_1 \geq 0\%$ vs non- responders)	Responders $\Delta\text{FEV}_1 \geq 12\%$	p Value (responders $\Delta\text{FEV}_1 \geq 12\%$ vs non- responders)	p Value (responders $\Delta\text{FEV}_1 \geq 0\%$ vs responders $\Delta\text{FEV}_1 \geq 12\%$)
Number of patients (n)	146	67	79	—	23	—	—
Severe asthmatics (n)	84	35	49	—	15	—	—
Age (y) (min–max)	47.5 $\pm$ 1.1 (18–72)	46 $\pm$ 1.82 (18–72)	49 $\pm$ 1.31 (26–69)	0.28	52 $\pm$ 2.27 (31–67)	0.97	0.27
Females (%)	57.5	56.7	58.2	0.715 ^a	60.9	0.64 ^a	0.82 ^a
FEV ₁ (% pred)	80.22 $\pm$ 1.84	88.0 $\pm$ 2.44	73.74 $\pm$ 2.5	0.0002	66.04 $\pm$ 4.3	0.0001	0.148
FEV ₁ /FVC	0.69 $\pm$ 0.010	0.73 $\pm$ 0.013	0.65 $\pm$ 0.015	0.0002	0.6 $\pm$ 0.03	0.0002	0.086
Reversibility	9.48 $\pm$ 0.509	10.01 $\pm$ 0.72	9.04 $\pm$ 0.71	0.215	8.29 $\pm$ 0.96	0.190	0.694
Atopy (%)	40.4	40.3	41.7	1.000 ^a	34.8	0.5 ^a	0.5 ^a
Age of onset	26.78	27.57	26.13	0.74	36.71	0.043	0.012
BMI (kg/m ²)	27.04 $\pm$ 0.407	27.07 $\pm$ 0.55	27.01 $\pm$ 0.6	0.631	27.13 $\pm$ 1.06	0.781	0.939
QoL (SGRQ)	35.50 $\pm$ 1.84	31.41 $\pm$ 2.43	39.11 $\pm$ 2.7	0.044	44.5 $\pm$ 4.16	0.007	0.293
ACQ (Juniper)	1.59 $\pm$ 0.094	1.44 $\pm$ 0.12	1.72 $\pm$ 0.141	0.24	2.13 $\pm$ 0.23	0.010	0.101
CRP (mg/L)	5.01 $\pm$ 0.64	5.05 $\pm$ 1.10	4.98 $\pm$ 0.742	0.59	4.06 $\pm$ 1.25	0.7	0.451
FeNO (ppb)	44.0 $\pm$ 4.16	48.0 $\pm$ 8.04	41.71 $\pm$ 4.7	0.9	70.86 $\pm$ 13.06	0.073	0.014
Sputum cells ($\times 10^6$)	2.6 $\pm$ 0.61	4.49 $\pm$ 1.26	1.02 $\pm$ 0.20	0.0003	0.77 $\pm$ 0.2	0.004	0.947
Sputum eosinophils (%)	12.06 $\pm$ 2.27	6.28 $\pm$ 2.08	16.8 $\pm$ 3.6	0.015	29.70 $\pm$ 8.31	0.002	0.088
Sputum neutrophils (%)	42.61 $\pm$ 2.96	42.84 $\pm$ 4.51	42.4 $\pm$ 4.0	0.95	41.4 $\pm$ 7.66	0.86	0.905
Blood eosinophils ($\times 10^9$)	0.35 $\pm$ 0.03	0.311 $\pm$ 0.043	0.38 $\pm$ 0.04	0.072	0.50 $\pm$ 0.092	0.025	0.205

p < 0.05 in bold.
^a Chi-square test.

Figure 6-1. Table from Kupczyk et.al. 2013 (Respiratory Medicine, 107:1521–1530). A comparison between responders and non-responders from the BIOAIR trial of oral corticosteroids. Response is defined as an improvement in baseline FEV1 $\geq 12\%$. Note age of onset as a significant predictor of response to oral corticosteroids along with FeNO and with sputum eosinophilia approaching significance.

The BIOAIR trial cohort while enriched for patients with eosinophilic inflammation, is representative of other severe eosinophilic asthma cohorts in the published literature

with persistent T2 inflammation despite ICS treatment.^{36 172} Indeed, the 2018 Lancet commissioners on asthma²⁹ proposed that evidence of persistent airway eosinophilia (in adults) despite the prescription of a moderate dose of inhaled corticosteroid should form part of the definition of severe asthma if there was a past history of exacerbation.

6.1.2 Inhaled corticosteroid resistant asthma

Why some patients demonstrate persistent T2 inflammation despite ICS is not fully understood.^{172 175} ICS treatment reduces levels of biomarkers of T2 inflammation such as fractional exhaled nitric oxide (FeNO), sputum and blood eosinophil counts.¹⁷⁶⁻¹⁸³ The clinical benefits of ICS treatment are often identified to be greater in the presence of higher levels of T2 inflammation.¹⁸⁴ The CAPTAIN trial of patients with moderate asthma for example, identified a benefit of higher doses of inhaled corticosteroids only in those patients with higher blood and FeNO levels.

The benefits of escalating inhaled corticosteroids in patients with refractory eosinophilic inflammation has been trialled in research settings aiming for FeNO suppression, often alongside attempts to assess adherence to treatment with chipped inhalers.^{172 185 186} In clinical practice, doses of ICS treatment are escalated in response to inadequate symptom control and subsequently severe eosinophilic patients are more frequently established on high dose ICS.¹⁸⁷

The benefits of escalating ICS in this way are disputed. Evidence from meta-analyses suggests that the majority of clinical benefit from ICS treatment occurs at

low doses (Figure 6-2).¹⁸⁸ Escalating to higher doses rarely increases clinical benefits but does increase incidence of adverse effects. In support of this, hypothalamic pituitary axis data suggest the majority of higher dose ICS therapy is systemically absorbed (Figure 6-3)¹⁸⁹ and OCS-sparing effects from ICS appear to be due to systemic absorption.¹⁹⁰ These data suggest there may be pharmacological and / or physiological limitations to dose-response from ICS treatment in the airways.

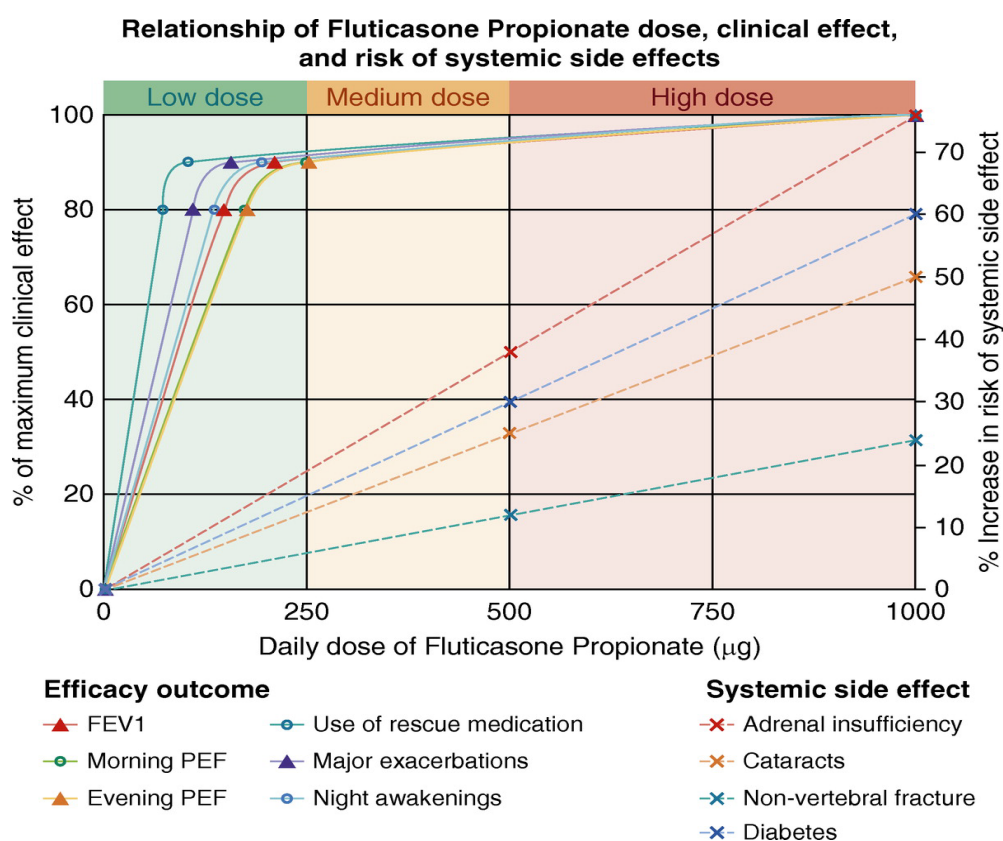


Figure 6-2. Figure from Beasley et.al. 2019 (American Journal of Respiratory and Critical Care Medicine 199 (12) 1471–1477 “Inhaled Corticosteroid Therapy in Adult Asthma: Time for a New Therapeutic Dose Terminology.” Schematic dose–response curves for different for different outcomes for efficacy and adverse effects with inhaled corticosteroids using data from randomized controlled trials of inhaled corticosteroids in steroid naïve patients.

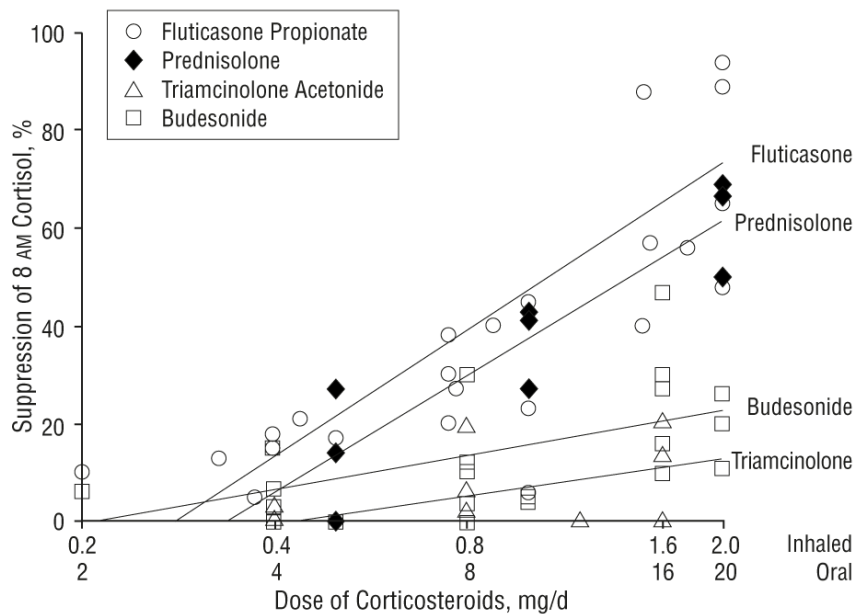


Figure 6-3. Figure from Lipworth et al. 1999 (JAMA Internal Medicine 159, 9: 941–955). A meta-analysis of studies that evaluated the effect of corticosteroid treatments on 8 AM plasma and serum cortisol levels.

6.1.3 The *additional* benefits of oral corticosteroid treatment

Following their introduction in the 1970s inhaled corticosteroids (ICS) have replaced oral corticosteroids (OCS) as the mainstay of asthma anti-inflammatory treatment. Systemic oral corticosteroids (OCS) are used to treat T2 high asthma that is incompletely controlled with inhaled corticosteroids (ICS). Reserving systemic corticosteroids for ICS refractory patients is understood given their indisputable side effects including infection, obesity, weight gain, cataracts, adrenal failure and hypertension.^{187 191 192} The introduction of steroid-sparing biologic therapies has played an important role in reducing the requirement for prescriptions of OCS as a maintenance treatment in the United Kingdom.¹⁹³ However, registry data as of 2021

recorded that 52% of patients with severe asthma were taking a median daily dose of 10 mg prednisolone.¹⁹⁴

The beneficial effects of oral steroids in providing symptomatic benefit to asthma patients with sputum eosinophilia (but not to those without sputum eosinophilia) were observed by Harry Morrow Brown in 1958 (Figure 6-4).¹³ Surprisingly perhaps, there are no modern large randomised controlled trials that definitively show benefits of chronic maintenance oral prednisolone over placebo (e.g., ≥3–6 months) or over high dose inhaled therapy as a maintenance asthma strategy.

90 CASES OF CHRONIC ASTHMA TREATED WITH PREDNISOLONE		
Relief of bronchospasm	Cases with eosinophilic sputum	Cases with a few or no eosinophils
Complete	56	1
Partial	7	3
Slight	—	7
No relief	—	16
Total	63	27

Figure 6-4. Data from Morrow Brown 1958 (Lancet, 272:7059 1245–1247) “Treatment of chronic asthma with prednisolone significance of eosinophils in the sputum.”

A small number of head-to-head trials between ICS and OCS were performed in the 1970s and 1980s following the introduction of ICS, limited to identifying equivalent doses for symptom control and lung function endpoints.¹⁹⁵ Despite their widespread

use, the mechanism of the beneficial effects of OCS *on top of* ICS as used in refractory severe eosinophilic asthma, is not understood. It has been suggested that a greater suppression of the systemic aspects of T2 inflammation may be important.¹⁹⁶ Another possibility is that OCS are effective against T2 airway inflammation in sites such as the distal airways that are poorly accessed by ICS and that patients with ICS-unresponsive T2 airway inflammation have greater involvement of these sites.¹⁹⁷

6.1.4 Plan

A better understanding of these mechanisms is important as it might allow ICS unresponsive disease to be recognized more promptly and before extensive OCS exposure. This chapter describes an observational study that was performed to compare the effects on clinical and laboratory markers of T2-inflammation from:

- (i) initiating low-dose inhaled steroids in corticosteroid naïve patients with T2-high asthma

with

- (ii) oral corticosteroids in patients who have T2-high asthma despite treatment with medium-high dose ICS.

Participants with T2 airway inflammation, as reflected by baseline fractional exhaled nitric oxide (FeNO) >45 ppb and/or blood eosinophil counts >0.30 x10⁹/L, were recruited in two groups. Patients with corticosteroid naïve asthma received 200 mcg

inhaled beclomethasone twice daily for 8 weeks. This dose was selected as the initial dose in UK treatment guidelines and to maintain a low likelihood of systemic absorption (Figure 6-3)⁹ and the duration was selected in light of the results of a systematic analysis of ICS effects.¹⁹⁸ Those patients already established on medium-high dose ICS treatment received prednisolone 30 mg daily for 10 days. This regimen is suggested in the literature as likely to identify steroid resistant asthma (e.g., Kamada et.al.¹⁹⁹). It had been used in previous acute asthma trials and it may have minimised side effects occurring at higher doses.²⁰⁰

Pre-and-post-treatment symptom scores, lung function, FeNO, blood and sputum eosinophils, and mediators of T2-inflammation in sputum and serum were assessed.

6.2 Methods

6.2.1 Study design

The study was sponsored by the University of Oxford and approved as part of the Oxford Airways Study OAS (REC 18/SC/0361) and NIHR CSP Study 88241 (REC 08/H0406/189). It was advertised locally and participants were recruited from the Oxford special airways clinic or via public advertisement between 2017 and 2024. Induction of sputum was halted between March 2020 February 2022 due to the COVID-19 pandemic.

6.2.2 Participants

All participants were adult men and women aged ≥ 18 years who provided written consent. Participants were recruited into three groups:

6.2.2.1 T2-high patients with asthma

Eligible patients for inclusion were never or former smokers with ≤ 10 pack year history who were non-smoking for ≥ 1 year. They had confirmed current or historic evidence of variable airflow limitation defined as any one of the following; FEV1 bronchodilator reversibility >200 ml; FEV1 percentage change $\geq 12\%$, methacholine challenge of less than 8 mg/ml; or peak expiratory flow (PEF) diurnal variation.

Additionally, patients had a FeNO of ≥ 45 ppb and/or blood eosinophil count at baseline of $\geq 0.30 \times 10^9/L$.

Exclusion criteria were; evidence of exacerbation of asthma at the time of enrolment; any history or radiographic evidence of alternative respiratory disease or evidence of respiratory infection at enrolment; and pregnancy or breastfeeding.

(i) Inhaled corticosteroid (ICS) group

In addition to the above inclusion/ exclusion criteria, patients in the ICS group were excluded if there was lifetime use of any bronchial anti-inflammatory agents.

Participants taking ICS were given beclomethasone dipropionate with HFA-134a propellant (Clenil Modulite) 200 mcg one puff twice daily via MDI device to be taken with an AeroChamber as per asthma treatment guidelines. Assessments were performed at baseline and after 8 weeks of treatment.

(ii) Oral corticosteroid (OCS) group

In addition to the above inclusion and exclusion criteria, patients in the OCS group were excluded at the baseline visit if: they were taking less than 400 mcg BDP equivalent inhaled corticosteroid daily dose; or if they had been taking inhaled corticosteroids for less than 3 months; or if there was any history of poor adherence to inhaled treatment based on prescription collection rates; or if their inhaler technique was assessed as inadequate. They were also excluded if they had taken any systemic corticosteroids within the preceding 6 weeks or if there was any history of previous biologic therapy. Participants taking OCS were given prednisolone 30 mg

daily for 10 days for clinical indications. Assessments were performed at baseline and on the 10th day of treatment.

6.2.2.2 Healthy control (HC) group

Eligible patients for inclusion were those with no history of asthma or other respiratory diseases and no history of previous inhaled treatments. Measurements were obtained at a single baseline visit.

6.2.3 Measurements and samples

At the baseline and post-treatment visits each participant underwent pre and post bronchodilator spirometry according to American Thoracic Society (ATS) standards using 3 reproducible measurements of FEV₁. Measurements of FeNO (NIOX, VERO Circassia Ltd, Oxford, UK), Full blood count (FBC) and Asthma Control Questionnaire– 5 (ACQ5)²⁰¹ were also taken and a physical examination and medication check, including treatment adherence enquiry, was conducted. Plasma and serum were collected in lithium heparin and serum separator tubes, respectively. Sputum was processed immediately.

6.2.4 Sputum processing⁵

Sputum was induced according to the technique outlined in Pizzichini et al ²⁰² using 4% hypertonic saline and a Devilbiss nebuliser set to 1 ml/min.^{203 204} This protocol is available in Appendix section 11.4. Sputum was examined within 2 hours. All opaque or dense portions that looked different from saliva (plugs) or were free of squamous cell contamination were selected from the expectorate, weighed and treated with Dulbeccos PBS (dPBS) without calcium and magnesium at x8 the mass of the expectorate retained. The sample was vortexed with shaking and centrifuged at 400 g for 5 minutes. The supernatant was then aspirated: this was the PBS supernatant. The eluted and aliquoted sputum PBS supernatant was frozen at -80 °C prior to analysis.

6.2.5 Differential cell counts

Following from the above steps, four times the initial sample weight of 0.2% dithiothreitol (DTT) was added to the cell pellet, and the sample was incubated on ice for 15 minutes with vortexing and shaking at 5-minute intervals. The sample was filtered through a 48 µm gauze. A total non-squamous cell count was performed from this suspension in a haemocytometer. The proportion of salivary squamous cells was

⁵ Sputum processing as described in this paragraph and differential cell counts were performed by Mahdi Mahdi

noted, and cell viability was determined by the trypan blue exclusion method. Two cyto-spins were made and stained with Diff-Quik and a differential non-squamous cell count was performed for 400 cells. The differential cell count was expressed as the percentage of the total non-squamous and bronchial epithelial cells. The specimen was considered adequate if total and differential cell counts could be obtained and was excluded from the study if the pre-differential cell count squamous cell contamination was >40%. This method has been reported reliable in several previous studies.^{202 205-207}

6.2.6 ELISA assays

Sputum supernatants and plasma were stored at -80 °C until processing. Samples from each visit were assessed for 10 canonical mediators of T2-inflammation via solid-phase immunoassay based on electrochemiluminescence; eotaxin-1, eotaxin-2, eotaxin-3, interleukin (IL)-1 β , IL-4, IL-5, IL-13, IL-33, TSLP and TARC (Meso Scale Discovery [MSD], Rockville, MD, USA, cat: K15067M-2). A further two mediators, PGD₂ and LTE₄, were assayed in sputum only using competitive ELISA kits (Cayman Chemical, Ann Arbor, MI, USA, cat: 501060 & 512031). Assays were performed according to the manufacturer's instructions. The washing buffer for the electrochemiluminescence assay was PBS with 0.02% Tween20.

6.2.7 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 10 and SPSS statistical package version 29.0.2.0 (SPSS Inc, Chicago, IL). Figures were generated using ggplot2 v3.5.2 and GraphPad Prism version 10.

Normally distributed data are presented as mean (SE) and log-normally distributed data are presented as geometric mean (coefficient of variation, %). Data failing tests of normality / lognormality are presented as median (interquartile range, IQR). All non-normal / log-normal distributed data underwent $\log_{10}$ transformation prior to statistical analyses.

All PBS mediator concentrations were multiplied x8 post ELISA and prior to analysis to account for dilution during processing. Mediator levels that were not quantifiable due to falling below the lower limit of detection (LLOD) were assigned the arbitrary value of 0.5 x LLOD to permit analyses. To permit logarithmic analyses sputum cell count values of 0 were assigned the arbitrary value of 0.2% and FeNO values above the machine threshold >300 ppb were assigned 300 ppb.

Power calculations for recruitment were informed by the most comparable studies in the literature to have evaluated the effects of ICS and OCS treatments on FeNO and sputum eosinophilia. Cowan et al. 2010, reported mean fold reductions of 2.6 for FeNO and 5.0 for sputum eosinophil percentage in a relatively T2-high cohort who received high-dose ICS therapy administered over 28 days (approximately 2000 mcg

BDP⁶ daily equivalent). This estimated Cohen's d values from this study are 1.3 for FeNO and 1.6 for sputum eosinophilia, respectively. In contrast, Demarche et al. 2017, observing treatment effects in a cohort with lower levels of T2 inflammation and receiving a range of ICS doses over one year (400–1200 mcg BDP daily equivalent) reported mean fold reductions in FeNO of 2.3 and in sputum eosinophil percentage of 3.5, corresponding to Cohen's d values of 1.0 and 0.6, respectively.

For OCS, Sousa et al. 2017, evaluated the effects of prednisolone (0.5 mg/kg/day for 14 days) in a moderately T2-high cohort and reported mean fold reductions of 1.5 for FeNO and 4.5 for sputum eosinophilia, corresponding to Cohen's d values of 0.8 and 0.9, respectively. The 33% reduction in FeNO reported in this study was closely replicated in a large meta-analysis of OCS effects in patients with higher mean baseline levels of T2 inflammation by Busby et al. 2020, which, report a substantially larger fold reduction in sputum eosinophil percentage (9.1) following OCS treatment.

Accordingly, using a conservative estimate of Cohen's d of 0.8 for anticipated ICS and OCS effects on both FeNO and sputum eosinophilia, and assuming $\alpha = 0.05$ (two-sided) with 80% power to detect an effect ($\beta = 0.2$), the required sample size was calculated as $n = 15$ for paired (within-subjects) samples for both ICS and OCS treatment.

Treatment effects were assessed using a linear mixed effects model with Bonferroni correction for the family wise error rate ($\alpha 0.05$).

⁶ Beclomethasone Dipropionate

6.3 Results

A total of 76 participants completed the study with 31 participants recruited to the healthy control (HC) group, 22 to the inhaled corticosteroid (ICS) group and 23 to the oral corticosteroid (OCS) group, respectively. Recruitment is detailed in Figure 6-5. The demographic and baseline parameters of participants are described in Table 6-1. For the OCS group adherence checks demonstrated a median number of ICS/LABA inhalers collected per month over a 12-month period of 1 (IQR 0.54–1.13).

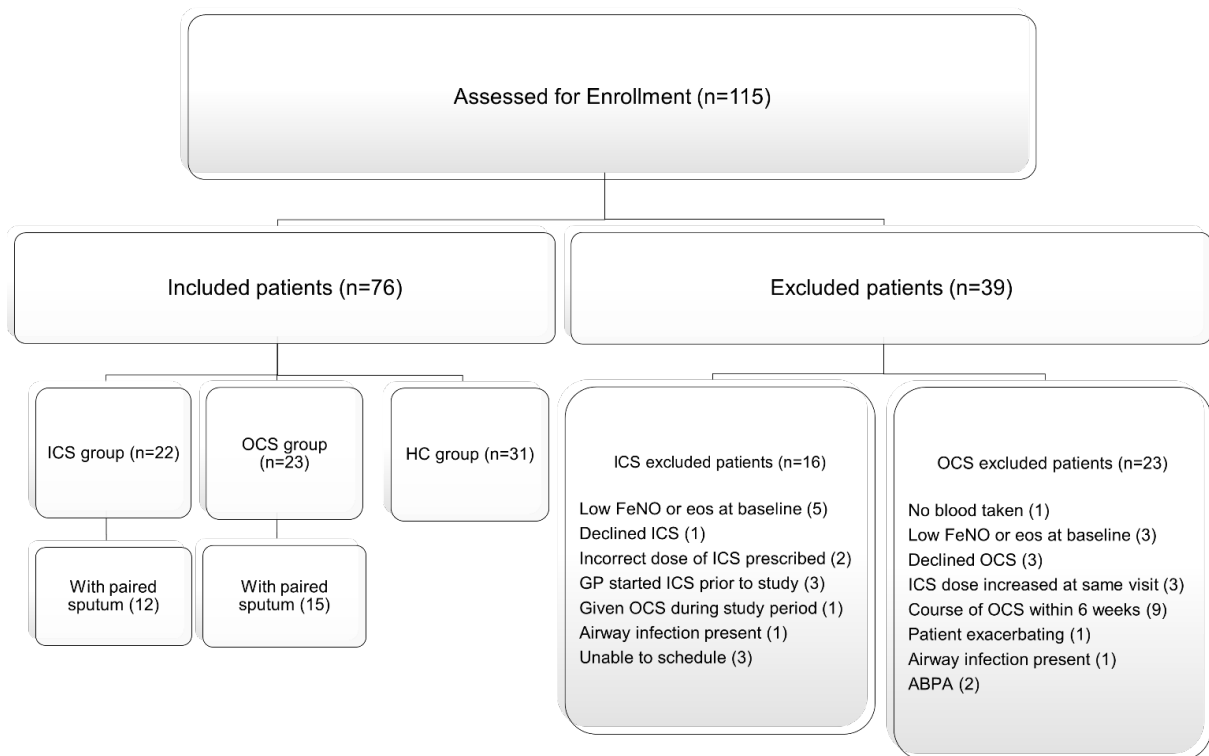


Figure 6-5. Consort diagram. Participant flow through the study. Abbreviations; ICS, inhaled corticosteroid; OCS, oral corticosteroid; HC, healthy control

Table 6-1. Participant baseline parameters

	Healthy Controls n = 32	ICS Group n = 22	OCS Group n = 23
Age (years)	40.1 (19–67)	56.4 (21–78)	56.4 (37–80)
Male : Female (n)	15 : 16	13 : 9	16 : 7
Age of onset symptoms (years)	n/a	43.1 (5–78)	38.5 (5– 75)
Sensitized to aero-allergen (n)	9 : 22	12 : 10	12 : 11
Diagnosis of eczema (n)	1 : 30	1 : 21	3 : 20
Nasal polyps (n)	0 : 31	3 : 19	7 : 16
Total IgE (IU/mL)†	22.6 (171%)	70.1 (130%)	247 (172%)
BDP equivalent daily dose (mcg)‡	n/a	n/a	800 (600–1600)
Pre-BD FEV ₁ (% predicted)	106.1 (±1.9)	88.4 (±3.9)	62.5 (±4.5)
BDR ≥200 ml or 12% FEV ₁ (n)	n/a	9 : 13	7 : 16
Bronchodilator response (mL)	n/a	220 (±44.6)	168 (±36.4)
FEF ₂₅₋₇₅ (% predicted)	96.1 (±13.6)	64.4 (±11.3)	34.2 (±7.9)
Sputum eosinophils (%)‡	0.2 (0.2–0.2)	8.3 (6.0–24.0)	22.1 (14.2–45.4)
Blood eosinophils (x10 ⁹ /L)†	0.11 (126%)	0.36 (44%)	0.58 (48%)
FeNO (ppb)†	17.9 (58%)	79.9 (59%)	73.9 (74%)

Values given as mean ± SE, except † = geomean (coefficient of variation, %), Age and Age of onset are given as mean (range) and ‡ = median (IQR). Abbreviations: BDP, beclometasone dipropionate; BD, bronchodilator; BDR, bronchodilator responsiveness; FeNO, fractional exhaled nitric oxide; FEV₁, forced expiratory volume in 1 second; IgE, immunoglobulin E.

6.3.1 Baseline sputum differential cell counts and mediator levels

The median squamous cell contamination for all samples was 4.0% (IQR 0.4–9.9%) and the median non-squamous cell viability was 70.8% (IQR 54.5–80.7%). One sputum sample was excluded from analysis due to high squamous cell count.

Baseline sputum differential cell counts are detailed in Figure 6-6 along with effects of treatment. Baseline sputum cell counts are representative of healthy subjects and moderate -to-severe eosinophilic asthma cohorts previously published. In the healthy control group there is a preponderance of macrophages followed by other cells in the order macrophages > neutrophils > lymphocytes > eosinophils. This is in keeping with several studies of sputum induction in healthy populations in the literature.²⁰⁸⁻²¹⁰ The ICS group is also macrophage predominant but with an increase in eosinophils while for the OCS group the order follows neutrophils > macrophages $\approx$ eosinophils > lymphocytes. This distribution corresponds with published sputum induction in other severe eosinophilic asthma cohorts.²¹¹

Baseline mediator levels are detailed in Appendix Table 11-5. While comparing baseline mediator levels across different platforms is problematic it is noted that the healthy control baseline sputum and serum mediator levels are similar to those published previously using the same platform.¹⁷² Baseline sputum and serum mediator levels for the OCS group are broadly similar to levels published for moderate-to-severe asthma, such as Jang et al.²⁰⁸

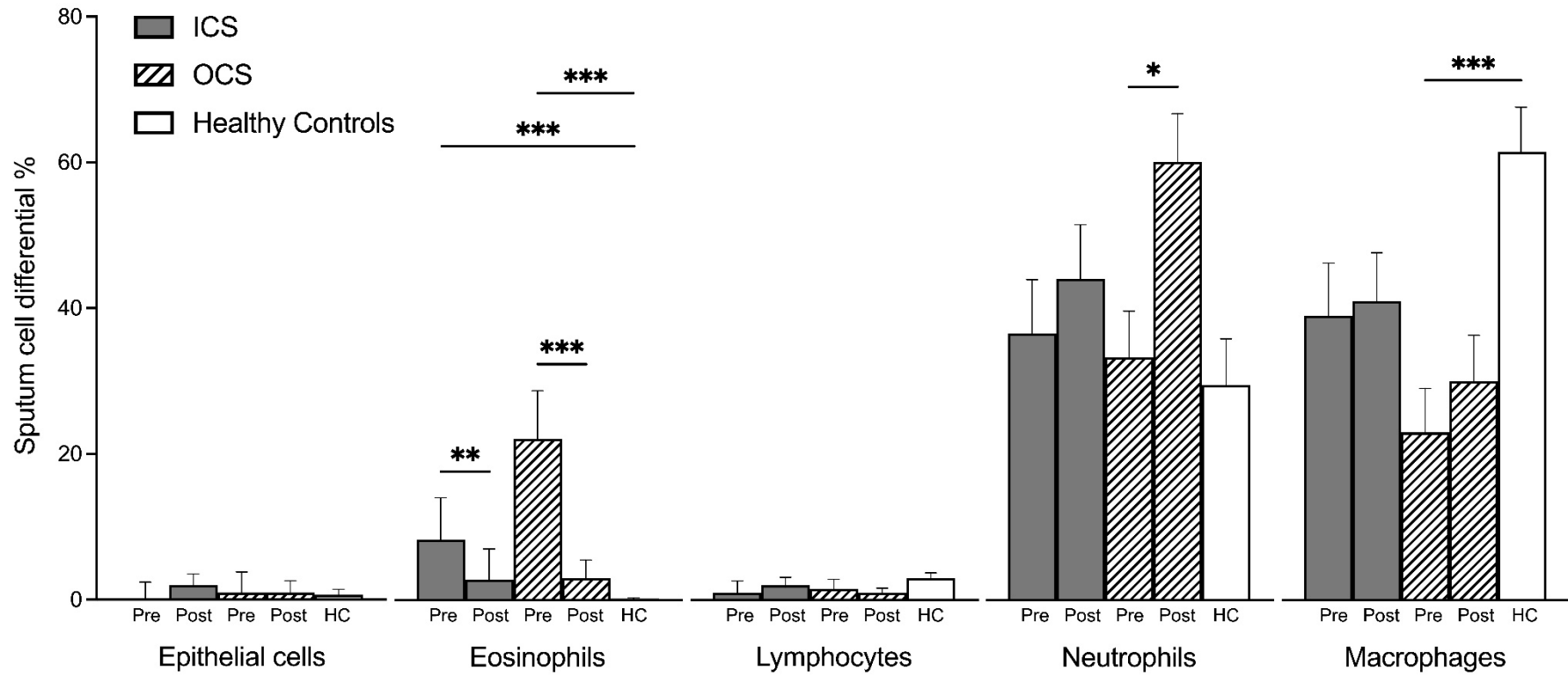


Figure 6-6. Effects of introducing ICS treatment and adding OCS treatment on sputum differential cell counts in T2-high asthma. Median values $\pm$ SE presented. Statistical comparisons were performed using a linear mixed effects model. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

6.3.2 Treatment effects on clinical measurements

Both ICS and OCS groups experienced a significant improvement in mean ACQ–5 symptom scores, above the minimum clinically meaningful difference.²¹² ACQ–5 scores for our ICS group before and after treatment were 2.1 and 0.7 respectively (mean reduction 1.4, 95%CI: 0.9 – 1.9, $p < 0.001$) while for our OCS group scores were 2.5 and 1.2 before and after treatment respectively (mean reduction 1.0, 95%CI: 0.8 – 1.7, $p < 0.001$) (Figure 6-7B).

Both ICS and OCS groups experienced significant improvement in FEV1. Mean FEV1 before and after treatment increased from 2.85 L to 3.00 L for the ICS group (mean increase 145 ml, 95%CI: 2–287 ml, $p < 0.05$). For the OCS group mean FEV1 increased from 2.05 L to 2.44 L (mean increase 397 ml, 95%CI: 187–606 ml, $p < 0.001$) (Figure 6-7E). For the OCS group there was also a large and significant mean improvement in FEF₂₅₋₇₅, a measure of small airways dysfunction (SAD). Mean FEF₂₅₋₇₅ increased from 1.12 L/s to 1.79 L/s for the OCS group (mean increase 0.49 L/s, 95%CI: 0.01–0.97 L/s, $p < 0.05$). There was no change in FEF₂₅₋₇₅ for the ICS group (Figure 6-7F).

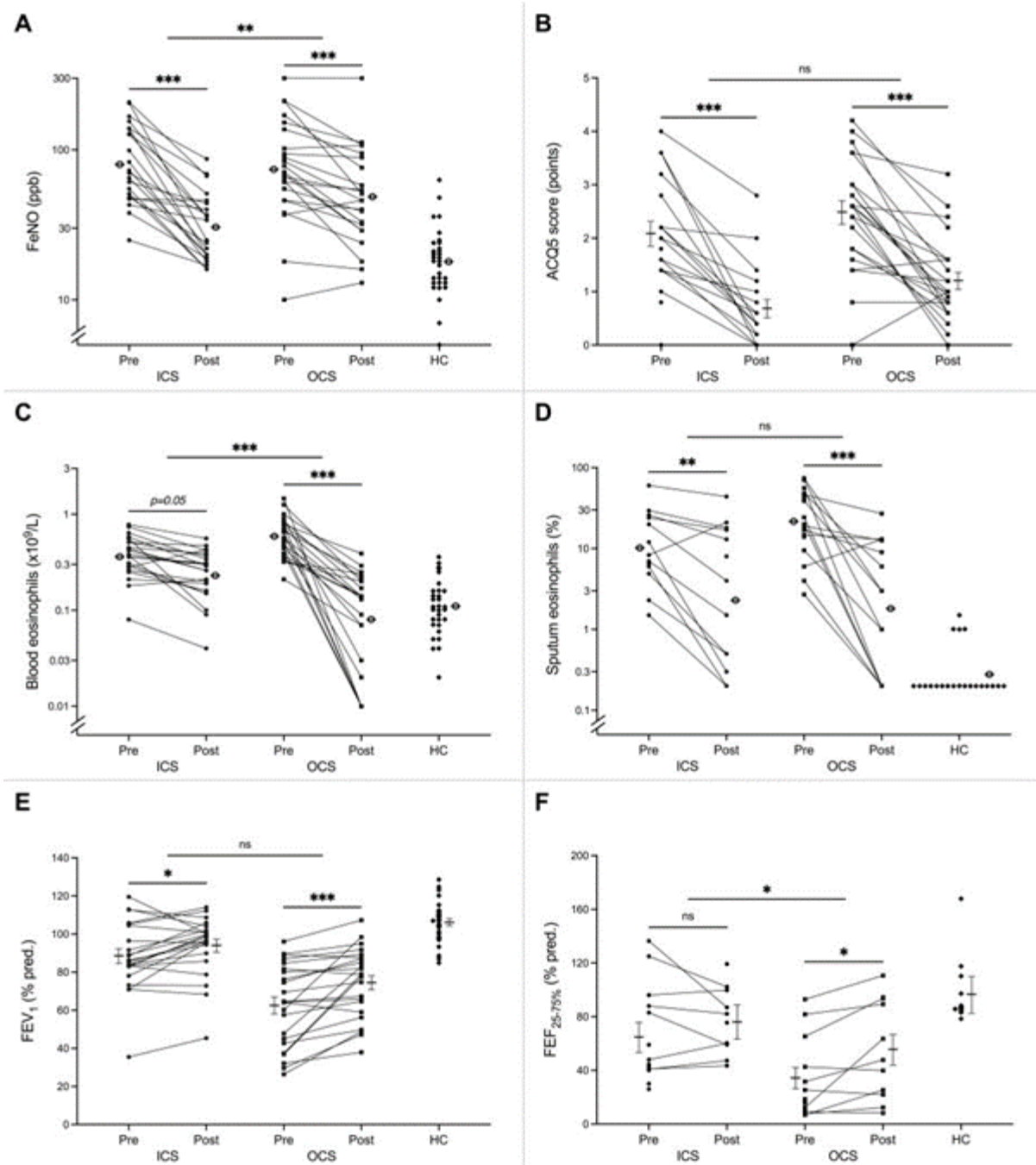


Figure 6-7. The effects of introducing ICS and of adding OCS treatment on (A) FeNO, (B) ACQ-5 score, (C) Blood eosinophil count, (D) Sputum eosinophil cell percentage, (E) FEV₁% predicted and (F) FEF₂₅₋₇₅% predicted. Symbol with horizontal bars denotes mean and standard error. Θ = geomean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Table 6-2. Fold reduction of T2 mediators in sputum from ICS and OCS

	Geomean Conc'n (pg/ml)	Geomean Conc'n (pg/ml)	Geomean Conc'n (pg/ml)		Fold Reduction & 95% CI (pg/ml)		Geomean Conc'n		Fold Reduction & 95% CI
	LLOD	H Ctrls	ICS Group Pre Post				OCS Group Pre Post		
IL-1B	0.12	88.7	104 86.2		1.21 (0.42 – 3.53)		132 180		0.74 (0.28 – 1.90)
IL-5	0.11	0.73	8.29 1.87		4.43 (1.67 – 11.8)		26.5 3.24		8.18 (2.63 – 25.4)
IL-4	0.01	0.17	0.38 0.31		1.22 (0.55 – 2.72)		1.19 0.44		2.73 (1.20 – 6.24)
IL-13	0.26	2.37	6.04 4.50		1.34 (0.63 – 2.86)		19.3 8.10		2.38 (1.16 – 4.86)
TARC	0.08	21.3	123 78.4		1.57 (0.40 – 6.25)		516 249		2.07 (0.62 – 6.94)
Eotaxin-1	7.55	71.6	187 98.9		1.89 (1.11 – 3.21)		701 730		0.96 (0.32 – 2.86)
Eotaxin-2	0.20	128	863 285		3.03 (0.53 – 17.3)		4033 1525		2.65 (0.77 – 9.13)
Eotaxin-3	0.48	16.0	333 87.8		3.79 (1.37 – 10.5)		1632 279		5.86 (1.30 – 26.5)
IL-33	0.29	1.29	1.54 1.29		1.19 (0.93 – 1.54)		4.50 2.25		2.00 (0.81 – 4.98)
TSLP	0.10	3.36	14.5 6.76		2.14 (0.74 – 6.22)		71.7 20.9		3.43 (0.99 – 11.9)
LTE4	19.5	192	420 339		1.24 (0.51 – 3.02)		1529 478		3.20 (1.26 – 8.09)
PGD2	16.3	466	1160 925		1.25 (0.69 – 2.28)		2327 1137		2.05 (0.59 – 7.11)

Table 6-3. Fold reduction of T2 mediators in serum from ICS and OCS

	Geomean Conc'n (pg/ml)	Geomean Conc'n (pg/ml)	Geomean Conc'n (pg/ml)		Fold Reduction & 95% CI (pg/ml)		Geomean Conc'n		Fold Reduction & 95% CI
	LLOD	H Ctrls	ICS Group Pre Post				OCS Group Pre Post		
IL-1 β	0.12	0.28	0.25 0.30		0.82 (0.60 – 1.12)		0.21 0.19		1.22 (0.77 – 1.63)
IL-5	0.11	0.52	0.76 0.71		1.07 (0.76 – 1.51)		0.92 0.51		1.82 (1.37 – 2.40)
IL-4	0.01	0.13	0.11 0.12		0.94 (0.79 – 1.12)		0.17 0.12		1.36 (1.12 – 1.64)
IL-13	0.26	2.71	2.66 2.71		0.98 (0.86 – 1.12)		2.97 2.67		1.11 (0.94 – 1.32)
TARC	0.08	208	228 182		1.25 (0.97 – 1.61)		278 166		1.67 (1.27 – 2.20)
Eotaxin-1	7.55	317	235 249		0.94 (0.79 – 1.13)		260 347		0.75 (0.65 – 0.87)
Eotaxin-2	0.20	1358	884 927		0.95 (0.86 – 1.05)		1015 1098		0.92 (0.80 – 1.07)
Eotaxin-3	0.48	22.5	38.9 37.1		1.05 (0.91 – 1.21)		47.6 30.9		1.58 (1.26 – 1.97)
IL-33	0.29	1.08	0.68 0.99		0.69 (0.39 – 1.23)		0.76 0.97		0.78 (0.45 – 1.36)
TSLP	0.10	3.18	3.65 3.39		1.08 (0.95 – 1.23)		4.44 3.89		1.14 (0.97 – 1.34)

Abbreviations: Con'c, concentration; H Ctrls, healthy controls; LLOD, lower limit of detection; ICS, inhaled corticosteroid; OCS, oral corticosteroid; IL, interleukin; LTE4, leukotriene E4; PGD2, prostaglandin D2; TARC, thymus and activation-regulated chemokine; TSLP, thymic stromal lymphopoietin

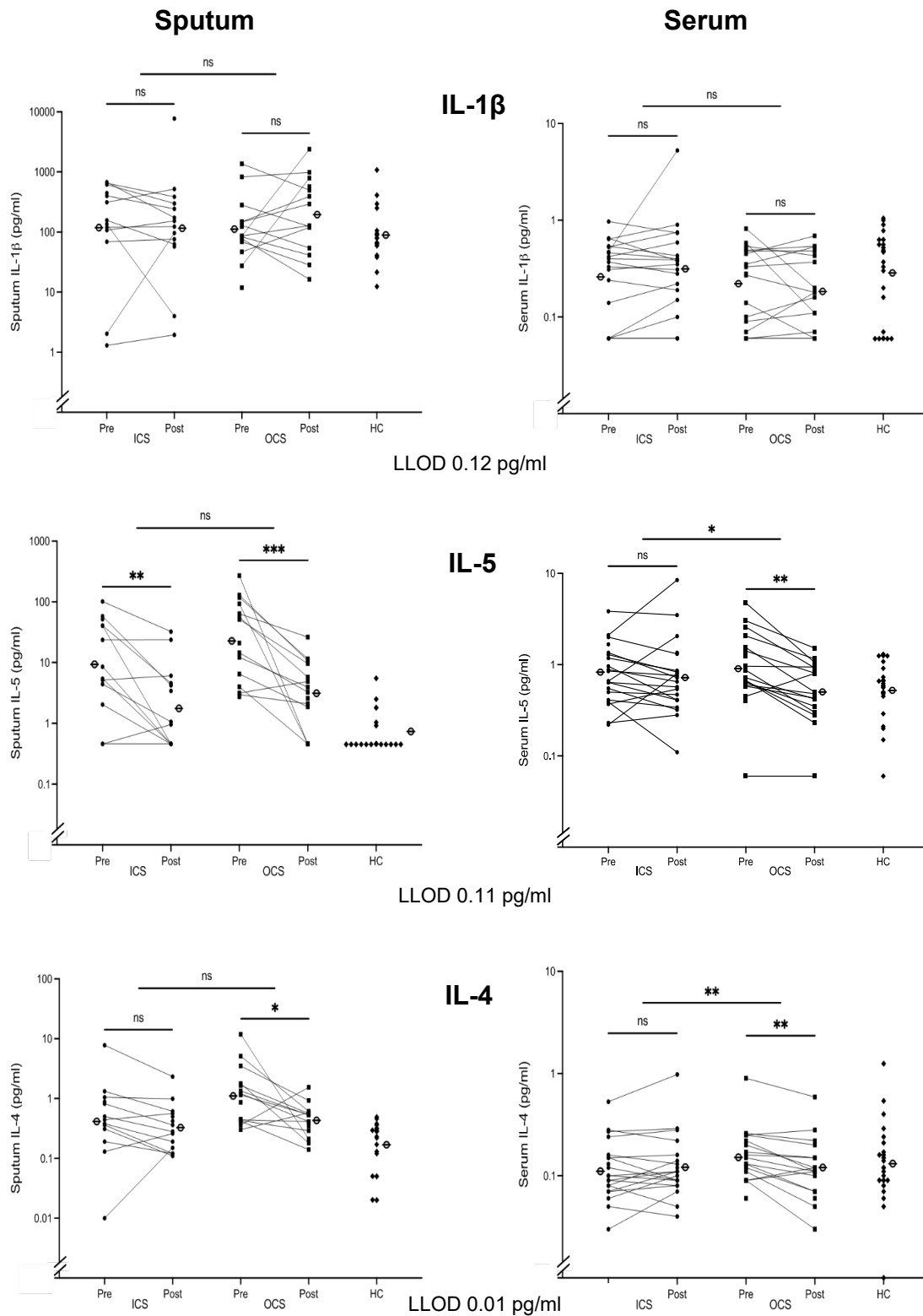


Figure 6-8. Effects of introducing low-dose inhaled corticosteroid treatment (ICS) and of adding oral corticosteroid treatment (OCS) in T2-high asthma on mediators in sputum (left) and serum (right). Abbreviations: IL, interleukin; HC, healthy controls; LLOD, lower limit of detection. Θ = geomean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

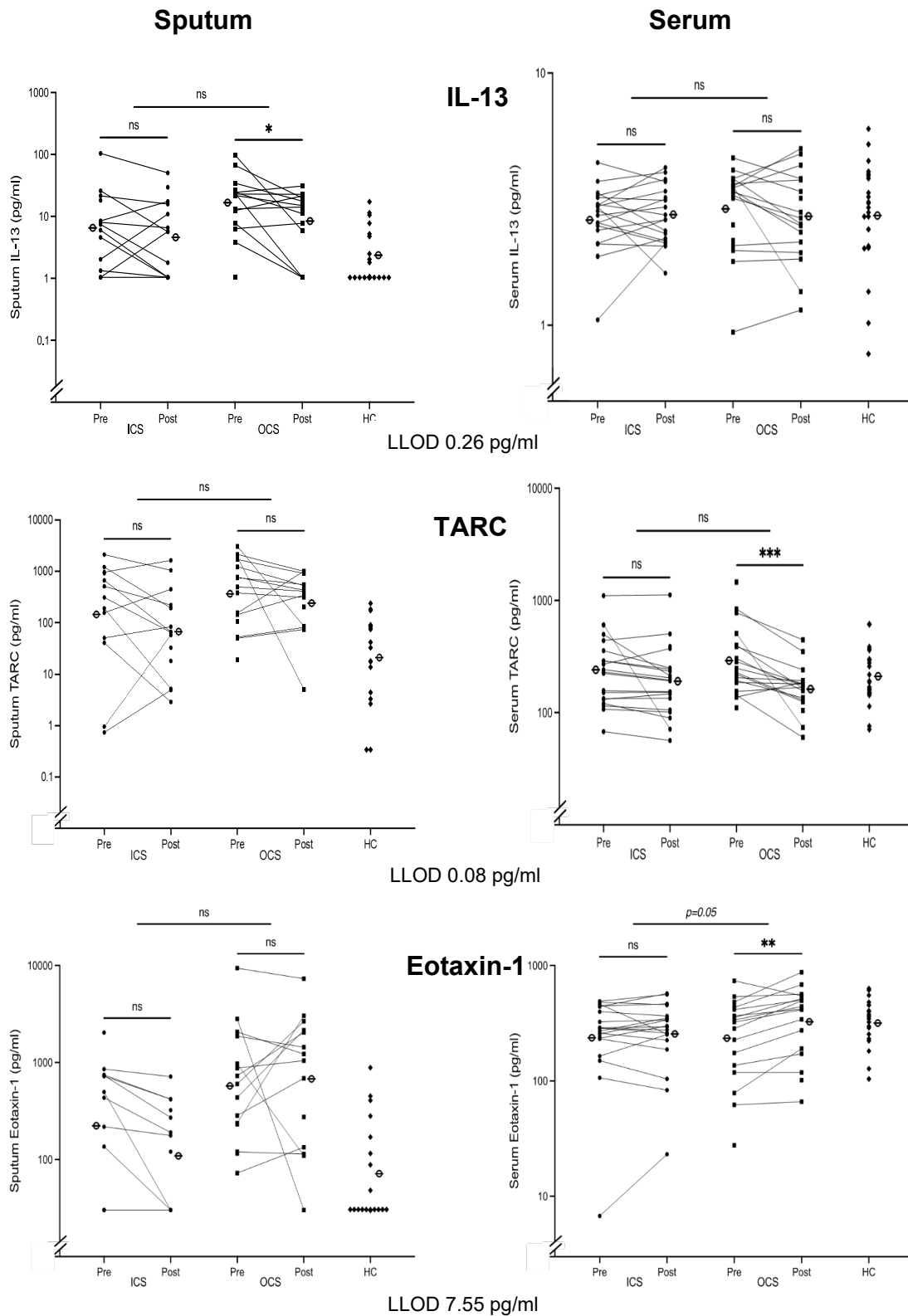


Figure 6-8 continued. The effects of introducing ICS and of adding OCS in T2-high asthma. Abbreviations: TARC, thymus and activation-regulated chemokine. LLOD, lower limit of detection. Θ = geomean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

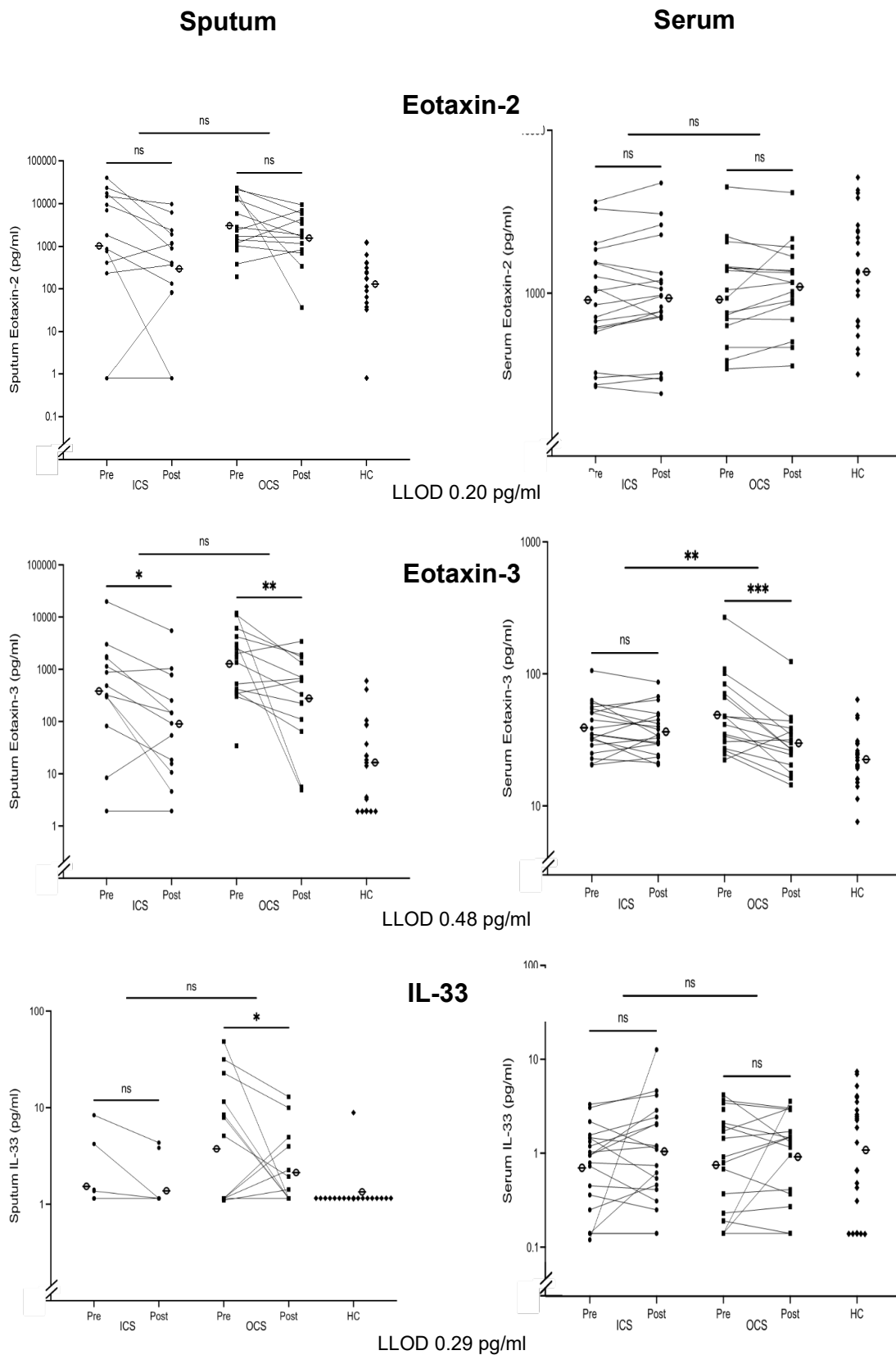


Figure 6-8 continued. The effects of introducing ICS and of adding OCS in T2-high asthma. Abbreviations: LLOD, lower limit of detection. Θ = geomean * p < 0.05, ** p < 0.01, *** p < 0.001

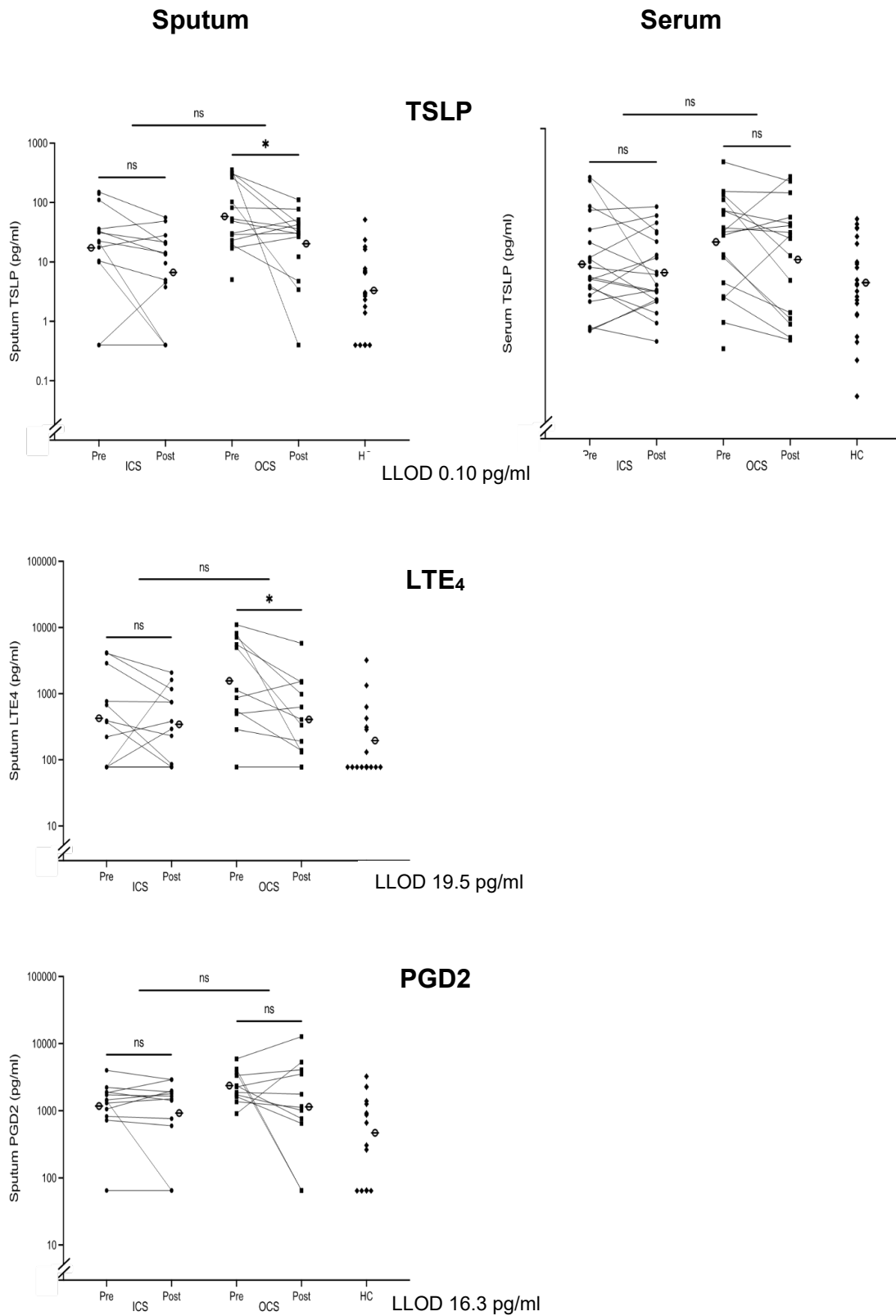


Figure 6-8 continued. The effects of introducing ICS and of adding OCS in T2-high asthma. Abbreviations: TSLP, thymic stromal lymphopoietin; LTE4, leukotriene E4; PGD2, prostaglandin D2; LLOD, lower limit of detection. Θ = geomean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

There was no significant difference between the reduction of sputum eosinophil counts for both OCS and ICS groups (Figure 6-7D). Before and after treatment sputum eosinophil counts for the OCS group were 21.4% and 1.8% (mean fold reduction 11.0, 95%CI: 3.79–31.7). Before and after sputum eosinophil counts for the ICS group were 10.0% and 2.3% (mean fold reduction 4.12, 95%CI: 1.77–9.60).

The ICS group demonstrated significantly more inhibition of FeNO than the OCS group (Figure 6-7A). FeNO reduced from 79.9 ppb to 30.4 ppb for the ICS group (mean fold reduction 2.63, 95%CI: 2.05–3.37, $p < 0.001$). By comparison, FeNO reduced from 73.9 ppb to 48.4 ppb for the OCS group (mean fold reduction 1.53, 95%CI: 1.26–1.85, $p < 0.001$). In contrast, the OCS group experienced significantly more inhibition of blood eosinophils (Figure 6-7C). Blood eosinophil counts reduced from 0.58 to 0.08 following OCS treatment (mean fold reduction 7.68, 95%CI: 4.22–13.97, $p < 0.001$). The introduction of ICS treatment reduced blood eosinophils from 0.36 to 0.23 (mean fold reduction 1.56, 95%CI: 1.27–1.92, $p = 0.05$).

6.3.3 Treatment effects on T2 mediators

Baseline levels of T2 mediators within sputum were consistently elevated for both ICS and OCS groups compared with healthy controls in a ubiquitous pattern $HC \text{ group} < ICS \text{ group} < OCS \text{ group}$ (Table 6-2). Fold reduction values for T2 mediators in sputum and serum are presented in Tables 6-2 and 6-3 and statistical comparisons of ICS and OCS treatment effects are presented in Figure 6-8. ICS treatment significantly reduced sputum levels of IL-5 and eotaxin-3 *only* and there

were no effects of low-dose ICS on any mediators in serum. By contrast, OCS treatment significantly reduced sputum levels of IL-5, IL-4, IL-13, IL-33, TSLP, eotaxin-3 and LTE₄ and significantly reduced serum levels of IL-5, IL-4, TARC and eotaxin-3. OCS treatment was associated with an increase in blood neutrophil counts and a decrease in blood basophil counts while there were no effects of ICS treatment on blood white cell counts, other than eosinophils (Appendix Figure 11-1). A combined correlation matrix showing changes in parameters before and after OCS treatment is presented in Figure 6-9. A similar matrix for changes with ICS treatment and baseline correlation matrices are provided in Appendix Figures 11-2 through 11-4.

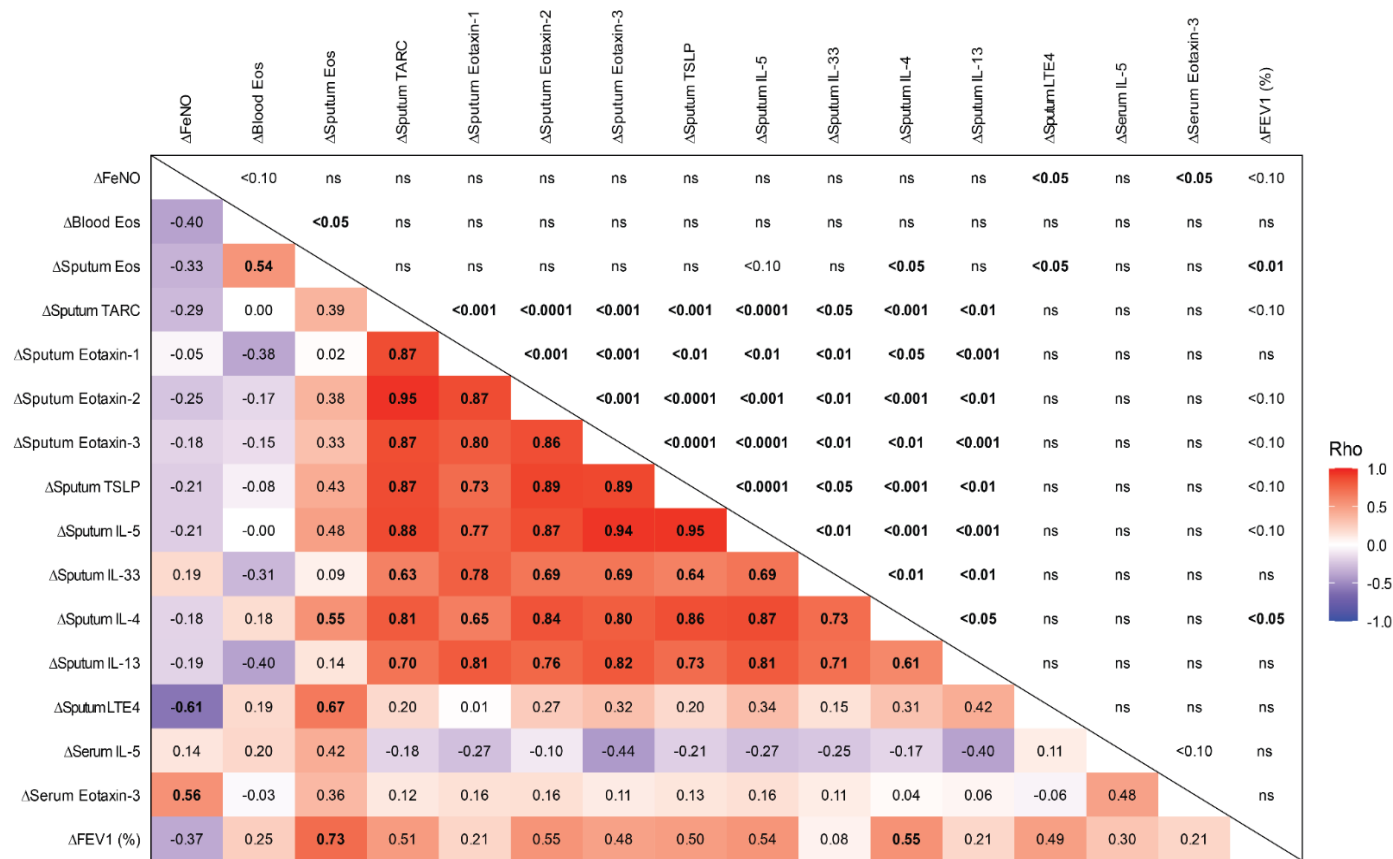


Figure 6-9. Associations in parameter changes following OCS treatment. Correlation matrix showing the relationship between changes (Δ) following OCS treatment. Spearman's r and uncorrected p values provided. Statistically significant values in bold.

6.4 Discussion

The introduction of ICS, and the addition of OCS in T2-high asthma, both reduced the sputum eosinophil count and resulted in similar clinical benefits. However, the pattern of the anti-inflammatory effects differed. OCS produced greater inhibition of blood eosinophils than ICS (mean fold reduction 7.68 versus 1.56, $p < 0.001$) while ICS produced greater inhibition of FeNO than OCS (mean fold reduction 2.63 versus 1.53, $p < 0.01$). OCS had a broader suppressive effect on T2 mediators in sputum and serum.

In patients with biomarker evidence of T2 airway inflammation, ICS treatment in corticosteroid-naïve patients and added OCS treatment in patients established on medium-high dose ICS both had beneficial effects on symptoms, lung function and on eosinophilic airway inflammation, as assessed by the induced sputum eosinophil count. However, ICS and OCS differed in their effects on FeNO and blood eosinophils. OCS had a larger relative effect on blood eosinophils and ICS had a larger relative effect on FeNO. These findings would be consistent with the suggestion that FeNO reflects an ICS-responsive airway epithelial and blood eosinophils reflect an OCS responsive reservoir of cells.¹⁹⁶

6.4.1 Hypotheses for the added benefits of OCS

The pattern of effects of ICS and OCS treatments observed here on FeNO and eosinophil levels and on T2 mediators in sputum and serum prompts a proposal for two mechanisms for these effects for further investigation.

Firstly, this study confirms the exquisite effect of ICS on FeNO. This is consistent with previous work *in vitro* demonstrating an important mechanism for the effect of ICS on FeNO by reducing mRNA expression of inducible nitric oxide synthase 2 (iNOS) from bronchial epithelial cells.²¹³ The study also showed ICS treatment reduced sputum levels of eotaxin-3, which is generated by bronchial epithelial cells in response to IL-13.^{214 215} Low-dose ICS treatment induced only a partial and incomplete effect on T2 cytokines, with no effect on sputum IL-4 and IL-13 or any T2 cytokines, chemokines or alarmins in serum. This could reflect insufficient activity in tissue harbouring cells known to produce high amounts of these cytokines in T2-high asthma patients, such as mast cells, CD4⁺ T-cells or ILC2s.²¹⁶ In contrast, OCS treatment had a broader suppressive effect on T2-inflammation with significant reduction in sputum IL-13 and IL-4 as well as sputum IL-5, IL-33, eotaxin-3, TSLP and LTE₄ and serum IL-5, IL-4, TARC and eotaxin-3. The most striking manifestation of this in terms of clinically accessible biomarkers was a marked reduction in blood eosinophils, which was not seen with ICS. The minor reduction of FeNO observed for our OCS group – already established on a topical treatment of the bronchial epithelia shown to inhibit iNOS – may reflect indirect inhibition of iNOS expression through reduction of IL-13. IL-13 is known to promote expression of iNOS expression from human bronchial epithelial cells in a dose-

dependent and donor-dependent manner, while IL-4 has also been identified to have a stabilising effect on iNOS mRNA.^{217 218} The relevance of IL-13 to FeNO production is further supported by data from the trials of the anti-IL-13 monoclonal antibody Lebrikizumab and the dual anti-IL-4R α /anti-IL-13 antibody Dupilumab, both of which reduced FeNO in severe asthma patients.^{94 95 219} The higher baseline sputum levels of IL-13 observed in the OCS group are consistent with this hypothesis.

The reduction of sputum eotaxin-3 levels following OCS treatment may be secondary to the reduction of IL-13. Eotaxin-3 mRNA expression has been reported to be strongly associated with IL-13 induced genes in endobronchial brushings²²⁰ and anti-IL-13 monoclonal antibody has been demonstrated to attenuate eotaxin-3 expression from bronchial epithelial cells *in vitro*.²²¹ Dupilumab trials have confirmed the suppressive effect of anti-IL-4R α /anti-IL-13 antibody on eotaxin-3 levels.⁹⁵ The reduction of sputum levels of TSLP following OCS treatment may similarly represent an indirect effect on bronchial epithelial and stromal cells known to produce TSLP via reduction of IL-4 and IL-13.^{222 223} TSLP is upregulated in epithelial cells by IL-4 and IL-13 in the presence of TNF α – and TNF α , not measured here, is elevated in eosinophilic asthma.^{224 225} In summary therefore, the first hypothesis proposed is that low dose ICS treatment predominantly inhibits FeNO production by epithelial cells directly, while OCS predominantly inhibits FeNO production indirectly through reduction of IL-13. This hypothesis is represented graphically in Figure 6-10. This supposition is supported by the findings of a previous study of low levels of prednisolone in the bronchoalveolar lavage fluid of patients taking this treatment.²²⁶

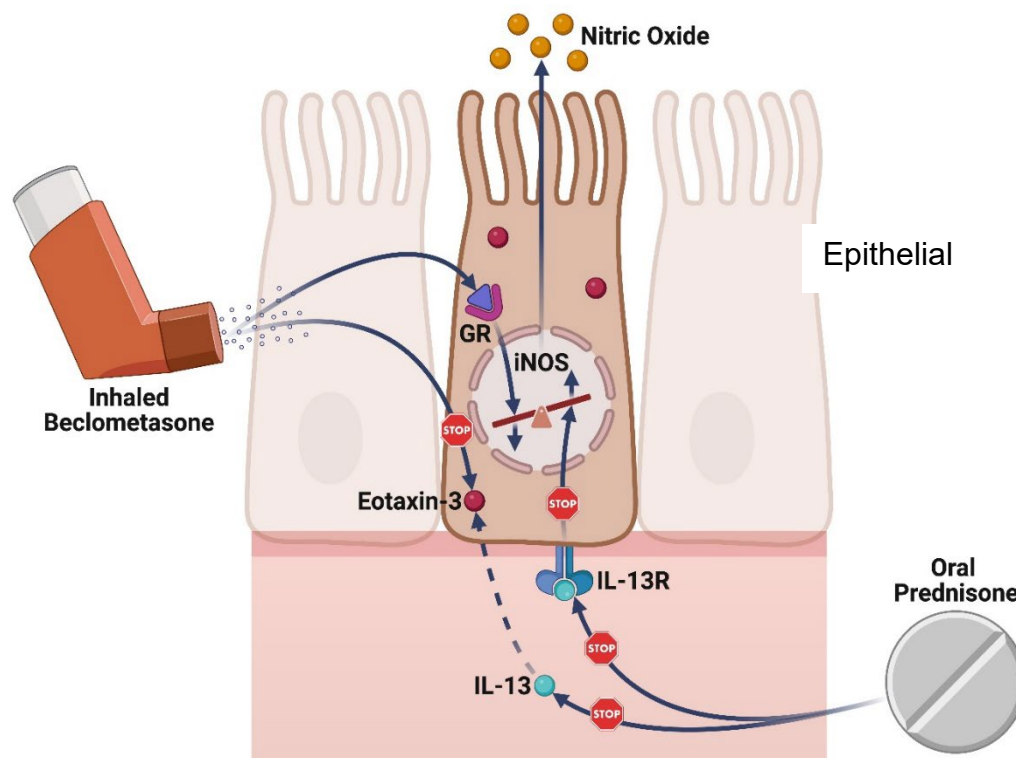


Figure 6-10. Hypothesised mechanisms of effects of low-dose inhaled corticosteroid (ICS) and of oral corticosteroid (OCS) treatments on FeNO. Low-dose ICS treatment predominantly reduces iNOS production in epithelial cells directly via glucocorticoid receptors (GR). Oral prednisolone (OCS) predominantly affects iNOS production through reduction of IL-13. OCS also simultaneously facilitates ICS delivery (and thereby iNOS reduction) by clearing sputum eosinophilia and mucous plugging. Image kindly generated by Corrado Pelaia in BioRender.

A second hypothesised mechanism for OCS and its inhibitory effects on FeNO and inflammatory mediators in the airway is provision of access to the small airways for ICS.²²⁷ OCS treatment induced a significant increase in FEV1 and a significant and large increase in FEF₂₅₋₇₅, the measure of small airways dysfunction (SAD) used. OCS treatment also significantly inhibited sputum eosinophil counts. There was a strong correlation between the reduction of sputum eosinophils and the

improvements in lung function in the OCS group ($r = 0.73$, $p < 0.01$, Figure 6-9) suggestive of an improvement in SAD linked to clearance of mucus plugging. The relationship between airway eosinophilia and SAD observed here is supported by other real-world studies including Abdo et al.²¹¹ and the ATLANTIS consortium²²⁸ who report sputum and blood eosinophilia as independent predictors of SAD. This relationship has also been elegantly illustrated in a hyperpolarised gas MRI study where sputum eosinophil counts were the strongest predictor of ventilation heterogeneity of the small airways.²²⁹

The effect of sputum eosinophil depletion in reducing mucus plugging has also been demonstrated in imaging data before and after use of the therapeutically ‘narrower’ anti-IL-5R α treatment Benralizumab.²³⁰⁻²³² Separately, one of the effects of ICS withdrawal in patients on Benralizumab, identified in the SHAMAL trial¹⁹⁴ is a rise in FeNO. Furthermore, increased distal mucus plugging (6th generation airways) has been identified via ultra-high-resolution CT for asthmatics with high blood eosinophils and high FeNO (>35 ppb) but not for patients with high blood eosinophils and moderate FeNO (20–35 ppb).¹⁹⁷ Taken together, these data suggest that reduction of sputum eosinophilia and peripheral mucus plugging helps provide enhanced access to the small airways for ICS allowing for inhibition of FeNO from ICS in areas of bronchial epithelia that topical ICS treatment was previously unable to reach. The effect size of OCS reduction in FeNO could be modulated by increases in measurable FeNO from small airways that now connect to conducting airways – a phenomena identified in bronchial challenge studies.²³³⁻²³⁵

6.4.2 Limitations of the study

The inherent limitations of this study, which was not a placebo-controlled trial, must be acknowledged. Firstly, the heterogeneity of T2 inflammation between patients is an important potential confounder.^{172 32} The excellent response of the corticosteroid naïve group to the introduction of ICS highlights the possibility that the relatively ICS resistant OCS group present distinct mechanisms of T2 inflammation. Secondly, the absence of systemic absorption in our low-dose ICS group is not certain without assays of hypothalamic function. Thirdly, it is possible that some of the effects achieved by the addition of OCS treatment on SAD might have been visible in our ICS group with greater statistical power, or with better ICS deposition in the small airways.²³⁶⁻²³⁸ The dose of Beclometasone dipropionate 400 mcg/daily was selected as a first line ICS treatment in clinical practice however, this may not be optimal. Also, FEF₂₅₋₇₅ may not be the optimal measure of small airway dysfunction. Notwithstanding these caveats, there was an excellent clinical and biological response to the introduction of low-dose ICS in the treatment naïve patient group.

6.4.3 How this study fits with the literature, specific effects on IL-5, and implications of baseline values for eosinophilic asthma

This study corroborates previous study findings regarding the *individual* effects of ICS and OCS in eosinophilic asthma. ICS reduce sputum eosinophilia in eosinophilic asthma^{176 179 184 239 240} while OCS reduces sputum eosinophils and blood

eosinophils.^{12 241} The effects of ICS in reducing FeNO have been well documented.^{198 240 242-244} Effects of OCS in reducing FeNO have also been reported and the degree of reduction seen here tracks the ~35% change reported in the meta-analysis of OCS effects by John Busby et.al.²⁴⁵

The increase in sputum neutrophilia following OCS treatment is also in keeping with previous reports^{176 178 211 241 246 247} and lends support to commentary that the mixed granulocytic sputum pattern found in patients with refractory eosinophilic disease and poor lung function is driven at least in part by corticosteroid use.^{248 249}

It is difficult to compare mediator values with other cohort studies due to differences in recruitment criteria. However, the reduction, but *not* normalisation, of sputum IL-5 following ICS treatment is consistent with the findings of the SARP program on the effects of ICS treatment in non-severe asthma patients.²⁵⁰ Indeed, small increases in airway IL-5 have been detected with back-titration of inhaled corticosteroids.²⁵¹ The absence of effect of ICS on serum IL-5 is also in keeping with previous studies.²⁵²

The modest effect of ICS on blood eosinophils (mean 1.6 fold reduction) in the absence of a serum IL-5 effect is potentially mechanistically interesting. OCS has been previously reported to significantly reduce IL-5 in sputum^{172 253 254} and, to a lesser degree, in serum.^{173 241} The data presented here fits with previous findings such that parenteral corticosteroids significantly reduce 'refractory' eosinophilic airway inflammation, but levels of T2 mediators in the airway are *not* normalised following OCS treatment.²⁵⁵

Perhaps the most striking observation for our OCS group is the pan-elevation of T2 mediators in sputum at baseline. This finding corroborates Couillard et al. (who

studied patients undergoing FeNO suppression treatment) that failure to suppress FeNO with ICS treatment identifies corticosteroid-resistant signalling in severe T2-high asthma. In this study, high levels of blood eosinophils and FeNO on ICS treatment selected for a group of patients who had consistently high levels of T2 mediators in the airway. This cohort was taking lower maintenance doses of ICS than the Couillard et.al. cohort and 39% of the group had never received a course of oral corticosteroids prior. High baseline levels of T2 mediators in the airway and an additive effect of oral steroids improving lung function and symptoms suggest trials of the initiation of oral-sparing systemic biologic treatments earlier in the disease course for such patients is warranted. These findings also support the proposal of the Lancet commissioners and others, that evidence of persistent airway eosinophilia (in adults) despite the prescription of a moderate dose of ICS should be included in the definition of severe asthma and / or be considered a phenotype of asthma.^{199 256}

257

6.5 Conclusions

This study compared the effects of introducing ICS treatment in T2-high asthma with the effects of adding oral corticosteroids in T2-high asthma. OCS have been used to treat asthma since the 1950s: since the 1970s they have been replaced by ICS as the mainstay of asthma anti-inflammatory treatment and reserved for ICS refractory disease. Despite this having been the case for some decades now there is no published work about the additional benefits on OCS on top of ICS in T2-high asthma, and there is little randomized control trial evidence regarding their use as an

adjunctive treatment. Understanding what the additional benefits of OCS are on clinical parameters and mediators of T2 inflammation has the potential to improve understanding of ICS refractory T2 high asthma.

The data presented here shows that the introduction of ICS and the addition of OCS in T2-high asthma resulted in similar clinical benefits. However, the pattern of the anti-inflammatory effects differed. OCS produced greater inhibition of blood eosinophils than ICS while ICS produced greater inhibition of FeNO than OCS. OCS had a broader suppressive effect on T2 mediators in sputum and serum than ICS. The pattern of results suggest that the additional benefits of OCS lie in provision of access to the small airways and from inhibiting lung inflammation in sites not accessed by ICS. From a clinical perspective these findings suggest that FeNO reflects an ICS responsive airway epithelia and blood eosinophils reflect an OCS responsive reservoir of cells. As well as prompting mechanistic hypotheses about the differential effects of ICS and OCS, these data would support conducting randomized controlled trials of biologic therapy for patients established on ICS who have high T2 biomarkers but who do not meet the current NHS England requirement of 3 clinically significant exacerbations requiring oral corticosteroids.

7 . What is the role of soluble IL5 receptor α (sIL5R α) in eosinophilic asthma?

In this chapter I measure sIL-5R α and eosinophil expression of mIL-5R α in the plasma and sputum of eosinophilic asthmatics for the first time to assess whether the regulatory effects of sIL-5R α on IL-5 seen *in vitro* may be applicable to eosinophilic asthma *in vivo*.

7.1 Introduction

Interleukin-5 (IL-5) is an important factor in eosinophilic asthma through its roles in eosinophil maturation, differentiation, survival and activation¹⁶⁷ and beyond, in eosinophil-adjacent and eosinophil-independent roles in the disease. The disease is characterised by the presence of elevated levels of IL-5 in the airway and in the peripheral blood.^{96 172 252 258-260} The strategy of targeting IL-5 in eosinophilic asthma has been a successful one, illustrated by the trials of the anti-IL-5 monoclonal antibody mepolizumab^{90 96 261} and a body of ‘real world’ evidence since its introduction into clinical practice.²⁶²⁻²⁶⁶

There are currently limited clinical trial data investigating whether it is possible to stop anti-IL-5 therapy after a period of treatment. The best available data come from the COMET²⁶⁷ randomized controlled trial whereby patients who had completed ≥ 3 years of mepolizumab treatment were randomized 1:1 with placebo for a further year: stopping treatment resulted in a clear return of eosinophilic inflammation and exacerbations (Figure 7-1).

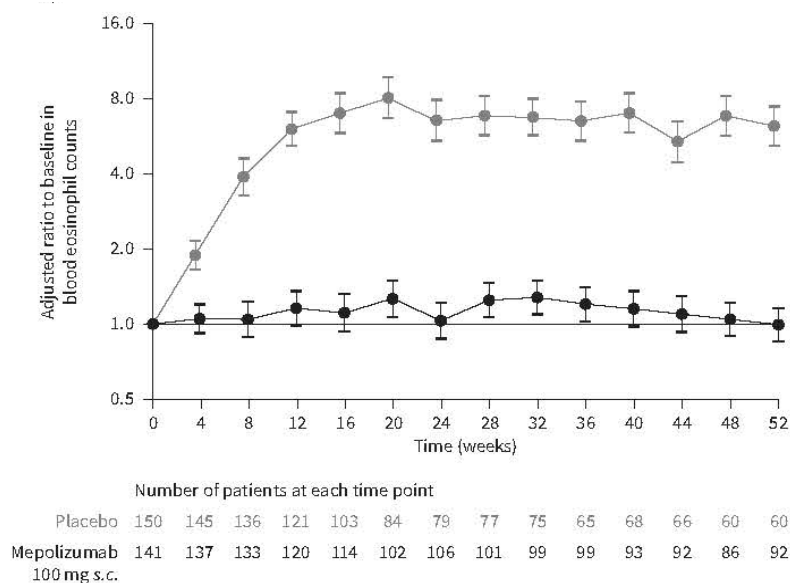


Figure 7-1. Data from Moore et al. 2022 (European Respiratory Journal 59: 2100396) “Stopping versus continuing long-term mepolizumab treatment in severe eosinophilic asthma (COMET) study.” Mean change from baseline in eosinophil counts is displayed. Patients switched to placebo from mepolizumab in grey, patients remaining on mepolizumab in black.

The mechanism of IL-5 over-production in late-onset eosinophilic asthma and the basal signalling maintaining its long-term dysregulation is unknown. As well as mechanisms of *increased* IL-5 production (discussed in Introduction) it is possible that eosinophilic asthma involves dysfunction in pathways which *suppress* IL-5 production. To draw analogies with other diseases, idiopathic multicentric Castleman disease is proposed to involve dysregulation of SOCS3 negative feedback to IL-6/JAK/STAT3 signalling resulting in IL-6 driven inflammation.²⁶⁸ Further, it is proposed that negative regulators of the IFN-1 pathway, such as *ISG15* and *USP18*, are insufficient in patients with Systemic Lupus Erythematosus (SLE).²⁶⁹ Among the mutations in genes which cause Aicardi-Goutieres syndrome, a disease with overlap

with SLE and that is characterised by overproduction of type-1 interferons²⁷⁰, are mutations in genes which function as suppressors of the cell intrinsic IFN-1 response, such as *ADAR1*.²⁷¹ Hypothesised points of dysfunction in IL-5 generation for eosinophilic asthmatics might include dysregulated expression of genes that regulate *GATA3*, the 'master regulator' of Th2 differentiation, such as *SOX4* and *ZFPM1* (mutations in these genes are examined in Chapter 3).

One potential point of dysfunction is a failure of active clearance of IL-5. There is accumulating evidence that some solubilised cytokine receptors act as *antagonists* by binding cytokines with high affinity thereby preventing cytokines binding to membrane receptors and signalling. This is a common and physiologically important regulatory mechanism. The solubilised receptors of the inflammatory cytokine TNF, sTNFR-1 and sTNFR-II, can bind TNF *in vitro* and *in vivo* and inhibit its downstream signalling functions.^{272 273} Rheumatoid arthritis patients have increased serum (and especially tissue) levels of TNF and sTNFR-II.^{272 274} The antagonist effect of the solubilized sTNFR receptors has been leveraged using drugs such as Etanercept (a TNFR-Fc fusion immunoglobulin protein) which bind TNF and block its interaction with TNF receptors on the cell surface, thereby preventing TNF-mediated inflammatory cellular responses.²⁷⁵⁻²⁷⁷ Etanercept has proven a successful treatment for rheumatoid arthritis and other autoimmune inflammatory diseases in which blocking TNF-mediated inflammatory cellular responses is desirable.²⁷⁸⁻²⁸¹

The principle of preventing interaction between cytokines and their receptors on the cell surface and blocking downstream signalling applies to several drugs across inflammatory diseases. Anakinra is a recombinant form of the natural anti-inflammatory cytokine IL-1 receptor (IL-1R α) which competes with IL-1 for binding to

its receptor and is used in diseases in which IL-1 is a key driver, such as Still's disease. In asthma, the monoclonal antibody astegolimab has been created to block the ST2 receptor (IL-1RL1) from binding the cytokine IL-33²⁸² while tozorakimab was purposely created to block ST2-IL-33 receptor ligand interaction by binding IL-33 with high affinity on the same face of the cytokine that normally engages the ST2 receptor (β -trefoil domain).²⁸³ There is of course, the highly successful example of anti-IL13/IL-4R α receptor blockade in eosinophilic asthma with dupilumab.⁹⁴

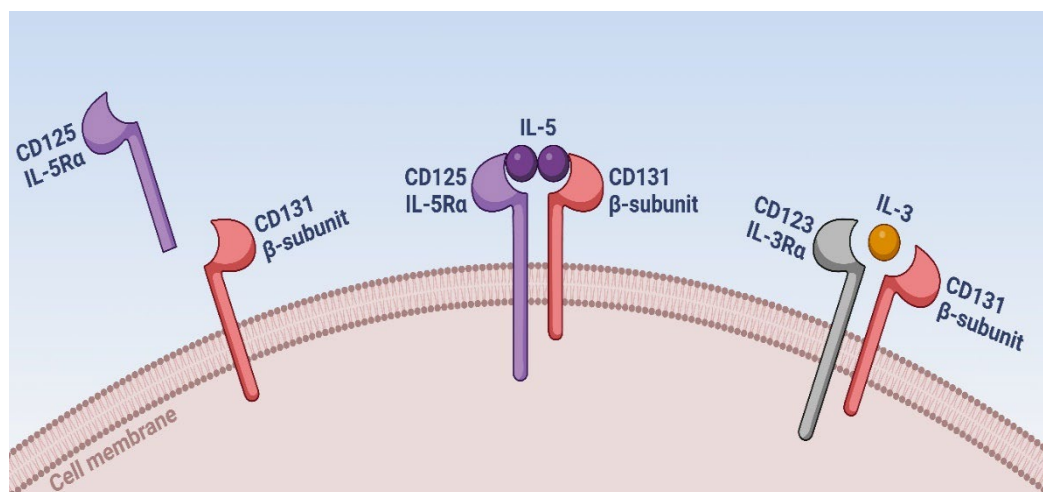


Figure 7-2. Schematic representation of the IL-5 receptor- α (IL-5R α) in a complex with its β sub-unit. On the left solubilized IL-5 receptor- α (sIL-5R α). Figure created in BioRender.

There is *in vitro* evidence from Monahan et al.²⁸⁴ that a solubilized, recombinant form of the extra-cellular portion of the IL-5 receptor (rsIL-5R α) can inhibit the effects of IL-5 on native membrane bound IL-5 receptor (mIL-5R α) on eosinophils through competitive inhibition (binding of IL-5 to mIL-5R α is shown in Figure 7-2). The inhibitory effects of their e-coli derived rsIL-5R were demonstrated on IL-5 induced

eosinophil proliferation and survival and on basophil release of leukotriene C4 (note: basophils also highly express mIL-5R α). Cameron et al.¹⁴⁴ have reported that sIL-5R α can inhibit local eosinophil differentiation within nasal mucosa tissue cultures from allergic patients. Recent *in vitro* work by Jian Luo using eosinophils derived from eosinophilic asthmatics confirms and extends this effect of rsIL-5R α on eosinophils to inhibiting features of eosinophil activation such as shape change and loss of CD62L expression (L-selectin) and inhibiting increase of CD63 expression a marker of activation-induced degranulation (Figure 7-3).^{285 286}

sIL-5R α could be generated by transmembrane cleavage of IL-5R α from the eosinophil cell surface or generated through direct production via alternative splicing.^{287 288} Soluble receptors that are generated through proteolytic cleavage are common in all cytokine and growth factor receptor families (e.g. IL6R, TNFR1, CXCR2, IL-1R2, ErbB4).²⁸⁹ The second mechanism of soluble receptor generation involves alternative splicing of a membrane receptor mRNA, that omits the receptor's transmembrane domain. The resultant soluble receptor is secreted from the cell into the extracellular space.²⁸⁹ sIL-5R α has been reported to be derived from both alternative splicing and proteolytic cleavage of mIL-5R α from the cell surface of eosinophils *in vitro*.^{287 288 290}

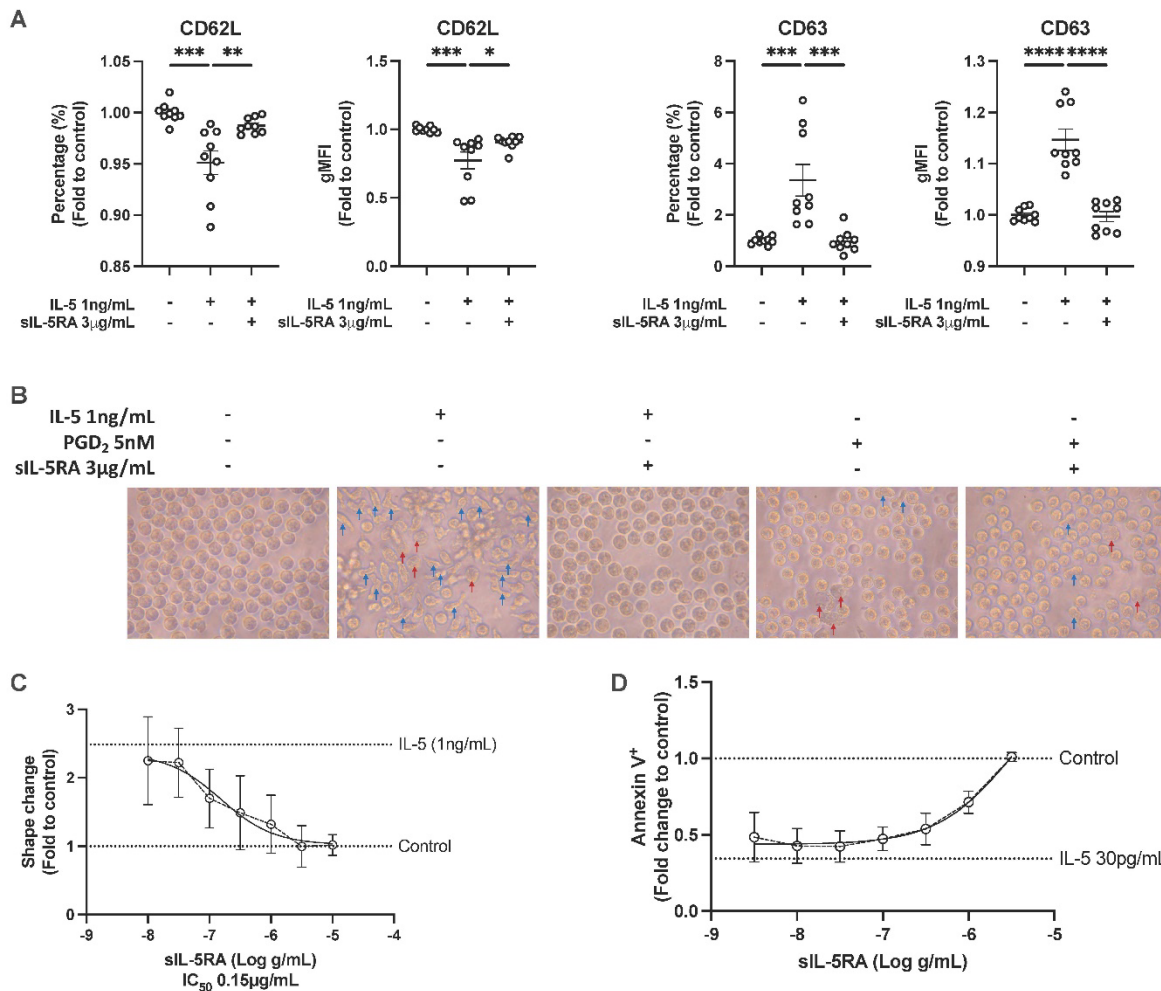


Figure 7-3. Unpublished work by Jian Luo (with permission). The inhibitory effect of rsIL-5R α on blood eosinophils. Peripheral blood eosinophils were isolated from eosinophilic asthmatics at steady state using a gradient density protocol, red-cell lysis and anti-CD16 microbeads. Cells were stimulated by IL-5 with or without the presence of rsIL-5R α . (A) rsIL-5R α attenuates IL-5-induced loss of CD62L expression and inhibits IL-5 increased CD63 expression on eosinophils. (B&C) rsIL-5R α inhibits IL-5-induced eosinophil shape change (D) rsIL-5R α blocks IL-5-sustained eosinophil survival.

Liu et al.²⁸⁸ using bronchoalveolar lavage eosinophils, demonstrated that IL-5 regulates its receptor through proteolytic release of mIL-5R α , with a corresponding increase of sIL-5R α in the supernatant following incubation of eosinophils with IL-5.

This process was partially inhibited by combination matrix-metalloproteinase (MMP) inhibitors suggesting that IL-5 mediated loss of mIL-5R α and increase in sIL-5R α is through proteolytic cleavage of mIL-5R α . While the mechanism of mIL-5R α cleavage is persuasive, it is yet to be confirmed that other mechanisms (e.g., endocytosis) are not involved in mIL-5R α downregulation. The use of broad matrix metalloproteinase blockade in these experiments may also interfere with other pluripotent MMP functions within eosinophils.²⁹¹

Assuming that sIL-5R α is generated from IL-5 ligand-receptor mediated cleavage of mIL-5R α , and that sIL-5R α antagonises the effects of IL-5 on eosinophils (e.g., through blockade of ligand-receptor interaction and signalling) one might hypothesize that increased IL-5 in eosinophilic asthma, along with blood and tissue eosinophilia, could represent dysfunction of the IL-5/IL-5R α axis. Loss of mIL-5R α from the eosinophil cell surface may have further inhibitory effects on eosinophils by leaving them refractory to stimulation from IL-5.²⁸⁸ The IL-5/IL-5R α axis could also represent a therapeutic target.

Eosinophil–IL-5–IL-5R α relationships *in vivo* were explored by Liu et al.²⁹² who measured mIL-5R α on blood and BAL eosinophils, and sIL-5R α in BAL fluid, both before and 48 hours after an antigen challenge to atopic (but not asthmatic) participants (Figure 7-4). The antigen challenge was associated with increased BAL IL-5 and an influx of eosinophils to the BAL fluid. The eosinophils in the BAL following the challenge expressed lower levels of mIL-5R α (in keeping with a similar experiment in mice by Tomakiet al.²⁹³) and there was increased sIL-5R α in the BAL. In this experiment there were strong positive correlations between BAL

eosinophils and sIL-5R α levels in BAL and between BAL IL-5 and sIL-5R α (Figure 7-4). The authors also noted that eosinophils in the BAL 48 hours following antigen challenge (with reduced mIL-5R α) were refractory to stimulation from IL-5 but not GM-CSFR α .

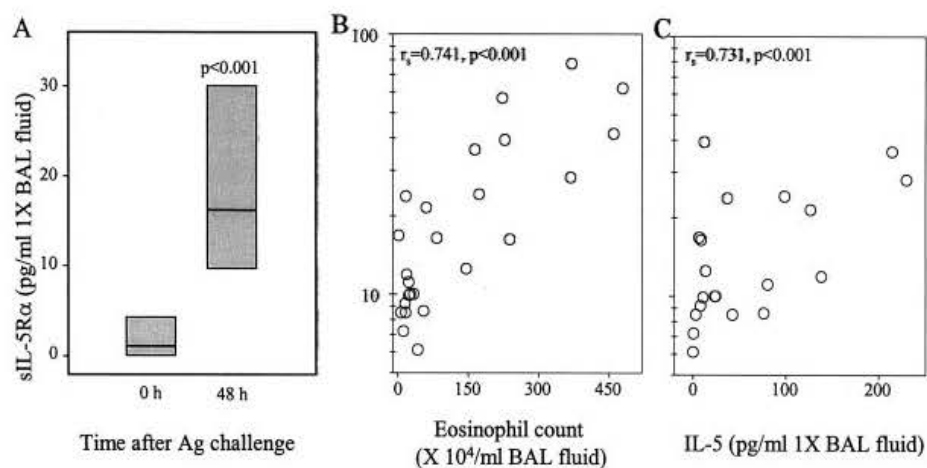


Figure 7-4. Data from Liu et al. 2002 (Journal of Immunology 169 (11):6452–6458) “Decreased Expression of IL-5 Receptor α on Human Eosinophils: 1. Loss of Membrane IL-5 receptor α on Airway Eosinophils and Increased Soluble IL-5 Receptor in the Airway After Allergen Challenge.” (A) sIL-5R α concentrations in BAL fluid before and after antigen challenge. Correlations post antigen challenge between (B) BAL eosinophil counts and sIL-5R α levels and (C) BAL IL-5 and sIL-5R α .

Yasruel et al.²⁹⁴ explored levels of mIL-5R α and sIL-5R α in the bronchial tissue of asthmatic patients who were *not* phenotyped for eosinophilic inflammation – none of the participants were taking inhaled or oral corticosteroids. They found higher levels of both mIL-5R α mRNA and sIL-5R α mRNA in the bronchial tissue of asthmatics compared to healthy controls and an inverse relationship between these two

variables. They also described a remarkably strong inverse correlation between the number of tissue cells expressing mL-5Ra mRNA with the patient's FEV1 ($r = -0.94$, $R^2 = 0.89$, $p < 0.001$). Given that 92% of the cells expressing mL-5Ra mRNA in the tissue in this experiment were EG2+ eosinophils this could be considered a correlation between tissue eosinophilia and FEV1. A positive correlation between number of cells expressing sIL-5Ra mRNA and FEV1 was also reported ($r = 0.72$, $R^2 = 0.52$, $p < 0.001$) which, the authors attribute to production of sIL-5Ra by alternate splicing. Interestingly, they also reported an inverse correlation between mL-5Ra mRNA and sIL-5Ra mRNA expression within cells in the bronchial tissue of asthmatics ($r = -0.66$, $R^2 = 0.43$, $p < 0.005$).

In a study of 34 patients with nasal polyposis, some with concomitant asthma, Gaevart et al.²⁹⁵ found the amount of sIL-5Ra mRNA expression in nasal polyp tissue from asthmatic patients was significantly higher than that in the nasal polyp tissue of non-asthmatics and controls. The amount of sIL-5Ra in tissue was also higher than in nasal secretions and in serum. sIL-5Ra protein levels were higher in the serum of asthmatics with nasal polyps than controls. mRNA levels of sIL-5Ra and mL-5Ra isoforms were again correlated positively and negatively with the degree of tissue eosinophilia, respectively. In nasal tissue sIL-5Ra was significantly correlated with concentrations of IL-5 ($R^2 = 0.405$, $p < 0.006$), with nasal tissue eosinophil percentage ($R^2 = 0.536$, $p < 0.001$) and with serum sIL-5Ra ($R^2 = 0.668$, $p = 0.006$). Gevaert and colleagues were unable to assess correlations between eosinophils, IL-5 and sIL-5Ra in serum due to many IL-5 measurements at the lower limit of detection.

The positive relationships described in these two studies between eosinophilia and sIL-5Ra, and between sIL-5Ra and IL-5, and inverse correlations between eosinophil mRNA expression and serum sIL-5Ra have also been reported in the serum of patients with hyper-eosinophilic diseases, such as idiopathic hyper-eosinophilia (HES), systemic mastocytosis (SM) and chronic eosinophilic leukaemia (CEL) by Wilson et al.²⁹⁶ (Figure 7-5).

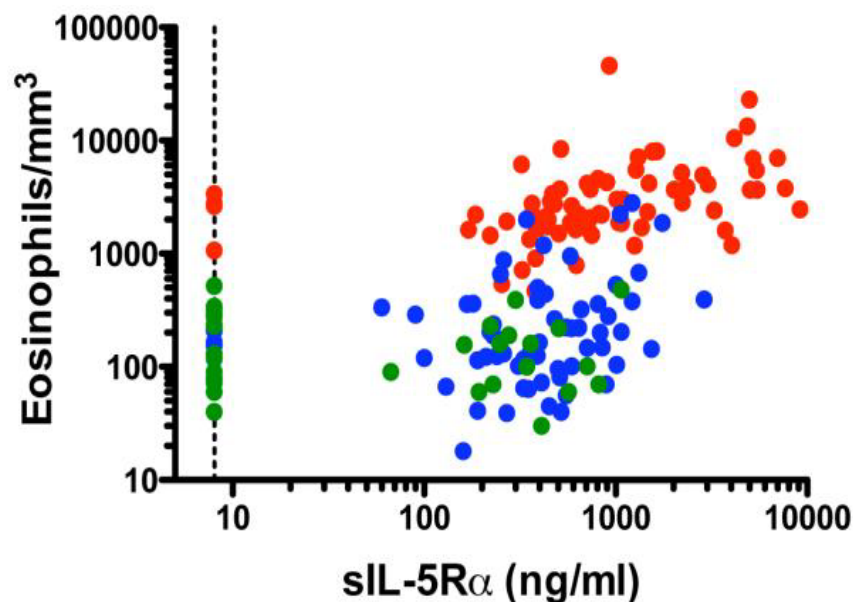


Figure 7-5. Data from Wilson et al. 2011 (Journal of Allergy and Clinical Immunology 128(5): 1086–1092) “Interleukin-5 receptor α levels in patients with marked eosinophilia or mastocytosis.” Serum levels of sIL-5Ra are positively correlated with blood eosinophilia ($r_s = 0.54$ $p < 0.0001$). Subjects with hyper-eosinophilic syndrome and chronic eosinophilic leukaemia are shown in red, those with mastocytosis in blue and healthy controls in green. Note units for sIL-5Ra should be pg/ml not ng/ml, confirmed with the last author of the publication (Amy Klion, personal communication May 19, 2022).

In totality, these results support the prospect of finding similar relationships between eosinophils–IL-5–IL-5Ra in the peripheral blood of patients with eosinophilic asthma and the potential for fruitful investigation of dysregulation of a sIL-5Ra negative feedback mechanism. Further *in vivo* support for this hypothesis comes from finding elevated levels of IL-5 in patients taking the anti-IL-5Ra treatment benralizumab. Benralizumab is an afucosylated IgG1 kappa antibody directed against IL-5Ra, which directly depletes eosinophils and their associated bone marrow progenitor cells through induction of antibody-dependent cell-mediated cytotoxicity via recruitment of natural killer cells.²⁹⁷ The Phase I and IIa studies of benralizumab reported serum levels of IL-5 were elevated at 90 days in patients on treatment while blood eosinophil levels were completely suppressed ~0.00 (Figure 7-6).²⁹⁸

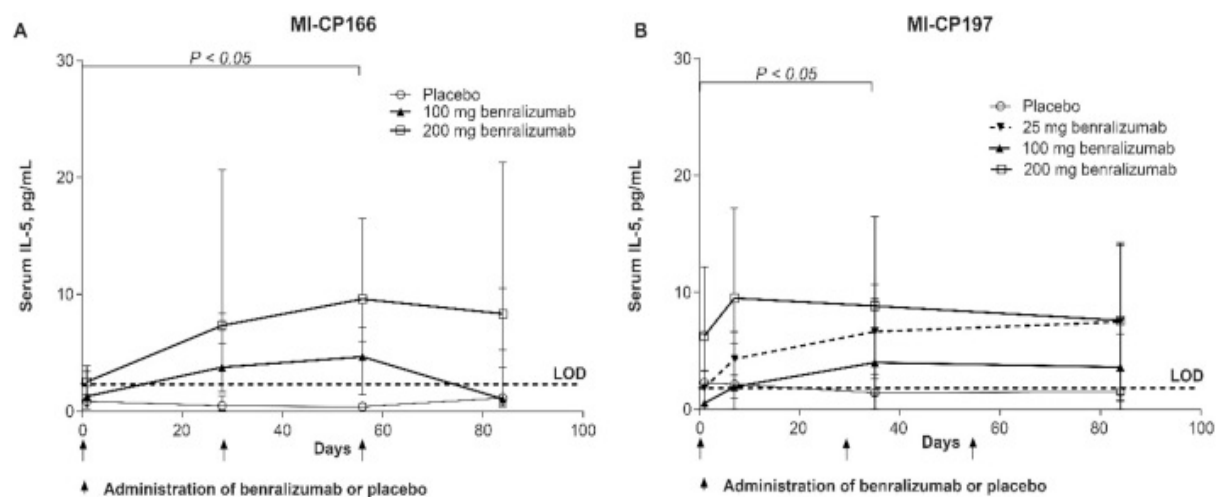


Figure 7-6. Data from Pham et al. 2016 (Respiratory Medicine, 111:21–29) “Reductions in eosinophil biomarkers by benralizumab in patients with asthma.” Data from early phase trials of benralizumab illustrate increases in serum IL-5 levels for those eosinophilic asthmatic patients on benralizumab compared to placebo. Note (i) eosinophil levels were completely

suppressed at 90 days in patients on benralizumab (ii) doses used in the above study are higher than the 30mg bi-monthly dose in current NHS use for eosinophilic asthma.

Similar findings of increased IL-5 concentrations with eosinophil reduction have been found in patients with eosinophilic asthma treated with mepolizumab. This could be due to an endogenous IL-5 auto-regulatory pathway responding to suppressed eosinophilia or, a view frequently opined in the literature is that this reflects measured IL-5-mepolizumab complexes (with a longer half-life) that are biologically inactive and a pharmacodynamic marker of target engagement, rather than increased free IL-5 activity.^{299 300} In the case of benralizumab however, IL-5R α is the drug target, not IL-5.

Increased IL-5 levels on anti-IL-5R α treatment could reflect the involvement of a negative feedback mechanism and an auto-regulatory increase of IL-5 in response to either (i) reduced levels of the target (eosinophil) or (ii) binding and removal of an IL-5 antagonist (sIL-5R α) (iii) complex formation with longer half-life than IL-5 (e.g., IL-5–sIL-5R α –anti-IL-5Ra complexes). As a prelude to the current experiments, IL-5 was measured in a small cohort of eosinophilic asthma patients maintained on benralizumab for a longer period (mean 439 days). This was to understand if the rise in IL-5 is a transient phenomenon, such as the formation of IgE complexes and their gradual long-term clearance in patients taking the anti-IgE therapy Omalizumab.³⁰¹ It was also done in mind of the lower 30 mg bi-monthly dose of benralizumab in use in the United Kingdom for severe eosinophilic asthma. Results are shown in Figure 7-7 and patient baseline parameters are available in Appendix Table 11-11. The

persistent elevation of serum IL-5 levels on anti-IL-5R α therapy are consistent with the effect of binding and removal of a hypothesized IL-5 antagonist (sIL-5Ra) *in vivo*.

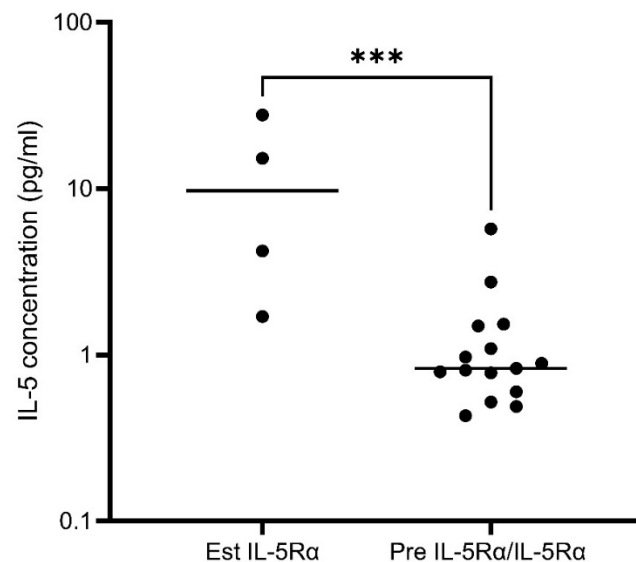


Figure 7-7. Pilot data prior to the current work. Comparison of serum IL-5 levels between patients with eosinophilic asthma taken directly before initiation of anti-IL-5R α /IL-5 monoclonal antibody therapy and patients established on anti-IL-5R α treatment (benralizumab). Bars represent the median. Data points are unpaired. Mean time on benralizumab 439 days. Patients established on benralizumab taking 30 mg bi-monthly. Untreated patients not taking oral corticosteroids. *** = $p < 0.001$. Abbreviations; Est, established on anti-IL-5R α treatment; Pre, sample taken before starting anti-IL-5R α /IL-5 treatment. Further details on the pilot including patient baseline parameters are available in Appendix section 11.3.1

The relationship between IL-5 and sIL-5R α in eosinophilic asthma has not been studied before. There is *in vitro* evidence that mIL-5R α is shed from eosinophils in

response to IL-5. Clinically, there is evidence that suggests mL-5R α is shed in the airways of atopic patients following allergen challenge. Given the inhibitory role of sIL-5 α on IL-5 mediated effects on eosinophils demonstrated *in vitro* and, given the increase in IL-5 levels for patients taking anti-IL-5R α antibody therapy (the reasons for which are unknown) it is possible that sIL-5R α has a regulatory role on IL-5 in eosinophilic asthma. This would have implications for therapeutic intervention. In order to begin assessment of this it is necessary to measure IL-5 and sIL-5R α and eosinophil levels in eosinophilic asthmatics at steady state.

7.1.1 Plan

The aims of the current study were to assess the relationships between eosinophil counts, IL-5 and sIL-5R α in eosinophilic asthmatics with high levels of eosinophilic inflammation, and to compare with healthy controls. The purpose is to understand:

- (i) Whether the relationships between eosinophil counts, IL-5 and sIL-5R α described in blood and serum for patients with hyper-eosinophilic disease are applicable to eosinophilic asthmatics at steady state.
- (ii) Whether sIL-5R α is elevated in the blood and / or sputum of eosinophilic asthmatics compared to healthy controls.
- (iii) Whether sIL-5R α is likely to have a role in regulating peripheral blood eosinophilia for eosinophilic asthmatics.
- (iv) If sIL-5R α may have a role as a biomarker of eosinophilic asthma.

7.2 Methods

7.2.1 Study design

The study was sponsored by the University of Oxford and approved as part of the Oxford Airways Study OAS (REC 18/SC/0361) and NIHR CSP Study 88241 (REC 08/H0406/189). Measurements were obtained at a single baseline visit.

7.2.2 Participants

7.2.2.1 Patients with eosinophilic asthma

Eligible patients for inclusion were never or former smokers who had abstained from smoking for at least one year. Participants were required to have a clinical diagnosis of eosinophilic asthma with confirmed current or historic evidence of variable airflow limitation, defined by at least one of the following: FEV1 bronchodilator reversibility >200 ml; FEV1 percentage change $\geq 12\%$; methacholine challenge of less than 8 mg/ml; or peak expiratory flow (PEF) diurnal variability. Additional inclusion criteria were: one or more recorded blood eosinophil counts $\geq 0.40 \times 10^9/L$ and willingness to undertake sputum induction. Exclusion criteria comprised any systemic corticosteroids within the preceding 2 months, evidence of exacerbation of asthma at the time of enrolment, and any history of previous biologic therapy.

7.2.2.2 Healthy control participants

Eligible patients for inclusion were never or former smokers with no history of asthma or other respiratory diseases and no history of previous inhaled treatments and willingness to undertake sputum induction.⁷

7.2.3 Measurements and samples

At the visit each participant underwent pre and post bronchodilator spirometry according to American Thoracic Society (ATS) standards using 3 reproducible measurements of FEV₁. Measurements of FeNO (NIOX, VERO Circassia Ltd, Oxford, UK), Full blood count (FBC) and Asthma Control Questionnaire–5 were also taken and a treatment enquiry, was conducted where relevant. Sputum was induced with hypertonic saline as described in section 6.2.4 and following the protocol described in full in Appendix section 11.4. Peripheral whole blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes. Plasma and serum were collected in lithium heparin and serum separator tubes, respectively. Sputum and whole blood were processed immediately.

⁷ 67% of patients were recruited and sputum inductions performed by the author with the remaining 33% performed by other members of the Respiratory Medicine Unit.

7.2.4 Sputum processing⁸

Sputum was induced and processed as described in section 6.2.4 with deviations from that protocol described below for the flow cytometry experiments. Sputum plugs were weighed and treated with Dulbeccos PBS (dPBS) without calcium and magnesium with 5mM EDTA at 8× the weight of the expectorate retained. EDTA was added during collection to equalize exposure of sputum eosinophils with that of blood eosinophils on collection. The sample was vortexed and centrifuged at 400 g for 5 minutes. The supernatant was aspirated (this was the PBS supernatant) and 4× the initial sample weight of 0.1% dithiothreitol (DTT) was added to the sample. DTT was used at 0.1% to reduce the potential for an effect on cell surface receptors. The sample was incubated on ice for 15 minutes with vortexing and shaking at 5-minute intervals. The sample was then filtered through a 48 µm gauze before the sputum cell pellet was re-suspended in RPMI 1640 with L-Glutamine and 10% FCS and 20 mM HEPES ('universal media') and placed on ice prior to staining simultaneously with blood leukocytes. An aliquot of this suspension was used for the cyto-spin and differential cell count performed as described in Chapter 1.

Pilot experiments were performed to consider whether the handling of sputum eosinophils and use of DTT may have resulted altered mIL-5Rα expression and/or eosinophil activation.

⁸ Sputum plug processing as described in this paragraph was performed by Mahdi Mahdi with supply of samples and buffers from the author.

7.2.5 ELISA assays

Sputum supernatants suspended in phosphate buffered saline and plasma were stored at -80 °C until processing. Samples were assessed using an IL-5 solid-phase immunoassay based on electrochemiluminescence (Meso Scale Discovery [MSD], Rockville, MD, USA, cat: K151QSD) and an sIL-5R α classic sandwich ELISA (Abcam, Cambridge, UK, cat: ab155435). Assays were performed according to the manufacturer's instructions. Samples were diluted 1:1 with the manufacturer's diluent for the IL-5 assay but not the sIL-5R α assay. Washing buffer was PBS with 0.02% Tween20.

Pilot assays were undertaken (i) to determine choice of plasma versus serum and PBS versus DTT supernatant for the sIL-5R α assay which, has limited reported use in the literature (plasma was selected). (ii) to assess whether to dilute phosphate buffered saline (PBS) samples per manufacturers' instructions (they were assayed undiluted) and (iii) whether to incubate samples prior to analysis (they were not). There were also (iv) heterologous spike-in experiments to assess interference and cross-reactivity within the two assays. Recombinant human IL-5 at defined concentrations (Miltenyi Biotec, Bergisch Gladbach, Germany, cat: 130-093-927) was spiked into the sIL5R α assay and recombinant human sIL-5R α (Biotechne, Minneapolis, MN, USA cat: 130-093-927) was spiked into the IL-5 assay. The vehicle used in these experiments was the manufacturer's provided diluent. A description of these pilot experiments and results is detailed in Appendix sections 11.3.2 and 11.3.3.

7.2.6 Blood leucocyte isolation

Cells were isolated from whole blood using a density gradient technique and red-cell hypotonic lysis adapted from a technique described in Akuthota. et al.³⁰² This was designed to avoid eosinophil activation. Whole blood was diluted with Dulbeccos PBS without calcium and magnesium and with 0.5% sterile foetal calf serum (FCS), layered on top of Lymphoprep™ (Axis-Shield/Cytiva, Oslo, Norway, cat: 1114547) then centrifuged at 600 g for 40 min at 4 °C. The mononuclear cell layers and granulocyte layers were aspirated and combined in a fresh falcon tube before a 10:1 volume of ice cold 0.18% hypotonic saline was added to the cellular solution for 30 seconds before cell 'rescue' by adding an equal total volume of 1.68% hypertonic saline containing 20 nM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 5% FCS at room temperature. The solution was centrifuged at 400 g for 6 minutes at 4 °C and the cell pellet retained before the process of red cell lysis and cell rescue was repeated for a second time. Following this red cell casts were aspirated from above the cell pellet (see Figures 7-8 and 7-9 for the leukocyte composition of the pellet and Appendix Figure 11-5 for an image of the red cell casts). Cells were then re-suspended in 2 ml of RPMI 1640 with L-Glutamine with 10% FCS and 20 mM HEPES ('universal media') and placed on ice⁹ prior to simultaneous staining with sputum cells.

⁹ Vortex and DTT controls were processed at this point

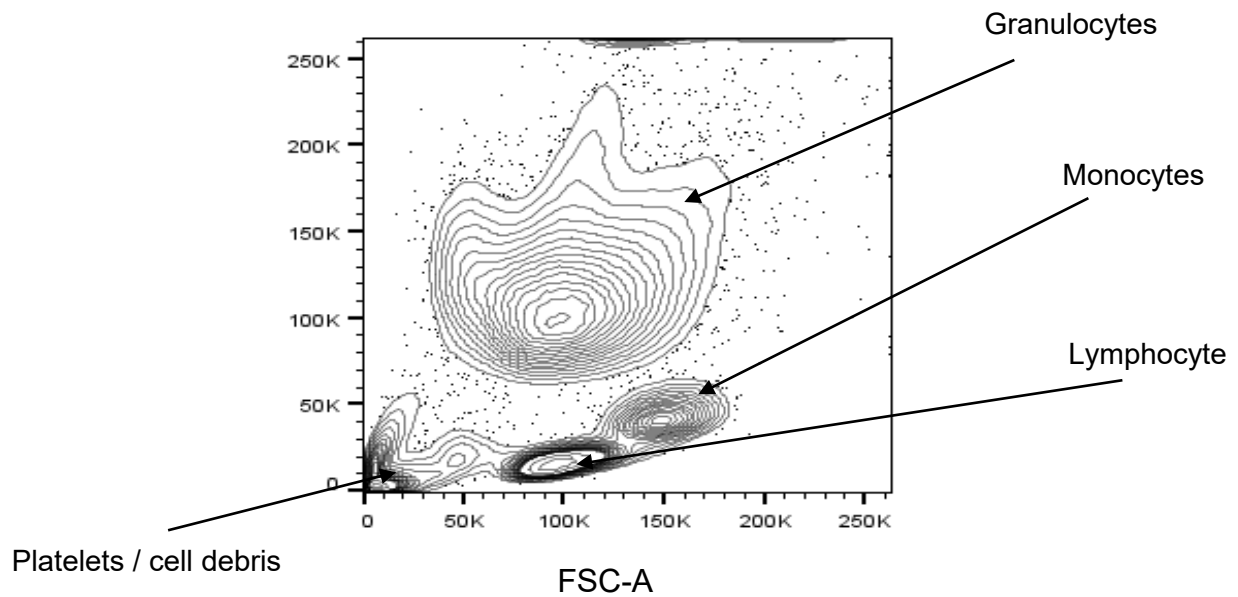


Figure 7-8. Flow cytometry plot of an unstained whole blood sample from an eosinophilic asthmatic patient after hypotonic red-cell lysis and processing through to FACS according to the methods described for stained samples. The pattern of cell distribution is commonly expected with the exception of fewer platelets / cell debris than typically seen in illustrations.

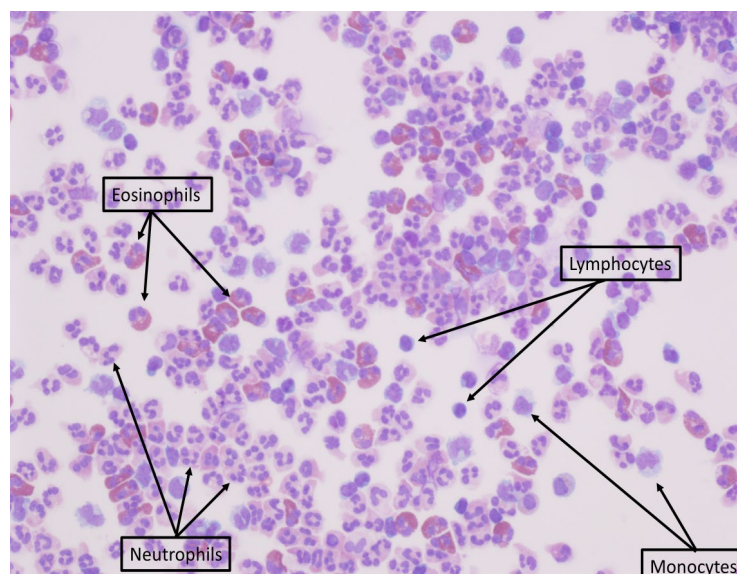


Figure 7-9. Image of cells following Wright-Giemsa staining of the above sample. An enriched population of eosinophils are identifiable from their distinctive red acidophilic granules and bilobed nuclei

7.2.7 Flow cytometry

For paired blood and sputum samples, cell counts were estimated using trypan blue and an automated haemocytometer. Fc receptors were blocked using 20 μ L of reagent per 1×10^7 cells according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany, Cat: 130-059-901) with mixing and incubation at 2–8°C for 20 minutes followed by centrifuging at 400 g for 6 minutes at 4 °C then resuspending the cells in universal media. Aliquots of one million blood or sputum cells were stained with master-mixes of: (i) anti-IL-5R α / CD125 (clone REA705, Miltenyi, cat: 130-110-544); (ii) anti-CD16 (clone 3G8, BioLegend, San Diego, CA, USA, Cat: 302046); (iii) anti-CD45 (clone HI30, Biolegend, Cat: 304012); (iv) anti-Siglec-8 (clone 7C9, Biolegend, Cat: 347112) used as markers of identification. Cells were also stained with (v) anti-CD123 (clone AC145, Miltenyi, cat# 130-113-325) as a marker of activation and as a negative control; (vi) anti-CD62L (clone DREG-56, Biolegend, cat# 304828) as marker of activation and as a positive control and (vii) the viability dye DRAQ7 (Biostatus Ltd., Shepshed, Leicestershire, UK, cat# DR7-1000). Staining with (i) - (vi) was followed with incubation at 4°C in the dark for 40 minutes followed by two cycles of washing and centrifugation at 400 g for 6 mins at room temperature. Cell washing was performed using Dulbeccos PBS without calcium and magnesium with 2% FCS. DRAQ7 was then added at 1:1000 followed by further incubation for 10 minutes in the dark at room temperature followed by one further cycle of cell washing, centrifugation and re-suspension in stain buffer (BD

Biosciences, San Jose, CA, USA, cat: 554657). Samples were then taken immediately for flow cytometry and FACS sorting using a BD FACS Aria III.¹⁰

The fluorochromes and concentrations of antibodies, dye and FC blocker are listed in Table 7-1. Calibration controls were run for all stains using human leucocytes prior to the experiment. Fluorescence minus one control (FMOs) used to define positive gating thresholds for each marker were run for all markers in one pilot experiment (Appendix Figure 11-7) and were run for CD125 (IL-5R α) for every participant.

7.2.8 Cytospins and Wright-Giemsa staining of eosinophils

Following FACS sorting purified eosinophil populations were re-suspended immediately in universal buffer at a concentration of 0.5×10^6 cells/ml. The cell solution was pipetted into the cyto-funnel(s) and centrifuged at 600 g for 5 minutes. Slides were air dried for 12 hours before fixing in 100% methanol for 10 minutes. Staining was performed the following day using a Wright-Giemsa kit (Abcam, Cambridge, UK, cat: ab245888) following manufacturer's instructions. Cell counts were calculated by hand with a light microscope at 20x magnification counting a representative sample of 400 cells per slide.¹¹

¹⁰ Flow sorting performed by Helen Ferry in the Experimental Medicine Division Flow Cytometry Facility

¹¹ These cell differential counts were performed by the author

Table 7-1. Antibodies, dyes and FC blocker used in the experiment

Fluorochromes, antibody concentrations and lasers utilized on the FACS Aria III are given. Spectral overlap is illustrated in Appendix Figure 11-6 and was corrected by compensation using single-stained controls for each fluorochrome prior to the current experiment. Antibodies were titrated using fluorescence minus one populations (FMO) prior to the current experiment. (FMO) populations are presented in Appendix 11-7. Abbreviations: Ex Max, excitation maximum; Em Max, emission maximum.

Role	Antibody	Fluorochrome	Ex Max	Em Max	Laser	Clone	Concentration used
Target	anti-CD125	PE	565	578	Yellow Green E	REA705	1:75
Gating	anti-CD16	BV785	405	785	Violet A	3G8	1:43
Gating	anti-CD45	BV605	405	602	Violet	HI30	1:30
Gating	anti-Siglec-8	PE-Cy7	565	781	Yellow Green A	7C9	1:30
Negative Control	anti-CD123	FITC	490	525	Blue	AC145	1:75
Positive Control	anti-CD62L	BV421	405	421	Violet	DREG-56	1:30
Live/Dead	DRAQ7	n/a	598	694	Red	n/a	1:1000
FC Blocking	–	n/a	–	–	–	n/a	20uL per 1 x 10 ⁷ cells

7.2.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 10 and SPSS statistical package version 29.0.2.0 (SPSS Inc, Chicago, IL). Figures were generated using ggplot2 v3.5.2, GraphPad Prism and FlowJo v10.10.0. Normally distributed data are presented as mean ($\pm$ SD); log-normally distributed data as geometric mean (coefficient of variation, %) and data failing tests of normality / lognormality are presented as median (interquartile range, IQR).

All supernatant mediator concentrations were multiplied x8 post ELISA while plasma and serum concentrations were multiplied x2 prior to analysis to account for dilution during processing. Mediator levels were log-normalized prior to analysis. Values that were not quantifiable due to falling below the lower limit of detection (LLOD) were assigned the LLOD to permit analyses. To permit logarithmic analyses sputum cell count values of 0 were assigned the arbitrary value of 0.2%

For flow cytometry comparisons the logarithm of the median fluorescence intensity is presented (logMFI) and stain index is calculated as the mean fluorescence intensity of positive cells minus the mean intensity of negative cells divided by $2 \times$ the relative standard deviation of the negative cell population. Multiple comparisons were performed using repeated measures ANOVA with Bonferroni correction for the family wise error rate ($\alpha = 0.05$).

7.3 Results

A total of 50 eosinophilic asthmatics and 17 healthy controls were recruited. Baseline parameters for the two groups are shown in Table 7-2. The eosinophilic asthma group includes the 7 patients whose samples were used in the flow cytometry experiment. Two patients were subsequently excluded from the eosinophilic asthma group following diagnoses of allergic broncho-pulmonary aspergillosis (ABPA), due to potential to confound the patient phenotype, and one healthy control was excluded due to suspected respiratory disease.

One sputum sample was discarded due to squamous contamination. Including this, sputum sample availability was 95.5%. Median sputum cell viability was 70.6% (IQR: 55.6–80.0) and squamous contamination was 1.3% (IQR: 0.1–5.9). Baseline differential cell count for the eosinophilic asthma group was similar to that of the eosinophilic asthma group recruited to the OCS arm in the previous experiment (section 6); median sputum neutrophils 44%, IQR 20–64%; eosinophils 27% IQR 7–48%; macrophages 21% IQR 8–30; lymphocytes 1% IQR 0–3%. Healthy controls sputum differential cell counts were very similar to those of the control cohort described in section 6; median sputum macrophages 60% IQR 41–75%; neutrophils 31% IQR 20–53%; lymphocytes 4% IQR 3–6% and eosinophils 0.2% IQR 0–0%.

Table 7-2. Participant baseline parameters

	Healthy Controls n=17	Eosinophilic Asthmatics n=50
Age (years)	30.5 (19–50)	57.1 (19–82)
Male : Female (n)	6 : 11	24 : 26
Age of onset symptoms (years)	n/a	34.6 (1–75)
Nasal polyposis (n)	0 : 17	14 : 36
Sensitized to aero-allergen (n)	10 : 7	35 : 15
Diagnosis of eczema (n)	0 : 17	9 : 41
Total IgE (IU/mL)†	38 (148%)	162 (184%)
BDP equivalent daily dose (mcg)‡	n/a	800 (400–1150)
Pre-BD FEV ₁ (% predicted)	104.5 (±12.3)	67.8 (±24.9)
Sputum eosinophils (%)‡	0.2 (0.2–0.2)	27.2 (7.0–47.6)
Blood eosinophils (x10 ⁹ /L)†	0.12 (64%)	0.53 (47%)
FeNO (ppb)†	16.9 (49%)	55.9 (68%)

Values given as mean ± SE except † = geomean (coefficient of variation, %) and Age/Age of onset are given as mean (range) ‡ = median (IQR). Abbreviations: BDP, beclometasone dipropionate; FeNO, fractional exhaled nitric oxide; Pre-BD FEV₁, pre-bronchodilator forced expiratory volume in 1 second; IgE, immunoglobulin E.

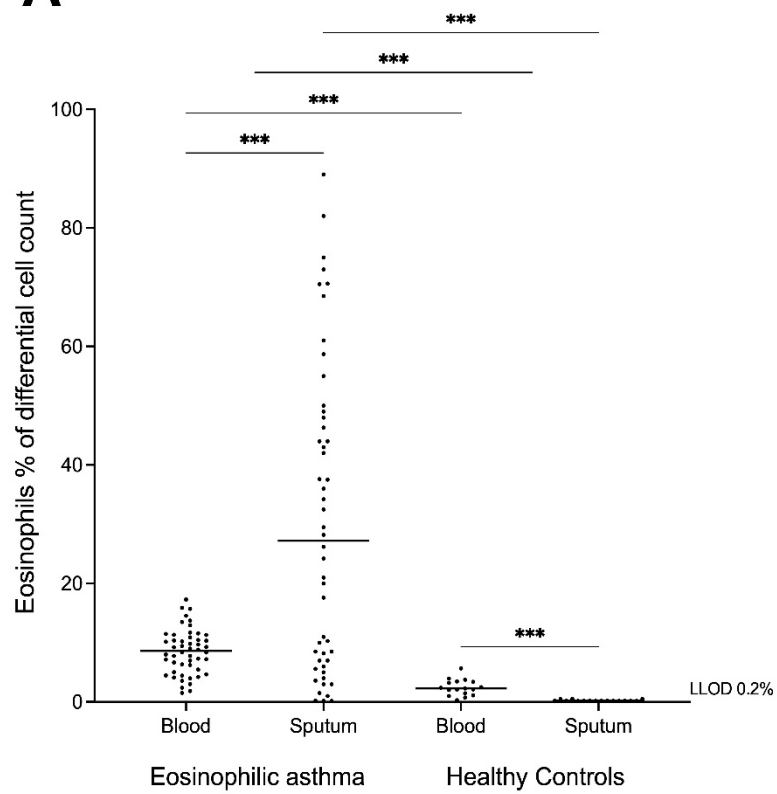
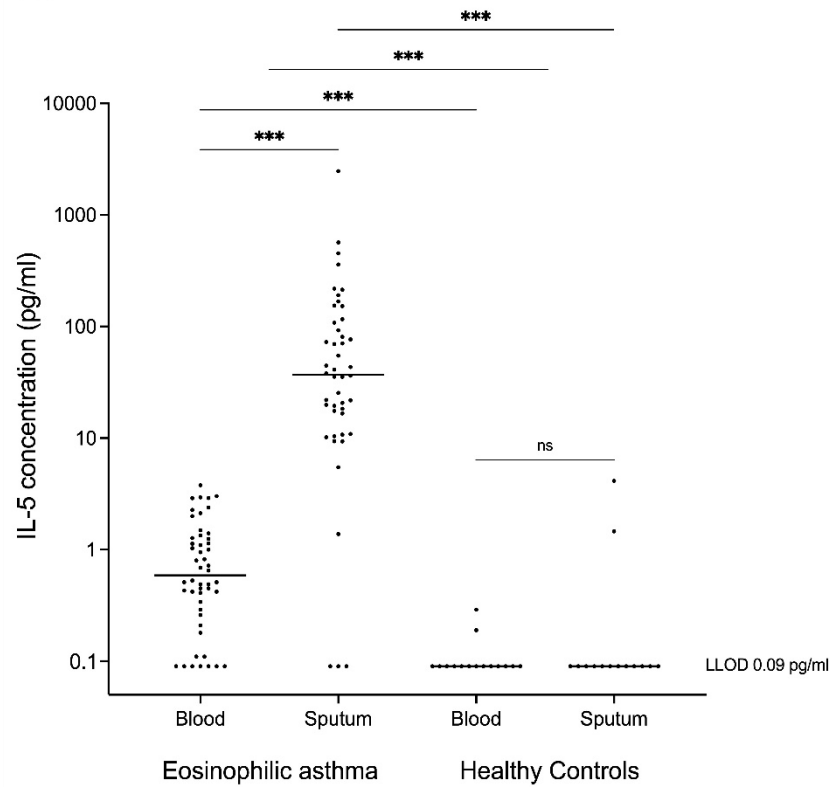
For the sIL-5R α assay the median coefficient of variance for replicate wells within all plates was 2.57% (IQR: 1.06–4.46%) while for the IL-5 assay median coefficient of variance was 3.75% (IQR 1.99–7.37%).

The results of preliminary experiments such as plasma vs serum and PBS vs DTT sputum supernatant and heterologous spike-in experiments are detailed in Appendix sections 11.3.2 and 11.3.3. PBS was selected ahead of DTT based on these results and the results of previous published work using PBS to allow for more direct comparison.^{172 206 303-305} PBS was used undiluted in light of pilot experiment results and in keeping with other published analyses.^{172 306-308} Plasma and serum showed minimal systematic bias so plasma was selected in view of other work suggesting plasma is a better matrix than serum for the evaluation of cytokines.³⁰⁹ Heterologous spike in experiments were performed for IL-5 and sIL-5R α assays using recombinant human IL-5 and recombinant human IL-5R α products that demonstrated agonist and antagonist effects on eosinophils in *in vitro* assays (unpublished experiments of Jian Luo). There was no effect of IL-5 on recognition of sIL-5R α for the sIL-5R α assay. There was increased IL-5 retrieval from spiking sIL-5R α into the IL-5 assay between 200 pg/ml and 2,000 pg/ml but no additional effect between 2,000 pg/ml and 40,000 pg/ml. In the context of physiological ranges of sIL-5R α and the experimental set-up this effect was considered more likely due to artefact (discussed in Appendix section 11.3.2).

7.3.1 The relationships between eosinophils, IL-5 and sIL-5R α .

The percentage of eosinophils within both the total peripheral blood white cell count and within the sputum cell count differential was significantly higher in eosinophilic asthmatics than healthy controls, as expected (Figure 7-10A). As a percentage of all white cells there were more eosinophils in sputum than in blood for eosinophilic asthmatics while this relationship was reversed for healthy controls, with a significantly higher percentage of eosinophils in blood than sputum. These results are in keeping with the current understanding of health and eosinophilic asthma: Under homeostatic conditions eosinophils circulate briefly and preferentially home to tissues including the gastrointestinal tract with disposal in the liver and spleen;^{137 310} in eosinophilic asthma, eosinophils accumulate in the airways and can be detected as increased sputum eosinophils, consistent with enhanced recruitment and transepithelial migration into the airway lumen.^{19 37 137 308 311}

The concentration of IL-5 within both plasma and sputum was significantly higher in eosinophilic asthmatics than healthy controls respectively, with IL-5 levels higher in sputum than in plasma for eosinophilic asthmatics, in keeping with the body of published sputum analyses of eosinophilic asthma.^{252 258-260} There was no difference between IL-5 concentration in plasma or sputum for healthy controls, with the majority of values for healthy controls at the lower limit of detection for both plasma and sputum.

A**B**

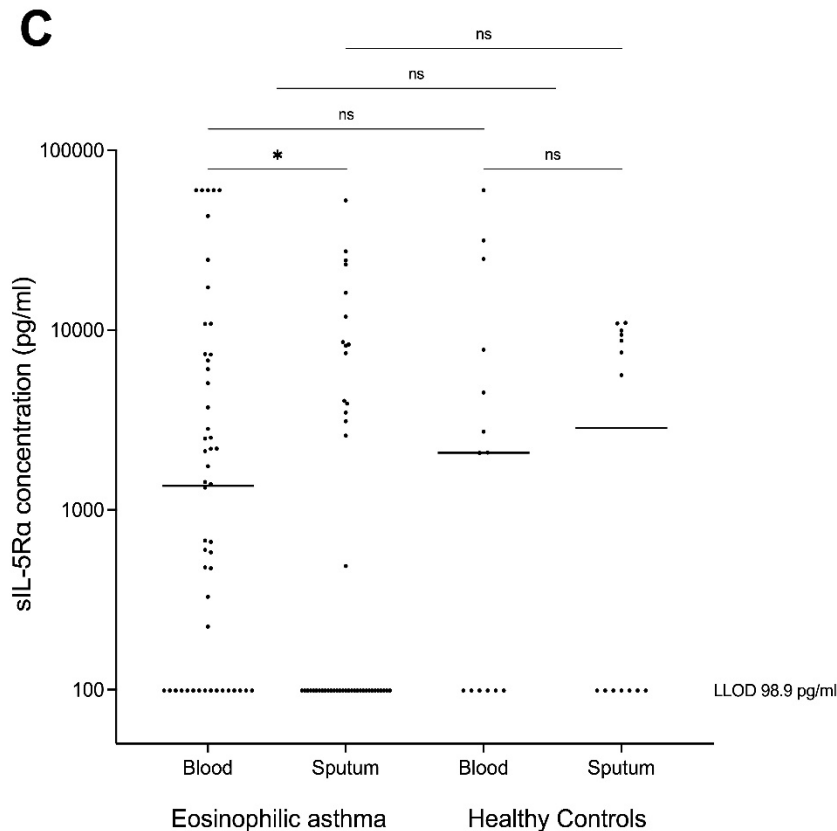


Figure 7-10. Distribution of (A) peripheral blood and sputum eosinophil cell percentages (B) plasma and sputum IL-5 concentrations and (C) plasma and sputum sIL-5Rα concentrations for eosinophilic asthma and healthy controls. Bar denotes the median. Data are paired samples from the same visit. A sub-analysis of measured clinical and laboratory parameters in eosinophilic asthmatics based on detectable sputum sIL-5Rα is presented below.

For the sIL-5Rα assay there was a wide dynamic range of values for eosinophilic asthmatics and healthy controls. Geometric mean values of sIL-5Rα in plasma were similar for eosinophilic asthmatics (1,208 pg/ml, CV% 1,980) and for healthy controls (1,230 pg/ml, CV% 181). This result contrasts with the Gevaert et al.²⁹⁵ study which, found higher levels of sIL-5Rα in the serum of patients with nasal polyposis and (likely eosinophilic) asthma than in the serum of healthy controls. In sputum, the

geometric mean sIL-5R α for eosinophilic asthmatics was 435 pg/ml (CV% 221). By comparison, the results published by Liu. et al.²⁹² showed levels of sIL-5R α in BAL were relatively low for atopic (non-asthmatic) participants at steady state but increased following the allergen challenge. This result might also be considered to contrast with the Gevaert et al. study where 10X the serum level of sIL-5R α was found in the nasal secretions of eosinophilic asthmatics with polyps. Eosinophilic asthmatics presented here were at steady state with relatively high sputum eosinophil counts and high sputum levels of IL-5. The geometric mean sIL-5R α in sputum for healthy controls, who have relatively low levels of sputum IL-5 and low sputum eosinophil counts, was higher than that of eosinophilic asthmatics (934 pg/ml, CV% 105): this difference was not statistically significant. There was no main effect of group (eosinophilic asthma vs healthy controls) in repeated measures ANOVA for sIL-5R α but there was a significant difference for plasma vs sputum concentration of sIL-5R α for eosinophilic asthmatics ($p < 0.02$, Figure 7-10C).

Correlations of blood eosinophil counts, sputum eosinophil percentages, IL-5 and sIL-5R α concentrations in eosinophilic asthmatics are presented in Figure 2.11.

There was a strong correlation between blood eosinophil counts and sputum eosinophil count percentages for eosinophilic asthmatics ($r_s = 0.66$, $p < 0.0001$, $n = 50$), similar to that reported by Shrimanker et al.³¹² from their cohort of 74 eosinophilic asthmatics ($r_s = 0.65$, $p < 0.0001$) and in keeping with other studies.^{19 312}

³¹³ There was also a moderate correlation observed between blood eosinophils and plasma IL-5 levels ($r_s = 0.41$, $p < 0.01$, $n = 50$) again, in keeping with the range of previously reported correlations between blood eosinophils and plasma or serum IL-5.^{172 258 314} A *post hoc* sub-analysis did not show increased strength of this

relationship for the late-onset phenotype of eosinophilic asthma versus the early onset eosinophilic phenotype (Appendix section 11.3.3).

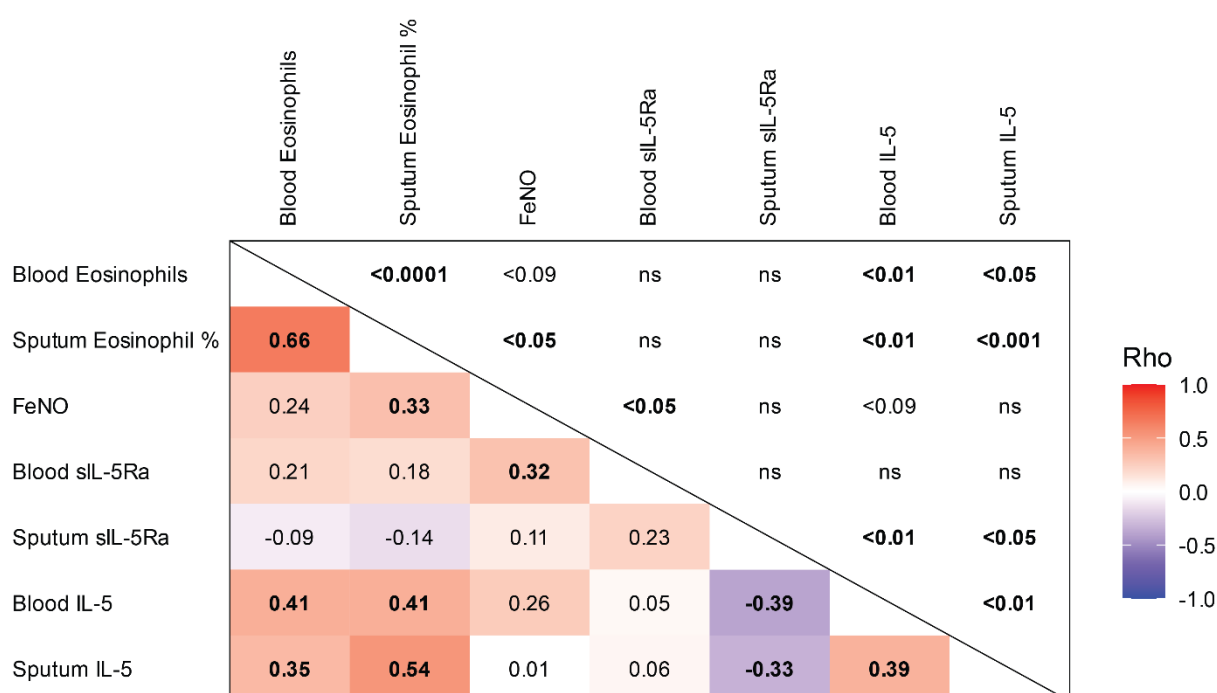


Figure 7-11. Spearman correlation matrix for blood counts; sputum eosinophil percentages; plasma and sputum IL-5 concentrations and plasma and sputum IL-5Rα concentrations for eosinophilic asthmatics. Statistically significant values in bold.

There was a moderate correlation between sputum eosinophils and sputum IL-5 levels ($r_s = 0.54$, $p < 0.001$, $n = 46$), again in keeping with other published data.^{19 208}

315

In contrast to the data published by Wilson et al.²⁹⁶ who examined sIL-5Rα for patients with hyper-eosinophilic diseases, there was no significant relationship observed between sIL-5Rα and blood eosinophils nor between sIL-5Rα and IL-5 for

eosinophilic asthmatics. There were however, weak-moderate inverse relationships between sIL-5R α and blood and sputum IL-5 levels (Figure 7-11).

Post hoc sub analyses were conducted to explore the apparent bimodal distribution of sputum sIL-5R α results for the eosinophilic asthma group. Firstly, the correlation matrix was re-run to include only those with results above the LLOD for sputum sIL-5R α (n = 16). This produced similar results to the overall correlation matrix (Figure 7-11), with the only statistically significant correlations between blood eosinophils and sputum eosinophils ($r_s = 0.76$, $p < 0.001$, $n = 16$); sputum eosinophils and sputum IL-5 ($r_s = 0.50$, $p = 0.05$, $n = 16$); and sputum eosinophils and plasma IL-5 levels. ($r_s = 0.54$, $p = 0.03$, $n = 16$). Secondly, a group-wise comparison of all clinical and laboratory parameters was conducted for those participants with sputum sIL-5R α levels that were undetectable (LLOD) (n=36) versus those with measurable sputum sIL-5R α levels (n=16). This analysis revealed that the group with measurable sputum sIL-5R α levels had higher sIL-5R α levels in plasma (geomean 2290pg/ml v 909 pg/ml; Mann-Whitney U test, $p = 0.02$). Interestingly, the group with measurable sputum sIL-5R α levels had lower sputum IL-5 levels than those with undetectable sputum sIL-5R α with this difference not reaching significance (196 pg/ml v 851 pg/ml, $p = 0.11$). All other clinical and laboratory parameter comparisons in this sub-analysis were non-significant.

For healthy controls there were no significant relationships between blood eosinophils, sputum eosinophils, plasma IL-5, plasma sIL-5R α , sputum IL-5 and sputum sIL-5R α . The relationship between sputum eosinophils and sputum IL-5 was trending towards but not reaching significance ($r_s 0.45$, $p = 0.10$, $n=14$). Many results for healthy controls for the IL-5 and sIL-5R α assays were at the LLOD (Figure 7-10).

7.3.2 Examining mIL-5Rα on paired blood and sputum eosinophils

The finding of significantly reduced levels of sIL-5Rα in the sputum of eosinophilic asthmatics compared to plasma – an effect not seen for healthy controls – appeared initially counter-intuitive when one considers the increased percentage of eosinophils and increased levels of IL-5 (which can induce mIL-5Rα cleavage) in the airways of these patients. It is unclear for example, if the environment within the lumen of the airway of eosinophilic asthmatic patients means that results for sIL-5Rα for these patients should be compared to the BAL sIL-5Rα results for the atopic (non-asthmatic) participants in the Liu. et al.²⁹² study, either before *or* after their antigen challenge.

One hypothesis is that eosinophils that have migrated into the lumen of the airway have reduced expression of mIL-5Rα. To test this flow cytometry was performed on blood and sputum eosinophils obtained at the same timepoint for 7 individuals with eosinophilic asthma. Handling of blood and sputum was adapted to equilibrate post-collection conditions and to minimise the potential for eosinophil activation. Markers were selected for eosinophil identification and sorting (CD45/CD16/Siglec-8). CD62L (L-selectin) was selected as a positive control for the hypothesis. Previous studies have reported reduced expression of CD62L on sputum eosinophils from eosinophilic asthmatics when compared to blood eosinophils³¹⁶ and reduced CD62L expression is considered a marker of eosinophil migration and activation and forms part of the 'Mesnil criteria' characterization of an inflammatory eosinophil subset.³¹⁷ IL-3Rα (CD123) was used as a negative control. It is reported to be up-regulated on sputum eosinophils (also part of the Mesnil criteria) and mIL-3Rα has shared

characteristics with IL-5R α as part of the same IL-5/IL-3/GM-CSF receptor family and as an α chain receptor that requires the same β chain (β c / CD131) (Figure 7-2).

A population of high purity eosinophils based on scatter profile and identification markers were flow sorted from blood and sputum and assessed for CD125 (mIL-5R α) expression: cyto-spins were performed on these populations to verify eosinophil purity (Figure 7-13). The gating strategy is presented in Figure 7-12. mIL-5R α (CD125) expression on blood and sputum eosinophils is presented in Figures 7-14 and 7-15. Purity of eosinophil populations was demonstrably high in both blood (median 100% purity, IQR: 100.0-100.0) and sputum (99.0% purity, IQR: 98.8-99.6).

Sputum eosinophils demonstrated increased autofluorescence, a recognised marker of activation.³¹⁸ Stain index – which, accounts for the increased auto-fluorescence of sputum eosinophils at baseline using the FMO condition and is a more quantitative measure of expression – is calculated using the equation $MFI_{(pos)} - MFI_{(neg)} / 2 * rSD_{(neg)}$. The stain index for mIL-5R α (CD125) was significantly lower in sputum eosinophils compared to blood. eosinophils ($p < 0.000006$, $n=7$). Indeed, there appears to be little staining of CD125 on sputum eosinophils (Figure 2.15). A comparative stain index for neutrophils gated as Granulocytes > Single cells > Live cells > CD45⁺ cells > CD16⁺ showed minimal staining of mIL-5R α (CD125) on neutrophils in blood or sputum (Appendix Figure 11-8). FMOs for all stains are presented in Appendix Figure 11-6.

Gating and FACS strategy for blood sputum eosinophils A-G

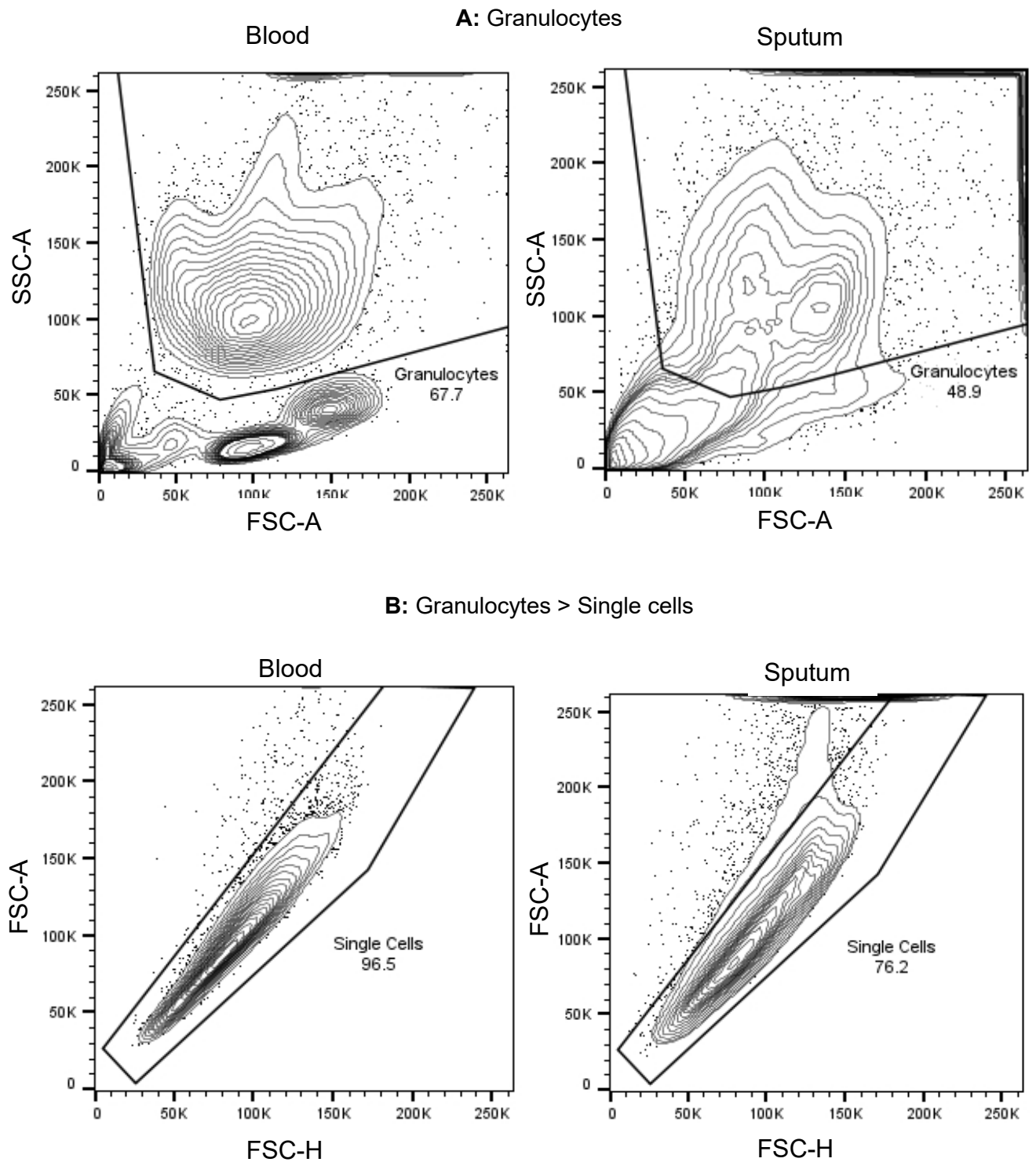
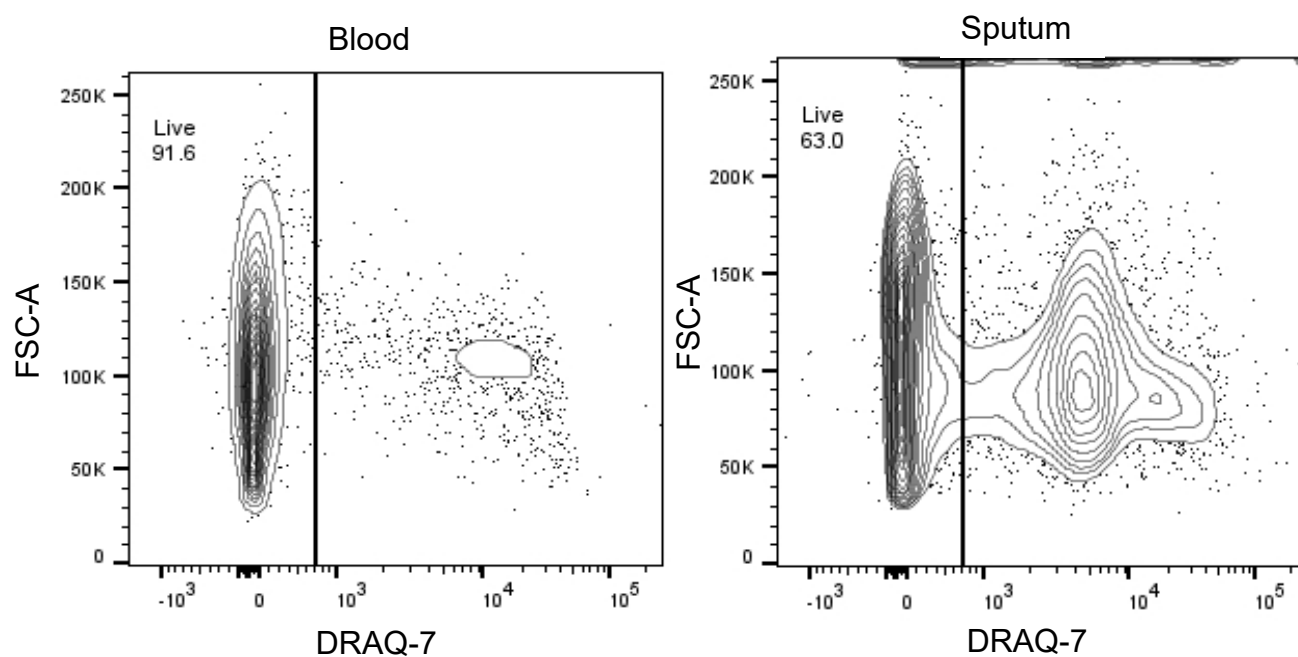


Figure 7-12 (A–B). The same gating and FACS strategy used for blood and sputum eosinophils. Cells were consecutively gated on A: Granulocytes (granulocytes retained); B: Single cells (doublets removed). Pooled samples, $n = 7$.

C: Granulocytes > Single cells > Live cells



D: Granulocytes > Single cells > Live cells > CD45⁺ cells

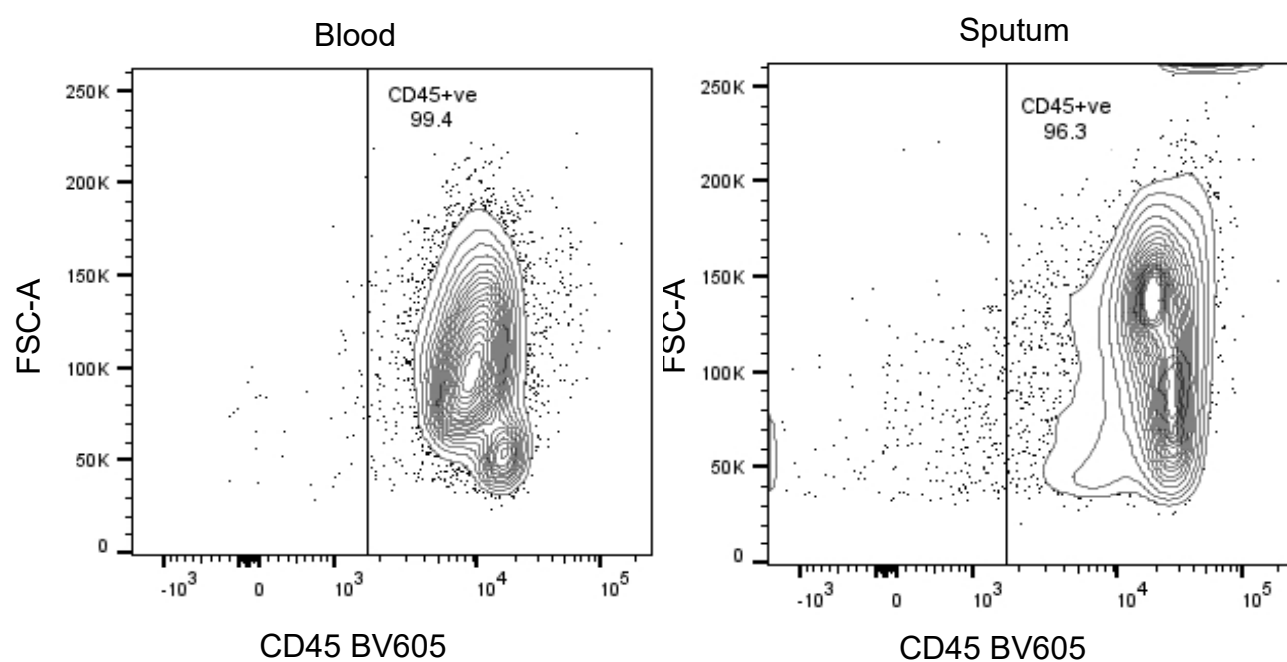
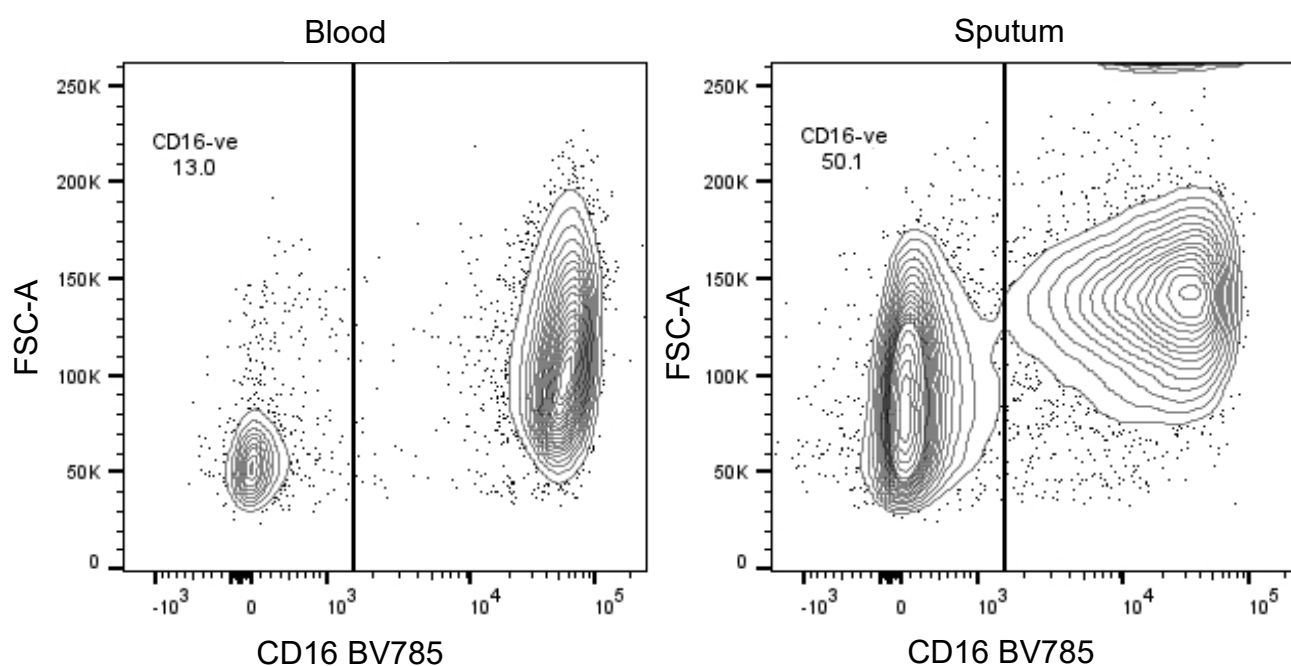


Figure 7-12 continued (C–D). C: Live cells (dead cells removed) D: CD45⁺ cells; (non-haematopoietic cells removed). Pooled samples, n = 7.

E: Granulocytes > Single cells > Live cells > CD45⁺ cells > CD16^{-ve} cells



F: Granulocytes > Single cells > Live cells > CD45⁺ cells > CD16^{-ve} cells > Siglec8⁺ cells

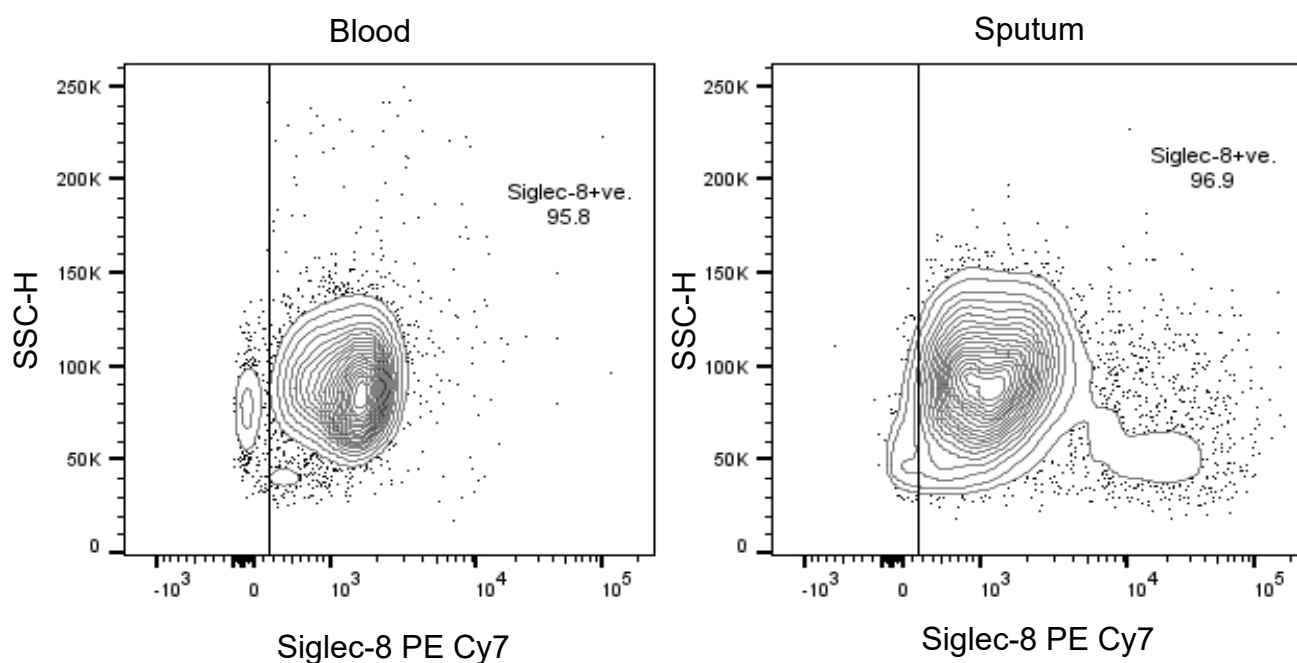


Figure 7-12 continued (E–F). E: CD16^{-ve} cells (neutrophils removed) and F: Siglec-8⁺ cells retained (eosinophil positive marker). Pooled samples, n = 7.

G: Granulocytes > Single cells > Live cells > CD45⁺ cells > CD16⁻ cells > Siglec8⁺ cells
> CD125⁺ (IL5Rα⁺ cells)

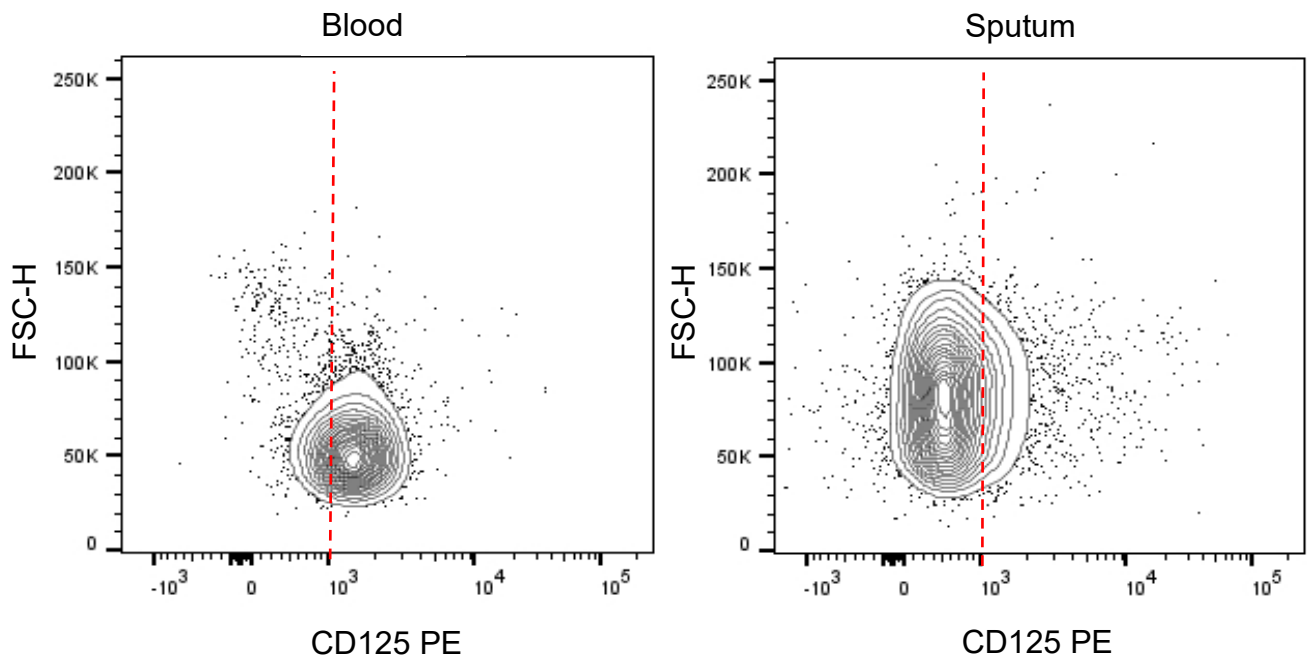


Figure 7-12 continued. Gating A-F resulted in retention of a purified population of Live, CD45⁺, CD16⁻, Siglec-8⁺ eosinophils. G. Expression of CD125 (IL-5Rα) on these eosinophil populations is demonstrated. A dotted red line is drawn at the same fluorescence a.u. to demonstrate the population shift. Pooled samples, n = 7.

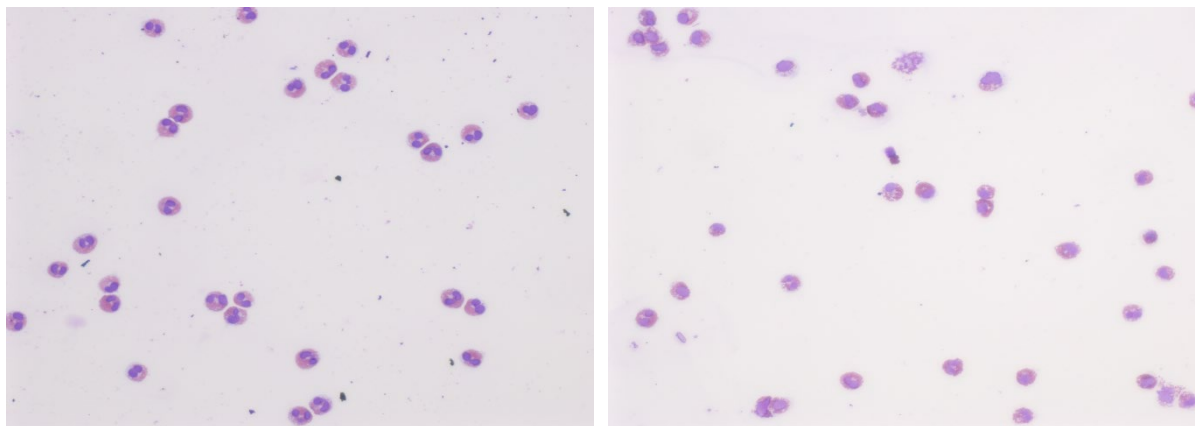


Figure 7-13. Example cyto-spins of eosinophils sorted using the above gates. Sorted cells from one participant (OAS#1546) from blood (left) and sputum (right). Median eosinophil purity of the sorted cells was very high for blood and sputum across the 7 participants:

median eosinophil purity in blood 100% (IQR: 100.0-100.0%) and sputum 99.0% (98.8-99.6%).

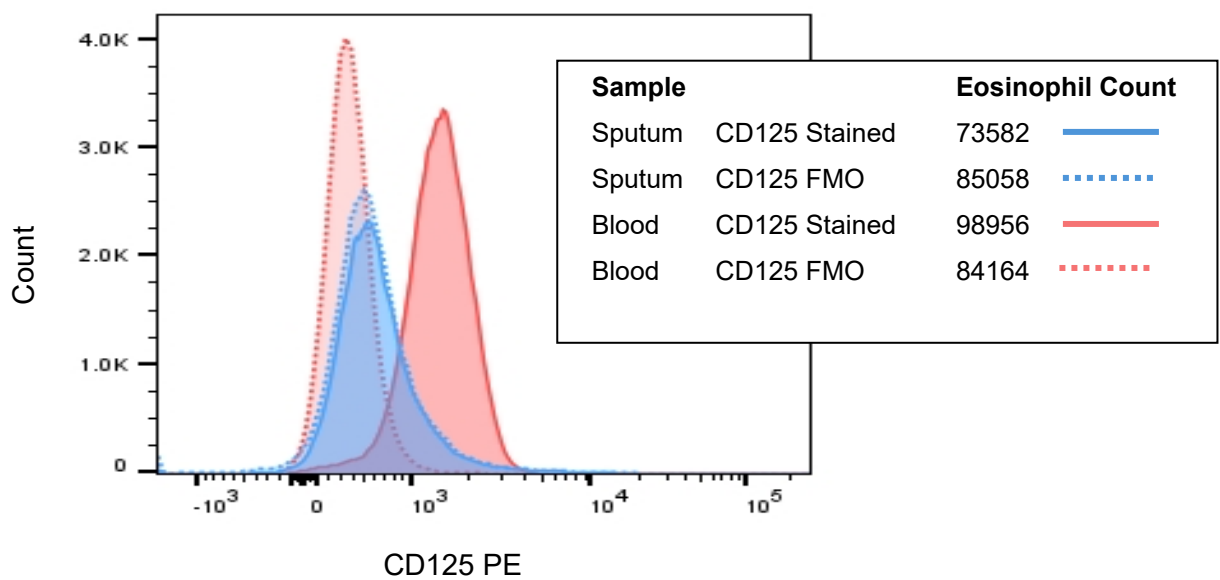


Figure 7-14. Histogram showing fluorescence of blood and sputum eosinophils and their respective fluorescence minus one condition (FMOs). This demonstrates the increased autofluorescence of sputum eosinophils compared to blood eosinophils (difference between blood and sputum FMOs). This is accounted for through the Stain Index. Histograms for FMOs for all markers are shown in Appendix Figure 11-6.

It remains possible that handling of sputum which, includes vortexing and filtering of sputum plugs through a gauze, could have caused loss of mIL-5R α expression on eosinophils. The reducing agent dithiothreitol (DTT) has also been demonstrated to alter the expression of cell surface proteins on leucocytes, such as CD11b, when treated *in vitro*.³¹⁹ The effect of adding 0.1% DTT during sputum processing could potentially disrupt disulphide bridges between receptor subunits. To assess this possibility further three additional conditions were created for blood leucocyte samples: these steps were intended to subject blood eosinophils to the same handling and treatments as sputum leucocytes. In one of three additional control

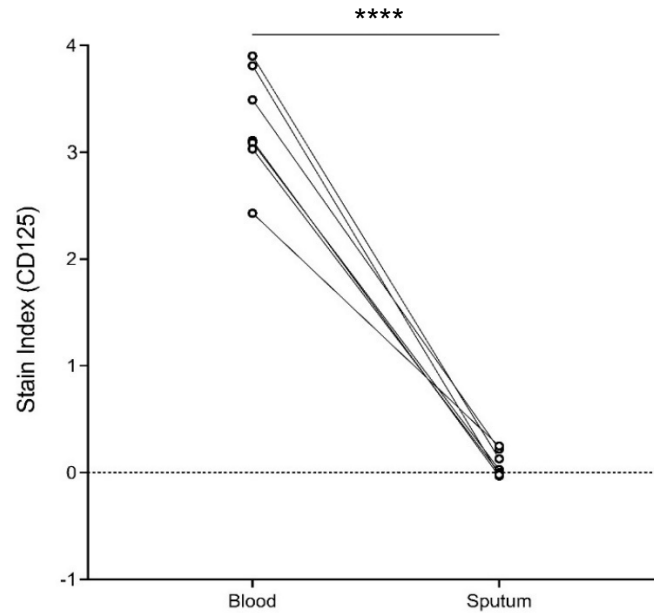


Figure 7-15. Stain index for IL-5R α (CD125) on paired blood and sputum eosinophils from individual participants with eosinophilic asthma. Stain index accounts for the increased auto-fluorescence of sputum eosinophils at baseline. It is calculated using the equation $MFI_{(pos)} - MFI_{(neg)} / 2 * rSD_{(neg)}$. **** = $p < 0.0001$

conditions 2×10^6 leucocytes were suspended in dPBS on ice for 30 minutes¹² while in the other two control conditions 2×10^6 leucocytes were suspended in dPBS + 0.1% DTT or vehicle. Both the DTT and vehicle conditions were vortexed vigorously at 5-minute intervals for 15 minutes and then remained on ice for a further 15 minutes. These conditions then proceeding to Fc receptor blocking with the other experimental conditions. There was no evidence of an effect of these additional

¹² This point in the protocol is denoted in the Methods section by a footnote

steps on expression of CD125 or on the expression of the positive (CD62L) or negative (CD123) control markers (Figure 7-16).

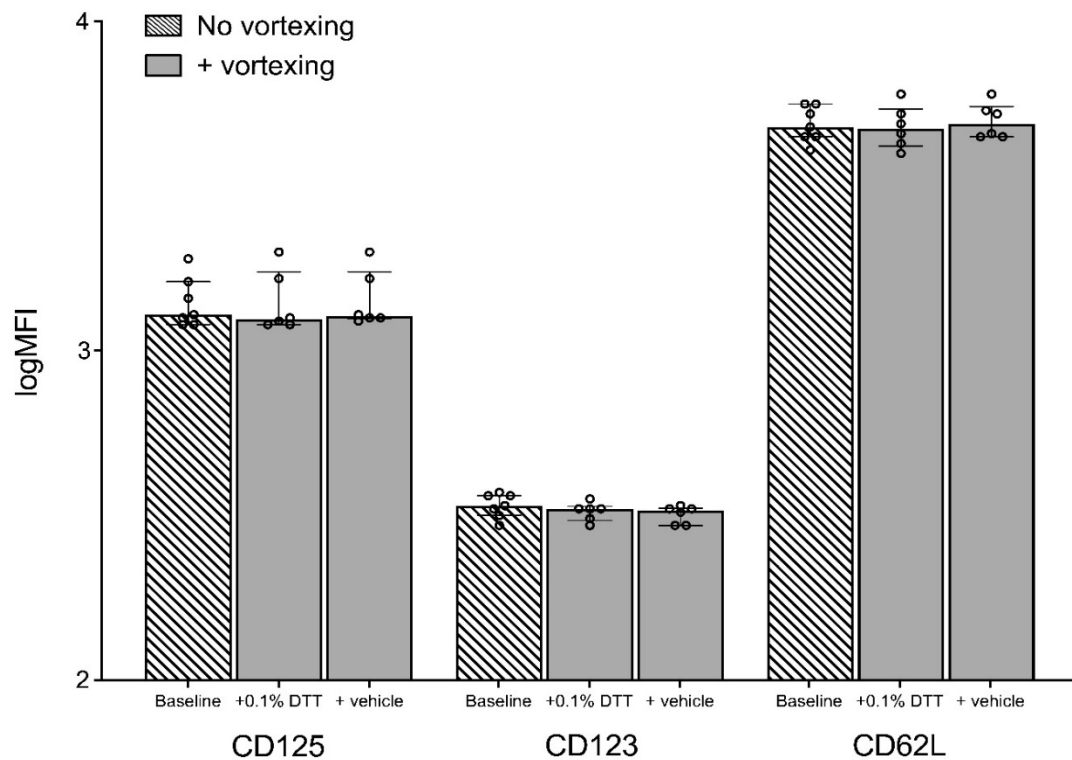


Figure 7-16. Control experiment to consider the effects of sputum handling on receptor expression by treating blood leucocyte samples per sputum samples. All samples were maintained in dPBS on ice for 30 minutes. In one condition 0.1% DTT was added to dPBS and vehicle was added to another. These two conditions underwent vigorous vortexing. No effect of vortexing +/- 0.1% DTT was seen. Paired samples presented.

Examination of receptor expression on blood and sputum eosinophils is presented in Figure 7-17. In addition to the reduced expression of mIL-5R α (CD125) on sputum eosinophils compared to blood there was also reduced expression of CD62L on

sputum eosinophils compared to blood (positive control) and increased expression of CD123 on sputum eosinophils compared to blood (negative control), as anticipated.

There were strong correlations of eosinophil expression of CD125 with CD123 and CD62L (Figure 7-18). Reduced expression of CD62L on eosinophils and increased expression of CD62L are both recognised markers of eosinophil activation: the negative Pearson's correlation between expression of these two markers is therefore anticipated ($r = -0.79$, $R^2 = 0.63$ $p < 0.001$, $n = 7$). The strongly negative correlation between CD123 loss of expression and CD125 increased expression ($r = -0.78$, $R^2 = 0.61$, $p < 0.001$) and the strongly positive correlation between CD62L and CD125 loss of expression ($r = 0.89$, $R^2 = 0.80$, $p < 0.0001$) suggest that CD125 loss of expression is correlated with eosinophil activation.

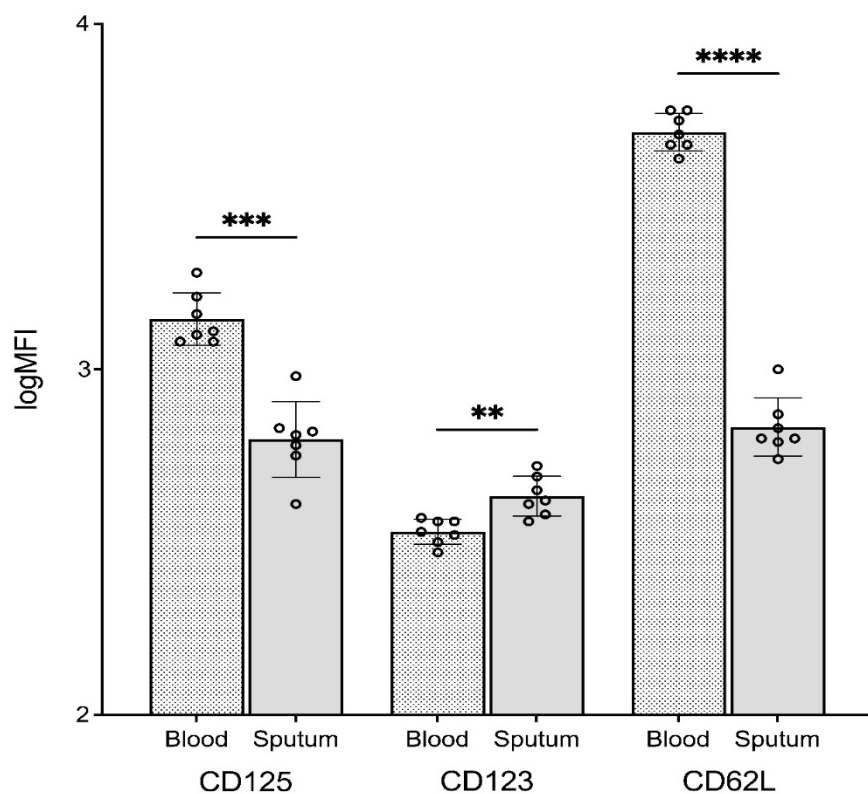


Figure 7-17. Expression of CD125, CD123 and CD62L on blood and sputum eosinophils. The increase in CD123 expression on sputum eosinophils (negative control) and decrease of CD62L expression on sputum eosinophils (positive control) were anticipated results. Paired data presented. ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

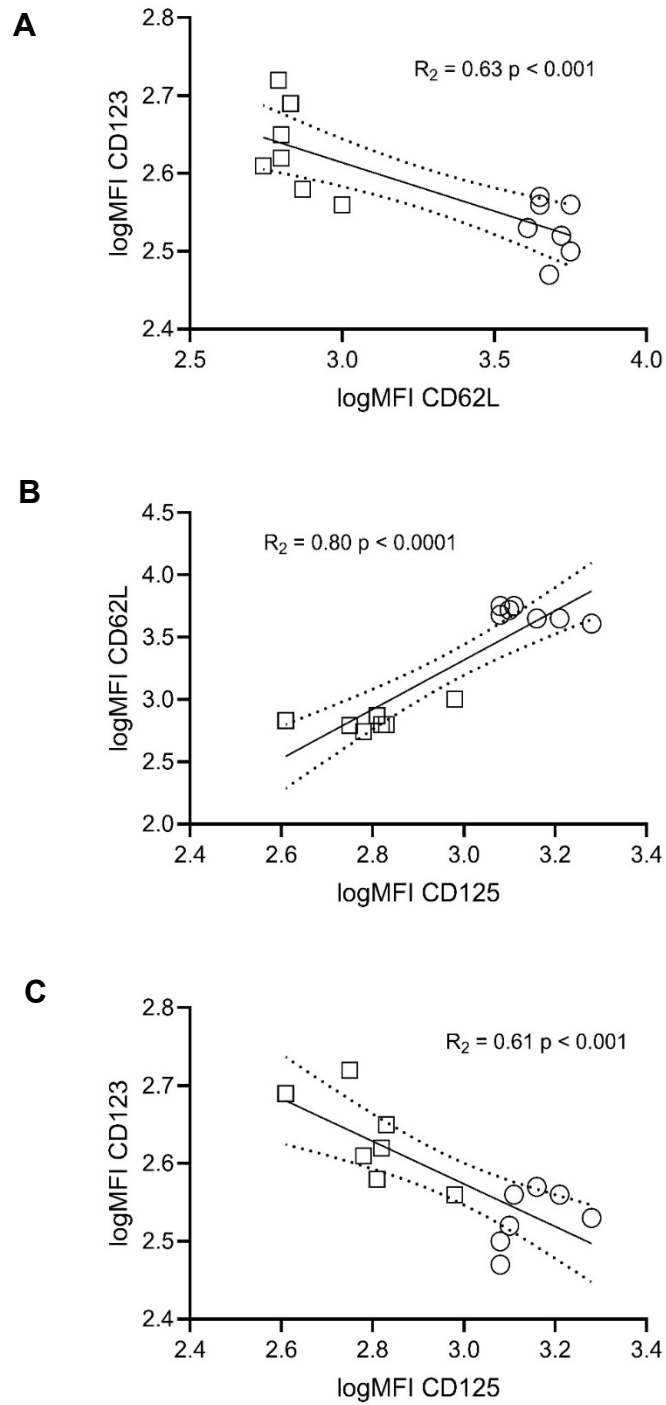


Figure 7-18. Correlations between the logMFI expression of CD125, CD123 and CD62L on eosinophils in blood and sputum. From top to bottom (A) CD62L and CD123 (B) CD125 and CD62L and (C) CD125 and CD123. $\square$ = sputum $\circ$ = blood.

7.4 Discussion

The main findings from this study of IL-5R α in eosinophilic asthmatics with high levels of eosinophilic inflammation at steady state are as follows:

- (i) There is no relationship between plasma levels of sIL-5R α and plasma levels of IL-5 or eosinophil counts. This directly contrasts with the findings of Wilson et al.²⁹⁶ in hyper-eosinophilic patients at steady state.
- (ii) Plasma levels of sIL-5R α are not higher in eosinophilic asthma than in healthy controls. This contrasts with the reported findings of Gevaert et al.²⁹⁵ in a group of nasal polyposis patients who also had eosinophilic asthma.
- (iii) Sputum levels of sIL-5R α are significantly lower than plasma levels for eosinophilic asthmatics. This contrasts with the finding of higher levels of sIL-5R α in the nasal secretions of polyp patients by Gevaert et al. and contrasts with the bronchoalveolar lavage of atopic participants in the Liu et al.²⁹² study *after* allergen challenge. For those participants, IL-5 and eosinophil levels were very high post challenge and correlated *positively* with the increased sIL-5R α in BAL. Here, there was a *negative* relationship between sputum sIL-5R α (low) and sputum IL-5 (relatively high) and no relationship between sputum sIL-5R α and sputum eosinophils. This finding

aligns more closely with the BAL of the Liu. et al. atopics *before* antigen challenge, when their eosinophil counts and IL-5 levels were low.

- (iv) Eosinophil expression of mIL-5R α is higher in blood than in sputum. Indeed, the stain index calculation which accounts for the increased auto-fluorescence of eosinophils in sputum, suggests little or no staining of mIL-5R α on sputum eosinophils. This is consistent with reduced mIL-5R α expression on BAL eosinophils in the Liu. et al. cohort *after* allergen challenge.
- (v) Loss of eosinophil mIL-5R α expression is strongly correlated with expression of other markers of eosinophil activation such as auto-fluorescence and CD62L ($r = 0.89$, $R^2 = 0.80$, $p < 0.0001$) and CD123 ($r = -0.78$, $R^2 = 0.61$, $p < 0.001$).

This study set out to consider the IL-5/sIL-5R α axis in eosinophilic asthma by examining the relationship between these two mediators at steady state and by comparison to the levels of these mediators and their relationship in healthy controls. Despite published and unpublished *in vitro* evidence of the inhibitory effects of sIL-5R α on IL-5-induced cleavage of IL-5R α from the eosinophil cell surface¹³, there was

¹³ Jian Luo has performed a series of *in vitro* experiments to confirm that sIL-5R α is generated from eosinophils via cleavage of mIL-5R α in response to ligand-receptor binding from IL-5 and other mediators and sIL-5R α is *not* produced in these conditions via alternate splicing. Luo*, Melhorn* et al., *in submission*

no evidence of a positive relationship between sIL-5R α and IL-5 levels for eosinophilic asthmatics in plasma. There was a moderate, but statistically significant, *negative* correlation between these markers in sputum. There was no difference between the level of sIL-5R α in plasma with that in healthy controls and there was a non-significant trend to lower sIL-5R α in sputum than healthy controls.

Healthy controls did not demonstrate any significant relationships between eosinophils/IL-5/sIL-5R α to suggest that this axis might be dysregulated in eosinophilic asthma and there was no clear evidence of binding and interference in the assays of IL-5 and sIL-5R α used that might have obscured this relationship.

By contrast, the main findings of this study are, 1. loss of eosinophil mIL-5R α expression is strongly associated with eosinophil activation and, 2. in eosinophilic asthma at steady state eosinophils in the lumen of the airway have reduced mIL-5R α expression, with implications for eosinophil stimulation, and potentially, asthma control.

7.4.1 Loss of mIL-5R α expression is associated with eosinophil activation

The migration of blood eosinophils into inflammatory tissue in eosinophilic asthma and encounters with cytokines and other factors (e.g., allergens) is associated with eosinophil activation. Airway (sputum) eosinophils exhibit a more activated phenotype than circulating eosinophils, characterized by reduced CD62L expression, increased CD123 expression, along with other activation markers, and enhanced degranulation.³²⁰ It has been proposed that this is why sputum eosinophils correlate

better with symptoms, airway remodelling and exacerbation risk than blood eosinophils.^{313 321 322} The loss of mL-5R α from sputum eosinophils and its association with the activation of eosinophils in eosinophilic asthmatics at steady state suggests this loss may be secondary to eosinophil diapedesis from the circulation and migration through the tissue into the lumen of the airway.

The cleavage of mL-5R α to sL-5R α by matrix metalloproteinases in eosinophilic asthma likely involves other mediators beyond IL-5 which, will confound simple correlations between IL-5 and sL-5R α in plasma, for example. Jian Luo has demonstrated that stimulation of eosinophils with eotaxin-3 and PGD₂ – markers which are both elevated in eosinophilic asthma – can result in mL-5R α cleavage which can be blocked by matrix metalloproteinase inhibitors (see unpublished data from Jian Luo in Figure 7-19 and eotaxin-3 and PGD₂ level data from section 6.3.3).

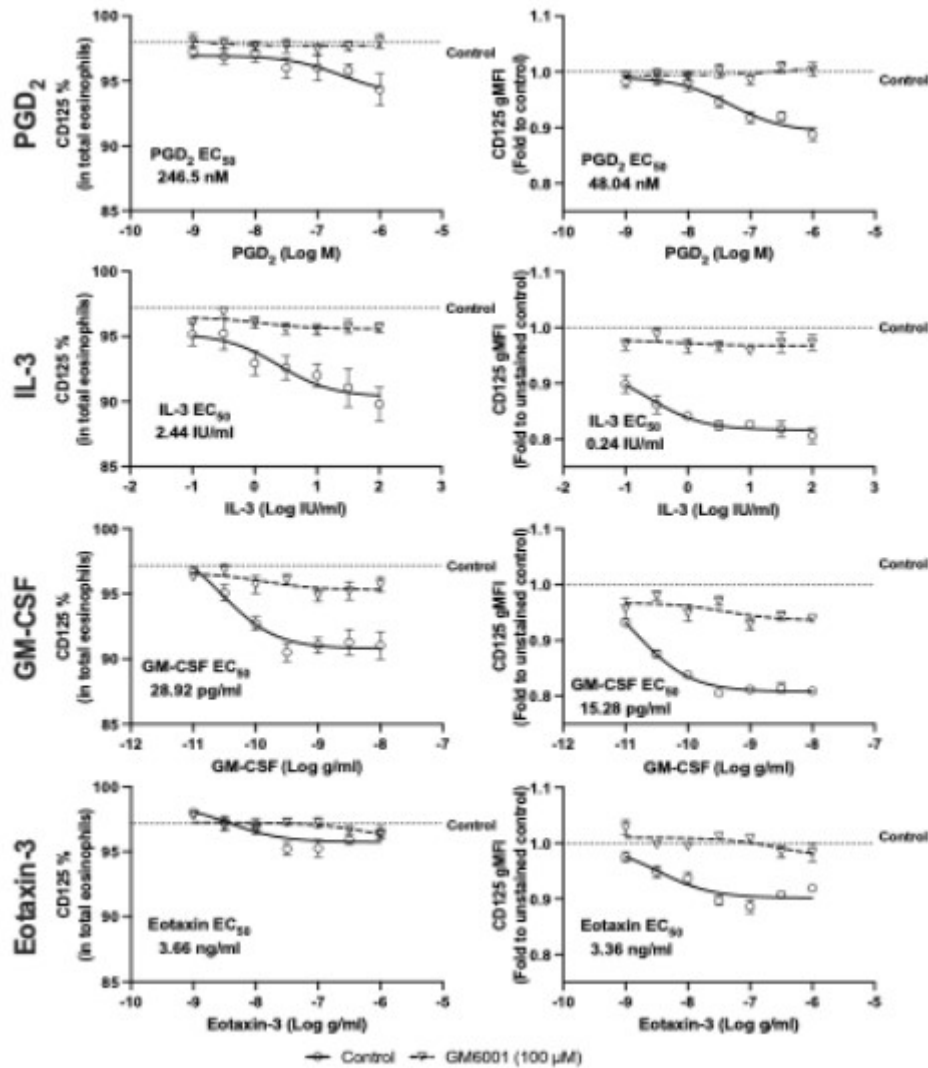


Figure 7-19. Unpublished work by Jian Luo (with permission). The combined matrix metalloproteinase inhibitor GM6001 inhibits dose dependent loss of mIL-5R α (CD125) from blood eosinophils following stimulation with PGD₂, IL-3, GM-CSF and Eotaxin-3.

It is suggested that the effects of other cytokines such as eotaxin-3 and PG2 are one of the reasons why the IL-5/IL-5R α axis as appears for hyper-eosinophilic patients in the Wilson et.al.²⁹⁶ study (e.g., with correlation between serum sIL-5R α and IL-5, $r = 0.40$, $p < 0.001$) was not replicated here for eosinophilic asthmatics. A perhaps greater factor is that systemic levels of IL-5 can be significantly higher in HES³²³⁻³²⁵

than in eosinophilic asthma, where IL-5 concentration is higher in the lung.^{167 172}

Systemic levels of IL-5 in the peripheral blood of eosinophilic asthmatics may not be sufficient to induce mIL-5R α cleavage and the IL-5/IL-5R α axis may only be relevant to the tissue and airway lumen in eosinophilic asthma where IL-5 levels are significantly higher (Figure 7-10). This explanation would also account for why relatively high levels of IL-5 are required to induce cleavage of mIL-5R α *in vitro*.²⁸⁴

7.4.2 Cleavage of mIL-5R α to sIL-5R α in eosinophilic asthma is hypothesized to occur in the tissue

Given the relative paucity of sIL-5R α found in sputum it seems likely that cleavage of mIL-5R α from eosinophils in eosinophilic asthma and generation of sIL-5R α is likely to be happening in the tissue. Encountered levels of IL-5 (and other mediators such as eotaxin-3) which stimulate cleavage are significantly higher in the tissue in eosinophilic asthma than in the systemic circulation.

Support from this proposition comes from the Gevaert et al.²⁹⁵ study of sIL-5R α protein levels in tissue homogenates from the nasal tissue of patients with polyps and eosinophilic asthma. Levels of sIL-5R α were significantly higher in the nasal tissue (median 26,096 pg/ml IQR: 17,640–34,287 pg/ml) than in the serum (759 pg/ml IQR: 475–1,030 pg/ml) or in the nasal secretion fluid (7,568 pg/ml IQR: 3,389–12,869) of these patients. The bronchial biopsy study of asthmatics by Yasriel. et al.²⁹⁴ also found significantly higher levels of sIL-5R α mRNA in the tissue of

asthmatics than controls. A graphical form of this hypothesis is presented in Figure 7.20.

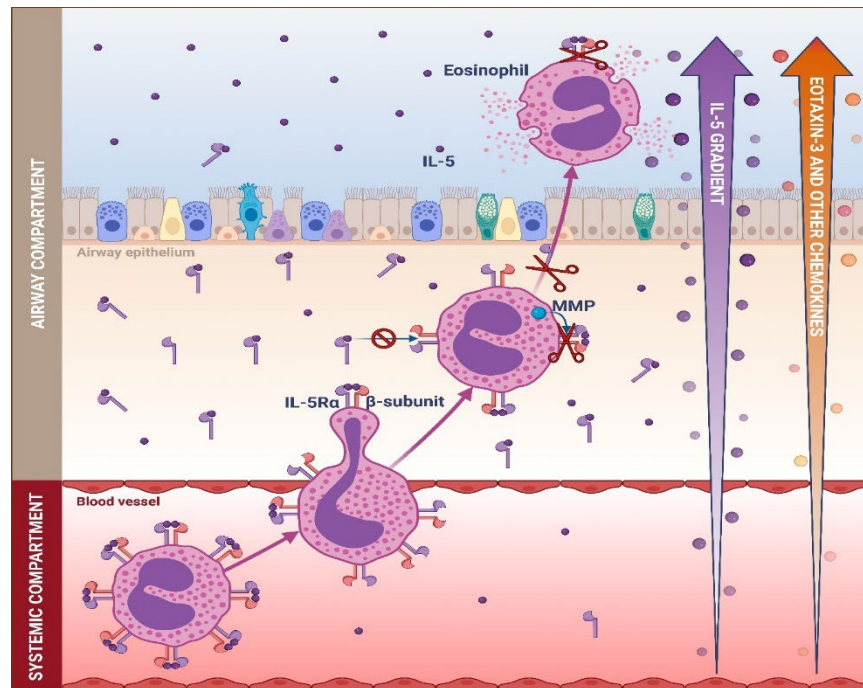


Figure 7-20. Graphical hypothesis based on data produced in this study and in vitro work by Jian Luo. In vivo cleavage of mIL-5Rα from the cell surface of eosinophils to form sIL-5Rα occurs predominantly in the tissue as eosinophils migrate from the blood to the lumen of the airway. Stimuli to mIL-5Rα cleavage include an increasing gradient of IL-5, eotaxin-3 and other mediators as the cell chemotaxes to the airway. Cleavage is mediated by matrix metalloproteinases. Image kindly generated by Corrado Pelaia in BioRender.

In their *in vitro* manipulation on BAL eosinophils Liu. et al.²⁸⁸ found that those eosinophils with reduced or absent mIL-5Rα had a significantly attenuated response to IL-5, but not to GM-CSF which stimulates eosinophils via the GM-CSF receptor (GM-CSFR): the GM-CSF receptor has a shared β common (βc) chain with IL-5

demonstrating the specificity of the ligand-receptor effect. The implications of this finding fits with the hypothesis proposed here: mIL-5R α cleavage from eosinophils in eosinophilic asthma at steady state occurs before eosinophils enter the airway and move beyond regulation by tissue-resident survival or retention signals. This might be particularly important in homeostasis for luminal eosinophils due to (i) their ability to be locally destructive (e.g., via EETosis¹⁴)³²⁶ (ii) the higher orders of IL-5 and other mediators present in the lumen of the airway with potential for eosinophil activation.

It would be desirable to test this as a hypothesis of a homeostatic mechanism by examining the blood and sputum eosinophils of healthy people. This was not done in the study here due to difficulty in obtaining adequate quantities of sputum to be able to flow-sort eosinophils efficiently. Given the efficient gating of pure populations of eosinophils performed here however, it would be reasonable to use the same gating strategy for flow cytometry assessment and forgo verification by cell sorting. It would also be desirable to perform a within-subjects analysis of eosinophilic asthmatics at the time of exacerbation, particularly to compare sputum eosinophil mIL-5R α expression and sIL-5R α levels at steady state and exacerbation. The Liu et al.²⁹² study found increased sIL-5R α levels in the lumen following antigen challenge and Rohde et al.³²⁷ found higher levels of sIL-5R α in the induced sputum of COPD patients experiencing acute viral induced exacerbation. A simplistic attempt to unify these findings with the data – and ignoring effects from differences in concentration gradients of cytokines in different compartments – would be to suggest that because

¹⁴ EETosis is a specialised form of eosinophil lytic cell death whereby eosinophils release web like structures called extra-cellular traps. Excessive EETosis contributes to tissue damage in asthma.

eosinophil transit time (and mIL-5R α cleavage time) from blood to airway is shorter during exacerbations eosinophils may arrive in the lumen of the airway with relatively higher expression of mIL-5R α . Such eosinophils would be exposed to very high levels of IL-5 which would (i) cleave eosinophil mIL-5R α to sIL-5R α in the lumen (with higher measured sIL-5R α in the lumen) and (ii) result in eosinophil hyper-activation secondary to high levels of IL-5 and other cytokines.²⁹² The shorter transit kinetics of eosinophils (as opposed to influx of numbers) has been suggested by existing allergen challenge studies but has not yet been demonstrably proven, however.^{310 328 329}

Should levels of sIL-5R α be increased in the airway at times of exacerbation, but more specifically, if increased levels of sIL-5R α should herald symptomatic exacerbation, then sIL-5R α may yet have a role as a biomarker in eosinophilic asthma. There are precedents for solubilised receptors as biomarkers in other diseases. Soluble CD163 (sCD163) for example, is a protein fragment generated by ectodomain shedding of the membrane bound receptor from the surface of monocytes and macrophages.³³⁰ sCD163 shedding occurs as a result of enzymatic cleavage by ADAM17 and sCD163 is upregulated in a large range of inflammatory diseases including macrophage activation syndrome, and rheumatoid arthritis.³³¹ The excretion of soluble CD163 into the urine is tightly associated with the presence of active glomerulonephritis in systemic lupus erythematosus and ANCA vasculitis and can be used to track response to therapy.^{332 333}

7.4.3 Other considerations and future directions

Aside the problem of not having a healthy control comparator group for the flow cytometry experiment further consideration might be given to other overlooked problems. Firstly, the assumption was made here, based on the inhibitory effects of recombinant human sIL-5R α on IL-5 in experiments *in vitro*, that endogenous sIL-5R α has similar effects *in vivo*. There are many examples of solubilised receptors that have pluripotent functions beyond acting as a decoy for their cytokine target. These include sIL-4R which can bind and stabilize IL-4, which is otherwise usually rapidly degraded, and release bioactive IL-4 several days after the initial complex formation *in vitro*.²⁸⁹ Secondly, no thought has been given thus far to other cellular actors and potential sources of sIL-5R α . These include ciliated epithelial cells which, single cell data from the Human Lung Cell Atlas suggest, highly express *IL5RA*.¹⁶³ Which surface(s) of these epithelial cells express mIL-5R α , whether it is cleaved to sIL-5R α as for eosinophils, and whether this can also have inhibitory effects on eosinophils have not yet been considered. Of note, it has recently been described that benralizumab can also target and deplete IL-5R α bearing cells in skin lesions.³³⁴ If the effects of sIL-5R α on IL-5 represent a homeostatic feedback mechanism that is relevant at higher concentrations of IL-5, then the effect of introducing benralizumab on that mechanism is not yet fully understood. The neutralization of eosinophils via mIL-5R α ³³⁵ might also neutralise the main source of sIL-5R α . Higher levels of IL-5 in patients taking benralizumab might represent a risk for rebound eosinophilia when patients stop treatment. Benrtalizumab therapy is also an opportunity to further probe the IL-5/IL-5R α axis – perhaps by testing the bioactive properties of the serum of patients established on benralizumab in culture with eosinophils +/- rsIL-5R α . Similar

co-culture experiments have been performed using serum from patients taking Reslizumab.³³⁶

The process of cleavage of mIL-5R α to form sIL-5R α and the inhibitory effects of sIL-5R α on IL-5 induced eosinophil activation may yet offer important therapeutic targets for eosinophilic asthma. Administration of sIL-5R α intravenously to mice in an OVA-sensitised model of asthma significantly alleviated the increase of eosinophils in bronchoalveolar lavage fluid that followed challenge with ovalbumin but had little effect on airway hyperreactivity once again demonstrating the dissociation between IL-5 and features of airway hyper-responsiveness.

Abnormalities of mIL-5R α cleavage have implications for asthma control and asthma pathogenesis. Eosinophils arriving in the airway with higher mIL-5R α expression may be more exposed to the effects of the higher airway concentrations of IL-5 in eosinophilic asthma. Mutations in genes regulating the cleavage process may have an impact on this process. Single nucleotide polymorphisms (SNP) in the *IL5RA* gene have been linked to increased eosinophil levels and increased tissue penetration by eosinophils in chronic eosinophilic leukaemia.³³⁷ Mutations in *IL5RA* and mutation in sheddase proteinases responsible for receptor cleavage such as matrix metalloproteinases (MMPs) are considered within a genetic analysis in the next chapter. The elevated IL-5 seen in patients taking benralizumab treatment will be investigated further in co-culture with eosinophils using eosinophil apoptosis assays.

8 . Searching for rare, high-Impact germline mutations and somatic mutations in late-onset eosinophilic asthma

In this chapter I look for rare, high-impact genetic mutations in genes that could influence basal signalling of IL-5 and T2 inflammation (e.g., *IL5RA*). I do this by deep sequencing a cohort of highly phenotyped late onset eosinophilic asthma patients using a targeted gene panel. This data is then aligned with a large publicly available dataset of sequenced healthy controls (gnomAD) to assess whether LOE asthma patients have a higher burden of rare variants in each gene relative to controls. This approach is intended to highlight important mechanistic pathways: it has not been used in asthma previously. I also consider whether somatic mutations in these genes, or in genes recognised to drive the development of haematopoietic clonal cell populations, could have a role in LOE asthma. I examine this with reference to a small pool of healthy controls I sequenced alongside the late onset eosinophilic asthma cohort. I also sequenced a very small group of younger healthy controls to help distinguish small clones from sequencing artefact. There have been no studies of clonal haematopoiesis in asthma to date.

8.1 Introduction

8.1.1 The 'missing heritability problem' in asthma

It is broadly accepted that asthma has a multi-factorial aetiology. The contribution of environmental factors to the pathogenesis of asthma is supported, for example, by the significantly higher prevalence of asthma among children in the United States of America (USA) from the Hutterite group of Anabaptist founder populations compared with their Amish counterparts.³³⁸ Although the Hutterite and Amish populations share a common genetic ancestry the Amish have preserved traditional farming practices that are thought to engage and shape the innate immune response resulting in a lower prevalence of asthma, compared with the modern mechanised and sociologically compartmentalised practices of the Hutterite farmers.³³⁹

Despite the strong influence of environmental factors there is substantial evidence supporting a heritable component to asthma. A family history of asthma is the strongest risk factor for the development of childhood onset disease.³⁴⁰ Monozygotic and Dizygotic twin asthma studies in Australia, Denmark and the Netherlands have demonstrated consistently high heritability of asthma in adults, ranging from 55 to 74%, with even higher estimates reported in young children.³⁴¹⁻³⁴⁴

Given this high heritability, there has been longstanding interest in identifying specific genetic risk factors for asthma. Since the first genome-wide association study (GWAS) of asthma was published by Moffatt et al. in 2007 more than 30 other GWAS of asthma have been conducted, identifying over 150 independent loci and

2,000 single nucleotide polymorphisms (SNP) associated with asthma.^{5 22 345-350}

However, despite the large scale of some of these studies – such as the analysis by Johansson, Rask-Andersen et al. (2019), which examined approximately 350,000 individuals of European ancestry from the UK Biobank³⁴⁸ – the reported odds ratios for associated variants are rarely greater than 1.20. Collectively, replicable SNP associations across GWAS studies are estimated to account for only 2.5–14% of asthma heritability.³⁵¹ This discrepancy between the predicted genetic contribution to asthma and the proportion explained by currently identified susceptibility loci has been termed the “missing heritability problem”.⁵

Several explanations have been proposed for this missing heritability. First, large-scale asthma GWAS have generally failed to account for the distinct endotypes and phenotypes of asthma and for its potentially heterogeneous underlying genetic architecture – in effect, these studies are insufficiently phenotyped.^{352 353} Some asthma GWAS have stratified by age of onset, distinguishing between childhood- and adult-onset disease.^{22 354-358} An example is the association between genetic variation in the 17q12-21 locus and childhood-onset asthma but not for adult-onset asthma, reported by the GABRIEL consortium.³⁵⁹ However, the depth of phenotyping in such studies remains limited. Asthma diagnoses are typically self-reported or based on primary care records without objective diagnostic data and T2 biomarkers are rarely reported.

Notable exceptions include the GWAS of Clay et al.³⁵⁶ which provided clinical data for 21,556 adult-onset cases (onset age 26-65 years), including mean FEV1% predicted (91.8) and mean eosinophil count ($0.22 \times 10^9/L$). However, reliance on self-reported physician diagnosed asthma and the preserved lung function and

modest eosinophil counts raise concerns regarding phenotype specificity. These values are not typical of late onset eosinophilic asthma as seen in secondary care settings^{35 36 41} – and likely reflect a heterogenous mixture of asthma phenotypes and potentially other respiratory conditions.

The GABRIEL consortium GWAS³⁵⁴ included a relatively small proportion of severe asthma patients attending UK secondary care clinics. Interestingly, this study identified polymorphisms located in or near genes implicated in T2 inflammatory pathways such as *IL1RL1*. More recently, GWAS investigating the shared genetic architecture of asthma and blood eosinophilia have identified variants associated with both traits, including in the *IL4R* and *TSLP* genes, highlighting genetically driven pathways relevant to eosinophilic asthma such as *STAT6* signalling.³⁶⁰⁻³⁶³ Nevertheless, these variants collectively explain less than 10% of asthma heritability. To date, no GWAS have been published that focus on carefully phenotyped, aggregated cohorts of patients with late-onset eosinophilic asthma.

8.1.2 Rare variants as a contributor to the missing heritability of asthma

A second, and potentially more profound reason for the ‘missing heritability’ observed in asthma GWAS is that a large proportion of identified SNPs are *common* non-coding variants that map intergenic or intronic regions and lack clear biological evidence of functional relevance.³⁶⁴ It is therefore possible that a substantial proportion of the genetic variance underlying a complex disease like asthma is attributable instead to relatively highly penetrant *rare* variants. The most deleterious alleles are typically located within protein-coding regions of the genome. Because

disease is deleterious to fitness, variants that increase disease risk are subject to negative selection; consequently disease-promoting alleles usually have frequencies below 1% (and rarer in the most damaging variants) and are often recently derived in the human population as a result of evolutionary selection.³⁶⁵ It has also been argued that relaxed selection pressures in modern human populations may permit the accumulation of deleterious rare alleles over hundreds of generations.

Analysis of HapMap and UK Biobank data illustrates that rare variants are more likely to be disease-predisposing than common variants. This is also true in relatively common and in late onset disease, where the effects of purifying selection are less pronounced.^{366 367}

An expanding body of empirical evidence supports the utility of rare variant analysis in identifying key disease-associated genes and biological pathways. Notable successes include: (i) the identification of aggregated rare loss-of function variants in *APOC3* associated with lower plasma triglyceride levels and reduced coronary artery disease risk;³⁶⁸ (ii) rare loss-of-function variants in the *SORL1* that substantially increase the risk of early-onset Alzheimer's disease (10-35 fold) and overall Alzheimer's disease (6-17 fold);³⁶⁹ (iii) an enrichment of rare damaging variants in *GCK* in maturity-onset diabetes of the young;³⁷⁰ and (iv) an excess of loss-of-function variants in *SETD1A* associated with Schizophrenia (OR~20-30) which were not detected by GWAS.³⁷¹

Many rare variant studies employ gene collapsing (or 'gene burden') approaches, in which the number of qualifying variants – typically loss-of-function variants in protein coding regions with very low allele frequencies (often <0.001) – is aggregated at the

gene level and compared between cases and controls. A consistent observation across recent studies of complex disorders is that the strongest association signals arise from the rarest variants.³⁷² One common limitation of smaller rare variant cohorts is the lack of sufficiently large, well matched control datasets. To address this, some studies leverage publicly available population-level sequencing resources such as gnomAD, using per-base-pair coverage summaries.³⁷³ These methods have been progressively refined over the past decade.^{367 372 374-377}

Typically, this requires careful harmonization of variant filtering, quality control and sequencing coverage between disease cases and control datasets to minimise artefactual signals arising from between-platform heterogeneity. Alignment by ancestry is also often considered although the impact of population stratification diminishes as variant rarity increases.³⁷² Following such harmonisation, rare synonymous variants – considered a near neutral class of genetic variation – are used to assess whether cases and controls' have comparable exome-wide or genome-wide variant burdens under the null hypothesis of no association (Figure 8-1).^{372 374 375} Gene burden results are further evaluated using tests of genomic inflation, such as λ_{GC} .³⁷⁵

To date, relatively little rare variant analysis has been performed in asthma. An early exome-wide (non-gene burden) study by Iguarta et al. in 2014³⁷⁸ did not find a significant role for rare variants with allele frequency <1% and concluded that associations with rare and low-frequency variants are likely to be ethnicity-specific and insufficient to explain a substantial proportion of the missing heritability of asthma. However, this study is limited by relatively shallow phenotyping and, more importantly, by its reliance on an exome genotyping array rather than direct

sequencing. Rare variants are known to be under-represented on genotyping arrays, which require pre-specified markers.^{365 372}

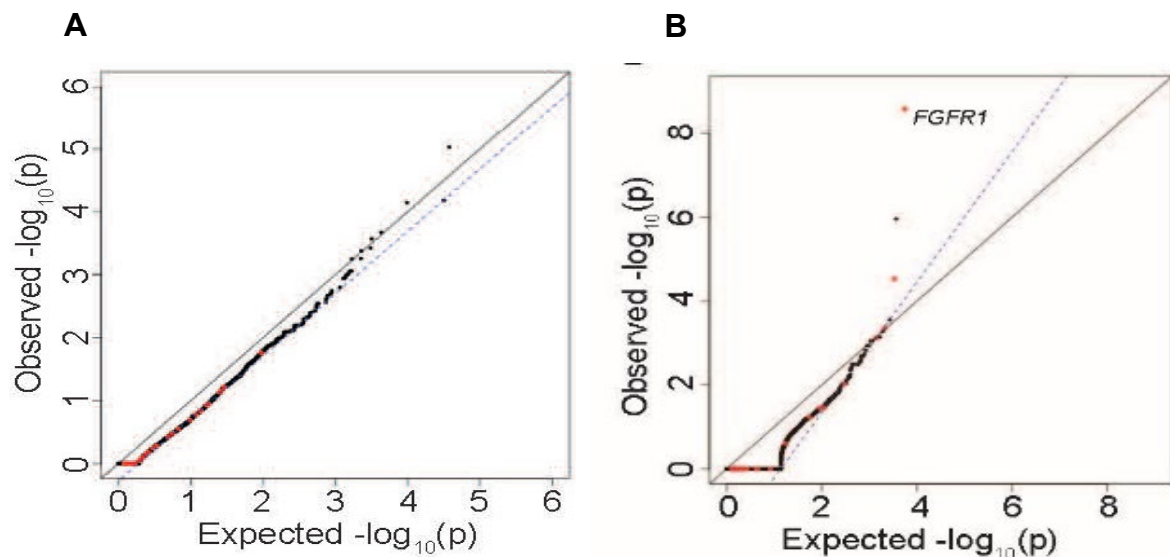


Figure 8-1. Two QQ plots from Guo et al. 2018, The American Journal of Human Genetics 103:522-534, “Burden testing of rare variants identified through exome sequencing via publicly available control data.” (A) Calibration of observed and expected variant probabilities, prior to burden testing, assessed using synonymous ‘neutral’ variants to evaluate comparability between targeted sequencing and a publicly available control dataset (gnomAD). (B) Gene burden analysis using this approach successfully ‘re-discovers’ known rare mutations in *FGFR1* associated with the Mendelian disorder idiopathic hypogonadotrophic hypogonadism.

There remains the possibility that very rare variants of large effect – predominantly affecting coding regions – may cluster within genes relevant to the late-onset eosinophilic asthma phenotype, thereby implicating key biological pathways amenable to therapeutic targeting. The recent identification of a rare, protective,

high-impact loss of function mutation affecting a splice acceptor site in *IL-33*, which confers reduced asthma risk and lower circulating eosinophil levels, provides a compelling case of this potential.³⁷⁹ Genetic mechanisms underlying other eosinophilic disorders have been elucidated, such as the *FIP1L1-PDGFR* fusion gene and related rearrangements involving *PDGRB* or *FGFR1* in myeloproliferative neoplasms.^{380 381}

8.1.3 Somatic mutations and clonal haematopoiesis of indeterminate potential

It is currently thought that lifelong blood cell production is driven by the activity of a relatively small number of haematopoietic stem cells (HSCs).³⁸² Estimates suggest that adults harbour approximately 10,000–50,000 HSCs, of which only a fraction actively contribute to haematopoiesis at any given time; this proportion declines with increasing age.³⁸³ These active cells were historically assumed to contribute roughly equally to blood production. However, over the past 15 years it has become clear that some haematopoietic stem and progenitor cells (HSPCs) contribute disproportionately to blood production, forming small clonal populations. This phenomenon is termed *clonal haematopoiesis*.

Clonal haematopoiesis (CH) arises when a single HSPC acquires a somatic (acquired) mutation that confers a competitive growth or survival advantage, enabling that cell and its progeny to expand disproportionately and contribute an unusually large fraction of circulating blood cells (Figure 8-2). Some clonal populations are detectable early in life and increase in size in a linear fashion with age, whereas others – such as those driven by mutations in spliceosome factors or

chromosomal anomalies – tend to emerge later in life and show exponential growth with ageing.^{384 385} The number of somatic mutations per cell also increases markedly with age, from fewer than 500 per cell in newborns to more than 3,000 per cell in centenarians.³⁸⁶ Consistent with this acquired, age-related accumulation, twin studies have shown that mosaic anomalies are often undetectable in younger pairs but increase sharply in twins older than 55 years.³⁸⁷

The mechanisms by which somatic mutations confer competitive advantage to stem cells are only partially understood.³⁸⁸ Somatic mutations can affect a wide number of genes,³⁸⁹ but there is a striking enrichment for mutations in genes that are also recurrently altered in overt myeloid malignancies such as acute myeloid leukaemia (AML) and myelodysplastic syndromes (MDS).^{385 390} These include *DNMT3A*, *TET2*, and *ASXL1*. Disruption of these genes in zebrafish models result in the emergence of clonal populations.³⁸⁸

Proposed mechanisms underlying clonal expansion differ according to the gene affected and include replication stress, reduced cellular quiescence, epigenetic dysregulation and altered inflammatory signalling; these processes remain areas of active investigation.³⁹¹⁻³⁹⁴

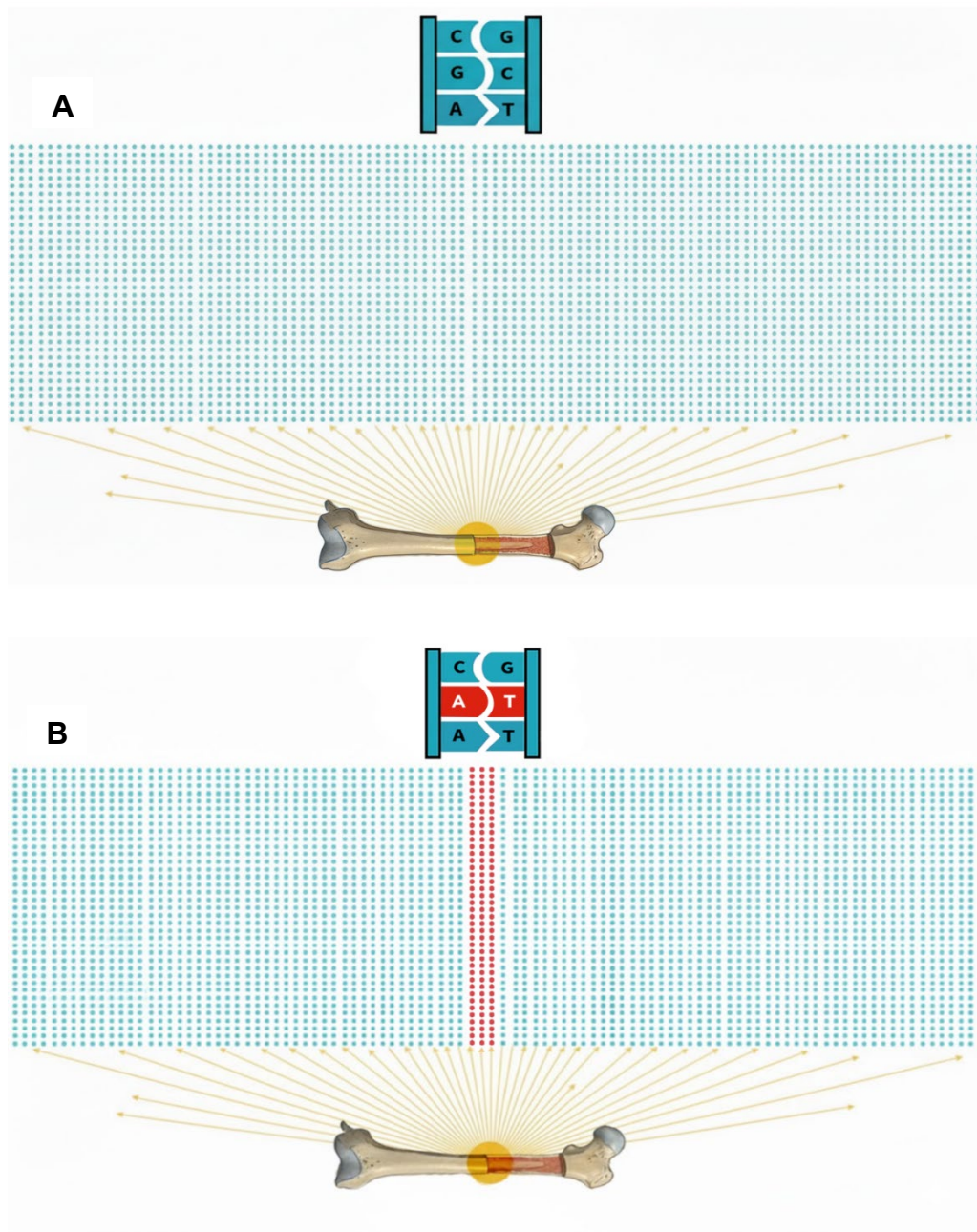


Figure 8-2. Schematic representation of a driver mutation resulting in clonal haematopoiesis. (A) Normal production of blood cells from bone marrow stem cells (blue circles). Three base pairs of DNA in a stem cell are highlighted. (B) Following a G→A transition (G–C → A–T) in the DNA of a stem cell, the cell gains a fitness advantage over other stem cells, begins to outcompete and contribute disproportionately to the circulating blood cell pool resulting in a small clonal population (red cells). Image created in Adobe Photoshop.

Mouse models of mutations affecting DNA methylation and de-methylation – such as *DNMT3A* and *TET2* – have demonstrated that loss of function of either of these genes leads to increased HSC self-renewal capacity and increased HSC numbers,³⁹⁵ albeit at the expense of loss of differentiation.³⁹⁶ Loss of *DNMT3A* is also associated with near-infinite serial transplantation capacity and functional immortalization of HSCs *in vivo*.³⁹⁷ It has been proposed that *DNMT3A* mutations may, in some contexts, play a benign role by helping to maintain apparently normal blood production later in life, when haematopoietic output would otherwise decline.³⁹⁸

There is also a growing body of evidence linking clonal haematopoiesis – particularly *DNMT3A* mutations – to aberrant inflammatory states.^{399 400} Inflammatory signalling appears to promote the expansion of mutant clones. Avagyan et al.³⁸⁸ demonstrated in zebrafish that *DNMT3A* and *AXL* knockout stimulates pro-inflammatory gene expression in mature myeloid cells while inducing anti-inflammatory gene expression in progenitor cell. This supports a model in which HSC clonal fitness is driven by resistance to inflammatory signals generated by differentiated progeny. This concept is further supported by single-cell analyses in humans by Jakobsen et al.⁴⁰¹ who showed that *DNMT3A* and *TET2*-mutant HSCs exhibit an attenuated inflammatory response compared with wild-type HSCs and concluded that CH clones are gradually selected because of their resistance to the deleterious impact of inflammation and ageing.

Unlike mutations in the checkpoint inhibitor *TP53* – which are more strongly associated with progression to leukaemia^{402 403} – the mechanisms by which the more commonly observed mutations in *DNMT3A*, *TET2* and *ASXL1* promote leukemogenesis are less well defined. Mutant clones, can, however, acquire

additional somatic mutations over time, and the sequential evolution of clones with multiple mutations has been observed during the development of AML.⁴⁰³⁻⁴⁰⁷

Since 2014, several studies have investigated CH mutations in approximately 160 genes associated with haematological malignancy at variant allele fractions of $\geq 1\%$ in individuals without known haematological cancer.^{383 408-412} A consistent finding across these studies is a strong age-related increase in the prevalence of CH (Figure 8-3).⁴⁰⁹ Detectable somatic mutations are rare in individuals younger than 40 years but rise substantially with age, reaching prevalences of approximately 18.4% in those older than 90 years.⁴⁰⁹ The most frequently mutated genes in healthy populations are DNMT3A, TET2 and ASXL1. The presence of CH is associated with a modest absolute increase in haematological cancer risk of approximately 1% per year and a small increase in all-cause mortality.⁴¹¹

The presence of somatic driver mutations at variant allele fraction $\geq 2\%$ in the absence of haematological malignancy has been termed *clonal haematopoiesis of indeterminate potential* (CHIP). Subsequent analyses, however, suggest that isolated mutations of DNMT3A, TET2 and ASXL1 alone may not be strongly predictive of malignant transformation.^{390 410 413} Advances in next generation sequencing technology have enabled the detection of clones at increasingly lower allele fractions,³⁹⁸ as well as studies examining interactions between CH and germline variation^{414 415} and investigations into the fitness landscape and longitudinal dynamics of clonal populations.⁴¹⁶⁻⁴¹⁹

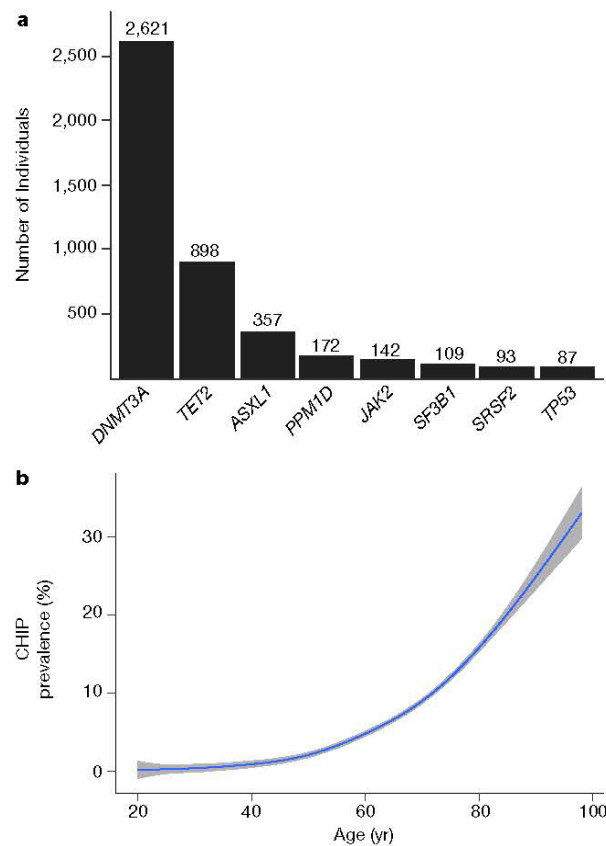


Figure 8-3. Data from Bick et al. 2020 (Nature 586: 763-768) “Inherited Causes of Clonal Haematopoiesis” in 97,691 whole genomes. CHIP was identified in 82,807 peripheral blood samples from donors without haematological malignancy through the curation of somatic mutations. (A) Counts for the eight most common driver genes. (B) CHIP prevalence increased with age at time of blood sampling. The central line represents a model spline; shaded areas denote the 95% confidence interval.

CHIP has also been implicated in non-malignant diseases. There is a significant association between CHIP and rheumatoid arthritis (odds ratio 2.1) as well as inferior overall survival in affected rheumatoid arthritis patients.⁴²⁰ In newly diagnosed rheumatoid arthritis, DNMT3A-mutated seropositive patients demonstrate increased inflammatory markers and disease activity compared with patients without CHIP.⁴²⁰ CHIP has also been associated with osteoporosis. In knockout mouse models, bone

loss appears to be mediated by pro-inflammatory cytokines including *IRF3-NF- κ B* dependent IL-20 expression from DNMT3A- mutant macrophages.⁴²¹

Two notable studies have examined CHIP in chronic obstructive pulmonary disease (COPD).^{399 422} Miller et al.³⁹⁹ identified CH mutations with a distribution similar to that observed in healthy adults (*DNMT3A* > *TET* > *ASXL1*) and reported a modest increase in the odds of CHIP with increasing COPD severity after adjustment for age, smoking history and sex. The authors also demonstrated accelerated emphysema development in a *TET2* knockout mouse model, associated with heightened inflammation and cytokine signalling.³⁹⁹ Lee et al.⁴²² reported CHIP prevalence at variant allele fractions $\geq 1.5\%$ to be 25.6% in a cohort of COPD patients. CHIP positivity was associated with cumulative smoking exposure and increased future exacerbation risk in former smokers with COPD. Unfortunately, there was no comparator healthy control population in this study. To date, no studies have examined CHIP in asthma.

8.1.4 Non ‘CHIP’ somatic mutations and late-onset eosinophilic asthma

The list of candidate driver mutations typically assessed in studies of CHIP is likely to represent a substantial under-estimate of the true number of clonal populations harbouring driver mutations. By surveying both CHIP mutations and synonymous (putatively neutral) mutations in healthy individuals – many of which are likely to be passengers of driver mutations – Poon et al.⁴²³ concluded that only around 30% of synonymous mutations could be explained as passengers of currently recognised

driver mutations. This observation suggests widespread positive selection acting on mutations elsewhere in the genome.

Non-CHIP somatic mutations in blood are known to play causal roles in a number of diseases, particularly auto-inflammatory and auto-immune disease.⁴²⁴ Examples include loss of function mutations in *STAT3* associated with autosomal dominant IgE syndrome,⁴²⁵ gain of function mutations in *STAT5B* associated with non-clonal eosinophilia, urticaria and dermatitis,⁴²⁶ and loss of function mutations in *UBA1* causing the late onset inflammatory VEXAS¹⁵ syndrome.⁴²⁷

My aim is to assess a highly phenotyped cohort of patients with late-onset eosinophilic (LOE) asthma for the presence of somatic mutations, including established CHIP driver mutations. Somatic mutations have been identified in multiple inflammatory diseases, and large populations studies have reported findings of synonymous mutations that likely represent passengers of non-CHIP driver mutations.⁴²³ In addition, recurrent, non-oncogenic non-missense mutations with increasing prevalence with age have been reported, with an average 50 year-old harbouring approximately 27 such mutations.⁴²⁸

I will investigate whether somatic mutations can be identified in genes that may drive LOE asthma, either as driver or passenger mutations, and compare with an age-matched control population. I will also assess whether LOE asthma – an inflammatory disorder – is associated with increased burden of CHIP driver

¹⁵ VEXAS stands for Vacuoles, E1 enzyme, X-linked, Autoinflammatory, Somatic

mutations, such as those affecting *DNMT3A*. Somatic clonal populations in LOE asthma may be present within the eosinophil lineage itself or within other disease-relevant lineages such as IL-5 secreting T-cell clones described in idiopathic eosinophilia⁴²⁹ or in mast cells, in which *in vitro* knockout of *DNMT3A* results in exaggerated responses to acute and chronic stimuli.⁴³⁰

8.1.5 Plan

8.1.5.1 Targeted ultra-deep sequencing of candidate LOE asthma genes

I will perform ultra deep sequencing (≥ 500 reads per base pair; $\geq 500X$ coverage) on a targeted gene panel designed to identify gain- or loss-of-function mutations that may drive the development of LOE asthma (referred to here as the “IDT panel” after Integrated DNA Technologies who produced my panel design). A cohort of highly phenotyped LOE asthma patients and healthy controls will be sequenced to identify germline and somatic variants associated with disease.

Ninety-five genes were selected for the IDT panel through a review of the literature, use of the ‘search tool for the retrieval of interacting genes / proteins’ (STRING),⁴³¹ and examination of differentially expressed genes in eosinophils (using distractor cell lines) via the ImmGen resource.⁴³² The panel includes transcription factors, splicing and RNA processing genes, signalling molecules, receptors, chemokines, cytokines and related regulatory genes. All genes included in the panel are listed in the

Methods section (Table 8-1). Exonic and 5' untranslated regions (UTRs) of each gene were sequenced.

Representative genes include *PTPN11* (encoding the protein tyrosine phosphatase SHP2) and *C/EBP α* , both of which are involved in eosinophil differentiation;^{131 433-435} Genes that have been demonstrated to provide negative feedback on eosinophils such as JAK2, Lyn and CCR3 are included.¹³⁷ *GATA3*, a master regulator of Th2 cell differentiation that binds directly to the *IL-5* promoter and positively regulates IL-5 transcription in Th2 cells is included.⁴³⁶⁻⁴³⁹ The upstream activator of GATA3, *STAT6*,^{440 441} as well as its activating cytokines *IL-4* and *IL-13*, are also included (Figure 8-4). Transcription factors *SOX4* and *ZFPM1*, which suppress GATA3-mediated Th2 differentiation, are likewise incorporated.^{442 443} The *IL-5* gene and its promoter region are sequenced in their entirety. Given the inhibitory feedback of the solubilised form of the IL5R α on IL-5 in vitro (discussed in section 7), *IL5RA* was included along with sheddases involved in cleavage of this and other receptors such as *MMP2* and *MMP9*.⁴⁴⁴

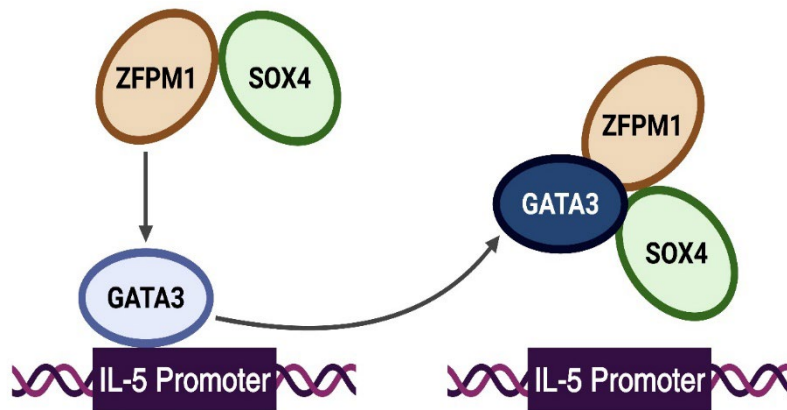


Figure 8-4. Schematic representation of GATA3 binding to the IL-5 promoter in Th2 cells, positively regulating IL-5 transcription. ZFPM1 suppresses GATA3 through protein-protein interaction while SOX4 acts via transcriptional regulation. Image created in BioRender

8.1.5.2 Targeted ultra-deep sequencing of CHIP driver genes

I will perform ultra deep sequencing ($\geq 500X$ coverage) using a targeted panel of putative clonal haematopoiesis driver genes (e.g., *DNMT3A*, *TET2*, *AXSL1*, *TP53*; referred to here as the “Roche panel” after the company that produced the panel) to examine whether LOE asthma is associated with increased rates of CHIP. The same cohort of LOE asthma patients and healthy controls will be sequenced using this panel. A small group of younger healthy individuals, in whom CHIP is unlikely, will serve as a “panel of normals” to filter sequencing artefact.

8.1.5.3 Rare-variant gene-burden analysis

I will perform rare-variant gene burden (“collapsing”) analysis for genes included in the IDT panel, comparing the burden of rare germline protein-truncating variants (variant allele frequency < 0.001) between LOE asthma patients and a large control

population drawn from the non-Finnish European (NFE) subset of gnomAD v4.1 exomes.³⁷³ This analysis will closely follow the framework described by Guo et al 2018³⁷⁵ which uses per-base-pair variant summary data and neutral synonymous variants to calibrate expected variant counts and assess type 1 error.

The gnomAD v4.1 exomes NFE population is well suited as a control cohort for a white-British LOE asthma population because:

- (i) 88.1% of individuals in this group self-report as white-British, with the majority of all samples in gnomAD v4.1 exomes (57%) coming from the UK Biobank;³⁷³ also, at very low variant allele frequencies (< 0.001) residual population stratification is unlikely to materially affect results.³⁷²
- (ii) The cohort is large with a median 518,515 participants sequenced per exons base pair across the targeted panels
- (iii) although read depth is lower than that achieved with targeted sequencing, coverage remains high, with 99.9% of sites exceeding 10X coverage and 94.6% exceeding 20X
- (iv) variants in gnomAD v4.1 exomes were called using GATK best practices⁴⁴⁵ with joint variant calling using the same truth and training sets, and variant quality score recalibration model closely mirroring the analytical pipeline used in this study.⁴⁴⁶

8.1.5.4 4. Analysis of somatic mutations in eosinophilopoiesis-related genes

Finally, I will outline an analysis plan to assess the prevalence and characteristics of somatic mutations in eosinophilopoiesis-related genes included in the IDT panel, comparing LOE asthma patients with healthy controls.

8.2 Methods

8.2.1 Study design

The study was sponsored by the University of Oxford and approved as part of the Oxford Airways Study OAS (REC 18/SC/0361) and NIHR CSP Study 88241 (REC 08/H0406/189). Measurements were obtained at a single baseline visit.

8.2.1.1 Late onset eosinophilic (LOE) asthma patients

Eligible patients for inclusion were never or former smokers who had abstained from smoking for at least one year. Participants were required to have a clinical diagnosis of eosinophilic asthma with confirmed current or historic evidence of variable airflow limitation, defined by at least one of the following; FEV1 bronchodilator reversibility >200ml; FEV1 percentage change $\geq 12\%$; methacholine challenge of less than 8 mg/ml; or peak expiratory flow (PEF) diurnal variability. Additional inclusion and exclusion criteria were as follows:

Inclusion criteria:

1. One or more recorded blood eosinophil counts $\geq 0.40 \times 10^9/L$
2. Age of onset of respiratory symptoms ≥ 18 years

Exclusion criteria:

3. Any clinical history of eczema or atopy

4. Established treatment with biologic therapy
5. Any history of malignancy

8.2.1.2 Age-matched healthy control (HC) group and healthy control panel of normals (PON) group

Eligible healthy control participants had no history of asthma or other respiratory diseases and no prior use of inhaled respiratory therapies. Additional exclusion criteria were as follows:

Exclusion criteria:

1. Evidence of airflow obstruction on spirometry
2. One or more recorded blood eosinophil counts $\geq 0.40 \times 10^9/L$
3. Any history of malignancy

Healthy participants aged ≥ 40 years old were recruited as aged-matched healthy controls for the LOE asthma group. A smaller group of healthy participants aged 18–32 years was recruited to act as a sequencing *panel of normals* (PON). The prevalence of clonal haematopoiesis in this younger age group is extremely low; therefore, the presence of sequencing reads supporting a variant allele in this cohort was used during variant calling to identify and exclude likely sequencing artefacts (see clonal haematopoiesis pipeline, section 8.2.7).

8.2.2 Targeted gene panels

Samples were sequenced for targeted variant detection using two distinct gene panels.

A. Roche panel for putative clonal haematopoiesis driver mutations

Targeted sequencing was performed using the SeqCap EZ Prime Choice Library (Roche Sequencing and Life Science, Pleasanton, CA, USA; cat. no. 08247480001). This commercially available panel comprises a pool of probes covering approximately 401kb and targets 108 genes, along with an additional 5 loci (defined by dbSNP reference ID) that are recurrently mutated in clonal haematopoiesis and myeloid malignancies. Previous versions of this panel have been used in published investigations of clonal haematopoiesis (Jackobsen et al 2024⁴⁰¹).

B. IDT panel of putative causal genes in late onset eosinophilic asthma

A custom NGS Discovery Probe Pool (Integrated DNA Technologies, Coralville, IA, USA; ID#043492322) was designed by the author in collaboration with integrated DNA technologies (IDT). Exonic regions were defined through manual inspection of protein-coding transcript coordinates in Ensembl GRCh38.p14 (https://www.ensembl.org/Homo_sapiens) and cross-referenced with the UCSC Table Browser tool (<https://genome.ucsc.edu/cgi-bin>) to include 5' untranslated regions (5'UTRs)

The panel targets exons from 95 genes as well as the entire IL-5 promoter region, covering approximately 478kb. A total of 7,899 probes are distributed

across two probe pools. A summary of all probe target co-ordinates is provided in Table 8-9, more detailed information on coverage is available in Appendix Table 11-7, and a comprehensive list of individual probes is available on request.

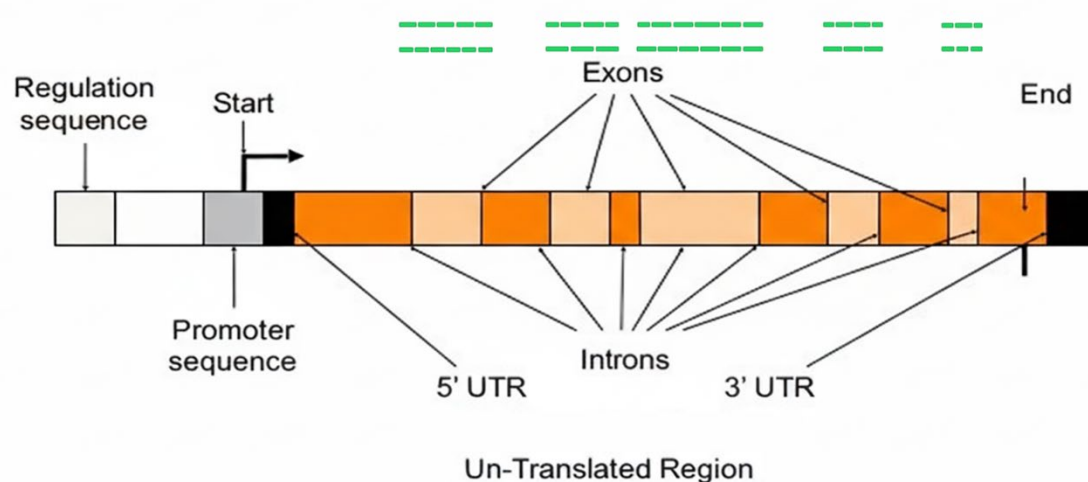


Figure 8-5. Schematic demonstrating the tiled configuration of capture probes (green) with 50% overlap across exonal regions. The IDT panel additionally includes 5'UTR regions and the full IL-5 promoter. Image created by the author in Adobe Photoshop.

8.2.3 Wet-laboratory workflow

8.2.3.1 Extraction of DNA

Whole blood was collected into ethylenediaminetetraacetic acid (EDTA) or heparin-coated tubes and stored at -80 °C until processing. Genomic DNA was extracted from 200 µl of whole blood using a spin column method (QIAamp® DNA Mini and Blood Mini, QIAGEN, Hilden, Germany; cat. no. 51106) and eluted in 100 µl of Buffer EB (QIAGEN, Hilden, Germany; cat. no. 19086). DNA concentration was assessed using the Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, USA) and fluorometrically using the Qubit dsDNA High Sensitivity Assay Kit (Life Technologies, Carlsbad, CA, USA; cat. no. Q32854). Samples yielding <200 ng of DNA were re-extracted. DNA concentrations are presented in Appendix Table 11-6.

Table 8-1. List of the 202 genes investigated in the study.

The Roche panel comprised 108 putative clonal haematopoiesis driver genes, together with an additional 5 target loci specified by their dbSNP reference identifiers. The custom designed IDT eosinophilia panel included 95 genes selected for their putative relevance to late-onset eosinophilic asthma. A list of coordinate regions of capture probe per gene is provided in Appendix Table A4.2.

Panel	Chr	Location	Gene	Ensembl ID	Full name	Functional Category
Roche 1		1q21.3	SETDB1	ENSG00000143393	SET domain bifurcated histone lysine methyltransferase 1	Epigenetic regulator
Roche 2		2p23.2	DNMT3A	ENSG00000119772	DNA methyltransferase 3 alpha	Epigenetic regulator
Roche 2		2p23.2	ASXL2	ENSG00000182585	ASXL transcriptional regulator 2	Epigenetic regulator
Roche 3		3p21.31	SETD2	ENSG00000143379	Set domain containing 2	Epigenetic regulator
Roche 4		4q24	TET2	ENSG00000168769	Tet methylcytosine dioxygenase 2	Epigenetic regulator
Roche 7		7q22.3	KMT2E	ENSG00000174469	Lysine methyltransferase 2E	Epigenetic regulator
Roche 7		7q36.1	EZH2	ENSG00000106462	Enhancer of zeste homolog 2	Epigenetic regulator
Roche 7		7q36.1	KMT2C	ENSG00000146463	Lysine N-methyltransferase 2C	Epigenetic regulator
Roche 11		11q12.3	MTA2	ENSG00000101247	Metastasis associated 1 family member 2	Epigenetic regulator

Table 8-1 continued

Panel	Chr	Location	Gene	Ensembl ID	Full name	Functional Category
Roche 11	11	11q23.3	KMT2A	ENSG00000118058	Lysine methyltransferase 2A	Epigenetic regulator
Roche 11	11	11q14.2	EED	ENSG00000104299	Embryonic ectoderm development	Epigenetic regulator
Roche 12	12	12p13.33	KDM5A	ENSG00000124212	Lysine demethylase 5A	Epigenetic regulator
Roche 12	12	12q13.12	KMT2D	ENSG00000167548	Lysine methyltransferase 2D	Epigenetic regulator
Roche 16	16	16p13.3	CREBBP	ENSG00000005339	CREB binding protein	Epigenetic regulator
Roche 16	16	16p11.2	SRCAP	ENSG00000198535	Snf2 related CREBBP activator protein	Epigenetic regulator
Roche 17	17	17q11.2	SUZ12	ENSG00000178691	SUZ12 polycomb repressive complex 2 subunit	Epigenetic regulator
Roche 17	17	17q21.31	KANSL1	ENSG00000164985	KAT8 regulatory NSL complex subunit 1	Epigenetic regulator
Roche 20	20	20q11.21	ASXL1	ENSG00000171456	ASXL transcriptional regulator 1	Epigenetic regulator
Roche 22	22	22q13.2	EP300	ENSG00000100393	E1A binding protein P300	Epigenetic regulator
Roche X	X	Xq26.2	PHF6	ENSG00000100219	PHD finger protein 6	Epigenetic regulator
Roche X	X	Xq28	BRCC3	ENSG00000147180	BRCA1/BRCA2 containing complex subunit 3	Epigenetic regulator
Roche X	X	Xp11.4	BCOR	ENSG00000183337	BCL6 co-repressor	Epigenetic regulator

Table 8-1 continued

Panel	Chr	Location	Gene	Ensembl ID	Full name	Functional Category
Roche X		Xp11.3	KDM6A	ENSG00000147099	Lysine demethylase 6A	Epigenetic regulator
Roche X		Xq21.1	ATRX	ENSG00000107796	ATRX chromatin remodeller	Epigenetic regulator
Roche X		Xq26.1	BCORL1	ENSG00000166199	BCL6 corepressor like 1	Epigenetic regulator
IDT	2	2p25.1	ID2	ENSG00000115738	Inhibitor of DNA binding 2	Transcription factor
IDT	2	2q32.2	STAT4	ENSG00000138378	Signal transducer & activator of transcription 4	Transcription factor
Roche 3		3q21.3	GATA2	ENSG00000179348	GATA binding factor 2	Transcription factor
IDT	6	6p22.3	SOX4	ENSG00000124766	Transcription factor SOX4	Transcription factor
Roche 6		6q21	PRDM1	ENSG00000137944	PR domain zinc finger protein 1	Transcription factor
Roche 7		7p12.2	IKZF1	ENSG00000185811	DNA-binding protein Ikaros	Transcription factor
Roche 7		7q22.1	CUX1	ENSG00000257923	Cut like homeobox 1	Transcription factor
Roche 8		8q24.21	MYC	ENSG00000136997	MYC proto-oncogene bHLH transcription factor	Transcription factor
Roche 10		10p14	GATA3	ENSG00000107485	GATA binding protein 3	Transcription factor
IDT	11	11p11.2	SPI1	ENSG00000066336	Spi-1 proto-oncogene	Transcription factor

Table 8-1 continued

Panel	Chr	Location	Gene	Ensembl ID	Full name	Functional Category
Roche	11	11p13	WT1	ENSG00000184937	WT1 transcription factor	Transcription factor
Roche	11	11q13.1	SF1	ENSG00000168081	Splicing factor 1	Transcription factor
IDT	12	12q13.3	STAT6	ENSG00000166888	Signal transducer & activator of transcription 6	Transcription factor
Roche	12	12p13.2	ETV6	ENSG00000139083	ETS variant transcription factor 6	Transcription factor
IDT	14	14q11.2	CEBPE	ENSG00000179344	CCAAT enhancer binding protein epsilon	Transcription factor
IDT	16	16q24.1	IRF8	ENSG00000140968	Interferon regulatory factor 8	Transcription factor
IDT	16	16q24.2	ZFPM1	ENSG00000163399	Zinc finger protein FOG family member 1	Transcription factor
Roche	16	16q22.1	CTCF	ENSG00000102974	CCCTC-binding factor	Transcription factor
IDT	17	17q21.1	NR1D1	ENSG00000126368	Nuclear receptor subfamily 1 group D member 1	Transcription factor
Roche	17	17p13.1	TP53	ENSG00000141510	Tumour protein 53	Transcription factor
Roche	17	17q21.1	IKZF3	ENSG00000161405	IKAROS family zinc finger 3	Transcription factor
Roche	17	17q21.2	STAT5B	ENSG00000173757	Signal transducer & activator of transcription 5B	Transcription factor
Roche	17	17q21.2	STAT3	ENSG00000168610	Signal transducer & activator of transcription 3	Transcription factor

Table 8-1 continued

Panel	Chr	Location	Gene	Ensembl ID	Full name	Functional Category
IDT	19	19p13.11	NR2F6	ENSG00000128524	Nuclear receptor subfamily 2 group F member 6	Transcription factor
Roche	19	19q13.11	CEBPA	ENSG00000140968	CCAAT enhancer binding protein alpha	Transcription factor
IDT	20	20q11.21	ID1	ENSG00000125952	Inhibitor of DNA binding 1	Transcription factor
Roche	21	21q22.12	RUNX1	ENSG00000159216	RUNX family transcription factor 1	Transcription factor
IDT	22	22q12.1	XBP1	ENSG00000100219	X-Box binding protein 1	Transcription factor
Roche	X	Xp11.23	GATA1	ENSG00000102145	GATA binding protein 1	Transcription factor
Roche	X	Xq13.11	MED12	ENSG00000115705	Mediator complex subunit 12	Transcriptional reg
Roche	2	2q33.1	SF3B1	ENSG00000115524	Splicing factor 3b subunit 1	Splicing / RNA process
IDT	4	4q12	FIP1L1	ENSG00000119711	Factor interacting with PAPOLA and CPSF1	Splicing / RNA process
Roche	4	4p15.2	DHX15	ENSG00000100453	DEAH-box helicase 15	Splicing / RNA process
Roche	5	5q35.3	DDX41	ENSG00000156103	Probable ATP-dependent RNA helicase DDX41	Splicing / RNA process
Roche	7	7q34	LUC7L2	ENSG00000165810	Putative RNA binding protein Luc7-like 2	Splicing / RNA process
Roche	9	9q21.32	HNRNPK	ENSG00000165119	Heterogenous nuclear ribonucleoprotein K	Splicing / RNA process

Table 8-1 continued

Panel	Chr	Location	Gene	Ensembl ID	Full name	Functional Category
Roche 12	12	12q13.12	PRPF40B	ENSG00000166913	Pre-mRNA processing factor 40 homolog B	Splicing / RNA process
Roche 13	13	13q21.33	DIS3	ENSG00000182318	DIS3 homolog, exosome endoribonuclease and 3'-5' exoribonuclease	Splicing / RNA process
Roche 17	17	17p13.3	PRPF8	ENSG00000158552	Pre-mRNA processing factor 8	Splicing / RNA process
Roche 17	17	17q25.1	SRSF2	ENSG00000161929	Serine and arginine rich splicing factor 2	Splicing / RNA process
Roche 19	19	19q13.42	U2AF2	ENSG00000160224	U2 small nuclear RNA auxiliary factor 2	Splicing / RNA process
Roche 21	21	21q22.3	U2AF1	ENSG00000160221	U2 small nuclear RNA auxiliary factor 1	Splicing / RNA process
Roche 22	22	22q12.2	SF3A1	ENSG00000141759	Splicing factor 3a subunit 1	Splicing / RNA process
Roche X	X	Xp22.2	ZRSR2	ENSG00000100485	Zinc finger CCCH-type RNA binding motif and serine / arginine rich 2	Splicing / RNA process
Roche X	X	Xp11.4	DDX3X	ENSG00000100219	DEAD-box helicase 3 X-linked	Splicing / RNA process
Roche 1	1	1p36.33	GNB1	ENSG00000078369	G protein subunit beta-1	Signaling
Roche 1	1	1p34.2	PTPRF	ENSG00000142945	Protein tyrosine phosphatase receptor type F	Signaling

Table 8-1 continued

Panel	Chr	Location	Gene	Ensembl ID	Full name	Functional Category
Roche 1		1p31.3	JAK1	ENSG00000162434	Janus Kinase 1	Signaling
Roche 1		1p13.2	NRAS	ENSG00000213281	NRAS proto-oncogene	Signaling
Roche 1		1q22	RIT1	ENSG00000143643	RAS like without CAAX	Signaling
Roche 2		2q34	IDH1	ENSG00000138413	Isocitrate dehydrogenase (NADP(+))1	Signaling
Roche 3		3p22.2	MYD88	ENSG00000172936	Myeloid differentiation primary response 88	Signaling
Roche 3		3q13.11	CBLB	ENSG00000103254	Cbl proto-oncogene B	Signaling
Roche 4		4q31.3	FBXW7	ENSG00000109670	F-box /WD repeat containing protein 7	Signaling
IDT	5	5q31.2	STING1	ENSG00000184557	Stimulator of Interferon response CGAMP interactor 1	Signaling
IDT	6	6q23.3	TNFAIP3	ENSG00000118503	Tumour necrosis factor alpha induced protein 3	Signaling
Roche 7		7q34	BRAF	ENSG00000157764	Proto-oncogene B-Raf	Signaling
IDT	8	8q12.1	LYN	ENSG00000254087	LYN Proto-oncogene	Signaling
Roche 9		9p24.1	JAK2	ENSG00000096968	Janus Kinase 2	Signaling

Table 8-1 continued

Panel	Chr	Location	Gene	Ensembl ID	Full name	Functional Category
IDT	10	10q23.31	PTEN	ENSG00000171862	Phosphatase and Tensin homolog	Signaling
Roche	11	11p15.5	HRAS	ENSG00000174775	HRas proto-oncogene	Signaling
Roche	11	11q23.3	CBL	ENSG00000110395	CBL proto-oncogene	Signaling
Roche	11	11q22.3	ATM	ENSG00000149311	ATM serine/threonine kinase	Signaling
Roche	12	12p12.1	KRAS	ENSG00000133703	KRAS proto-oncogene GTPase	Signaling
Roche	12	12q24.12	SH2B3	ENSG00000111218	SH2B adaptor protein 3	Signaling
Roche	12	12q24.13	PTPN11	ENSG00000179295	Protein tyrosine phosphatase non-receptor type 11	Signaling
Roche	15	15q26.1	IDH2	ENSG00000182054	Isocitrate dehydrogenase (NADP(+))2	Signaling
IDT	16	16p13.13	SOCS1	ENSG00000185338	Suppressor of cytokine signalling 1	Signaling
Roche	16	16p12.3	SMG1	ENSG00000157181	SMG1 nonsense mediated mRNA decay associated PI3K related kinase	Signaling
Roche	17	17q11.2	NF1	ENSG00000196712	Neurofibromin 1	Signaling

Table 8-1 continued

Panel	Chr	Location	Gene	Ensembl ID	Full name	Functional Category
Roche	17	17q23.3	PPM1D	ENSG00000166265	Protein phosphatase Mg ²⁺ /Mn ²⁺ dependent 1D	Signaling
Roche	19	19p13.11	JAK3	ENSG00000105639	Janus kinase 3	Signaling
Roche	20	20q13.32	GNAS	ENSG00000112062	GNAS complex locus	Signaling
Roche	22	22q12.1	CHEK2	ENSG00000183765	Checkpoint kinase 2	Signaling
IDT	1	1p31.3	IL12RB2	ENSG00000123496	Interleukin-12 receptor subunit beta 2	Receptor
IDT	1	1q23.2	FCER1A	ENSG00000172270	FC Epsilon receptor 1a	Receptor
IDT	1	1q23.3	FCER1G	ENSG00000158869	FC Epsilon receptor 1g	Receptor
Roche	1	1p34.3	CSF3R	ENSG00000119535	Colony stimulating factor 3 receptor	Receptor
Roche	1	1p34.2	MPL	ENSG00000117400	MPL proto-oncogene thrombopoietin receptor	Receptor
IDT	2	2q11.1	IL1R1	ENSG00000115594	Interleukin-1 receptor type 1	Receptor
IDT	2	2q22.1	CXCR4	ENSG00000121966	C-X-C motif chemokine receptor 4	Receptor
IDT	3	3p26.2	IL5RA	ENSG00000134460	Interleukin-5 receptor subunit alpha	Receptor
IDT	3	3p22.3	CCR4	ENSG00000183813	C-C motif chemokine receptor 4	Receptor

Table 8-1 continued

Panel	Chr	Location	Gene	Ensembl ID	Full name	Functional Category
IDT	3	3p21.31	CCR1	ENSG00000163455	C-C motif chemokine receptor 1	Receptor
IDT	3	3p21.31	CCR3	ENSG00000183625	C-C motif chemokine receptor 3	Receptor
IDT	3	3p21.31	CCR2	ENSG00000121807	C-C motif chemokine receptor 2	Receptor
IDT	3	3p21.31	CCR5	ENSG00000160791	C-C motif chemokine receptor 5	Receptor
IDT	3	3p21.1	IL17RB	ENSG00000115604	Interleukin-17 receptor B	Receptor
Roche	4	4q12	PDGFRA	ENSG00000134853	Platelet derived growth factor receptor A	Receptor
Roche	4	4q12	KIT	ENSG00000157404	Proto-oncogene c-KIT	Receptor
IDT	5	5p13	IL7R	ENSG00000168685	Interleukin 7 receptor	Receptor
IDT	5	5q11.2	IL31RA	ENSG00000163349	Interleukin 31 receptor A	Receptor
IDT	5	5q32	ADRB2	ENSG00000169252	Beta 2 adrenergic receptor	Receptor
Roche	5	5q32	CSF1R	ENSG00000182578	Colony stimulating factor 1 receptor	Receptor
IDT	9	9p13.3	CD72	ENSG00000169251	Lyb-2	Receptor
Roche	9	9q34.3	NOTCH1	ENSG00000148400	Notch receptor 1	Receptor

Table 8-1 continued

Panel	Chr	Location	Gene	Ensembl ID	Full name	Functional Category
IDT	11	11q12.2	PTGDR2	ENSG00000125844	Prostaglandin D2 receptor 2	Receptor
IDT	11	11q23.3	IL10RA	ENSG00000166507	Interleukin 10 receptor subunit alpha	Receptor
Roche	13	13q12.2	FLT3	ENSG00000122025	FMS related receptor tyrosine kinase 3	Receptor
IDT	16	16p12.1	IL4R	ENSG00000109471	Interleukin-4 receptor	Receptor
IDT	19	19p13.2	FCER2	ENSG00000184451	FC epsilon receptor 2	Receptor
IDT	19	19p13.11	IL12RB1	ENSG00000138735	Interleukin 12 receptor subunit beta 1	Receptor
IDT	21	21q22.11	IL10RB	ENSG00000100264	Interleukin 10 receptor subunit beta	Receptor
IDT	22	22q11.1	IL17RA	ENSG00000177663	Interleukin-17 receptor alpha	Receptor
IDT	22	22q12.3	CSF2RB	ENSG00000163069	Colony stimulating factor 2 receptor subunit beta	Receptor
IDT	X	Xp22.33	CRLF2	ENSG00000105369	Cytokine receptor like factor 2	Receptor
IDT	X	Xp22.33	IL3RA	ENSG00000185291	Interleukin 3 receptor subunit alpha	Receptor
IDT	X	Xq24	IL13RA1	ENSG00000131747	Interleukin 13 receptor subunit alpha 1	Receptor
IDT	X	Xq28	IL9R	ENSG00000173585	Interleukin-9 receptor	Receptor

Table 8-1 continued

Panel	Chr	Location	Gene	Ensembl ID	Full name	Functional Category
IDT	3	3p25.2	TIMP4	ENSG00000136999	TIMP metalloproteinase inhibitor 4	Protease / ECM
IDT	5	5q33.3	ADAM19	ENSG00000135048	A Disintegrin and Metalloproteinase 19	Protease / ECM
IDT	8	8p11.22	ADAM9	ENSG00000168615	Disintegrin and metalloproteinase domain containing protein 9	Protease / ECM
IDT	8	8q21.3	MMP16	ENSG00000198681	Matrix metalloproteinase-16	Protease / ECM
IDT	14	14q11.2	MMP14	ENSG00000157227	Matrix metalloproteinase 14	Protease / ECM
IDT	15	15q21.3	ADAM10	ENSG00000170345	A disintegrin and metalloproteinase domain 10	Protease / ECM
IDT	16	16q12.2	MMP2	ENSG00000112715	Matrix metalloproteinase 2	Protease / ECM
IDT	16	16q21	MMP15	ENSG00000198964	Matrix metalloproteinase 15	Protease / ECM
IDT	17	17q25.3	TIMP2	ENSG00000035862	TIMP metalloproteinase inhibitor 2	Protease / ECM
IDT	20	20q11.22	MMP24	ENSG00000162631	Matrix metalloproteinase 24	Protease / ECM
IDT	20	20q13.12	MMP9	ENSG00000100985	Matrix metalloproteinase 9	Protease / ECM
IDT	22	22q12.3	TIMP3	ENSG00000100234	TIMP metalloproteinase inhibitor 3	Protease / ECM

Table 8-1 continued

Panel	Chr	Location	Gene	Ensembl ID	Full name	Functional Category
IDT	X	Xp11.3	TIMP1	ENSG00000102265	TIMP metalloproteinase inhibitor 1	Protease / ECM
IDT	16	16q21	CCL22	ENSG00000102970	C-C motif chemokine ligand 22	Chemokine
IDT	16	16q21	CCL17	ENSG00000102962	C-C motif chemokine ligand 17	Chemokine
IDT	17	17q12	CCL11	ENSG00000172156	C-C motif chemokine ligand 11	Chemokine
IDT	17	17q12	CCL5	ENSG00000161570	C-C motif chemokine ligand 5	Chemokine
Roche 3		3q22.3	STAG1	ENSG00000101972	Cohesin subunit SA-1	Chromatin / cohesin
Roche 5		5p13.2	NIPBL	ENSG00000149295	Nipped-B-like protein	Chromatin / cohesin
Roche 8		8q24.11	RAD21	ENSG00000164754	Double-strand-break repair protein rad21 homolog	Chromatin / cohesin
Roche 10		10q23.2	WAPL	ENSG00000154027	WAPL cohesin release factor	Chromatin / cohesin
Roche 10		10q25.2	SMC3	ENSG00000108055	Structural maintenance of chromosomes 3	Chromatin / cohesin
Roche 13		13q13.1	PDS5B	ENSG00000136492	PDS5 cohesin associated factor B	Chromatin / cohesin
Roche 19		19p13.11	MAU2	ENSG00000163827	MAU2 sister chromatid cohesion factor	Chromatin / cohesin
Roche X		Xp11.22	SMC1A	ENSG00000142197	Structural maintenance of chromosomes 1A	Chromatin / cohesin

Table 8-1 continued

Panel	Chr	Location	Gene	Ensembl ID	Full name	Functional Category
Roche X		Xq25	STAG2	ENSG00000101972	STAG2 cohesin complex component	Chromatin / cohesin
IDT	1	1q32.1	IL10	ENSG00000136634	Interleukin-10	Cytokine
IDT	3	3q25.33	IL12A	ENSG00000168811	Interleukin-12 subunit alpha	Cytokine
IDT	4	4q31.21	IL15	ENSG00000164136	Interleukin-15	Cytokine
IDT	5	5q22.1	TSLP	ENSG00000145777	Thymic stromal lymphopoietin	Cytokine
IDT	5	5q31.1	IL3	ENSG00000164399	Interleukin 3	Cytokine
IDT	5	5q31.1	CSF2	ENSG00000164400	Granulocyte-macrophage colony stimulating factor	Cytokine
IDT	5	5q31.1	IL5	ENSG00000113525	Interleukin-5	Cytokine
IDT	5	5q31.1	IL13	ENSG00000169194	Interleukin-13	Cytokine
IDT	5	5q31.1	IL4	ENSG00000113520	Interleukin-4	Cytokine
IDT	5	5q31.1	IL9	ENSG00000113524	Interleukin-9	Cytokine
IDT	5	5q33.3	IL12B	ENSG00000113302	Interleukin-12 subunit beta	Cytokine
IDT	6	6p21.33	TNF	ENSG00000232810	Tumour necrosis factor	Cytokine

Table 8-1 continued

Panel	Chr	Location	Gene	Ensembl ID	Full name	Functional Category
IDT	6	6p12.2	IL17A	ENSG00000112115	Interleukin-17A	Cytokine
IDT	7	7p15.3	IL6	ENSG00000136244	Interleukin-6	Cytokine
IDT	7	7q11.2	CCL26	ENSG00000183318	Eotaxin-3	Cytokine
IDT	7	7q11.23	CCL24	ENSG00000183644	Eotaxin-2	Cytokine
IDT	9	9p24.1	IL33	ENSG00000137033	Interleukin-33	Cytokine
IDT	12	12q24.31	IL31	ENSG00000138642	Interleukin-31	Cytokine
IDT	19	19q13.2	TGFB1	ENSG00000105329	Transforming growth factor beta 1	Cytokine
Roche	1	1q31.1	BRINP3	ENSG00000151240	BMP/retinoic acid inducible neural specific 3	Other
IDT	3	3p26.2	TRNT1	ENSG00000164061	tRNA nucleotidyl transferase 1	Other
Roche	5	5q35.1	NPM1	ENSG00000181163	Nucleophosmin	Other
Roche	5	5p15.33	TERT	ENSG00000164362	Telomerase reverse transcriptase	Other
IDT	7	7p14.1	TRGC2	ENSG00000211680	T cell gamma receptor constant 2	Other
IDT	7	7p14.1	TRGC1	ENSG00000211679	T cell gamma receptor constant 1	Other

Table 8-1 continued

Panel	Chr	Location	Gene	Ensembl ID	Full name	Functional Category
IDT	7	7q34	TRBC2	ENSG00000211703	T cell receptor beta constant 2	Other
IDT	8	8q12.1	SDCBP	ENSG00000161202	Syndecan binding protein	Other
Roche	9	9p21.3	CDKN2A	ENSG00000147889	Cyclin dependent kinase inhibitor 2A	Other
IDT	11	11q12.1	PRG2	ENSG00000198479	Proteoglycan 2, Pro eosinophil major basic protein	Other
Roche	12	12p12.1	ETNK1	ENSG00000162441	Ethanolamine kinase 1	Other
Roche	13	13q14.2	RB1	ENSG00000139687	RB transcriptional co-repressor 1	Other
IDT	14	14q11.2	RNASE3	ENSG00000197956	Ribonuclease A family member 3	Other
IDT	14	14q11.2	RNASE2	ENSG00000197955	Ribonuclease A family member 2	Other
IDT	14	14q11.2	TRDC	ENSG00000211747	T cell receptor delta constant	Other
IDT	14	14q11.2	TRAC	ENSG00000211677	T cell receptor alpha constant	Other
IDT	14	14q32.33	IGHE	ENSG00000211893	Immunoglobulin heavy constant epsilon	Other
Roche	14	14q32.13	TCL1A	ENSG00000100721	TCL1 family AKT co-activator A	Other
IDT	17	17q22	EPX	ENSG00000100565	Eosinophil peroxidase	Other

Roche 18	18q12.3	SETBP1	ENSG00000152284	SET binding protein 1	Other
IDT 19	19q13.2	CLC	ENSG00000105245	Charcot Leyden crystal galectin	Other
Roche 19	19p13.13	CALR	ENSG00000179218	Calreticulin	Other
Roche X	Xp22.2	PIGA	ENSG00000165168	Phosphatidylinositol glycan anchor biosynthesis class A	Other
Roche 1	1q43	rs199822819-1		dbSNP variant locus	Other
Roche 3	3q25.33	rs1210060191-1		dbSNP variant locus	Other
Roche 8	8q21.3	rs1187082186_rs777460725-1		dbSNP variant locus	Other
Roche 11	11q21	rs587781384-1		dbSNP variant locus	Other
Roche 15	15q26.3	rs754640606-1		dbSNP variant locus	Other

Abbreviations: Chr, chromosome; Location, cytogenetic location. Ensembl ID per Genome Reference Consortium Human Build 38 (GRCh38)

8.2.4 Library preparation and panel capture

DNA libraries were prepared using the KAPA HyperPlus DNA Library Preparation Kit (Roche Sequencing and Life Science, Pleasanton, CA, USA; cat. no. KK8514) according to the manufacturer's protocol. Two hundred nanograms of genomic DNA was enzymatically fragmented followed by end repair, A-tailing, adapter ligation and clean up using Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA; cat. no. A63881) to generate fragments of approximately 320 bp.¹⁶

Libraries were indexed using unique dual-Indexed (UDI) primers (Roche; cat. no. 09134336001) to minimize index hopping and cross-sample contamination and amplified by ligation-mediated PCR for 6 cycles. Library concentration was quantified using Qubit and size distribution and quality analysed using an Agilent 2100 Bioanalyzer with DNA High Sensitivity chips (Agilent Technologies, Santa Clara, CA, USA cat. No. 5067-4626). Sample results are presented in Appendix Figures 11-9 and 11-10.

Target enrichment was performed separately for each panel

(1) Roche panel capture

Libraries were hybridised to the Roche biotinylated probe pool using the KAPA HyperCap workflow v3.0 in pools of 12 samples. Captured libraries

¹⁶ I am grateful to Marlen Metzner who guided me through library preparation and hybridization and performed the AMPure bead clean-up.

were amplified by 16 PCR cycles using KAPA HiFi HotStart DNA polymerase (Roche; cat. no. KK2602) and purified using AMPure XP Beads.

(2) IDT panel capture

Libraries were hybridised to the IDT probe pool using the xGen Hybridization workflow, also in pools of 12 samples. Captured libraries were amplified by 10 PCR cycles using KAPA HiFi HotStart DNA polymerase and the xGen Library Amplification Primers Mix (IDT; cat. no. 1077675) followed by AMPure XP bead purification.

Post-capture libraries were quantified and assessed for size and quality using Qubit and Bioanalyzer. Libraries were pooled in equimolar concentrations to a final concentration of 4 nM and sequenced on an Illumina NextSeq 500 using 2 × 75 bp paired-end reads targeting approximately 1000 reads per base pair within targeted regions. Six sequencing runs were performed in total. A summary of the wet-lab workflow is presented in Figure 8-6

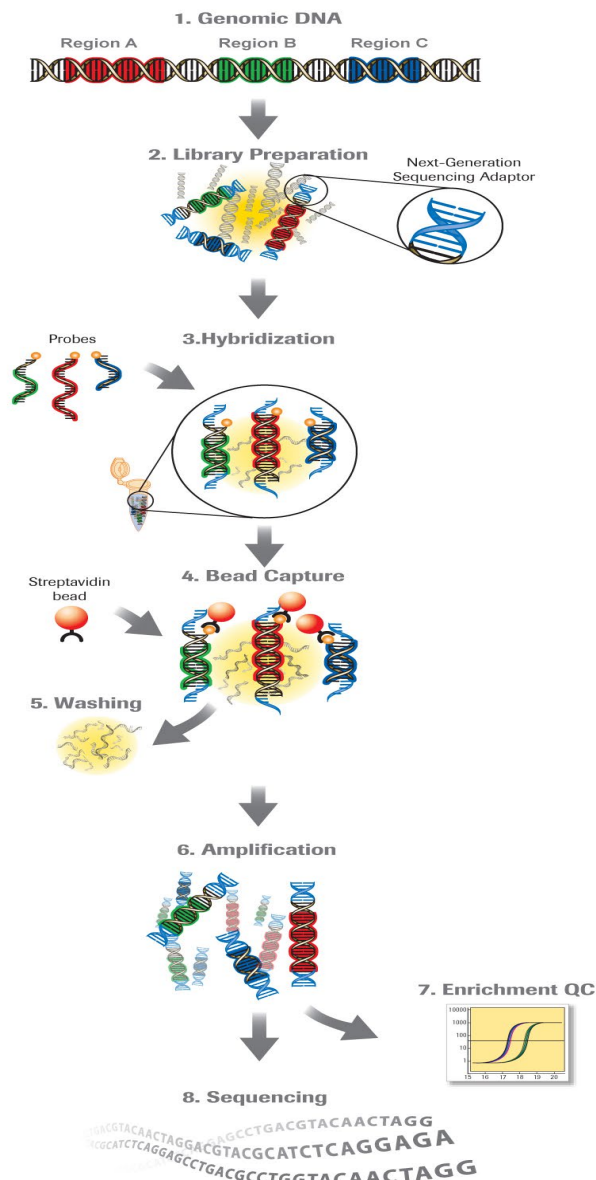


Figure 8-6. Schematic overview of the wet-laboratory workflow: (1) genomic DNA extraction (2) enzymatic fragmentation, end repair, A-tailing and adapter ligation (library preparation); (3–4) hybridisation of libraries to biotinylated capture probes and capture with streptavidin-coated beads; (5) washing to remove non-specifically bound fragments; (6) post-capture PCR amplification; (7) library quality control; (8) equimolar pooling and next generation sequencing. Image courtesy of Niels Asger Jakobsen

8.2.5 Initial germline & somatic variants bioinformatics workflow¹⁷

Sequencing data were analysed in custom pipelines based on the GATK Best Practices⁴⁴⁵ (GATK v4.3.0.0 and Picard v2.23.0). Full details of germline and somatic pipelines are available at: <https://github.com/hinks-lab/CHIP-project-pipeline/>.

Raw sequencing reads were converted to unmapped BAM files and adapter sequences were soft-clipped using Picard *MarkIlluminaAdapters*. Reads were then converted back to FASTQ format and aligned to the hg38 human reference genome assembly using the Burrows–Wheeler Aligner (BWA v0.7.18) with alternate contig-aware alignment enabled. Mapped and unmapped BAM files were merged using *MergeBamAlignment* and reads from different sequencing lanes were combined. Duplicate reads were marked using Picard *MarkDuplicates* and base quality scores recalibration (BQSR) was performed using GATK *BaseRecalibrator* and *ApplyBQSR*. Target enrichment metrics and coverage statistics were calculated using Picard *CollectHsMetrics* and custom scripts.

8.2.6 Germline rare variant analysis bioinformatics workflow

Germline single-nucleotide variants (SNVs) and small insertions and deletions (indels) were identified using *HaplotypeCaller* (GATK v4.3.0.0) following established best practices. Following alignment, duplicate removal and BQSR variants were

¹⁷ I acknowledge the considerable and indispensable assistance of Noah Tom Jones in production of the upstream germline and somatic pipelines which we wrote together.

called in genomic VCF (gVCF) mode on a per-sample basis to retain genotype likelihoods across both variant and non-variant sites. Individual gVCFs were subsequently combined and jointly genotyped across all samples using *GenotypeGVCFs* to ensure consistent genotype assignment and to improve sensitivity for low-frequency variants.

Variant quality score recalibration (VQSR) was applied to distinguish true variants from sequencing artifacts using high-confidence truth and training datasets. These included, HapMap v3.3 (very high-confidence SNPs) Omni 2.5M SNP array data (high-confidence SNPs), 1000 Genomes Phase 1 high-confidence SNPs (to improve model generalization) and dbSNP (to label known versus novel variants). VQSR was also performed in indel mode using Mills and 1000 Genomes Gold Standard Indels as training data, with dbSNP used for annotation only. A Gaussian mixture model was trained to assign VQSLOD scores. Variants were subsequently annotated using ANNOVAR,⁴⁴⁷ variant effect predictor (VEP⁴⁴⁸), SnpSift,⁴⁴⁹ and gnomAD v4.1.0.

8.2.6.1 Post VQSR filtering

Variants falling within the most permissive VQSR tranche (VQSR99.90to100.00) corresponding to approximately 0.1% of the truth set, were removed, accounting for 2.8% of all called variants. Additional hard filters were applied to exclude variants with (i) an alternative allele depth of <10 and/or (ii) an alternative allele Phred-scaled quality score <40.

8.2.6.2 Alignment of targeted sequencing with gnomAD v4.1 exomes for rare variant gene burden testing

Alignment of targeted sequencing with gnomAD v4.1 exomes followed a process similar to that described in Guo et al. (2018) “Burden testing of rare variants identified through exome sequencing via publicly available control data.” Summary variant data from gnomAD v4.1 exomes corresponding to each base pair position covered by the targeted sequencing were obtained using (i) publicly available data from gnomAD v4.1.0 exome data (ii) BED files defining the targeted regions, and (iii) Hail v0.2.130.

gnomAD v4.1 exome data were annotated using ANNOVAR, VEP and SnpSift in parallel with the targeted sequencing data and filtered to include only the non-Finnish European population for compatibility. Both datasets were divided into the 203 target genes (and the IL-5 promoter region) and analysed jointly on a per-gene basis using R v4.3.0. Analyses were restricted to exonic regions and variants with germline allele frequency > 0.001 were excluded.

Variants annotated by VEP v112 as synonymous and presumed neutral with respect to protein function (but comparably rare) were counted within each gene for LOE asthma cases, healthy controls and gnomAD v4.1 exome controls to assess the risk of type 1 error. Rare damaging variants were defined as those annotated by VEP as having a “HIGH” impact on protein coding (including transcript ablation, splice donor or acceptor variants, stop gained, frameshift variants, stop lost, and start lost) and were counted to generate gene-level burden estimates.

As only an estimated ~25% of missense variants are strongly deleterious, ~50% weakly deleterious and ~25% neutral^{450 451} only missense variants with a combined annotation dependent depletion (CADD⁴⁵²) Phred score¹⁸ ≥ 20 – placing them in the predicted 1% most deleterious variants – were included. Visualisation of the variant distribution and protein domain localization was performed using Integrated Genomics Viewer (IGV) v2.19.7. An example of a rejected variant is shown in Figure 8-7, with examples of accepted variants provided in Appendix Figures 11-11 and 11-12.

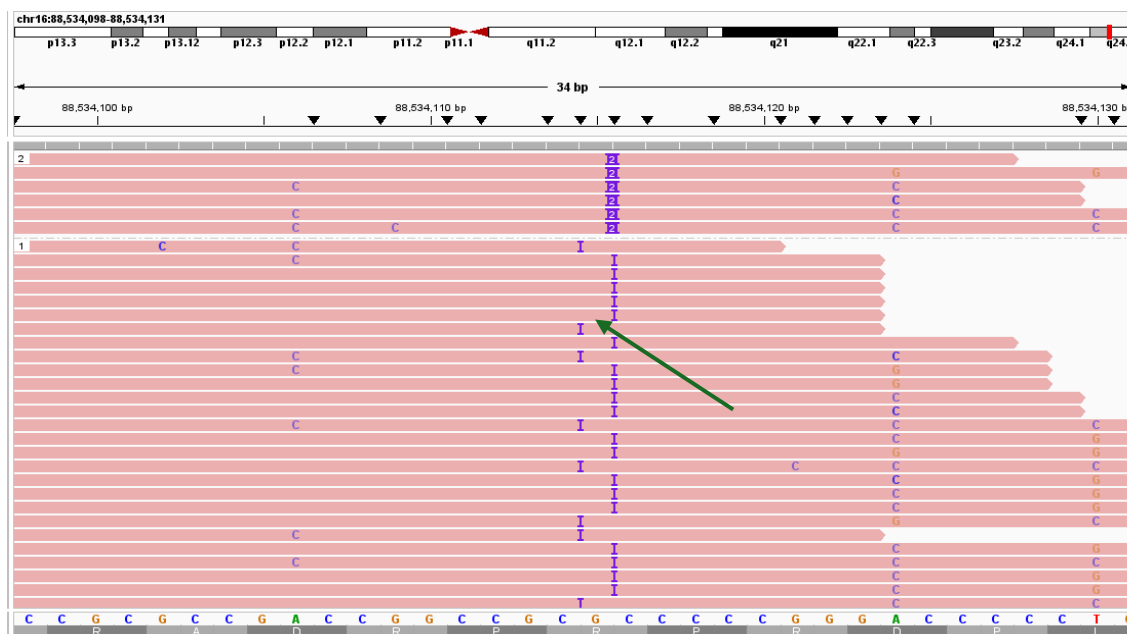


Figure 8-7. Example of a putative germline mutation call rejected following unsatisfactory visual inspection of the BAM file in Integrative Genomics Viewer (IGV). A putative G>GC single base pair frameshift insertion at chr 16:88534115 in the ZFP1 gene is indicated by the purple symbol **I** (green arrow). The variant was rejected as likely artefact for several

¹⁸ The CADD Phred score is a scaled version of the raw CADD measure of deleteriousness.

reasons; (i) insertions are present at different genomic positions on multiple other reads (ii) additional single-base mutations are observed on reads supporting the insertion, with variability across these reads (iii) there is a strong strand bias, with all the reads shown mapping to the forward strand (forward-strand reads in background pink) (iv) the variant occurs within a GC rich region. A subset of total reads is shown.

8.2.7 Clonal haematopoiesis analysis bioinformatics workflow

Clonal haematopoiesis variant analysis pipelines closely followed the procedure described by Jackobsen et al 2024⁴⁰¹. Somatic variants were called from the pre-processed BAM files using VarDictJava v1.7.0 and Mutect2 in tumour-only mode. For VarDict, variants were called with a minimum variant allele frequency (vAF) of 0.01, a minimum base quality score of 25, and a minimum of two supporting reads, with indel realignment and adapter removal enabled. For Mutect2, a minimum tumour LOD score of 1 was applied, and variants were filtered using *FilterMutectCalls* and *FilterByOrientationBias* to remove context dependent artefacts. Indels were left-aligned and normalized using bcftools (v1.17). Variants were annotated using ANNOVAR. VarDict and Mutect2 calls were analysed independently to derive a consensus of high-confidence variants.

Post-processing filters for VarDict calls included:

- (1) A minimum of 5 variant-supporting reads for SNVs (with ≥ 2 reads on each strand), or 10 reads for indels (with ≥ 4 reads on each strand).
- (2) A minimum base quality score of 30.

- (3) A minimum mapping quality score of 40, except for variants in *U2AF1/U2AF1L5*, where mapping quality was ignored due to duplication of *U2AF1* on chromosome 21 in hg38.
- (4) A maximum strand bias Fisher's exact test p-value of 0.0001.
- (5) A mean variant position ≥ 12 base pairs from read end.
- (6) Exclusion of variants with ≥ 2 supporting reads in any panel of normals.

Post-processing filters for Mutect2 calls included:

- (1) Passing all default Mutect2 filters or only failing only the *clustered_events* filter.
- (2) The same read support and strand balance requirements as VarDict (above).
- (3) A mean variant position ≥ 12 base pairs from read end.
- (4) Exclusion of variants with ≥ 2 supporting reads in any panel of normals.

Variants were flagged as likely sequencing artefacts and removed if they were:

- (i) Recurrent within the cohort unless reported ≥ 5 times in COSMIC v97 or ≥ 2 times in a curated list of clonal haematopoiesis-associated mutations compiled from five large studies.^{403 408 411 453 454}
- (ii) Present in a panel of normal cord blood samples (Jakobsen et al 2024).⁴⁰¹

(iii) VarDict called variants with a microsatellite instability score ≥ 4 unless reported in COSMIC

Variants were flagged as likely germline and removed if they:

(i) had a non-Finnish European allele frequency >0.001 and were not recurrent COSMIC or present in the curated list of clonal haematopoiesis-associated mutations.

(ii) exhibited a variant allele frequency between 0.4-0.6 or >0.9 unless recurrent in COSMIC ≥ 5 times or present in the curated list of clonal haematopoiesis-associated mutations.

(iii) were independently identified as germline by the germline pipeline unless recurrent in COSMIC ≥ 5 times or present in the curated list of clonal haematopoiesis-associated mutations.

Visualisation of coding variants passing all filters was performed using IGV.

8.2.7.1 Annotation of driver variants

Samples were annotated as having clonal haematopoiesis based on the presence of driver mutations in the exonal regions of genes in the Roche panel. Variants were classified as driver mutations according to the following criteria:

(1) Truncating mutations (e.g., missense, splice site or frameshift indel) in the following genes: DNMT3A, TET2, ASXL1, NF1, IKZF1, RAD21, WT1, KMT2D, SH2B3, TP53, CEBPA, RUNX1, BCOR, KDM6A, STAG2, PHF6, KMT2C, KMT2E, PPM1D and ZRSR2.

- (2) Truncating mutations in other panel genes if falling within annotated Interpro domains.
- (3) Truncating variants in *CALR* exon 9.
- (4) Non-synonymous variants at the following hotspot residues: CBL E366, L380, C384, C404, R420 and C396; DNMT3A R882; FLT3 D835; IDH1 R132; IDH2 R172 and R140; JAK2 V617F; KIT W557, V559 and D816; KRAS A146, Q61, G13 and G12; MPL W515; NRAS Q61, G12 and G13; SF3B1 K700 and K666; SRSF2 P95; U2AF1 Q157, R156 and S34.
- (5) Non-synonymous variants occurring within the following residues of *DNMT3A*: p.292-350, p.482-614 and p.634-912; or *TET2*: p.1104-1481 and p.1843-2002.
- (6) Other non-synonymous variants with a variant allele frequency <40% if occurring in InterPro domain *or* reported ≥ 5 times in COSMIC
- (7) Other non-synonymous variants if reported >100 times in COSMIC.
- (8) *FLT3* internal tandem duplication.

8.2.8 Statistical analyses

Statistical analyses were performed using R v4.3.0. Figures were generated using ggplot2 v3.5.2 and GraphPad Prism version 10.4.2. Normally distributed data are presented as mean ($\pm$ SD); log-normally distributed data as geometric mean (coefficient of variation, %); and non-normally distributed data as median (interquartile range, IQR).

8.2.8.1 Germline rare variant burden testing

All 30 healthy control participants were used as controls in germline analyses. Gene burden testing for LOE asthma patients versus gnomAD v4.1 non-Finnish European controls followed the approach of Guo et al.³⁷⁵ and Chen et al.³⁷⁴ and was confirmed with the author of Chen et al. (Wenan Chen, personal communication, March 8, 2024) For each comparison a 2 x 2 contingency table was constructed using: (i) the total number of alternate alleles within a gene and (ii) half the total allele number (AN/2).

All gene burden comparisons were performed using Fishers exact test. Results were assessed against a Bonferroni-corrected significance threshold for $\alpha = 0.05/99$ tests ≤ 0.00053 [95 IDT panel genes plus JAK2, KIT, GATA3 and STAT5B from the Roche panel]. The genomic control metric λ_{GC} was calculated for each set of tests to monitor statistic inflation or deflation. λ_{GC} was calculated as the median of observed test statistics divided by the median of expected statistics under the null hypothesis (0.456).

Variants within the IL-5 promoter region (Chr5: 132069001–13273907), not covered by gnomAD v4.1 exomes, were analysed separately using per-variant 2 x 2 contingency tables, with a Bonferroni corrected significance threshold of 0.0014 for 35 tests.

8.2.8.2 Driver mutation (clonal haematopoiesis) testing

Comparisons of driver mutation prevalence at $vAF \geq 1\%$ and $\geq 2\%$ between LOE asthma patients and healthy controls were performed using Fisher's exact test on 2 x 2 contingency tables. Demographic comparisons were performed using Welch's t-test due to unequal group sizes. Given the unequal group sizes of LOE asthma patients and healthy controls and the established importance of age to clonal haematopoiesis, a pre-specified sub-analysis will be performed for those age 40–70 years only, where there is the greatest overlap in age between groups.

8.3 Results

A total of 123/127 (96.8%) LOE asthma patients and 25/25 (100%) aged-matched healthy controls (HC) and 5/5 (100%) panel-of-normals (PON) controls recruited to the study self-identified as White British. As no sequencing control data were available for non-White British participants, samples from the 4 non-White British LOE asthma patients were not forwarded for genetic testing in order to avoid confounding effects of ancestry in the interpretation of germline and somatic variants. One sample (ID:'C16') failed sequencing quality control on the Roche panel (Appendix Figure 11-15) and was therefore excluded from subsequent analyses.

Baseline parameters for the late onset eosinophilic asthmatics, healthy control participants and panel of normal controls are presented in Table 8-2. There was no statistically significant difference in age between the LOE asthma group and healthy control group (Welch's t (39.5) = 1.79, p = 0.08).

8.3.1 Participant baseline parameters

Table 8-2. Participant baseline parameters

	LOE asthmatics n=123	Healthy Controls n = 25	Panel of normals n = 5
Age (years)	59.4 (±12.1)	55.9 (±9.9)	27.5 (±2.8)
Age of onset symptoms (years)	46.3 (±15.0)	n/a	n/a
Male : Female (n)	64 : 59	14 : 11	1 : 5
Nasal polyposis (yes : no)	36 : 87	0 : 25	0 : 6
Total IgE (IU/mL)†	89.2 (100%)	18.8 (160%)	19.9 (61%)
Highest blood eosinophils (x10 ⁹ /L)†	0.88 (60%)	n/a	n/a
FeNO on day (ppb)†	50.2 (±1.7)	18.8 (±0.9)	13.5 (±1.5)
Maintenance prednisolone (n, %)	22 (17.8)	n/a	n/a
Daily dose prednisolone (mg) ‡	12.5 (8.1–15.1)	n/a	n/a
Smoking history, pack years ‡	0 (0–4.3)	0 (0–0.9)	0 (0–0)

Values given as mean ± SD, except except † = geomean (coefficient of variation, %) and ‡ = median (IQR). Abbreviations: FeNO, fractional exhaled nitric oxide; IgE, immunoglobulin E.

The 40–70 years subset included 92% of aged-matched healthy controls and 77% of LOE asthma patients (Figure 8-8). Within this age range the mean age was 57.4 ± 7.7 for LOE asthma patients and 55.6 ± 8.6 for healthy controls.

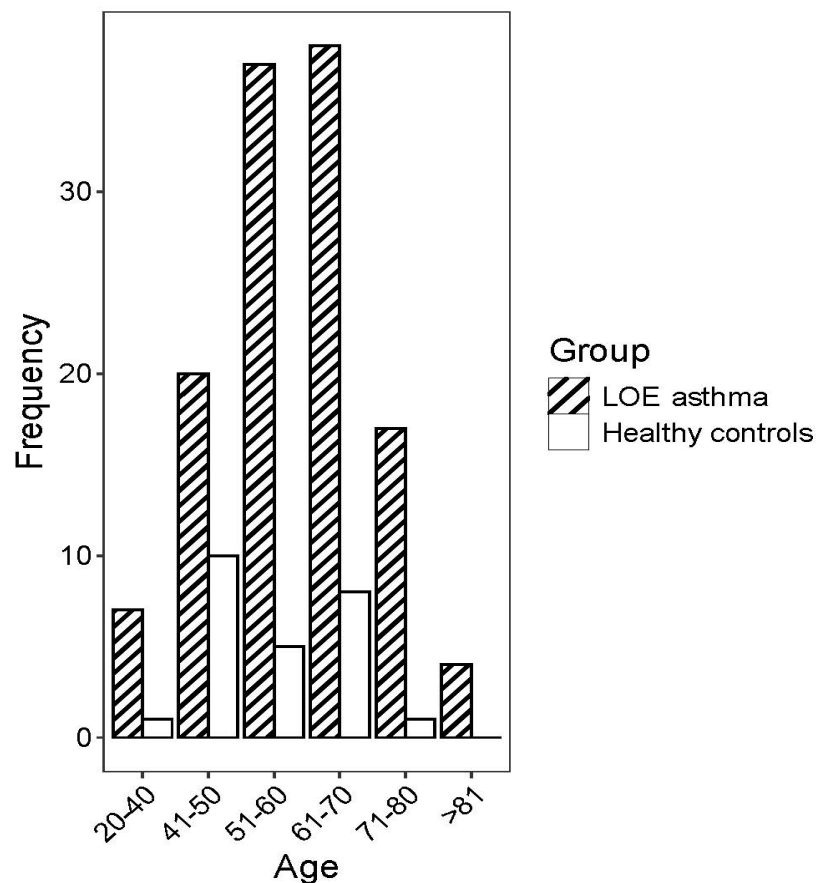


Figure 8-8. Histogram showing the age distributions of LOE asthma patients and healthy control participants. Because age is a key correlate of clonal haematopoiesis, statistical comparisons were additionally performed in the 40–70 year age range where the two groups are most closely matched.

8.3.2 Sequencing base and coverage quality control

Base call quality for the IDT panel showed that > 89% of base calls had a Phred score >30 (corresponding to 99.9% accuracy) while for the Roche panel >81% of calls achieved a Phred score >30 (Appendix Figure 11-13). Mean coverage across all base pairs targeted by the probes – including intronic and intergenic areas flanking targeted exons – is illustrated by gene and by sample for both panels in Appendix Figures 11-14 and 11-15. Mean coverage of high-quality reads within exonic regions was 1387 ($\pm$ 430) reads per base pair for the IDT panel and 987 ($\pm$ 416) reads per base pair for the Roche panel.

8.3.3 Germline rare variant analysis

Read depth in the targeted sequencing panels was substantially higher than in the corresponding regions of gnomAD v4.1 exomes, reflecting the additional depth required to sensitively detect somatic mutations (Figure 8-8). The mean quality-by-depth (QD; Phred-scaled confidence that a variant exists at a given site divided by the total read depth contributing to the variant call) in the targeted sequencing was 14.2 ± 5.6 . By comparison, the mean QD in gnomAD v4.1 exomes across the same sites was 12.1 ± 3.9 .

Similarly, the mean variant quality score log odds (VQSLOD) in the targeted sequencing was 14.1 ± 8.5 , compared with 5.2 ± 1.9 in directly comparable regions of gnomAD v4 exomes (Figure 8-9).

More than 96% of individuals in the non-Finnish European population of gnomAD 4.1 exomes had $\geq 20\times$ coverage across the exonal regions targeted by the sequencing panels. Given this level of coverage, together with the higher mean QD and VQSLOD observed in the targeted sequencing data – particularly after exclusion of variants in the VQSR 99.90to100.00 tranche – no down-sampling of the gnomAD dataset was performed for harmonization. This approach ensured complete representation of all callable sites from gnomAD and resulted in a more conservative statistical test, consistent with previously published methodologies.³⁷⁵

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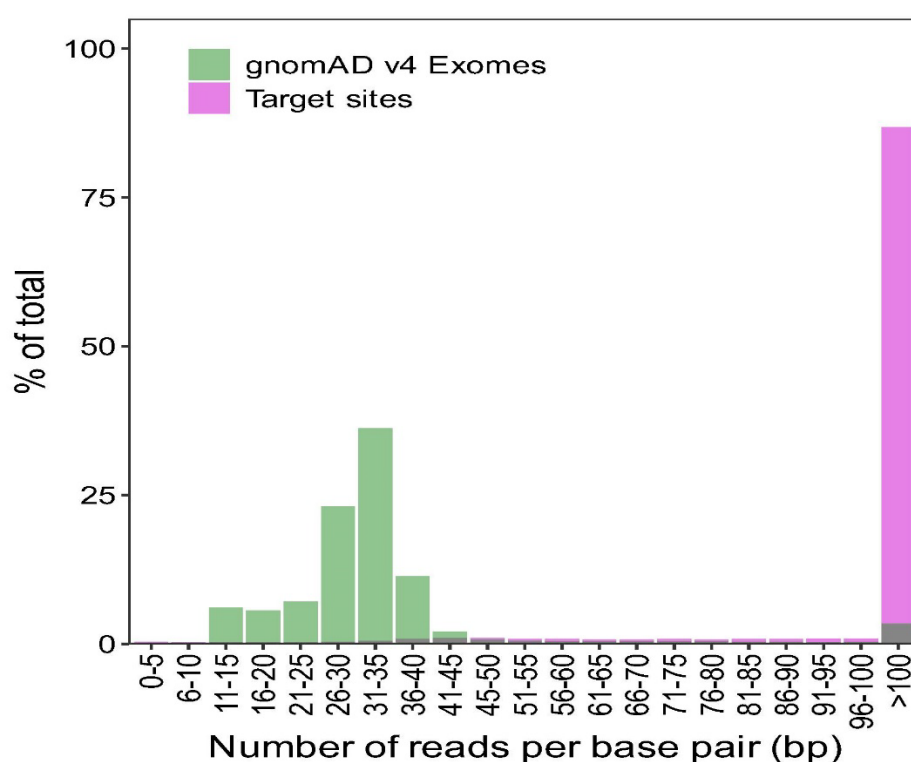


Figure 8-9. Histogram displaying read depth per base pair for the targeted sequencing performed in this study and for aligned sequencing data from gnomAD v4.1 whole exomes. Reads in both datasets include only those passing all quality control steps. Darker shaded areas indicate areas of overlap between the two datasets. Note that this figure represents coverage across all base pairs aligned to the sequencing panel, rather

than coverage restricted exclusively to exonic regions, which was higher in both the targeted sequencing data and the gnomAD 4.1 exomes.

Comparison of the gene burden of rare exonic synonymous variants (variant allele frequency < 0.001) between LOE asthma and gnomAD controls did not reveal any systematic differences. The absence of differences among synonymous ‘neutral’ variants is reassuring of a low probability of ‘over-call’ when assessing gene burden of deleterious variants. The overall burden of rare synonymous mutations in the two populations across the sequenced regions was also very similar (136 mutations in 123 LOE asthma patients vs 602,722 mutations in 555,877 gnomAD NFE controls, $p = 0.75$). The genomic correction metric λ_{GC} was consistent with conservative testing parameters, indicating a low chance of type 1 error (Figure 8-10). The same parameters were therefore carried forward to testing the gene burden of rare, highly deleterious variants, including transcript ablation, splice donor or acceptor variants, stop gained, frameshift variants, stop lost, and start lost variants. There was no evidence that any gene harboured a significantly increased burden of these variants in either group (Figure 8-12).

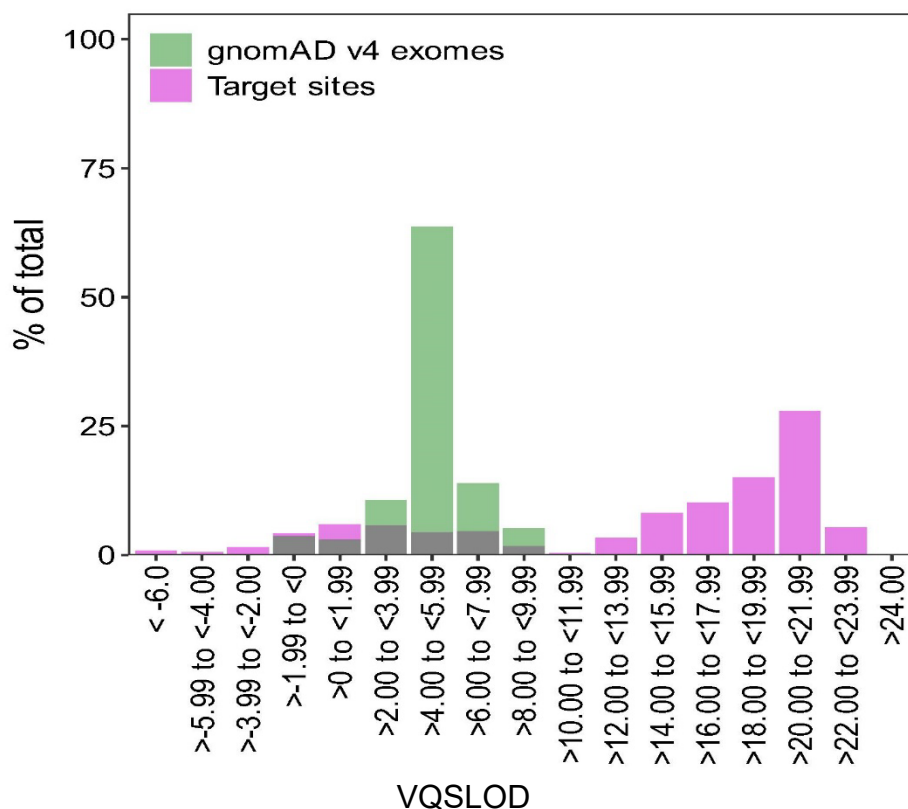


Figure 8-10. Distribution of variant quality score log odds (VQSLOD) for variants identified in targeted sequencing and in aligned regions of gnomAD v4.1.0 exomes. Darker shaded areas indicate overlap in VQSLOD scores between the two datasets. Variant calls falling within the lowest VQSLOD of targeted sequencing (VQSR 99.90to100.00) were subsequently excluded from downstream analyses. This represented 2.8% of called variants.

For missense variants with a CADD Phred score of ≥ 20 (i.e. predicted to be among the most deleterious 1% of variants), a higher burden was observed for the *IL17RA* gene in LOE asthma patients compared with controls ($p = 0.0007$, Figure 8-13). However, this finding did not meet the pre-specified Bonferroni-corrected significance threshold ($p = 0.00053$). Details of the *IL-17RA* missense variants identified in 6 LOE asthma patients are provided in Table 8-3.

When high impact variants were combined with the most deleterious missense variants, an increased burden for *IL17RA* was again observed for LOE patients (OR = 5.65, 95%CI: 2.5–12.8, $p = 0.0009$), but this result likewise did not reach Bonferroni corrected significance. The overall burden of high impact and deleterious missense variants across the sequenced regions was very similar for the two populations (151 mutations in 123 LOE asthma patients vs 634,657 mutations in 555,877 gnomAD NFE controls, $p = 0.18$). The genomic correction metric λ_{GC} for this comparison of 0.76 was consistent with conservative testing parameters. A comparison of high impact + deleterious missense variants for LOE asthma patients compared with the recruited healthy controls ($n=30$) is presented in Appendix Figure 11-16.

Gene burden of synonymous variants for LOE asthma versus gnomAD

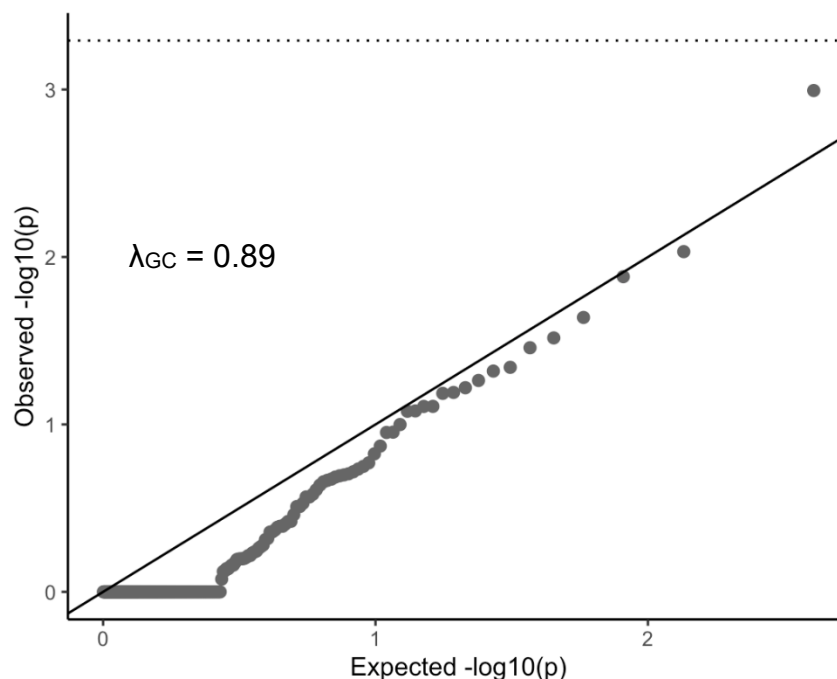


Figure 8-81. QQ plot comparing the gene burden of rare exonic synonymous variants between LOE asthma patients and a large population of non-Finnish European controls from gnomAD v4.1 exomes across genes targeted by the IDT and Roche panels. Each grey circle represents a gene. The dotted horizontal line indicates the Bonferroni corrected significance threshold ($p \leq 0.00053$). Synonymous variants are considered a near neutral class of mutation and can therefore be used to assess the risk of type 1 error. There is no evidence of an excess burden of synonymous variants in the targeted gene set, and the genomic control metric λ_{GC} of 0.89 indicates a moderately conservative test.

A higher gene burden was also observed for LOE asthma patients in the *THRA* gene on 17q21.1, although this also failed to meet the Bonferroni corrected significance threshold (OR = 32.0, 95%CI: 7.9–130, $p < 0.002$). Notably, *THRA* was not a gene explicitly targeted by either sequencing panel. One frameshift mutation and one missense mutation with CADD Phredd score ≥ 20 were identified in two LOE asthma

cases (Table 8-4). Both variants were located within exon 10 of *THRA*, which was captured incidentally by the Roche panel due to probe overlap with the 5'UTR region of the neighbouring *NR1D1*. The *THRA* (thyroid hormone receptor alpha) gene has previously been implicated in asthma and the potential biological implications of this finding are explored further in the Discussion section.

Gene burden of high-impact variants for LOE asthma versus gnomAD

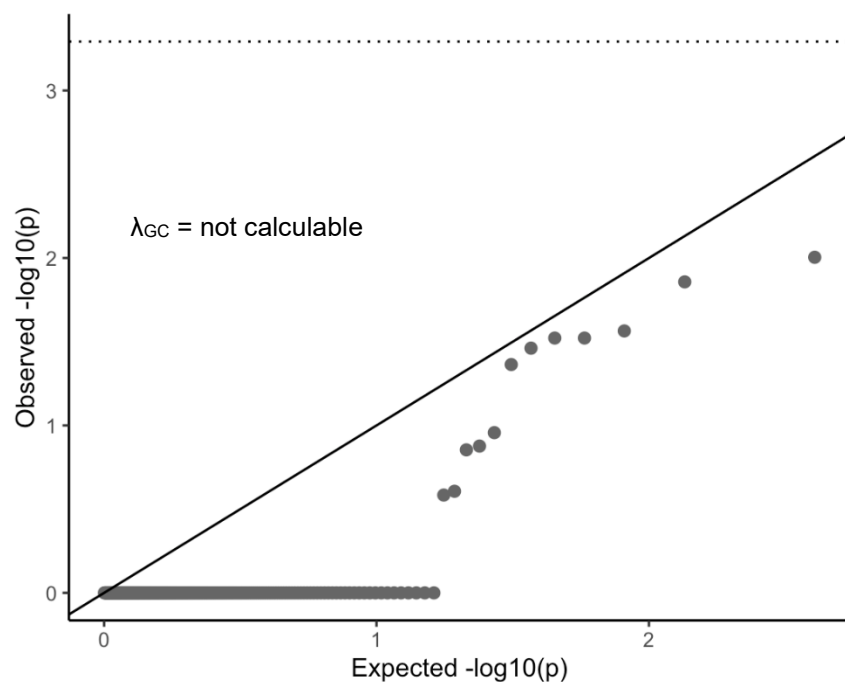


Figure 8-92. QQ plot comparing the gene burden of the most protein-deleterious classes of variants (including transcript ablation, splice donor or acceptor variants, stop gained, frameshift variants, stop lost, and start lost variants) between LOE asthma patients and gnomAD controls. No significant associations were observed. The λ_{GC} could not be reliably calculated due to the extreme rarity of alleles in the above classes with variant allele frequency < 0.001.

Gene burden of missense variants (CADD Phred ≥ 20)

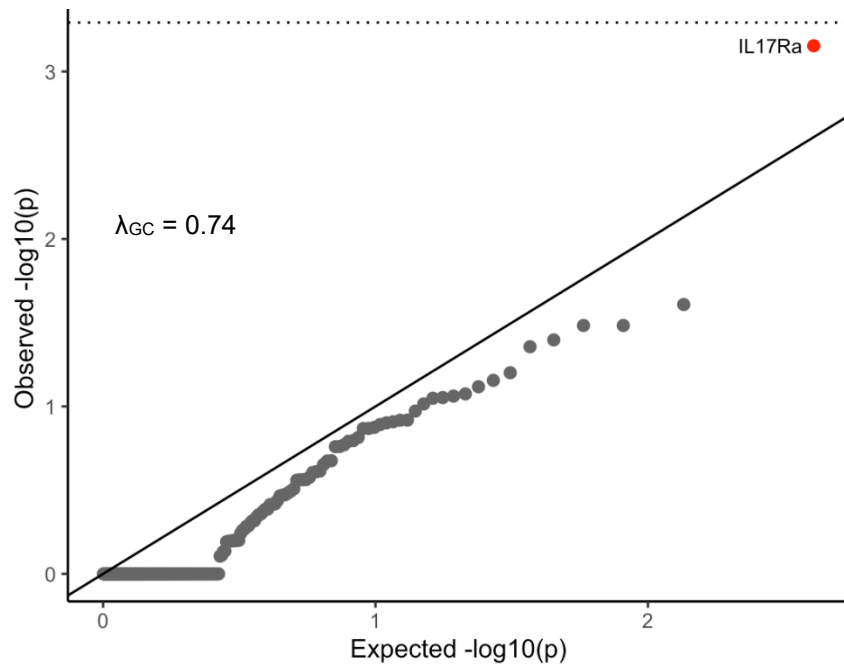


Figure 8-103. QQ plot comparing the gene burden of missense variants with a CADD score ≥ 20 (i.e. missense variants predicted to be among the most deleterious 1% of variants) between LOE asthma patients and gnomAD controls. The most significant association was observed for *IL-17RA* ($p = 0.0007$) but this did not reach the Bonferroni-corrected significance threshold ($p \leq 0.00053$). The genomic control metric λ_{GC} of 0.74 indicates that the distribution of observed test statistics is more conservative than expected under the null hypothesis.

A total of 35 variant sites were found within the IL-5 promoter region (Chr5: 132069001–13273907). These are not covered by gnomAD v4.1 exomes and were analysed separately per variant site using 2 x 2 contingency tables for LOE asthma patients versus the 30 healthy controls and a Bonferroni corrected significance threshold of 0.0014 for 35 tests. There was no evidence of a statistically significant burden at variant sites. There was also no evidence of increased total burden for

either group across the IL-5 promoter region. Because multiple events occurred within individuals, event rates were compared using a count-based regression framework rather than raw event totals. Event rates per individual were similar between groups (mean events per person: LOE asthma 15.1 vs healthy controls 15.3), and there was no evidence of a statistically significant difference in event rates between groups.

Gene burden of high-impact & missense variants (CADD Phred ≥ 20)

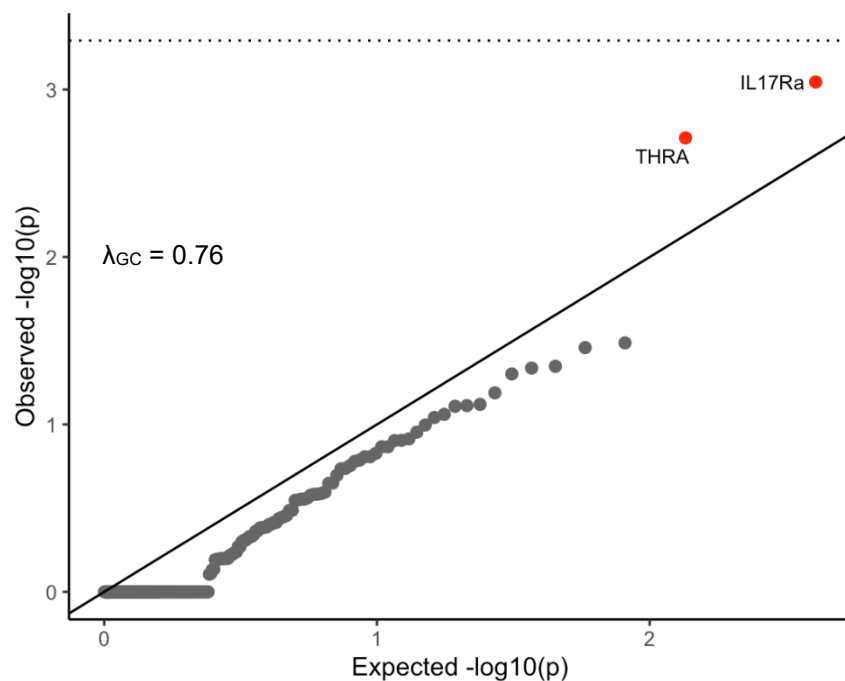


Figure 8-14. QQ plot comparing the gene burden of combined high impact variants (e.g., transcript ablation, splice donor or acceptor variants, stop-gained, frameshift, stop-lost, and start-lost variants) together with missense variants with a CADD Phred score ≥ 20 between LOE asthma patients and gnomAD controls. The most significant findings were for *IL-17RA* ($p = 0.0009$) and *THRA* ($p = 0.002$); however, neither reached the Bonferroni-corrected significance threshold ($p \leq 0.00053$). The λ_{GC} value of 0.76 indicates that the distribution of observed statistics is more conservative than expected under the null hypothesis.

Table 8-3. Missense mutations identified in the *IL17RA* gene

Reference genome GRCh38/hg38

Chr	Pos	Ref	Alt	vAF	Transcript(s) ID	Exon	codingDNA	Amino acid	Popmax AF
22	17100413	T	C	0.46	ENST00000319363.10	5	c.482T>C	p.Val161Ala	0.0001
					ENST00000612619.1	5	c.482T>C	p.Val161Ala	
22	17108441	A	T	0.48	ENST00000319363.10	13	c.1222A>T	p.Thr408Ser	0.00001
					ENST00000612619.1	12	c.1120A>T	p.Thr374Ser	
22	17109077	C	T	0.49	ENST00000319363.10	13	c.1858C>T	p.Arg620Trp	0.00004
					ENST00000612619.1	12	c.1756C>T	p.Arg586Trp	
22*	17109702	T	A	0.50/0.50	ENST00000319363.10	13	c.2483T>A	p.Leu828His	0.0005
					ENST00000612619.1	12	c.2381T>A	p.Leu794His	
22	17109076	G	C	0.48	ENST00000319363.10	13	c.1857G>C	p.Lys619Asn	0.000009
					ENST00000612619.1	12	c.1755G>C	p.Lys585Asn	

*2 patients with this mutation Abbreviations: Chr, chromosome; Pos, position; Ref, reference allele, Alt, alternate allele; codingDNA, coding DNA change; Amino acid, amino acid substitution, Popmax AF, maximum population variant allele frequency across genetic ancestry groups in gnomAD v4.1.0

Table 8-4. High-impact mutations identified in *THRA*

An A>G frameshift mutation and a G>A missense mutation were identified coincidentally in the gene *THRA* for 2 patients in the LOE asthma cohort, respectively. Reference genome GRCh38/hg38

Chr	Pos	Ref	Alt	vAF	Transcript(s) ID	Exon	codingDNA	Amino acid	Popmax AF
17	40093306	AG	A	0.51	ENST00000264637.8	10	c.1399delG	p.Ala467fs	0.00001271
					ENST00000584985.5	10	c.1282delG	p.Ala428fs	
17	40093234	G	A	0.48	ENST00000264637.8	10	c.1325G>A	p.Arg442His	0.00002712

Abbreviations: Chr, chromosome; Pos, position; Ref, reference allele; Alt, alternate allele; codingDNA, coding DNA change; Amino acid, amino acid substitution, Popmax AF, maximum population variant allele frequency across genetic ancestry groups in gnomAD v4.1.0

8.3.4 Clonal haematopoiesis analysis

The mean tumour log odds (TLOD), representing the likelihood that a variant is real rather than sequencing noise, for Mutect2 variant calls passing post processing filters was 666.4 ± 1499 . TLOD values ≥ 20 indicates high confidence calls, while values ≥ 30 indicate very high confidence.⁴⁵⁵ Following removal of recurrent variants and variants suspected to be germline (typically characterised by higher variant allele read counts) the mean TLOD decreased to 46.4 ± 73.6 .

For VarDict the mean quality score (QUAL) for calls passing post-processing filters was 272.4 ± 66.3 . QUAL scores >40 indicate high confidence somatic variants, and scores >100 indicate very strong read evidence.⁴⁵⁶ After removal of recurrent variants and suspected germline variants mean QUAL was 178.8 ± 72.5 .

8.3.4.1 CHIP mutation occurrence across groups

A total of 68 driver mutations with a variant allele frequency (vAF) $\geq 1\%$ were identified in LOE asthma patients and healthy controls using the targeted Roche gene panel. These are detailed in full in Appendix Table 11-8. Of these 68 mutations, 62 occurred in the 123 LOE asthma patients and 6 occurred in the 25 healthy controls (Figure 8-14). The most frequently observed mutations were in DNMT3A, followed by TET2 in keeping with previous studies across other populations.^{408 409 411}

⁴⁵⁴ Mutations within healthy controls were restricted to 4 genes (DNMT3A, TET2, BRINP3 and SMC1A) while mutations in LOE asthma patients were distributed across 31 genes. The odds of observing a driver mutation with vAF $\geq 1\%$ were

significantly higher in the LOE asthma group than in healthy controls (odds ratio [OR] = 3.22, 95% confidence interval [CI] 1.20–8.62; Fisher’s exact test, two-sided $p < 0.02$).

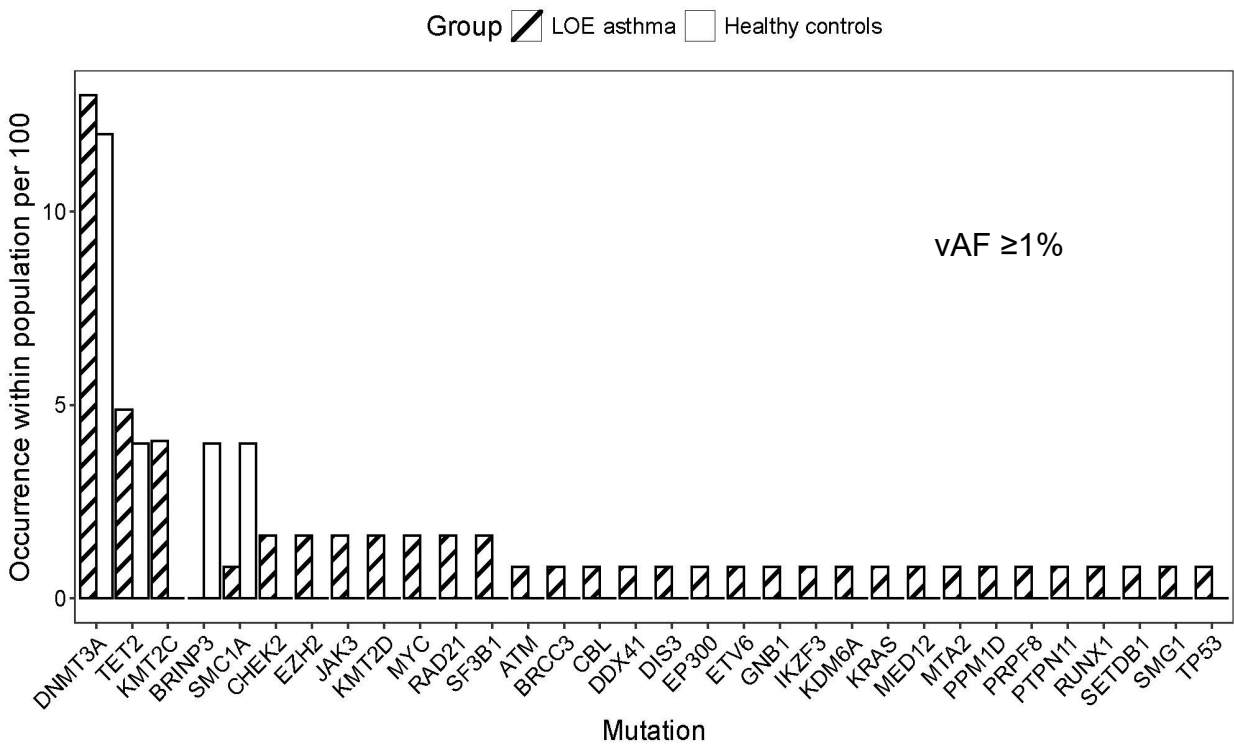


Figure 8-15. Occurrence of driver mutations with a variant allele frequency (vAF) of $\geq 1\%$ in LOE asthma patients and healthy controls. This includes all mutations across the total populations. The odds ratio of observing ≥ 1 driver mutation with vAF $\geq 1\%$ were significantly higher in the LOE asthma group compared with healthy controls (odds ratio 3.22, $p < 0.02$). Abbreviations: vAF, variant allele frequency.

At a higher threshold of vAF $\geq 2\%$ significantly more driver mutations were again observed in the LOE asthma population (26 mutations amongst 123 patients) compared with healthy controls (1 mutation amongst 25 participants) corresponding to an OR of 6.43 (95% CI 0.83–49.8; Fisher’s exact test, $p < 0.05$) (Figure 8-15).

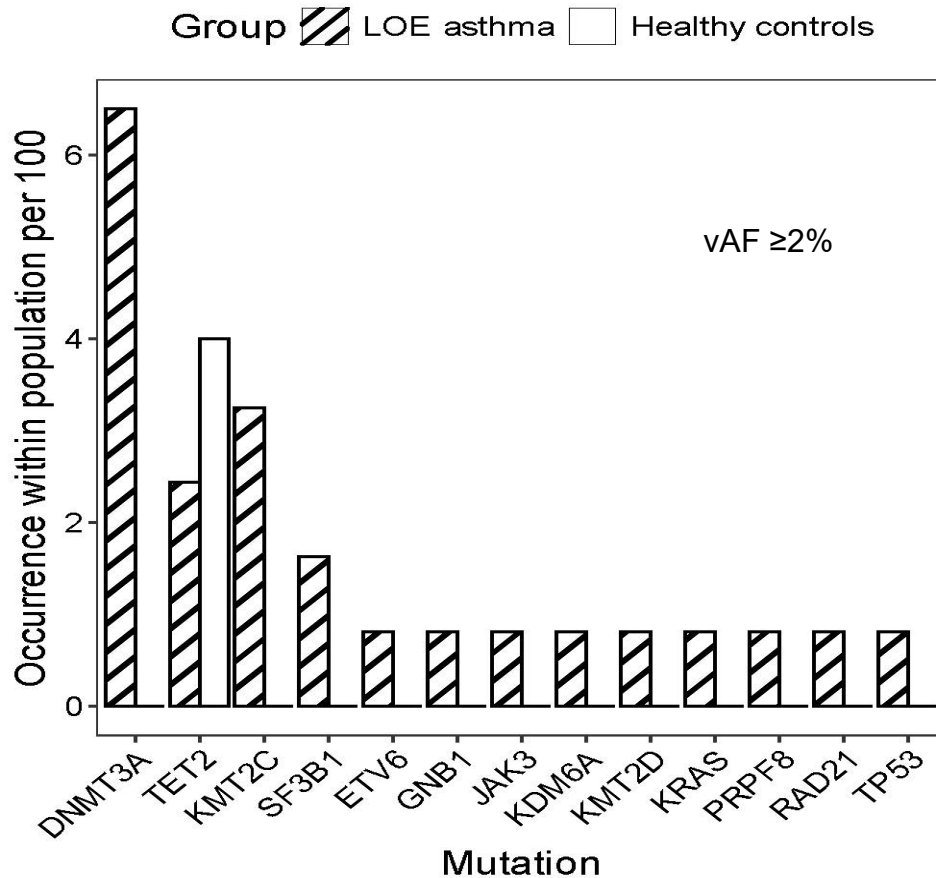


Figure 8-16. The Occurrence of driver mutations with a variant allele frequency (vAF) of $\geq 2\%$ in LOE asthma patients and healthy controls. The odds of observing a driver mutation at this threshold were significantly higher in the LOE asthma group compared with healthy controls (odds ratio 6.43, $p < 0.05$). Abbreviations: vAF, variant allele frequency.

8.3.4.2 CHIP driver mutation burden

The presence of 2 or more mutations within a single individual was observed exclusively in LOE asthma patients (12/123, 9.8%) and was not seen in healthy controls [0/25, 0%] (Figure 8-16). Of these LOE patients nine harboured two mutations and 3 harboured three driver mutations at $vAF \geq 1\%$. The odds ratio for having a mutation burden of ≥ 2 driver mutations was not estimable (infinite), due to

the absence of events in the control group, and the difference did not reach statistical significance (Fisher's exact test, two-sided $p = 0.22$). The mean age of LOE asthma patients with ≥ 2 mutations at $vAF \geq 1\%$ was 64.1 years with seven of these individuals being younger than 70 years. A Bland Altman analysis of agreement across vAF for paired clones at a threshold of $\geq 2\%$ showed good mean agreement within the vAF 2-3% range where the majority of clones cluster (mean agreement 0.05 (95% limits of agreement -0.12–0.22) but agreement breaks down above this vAF . Among the 9 LOE asthma patients with two CHIP mutations 3 had one DNMT3A mutation and 3 had one KMT2C mutation (including one individual with both a DNMT3A and KMT2C mutation). There were 2 individuals with mutations in MYC alongside another mutation. The remaining 2 individuals had combinations of mutations in different genes. Among the 3 LOE asthma patients with three CHIP mutations 2 of these 3 cases included mutations in TET2 while the third case was a patient with two separate mutations in DNMT3A (Appendix Table 11.8).

8.3.4.3 CHIP frequency within individuals

Overall, 47 of 123 LOE asthma patients (38.0%) harboured CHIP mutations at $vAF \geq 1\%$ compared with 6 of 25 healthy control participants (24.0%). This corresponded to an OR of 1.96 (95%CI: 0.73 to 5.26), which was not statistically significant (Fisher's exact test $p = 0.25$) (Figure 8-17). The mean age of patients with CHIP at $vAF \geq 1\%$ was 63.8 ± 10.8 in the LOE asthma group and 58.8 ± 9.3 in healthy controls; this difference was not statistically significant (Welch's t-test, $p = 0.26$).

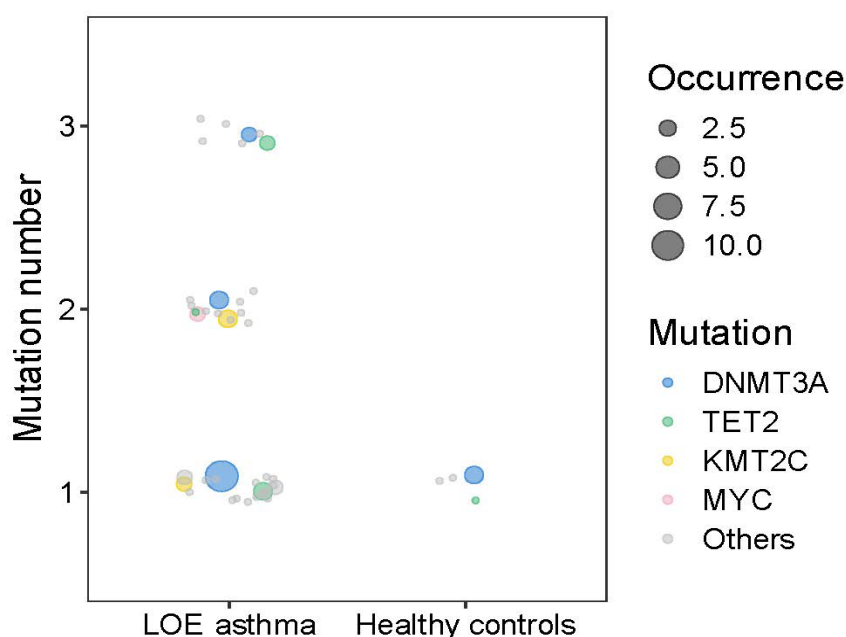


Figure 8-17. Mutation burden comparison between late onset eosinophilic (LOE) asthma and healthy control groups. Individual mutations are shown per participant with recurrent driver genes highlighted in colour (DNMT3A, TET2, KMT2C, MYC). Multiple mutations within the same individual are visualised, illustrating a higher mutation burden in the LOE asthma group compared with healthy controls.

Restricting the analysis to participants aged 40-70 years as pre-specified, CHIP at $vAF \geq 1\%$ was observed in 33 of 95 LOE asthma patients (34.4%) and in 4 of 24 healthy controls (16.7%). LOE asthma patients had numerically higher odds of CHIP than healthy controls, OR = 2.53 (95%CI: 0.79–8.05) although this difference did not reach statistical significance (Fisher's exact test, $p = 0.136$).

At a vAF threshold of $\geq 2\%$, 22 of 123 LOE asthma patients (17.9%) had CHIP compared with 1 of 25 healthy controls (4.0%). The odds ratio of having CHIP in the LOE asthma group was 5.23 (95%CI: 0.67–40.7), but this comparison was not statistically non-significant, (Fishers exact test $p = 0.127$) (Figure 8-17). The single

healthy control with CHIP at $\geq 2\%$ vAF was aged 67 years. In comparison, the mean age of LOE asthma patients with CHIP at $\geq 2\%$ was 65.4 ± 12.3 years.

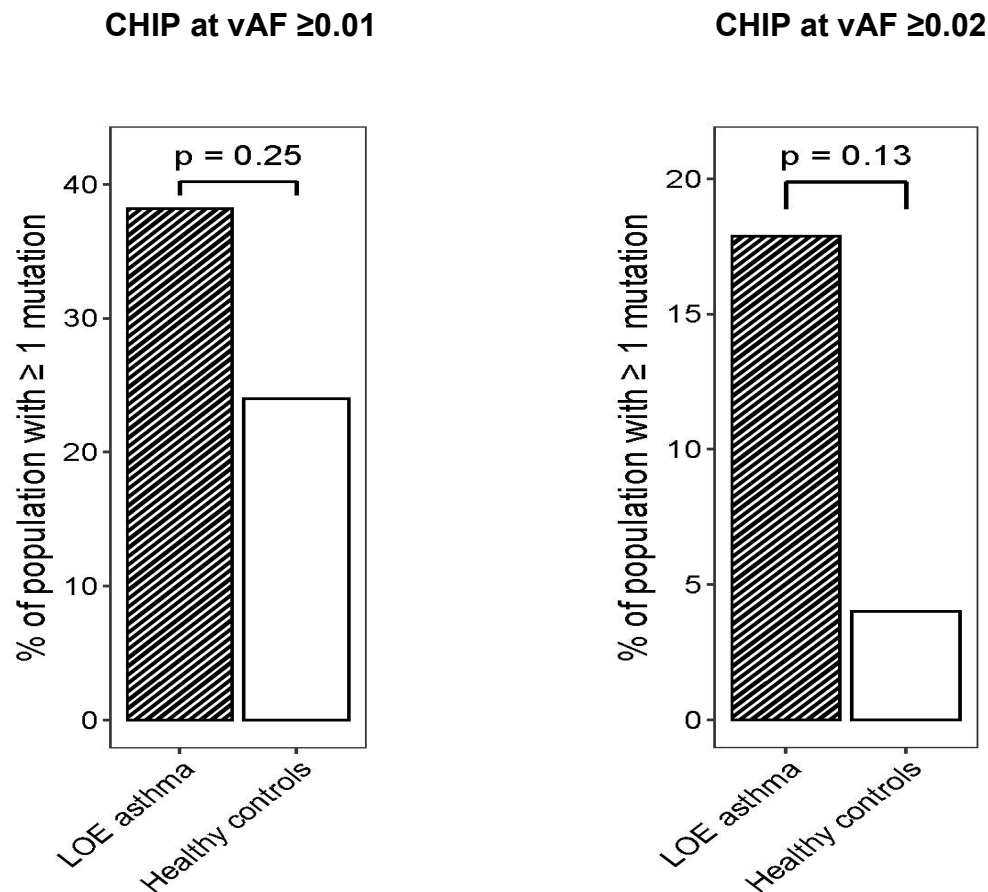


Figure 8-18. The prevalence of clonal haematopoiesis of indeterminate potential in late onset eosinophilic (LOE) asthma and healthy control individuals. There was a tendency towards a higher prevalence of CHIP in the LOE asthma group at a variant allele frequency threshold of $\geq 2\%$. However, this comparison was underpowered, partly owing to only a single event in the control group. Abbreviations: vAF, variant allele frequency.

When restricting the $\geq 2\%$ vAF analysis to participants aged 40–70 years, CHIP was observed in 14 of 95 LOE asthma patients and in 1 of 23 healthy controls 1/23 (OR = 3.8, 95%CI: 0.47–30.52; Fishers exact test, $p = 0.29$). The mean age of the 15 patients with CHIP at $\geq 2\%$ vAF in this age restricted group was 59.3 ± 10.7 .

Notably, there was one LOE asthma patient aged 28 years with a driver mutation at a vAF of 9% in the *RAD1* gene (see Appendix Table 11-8).

8.4 Discussion

8.4.1 Rare variant analysis

The main findings from rare variant burden analysis are:

- (i) It is possible to align a large dataset of controls such as gnomAD 4.1 exomes with the eosinophilic (IDT) and clonal haematopoiesis (Roche) targeted sequenced panels to produce a conservative test of rare variant gene burden. The legitimacy of this approach is supported by the non-significant differences for neutral synonymous variants across both datasets and the conservative (but acceptable) genomic constraint statistic of λ_{GC} of 0.89 for synonymous variant testing.
- (ii) There were no significant differences in gene burden identified for the rarest and most protein-damaging classes of variants ($vAF < 0.001$) which, it was proposed would have the largest disease effect. The most damaging classes of variants included transcript ablation, splice donor or acceptor variants, stop gained, frameshift variants, stop lost, and start lost variants and those missense variants in the 1% most deleterious variants by CADD Phredd score. The genomic constraint statistics for gene burden testing of these variants (λ_{GC} 0.74–0.76), suggests observed statistics were more conservative than expected under the null hypothesis and a

very conservative test. This is likely due to the limited number of variants of this rarity in the LOE asthma group and possibly also due to heterogeneity in the gnomAD control sample group.

- (iii) Six high-impact missense variants in *IL17RA* in patients with LOE asthma (OR = 5.65, 95%CI: 2.5–12.8, $p = 0.0009$) however, the gene burden for LOE asthma patients approached but did not reach Bonferroni-corrected significance ($p = 0.00053$). One frameshift mutation and one missense mutation were incidentally identified in *THRA* (OR = 32.0, 95%CI: 7.9–130) which, also did not reach Bonferroni threshold. *THRA* was not explicitly part of the targeted gene panels but exon 10 (chr17: 40,093,020–40,093,867) was covered due to overlap with the 5'UTR region of *NR1D1*.
- (iv) There were 32 variant sites identified within the IL-5 promoter area across LOE asthma patients and recruited healthy controls. There was no association of increased variant burden at these sites for either group. There was no association of increased burden of variants across the IL-5 promoter region for LOE asthma patients or healthy controls.

There are some important limitations to the methods which should be discussed.

Firstly, the gene burden method makes a strong assumption that all rare variants in a set are causal and associated with a trait with the same direction and magnitude of effect. Violation of these assumptions can result in a substantial loss of power.^{364 367}

³⁷⁷ The IDT gene panel contains both genes that (a) one might hypothesize to contain loss-of function variants in late-onset eosinophilic asthma patients e.g, *ZFPM1* and *SOX4* due to their ability to suppress *GATA3* mediated Th2 differentiation, as well as (b) genes one might hypothesize to contain gain-of-function mutations such *GATA3*, downstream targets such as IL-5, and upstream regulators such as *STAT6*. This would not be a problem if loss-of-function and gain-of-function mutations could be differentiated easily. Unfortunately, there are no protein damage predictors (e.g. CADD, PolyPhen-2, SIFT) that reliably predict gain-of-function variants. VEP, CADD and other variant tools are much better at identifying deleterious loss-of-function rather than gain of function mutations.⁴⁵⁷ They predict *disruption*, not enhancement, activation, or neomorphic effects. CADD was used here and is the “least bad” single predictor among this group, but it still does not predict gain-of-function reliably.⁴⁵⁷ This is an issue for those genes in the IDT panel for which gain of function mutations might be hypothesized and for identified missense variants which are the most common gain-of-function mutation type.^{451 458}

⁴⁵⁹ Instead of stratifying variants using predictors of deleteriousness (as done here) it may be preferable to stratify missense mutations based on occurrence in regulatory or functional hotspots where small changes can increase activity or remove inhibition, rather than destroy the protein e.g., within a kinase activation loop or ion channel pores / voltage sensors.

The 6 missense mutations identified in *IL17RA* are unlikely to be gain-of-function as the IL-17R α receptor has no catalytic activity and signals via IL-17 binding, receptor oligomerization, and adaptor recruitment (ACT1).⁴⁶⁰ Recognised loss-of-function mutations in *IL17RA* include protein truncating variants, frameshifts, splice disrupting

variants and missense variants that specifically destabilise the SEFIR domain: these are reported as part of the disease-causing spectrum for autosomal recessive *IL-17RA* deficiency / Immunodeficiency 51⁴⁶¹ 462 a key clinical manifestation of which is chronic mucocutaneous candidiasis. None of the 6 missense mutations in *IL17RA* identified here are within the SEFIR domain. The p.Val161Ala mutation is in the extracellular domain of the receptor, the p.Thr408Ser and p.Lys619Asn mutations are in the intra-cellular region of the receptor distal to the SEFIR domain, p.Arg620Trp is in the intra-cellular cytoplasmic domain and p.Leu828His (2 mutations) lies in the C-terminal cytoplasmic region. All 6 mutations are listed in ClinVar as 'variants of uncertain significance' (VUS).⁴⁶³ Lack of recorded pathogenicity for these variants does *not* mean that they are *not* causal – these alleles are very rare and have not been studied in the context of eosinophilic asthma before. Examples of clinically important mutations in receptors have been described for the TNF receptor and inflammatory bowel disease, for example.⁴⁶⁴

However, the lack of plausible gain-of function effect or established loss-of function mechanisms and the distributed effects within the IL-17R α receptor makes a causal role less likely. It also remains possible these variants could be in linkage disequilibrium with other variants in *IL17RA* or variants nearby on the same chromosome.⁴⁶⁵

By comparison, the incidental findings for the *THRA* gene are established loss-of-function variants. Both the c.1399delG frameshift variant and c.1325G>A missense variant listed in ClinVar as pathogenic. The former frameshift variant lies in the ligand-binding domain (LBD) of *THRA* and is critical for thyroid hormone binding and transcriptional repression. This is a well-established pathogenic hotspot for human

THRA disease often referred to as Resistance to Thyroid Hormone α (RTH α).⁴⁶⁶ Variants in this hotspot act by impairing triiodothyronine-3 (T3) from binding, resulting in dominant-negative repression and not loss-of-function by haploinsufficiency, with this mechanism demonstrated in mice⁴⁶⁷ and humans.⁴⁶⁶ The other missense mutation also lies in the (LBD) hotspot. Arg442 is highly conserved and involved in hormone binding pocket integrity and receptor conformational change upon T3 binding. This variant is also projected to act through dominant-negative activity of a mutated receptor and has been tested *in vitro*.⁴⁶⁸

Mutations in *THRA* are firmly related to manifestations of thyroid disease and there is correlation between *in vitro* severity and clinical phenotype/therapy response.⁴⁶⁸ It is important to note however, that RTH α is a disorder of tissue sensitivity, not hormone levels. Serum thyroid tests can look deceptively normal. The two patients with mutations in the LOE asthma cohort did not have thyroid disease listed or thyroid medications on their Case Report Forms. Hypothyroidism is linked with asthma, with a large recent epidemiological study by Huang et al.⁴⁶⁹ reporting increased levels of new diagnoses of hypothyroidism in (non-phenotyped) asthma patients when compared to non-asthma groups. In a non-eosinophilic asthma cohort S Birring et al.⁴⁷⁰ found patients with treated hypothyroidism report more respiratory symptoms and have evidence of higher levels of airway dysfunction and inflammation than those without hypothyroidism. It is also noteworthy that *THRA* is located within a locus of the genome that has been implicated in asthma by multiple GWAS (Figure 8-18).⁴⁷¹

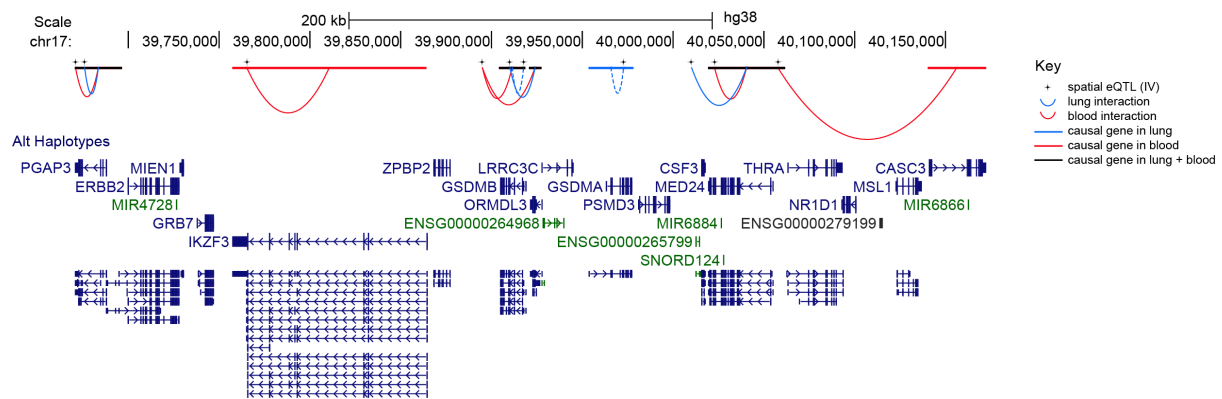


Figure 8-19. Figure reproduced from Zaied et al. 2024 (Front Immunol 15:1412032)

“Unspecified asthma, childhood-onset, and adult-onset asthma have different causal genes: a Mendelian randomization analysis” UCSC browser genome tracks spanning chr17:39,670,900-40,172,190 show 11 spatial eQTLs regulating seven protein-coding genes that are causal for asthma in either lung (blue) blood (red) or both tissues (black). *THRA* gene on right.

Physiologically there is evidence that T3 tends to favour Th1-type cellular immunity, while hypothyroid states skew toward Th2.⁴⁷² Both T cells and B cells express thyroid receptors (predominantly TR α but also TR β): T3 enters T cells via transporters (e.g., MCT8) and alters gene transcription.^{473 474} Adequate T3 is linked to sustained or increased T-bet signalling in T cells and dendritic cells and to IL-2 and IFN- γ production consistent with Th1 differentiation and responses whereas reduced T3 is linked with reduced T-bet signalling, and a relative increase in GATA-3 activity and Th2 differentiation and responses.⁴⁷⁴⁻⁴⁷⁶ There is also evidence that T3 pushes macrophages towards an M1-like (Th1 supporting) signature.⁴⁷⁷

In summary therefore, the finding of pathogenic mutations in *THRA* in LOE asthma patients is interesting due to the subclinical nature of the mutation, for the potential

mechanisms in immune cells relevant to Th2 phenotypes and for the potential epidemiological associations between hypothyroid states and asthma.

Returning to the problems of the gene burden variant analysis performed – a second issue is the possible heterogeneity underlying available gnomAD exomes control data. In this dataset variant counts and features are summarised per base pair site: individual level data are not publicly available. Although it is possible to account for individuals who are homozygous for a variant in gnomAD from summary data it is not possible to appreciate whether any individuals in gnomAD have ≥ 2 variants on the same haplotype within the same gene. Some individuals might therefore have been ‘counted twice’, or more frequently. This could result in over-inflated variant counts for the gnomAD controls and deflated λ_{GC} statistics in gene burden counts.

Furthermore, gnomAD is a general population and not a disease-free population. It is likely there are a number of participants with late-onset eosinophilic asthma among the gnomAD exomes 4.1 non-Finnish European dataset of 518,515 participants, bearing in mind an estimated population prevalence of LOE asthma within the UK of between 1.5-in-1000 and 1-in-200 people (see Introduction). It is suggested that rare variant gene burden analyses using very large general population control datasets such as this is robust to such an effect however, this is typically stated in the context of Mendelian and/or very rare diseases such as idiopathic hypo-gonadotrophic hypogonadism³⁷⁵ and glioblastoma multiforme,³⁷⁴ diseases which have much lower population prevalences of approximately 1 to 10-in-100,000.^{478 479}

A third suggested problem for the ‘underpowering’ of the collapsing analysis is that no down-sampling of the gnomAD sequencing coverage was performed in order to align coverage with the targeted sequencing panel(s). Typically, this is done using

QD and VSLOD with both the targeted sequencing data and public control data down-sampled to higher quality tranches to better align the datasets. Given the higher read coverage, higher QD and higher VSLOD values in the targeted sequencing this was not done in order (i) to begin with a conservative test and (ii) due to concern that VQSLOD scores in the targeted panels were higher due to ultra-high read coverage for a number of SNPs that matched truth set variant SNPs resulting driving the increase in VQSLOD scores (see the bimodal distribution of VQSLOD scores in Figure 8-9). Not doing so may mean variants were called more conservatively than variants in gnomAD exomes, particularly SNPs where coverage was not as high as median values.

8.4.1.1 How will the rare variant analysis be developed from here?

One (theoretical) approach to ascertain the validity of the *IL17RA* and *THRA* mutations for LOE asthma would be to ascertain if these are *de novo* mutations by comparing the DNA of those affected with their parents, assuming the parents do not have LOE asthma. This was done in a cohort of parents with autistic children and was able to demonstrate the nearly complete penetrance of mutations in *CHD8* following the finding of only 3 cases of mutation in *CHD8* in 1078 children.³⁶⁴ Given the ages of the LOE patients here, plus the absence of an *a priori* ethical approval framework, this will not be practically possible.

A reasonable approach would be to liberalise parameters in a second burden test given the conservative testing parameters of the initial analysis. This could be done by raising the vAF threshold from 0.001 to 0.005 and/or by incorporating rare 5'UTR

variants which can functionally behave like loss-of-function mutations in some diseases.⁴⁸⁰⁻⁴⁸² Such an approach is considered preferable to down-sampling the gnomAD control dataset for example, due to the risk of omitting variant sites and false positives.

Further improvement to the analysis could be made by utilizing UK Biobank healthy control data rather than gnomAD control data. UK Biobank data is available on a per-individual basis rather than variant summary basis, along with some phenotypic information. This will permit removal of individuals with eosinophilia from the control group and permit adjustment for any individuals with ≥ 2 variants within the same haplotype / gene. This adjustment could have a significant impact in a relatively more 'common' disease like LOE asthma. The ancestry of selected individuals from the UK Biobank will also more closely represent that of the LOE asthma cohort here - acknowledging that any findings would ultimately need to be replicated in an ethnically diverse sample.

Lastly, it would be desirable to pursue the hypothesis that (mostly subclinical) tissue thyroid resistance and/or hypothyroidism is associated with LOE asthma. This cannot be done as a *post hoc* analysis within this experiment as thyroid related genes such as *THRA* were not included in the targeted panels and only a portion of exon 10 of *THRA* was captured. This can be done in future by sequencing the library prepared samples using a smaller targeted panel focused on genes such as *THRA* (in its entirety), *THRB*, *SLC16A2* (MCT8) and those signalling genes such as *TBX21* (T-bet) which convey messages from these receptors. The effect of T3 on human T cells has been documented, but specifically in the context of Th2 differentiation^{483 484}, and this could be a prelude to further work.

8.4.2 Clonal haematopoiesis analysis

The main findings from the clonal haematopoiesis somatic driver mutation analysis are:

- (i) There was a higher occurrence of CHIP mutations at $vAF \geq 1\%$ identified in the LOE asthma patient group compared with age-matched healthy controls (62 in 123 LOE patients versus 6 in 25 healthy controls, $p < 0.02$). There was also a greater occurrence of mutations at $vAF \geq 2\%$ in LOE asthma patients (26 in 123 vs 1 in 25, $p < 0.05$).
- (ii) There was a higher burden of mutations among individuals in the LOE asthma patient group with 12 of 123 harbouring ≥ 2 mutations compared with 0 of 25 healthy controls. However, this was not statistically significant ($p = 0.22$).
- (iii) Within individuals aged 40-70 years, CHIP at $vAF \geq 1\%$ was observed in 33 of 95 LOE asthma patients (34.4%) and in 4 of 24 healthy controls (16.7%). However, this difference did not reach statistical significance (OR of 2.53, 95%CI: 0.79–8.05, $p = 0.136$).
- (iv) Within individuals aged 40-70 years, CHIP at $vAF \geq 2\%$ was observed in 14 of 95 LOE asthma patients and in 1 of 23 healthy controls. Again, this did not reach statistical significance (OR = 3.8, 95%CI: 0.47–30.52, $p = 0.29$).

- (v) The most common CHIP mutations in LOE asthma and healthy controls followed the pattern seen in other general population studies of CHIP, of *DNMT3A* > *TET2*.^{408 409 411}

The most obvious limitation to the clonal haematopoiesis analyses is underpowered statistical testing. There was only one mutation event at vAF $\geq 2\%$ in healthy controls with subsequently wide confidence intervals for the odds ratio. Augmenting the dataset with controls from other studies is more problematic for somatic mutations than for germline mutations due to the variation and sensitivity of different probe sets and sequencing platforms when distinguishing artefact from genuine variants at low allele frequencies. Obtaining data sequenced using the same Roche panel and processed using the same bioinformatic pipeline would be the preferred way to address this problem.

In summary, there are tendencies towards higher numbers of vAF mutations and multiple mutations in LOE asthma compared with healthy controls, but this does not reach statistical significance.

Comparisons may be drawn between the results here and the investigation of CHIP in a cohort of 125 COPD patients sequenced very deeply (median coverage 2,225X) in the Lee et al.⁴²² (2025) study. The gene panel used in this study was smaller (25 genes) but nonetheless encompasses an estimated 90-95% of currently recognised CHIP mutations.⁴⁸⁵ The vAF threshold for CHIP of $\geq 1.5\%$ chosen by the authors is likely to be sensitive enough to detect CHIP but sufficiently above strata where artefact is often found. This COPD cohort were aged approximately a decade older than the LOE asthma patients examined here (68.1 ± 8.1 vs $59.4 \pm$ years); the COPD

patients had greater smoking intensity than the LOE asthma group (mean 42.8 vs 3.8 pack years) and included current smokers in their number (27.2% vs 0%), with smoking strongly associated with CHIP, particularly *ASXL1* mutations.^{486 487} Given these group differences, one might anticipate the prevalence of CHIP (at $vAF \geq 1.5\%$) to be higher in the Lee et al COPD cohort than in the LOE asthma patients here. Instead, they are quite similar at 25.6% in the COPD cohort compared with 22.8% in LOE asthma patients. We must caveat the comparison by saying that no healthy control group was assessed in the Lee et al study and there may have been a lower baseline rate of CHIP in healthy controls than seen here.

The most frequent genes mutated in the Lee et al. COPD cohort follow a similar pattern to that seen in general populations *DNMT3A* > *TET2* > *ASXL1* also seen in the LOE asthma cohort here with the exception that no mutations were seen in *ASXL1* for the LOE asthma group. This may relate to the lower rates of smoking in the LOE asthma group.

The prevalence of multiple CHIP mutations in the LOE asthma group is at the upper end of prevalence cited in general population studies. At $vAF \geq 1\%$ 12 of 47 LOE asthma patients (25.5%) had ≥ 2 mutations: at $vAF \geq 2\%$ 4 of 22 individuals (18.2%) had ≥ 2 mutations. In large general population studies such as Bick et al.⁴⁰⁹ less than 10% of individuals have more than one mutation. The data from Lee et al do not permit comparison of multiple mutations with their COPD cohort. However, in a longitudinal study of CHIP in participants at-risk of atherosclerosis by Uddin et al.⁴⁸⁸ of whom 54.2% were former smokers, found 16.2% of CHIP carriers had more than one mutation at $vAF \geq 2\%$.

Among the LOE asthma patients with ≥ 2 mutations none had TP53 or KRAS co-mutations and there was one instance of a spliceosome gene (*SF3B1*) co-mutated with *DNMT3A*. It is not possible to know if, for those individuals with ≥ 2 mutations, whether these mutations represent independent competing clones or sequential hits within an expanding clone. Sequential hits within a competing clone represent an increased risk of haematological malignancy. There is no established increased risk of haematological malignancy in people with a diagnosis of asthma⁴⁸⁹ although the risk of malignancy in the LOE asthma phenotype has not been assessed to date. A Bland Altman analysis of variant allele frequencies in paired and trio clones showed good agreement at lower vAFs – where the majority of clones are clustered (see Appendix Table 11-8) – but breaking down at higher vAFs. It seems more likely therefore, that these pairs and trios represent competing clones within the same individual(s) rather than sequential hits.

It is reasonable to hypothesize that a higher overall mutation rate in LOE asthma compared to healthy controls reflects increased clonal selective pressure and competition in the bone marrow of LOE asthma patients. An increased presence of multiple mutations (if proven) would reflect parallel competing clones with individual stem cells independently acquiring mutations and expanding at the same time (at variable rates) due to a marrow environment that favours mutant cells over wild type HSCs. This would be consistent with the recognised associations between clonal haematopoiesis and other aberrant inflammatory states.^{399 400 490} It would be consistent with the mechanism proposed by Avagyan et al.³⁸⁸ in which HSC clonal fitness is driven by resistance to inflammatory signals generated by their differentiated progeny. According to this hypothesis, CHIP in LOE asthma results

due to the anti-inflammatory effects of eosinophilic asthma. CHIP could also further drive disease progression through increased production of pro-inflammatory cytokines from DNMT3A mutant macrophages,⁴²¹ for example. One might also speculate that treatments which reduce eosinophilic inflammation, such as inhaled corticosteroids or biologic therapies, can reduce competition pressures within the bone marrow and reduce the number and/or size of emergent clones.

An alternative hypothesis is that causality operates in the opposite direction and CHIP *causes* LOE asthma. It is possible that the LOE patients studied here have driver mutations which are yet to be identified, perhaps in a gene or genes specific to LOE asthma. This is not unreasonable given that the majority of driver mutations are likely not yet known.^{423 491} In this scenario, clonal populations would drive LOE asthma, perhaps via production of IL-5 and T2 mediators. In this hypothesis LOE asthma, for which spontaneous remission *off* treatment is not reported, could be viewed as a disease of the blood. This hypothesis seems unlikely however, for several reasons; Firstly, CHIP is identified here for LOE patients in driver mutations typical of other populations i.e., *DNMT3A* > *TET2*, rather than in disease-specific drivers. Secondly, the effect of *DNMT3A* mutations in general populations has been well studied in single-cell DNA and RNA paradigms: *DNMT3A* mutations have been found across multiple lineages and are not restricted to a single output lineage⁴⁹² while *TET2* loss-of-function is accompanied by a skew towards myeloid programs but not eosinophil or mast cell lineages.⁴⁹³ There is a suggestion of increased burden of *KMT2C* found here for LOE asthma not found for healthy controls or in general populations but mouse work to date suggest that the *KMT2C* mutation effects are generic such as increased cell fitness under genotoxic and proliferative stressors.

Finally, although this hypothesis would be compatible with a ‘two-hit’ model of asthma, it does not address the organ-specific nature of asthma as a disease of the lung in the absence of CHIP in idiopathic eosinophilia and other eosinophilic diseases.

The interpretation of CHIP as a secondary response to eosinophilic inflammation is therefore preferred.

8.4.2.1 How will the clonal haematopoiesis analysis be developed from here?

To increase statistical power of comparisons between LOE asthma patients and healthy controls, additional age-matched healthy controls will be recruited. Where possible this will be incorporating existing sequencing data from age-matched controls generated from peripheral whole blood and the same Roche gene panel. These data can then be processed through the bioinformatic pipeline used in this study. For example, healthy controls sequenced from peripheral blood in Jakobsen et al.⁴⁰¹, would be suitable for inclusion. Notably, this cohort includes individuals with osteoarthritis, another inflammatory condition, which provides an opportunity to compare rates of CHIP in LOE asthma with those in other inflammatory diseases. This comparison may help determine whether CHIP prevalence in LOE asthma is disproportionately elevated relative to other inflammatory conditions.

If an increased rate of CHIP in LOE asthma is demonstrated I will perform a binomial regression of CHIP vs non-CHIP in LOE asthma patients to understand which features of the disease are most strongly linked with CHIP (other than age). In view of the proposed inflammaging mechanism of CHIP it may be reasonable to

hypothesize that duration of disease from onset to blood sampling would be one feature that may demonstrate a positive relationship with the emergence of CHIP.

Further studies might consider the relationship of CHIP to anti-inflammatory treatments. The large majority of LOE patients here were established on biologic therapy following phlebotomy. It would be interesting to resample subsets of these patients with and without CHIP at ≥ 5 years of biologic treatment to consider the longitudinal emergence and disappearance of clonal populations on these anti-inflammatory treatments.

Other potential *in vivo* and *in vitro* studies include examining specific cell populations in LOE asthma patients with and without CHIP. The inflammaging effects (as opposed to cause) of CHIP have been linked to increased cardiovascular risk through altered cell behaviour⁴⁹⁴ and CHIP has been linked to increased exacerbation risk in COPD.⁴²² One might begin examining CHIP as an agonist of eosinophilic inflammation through single cell DNA and RNA sequencing of critical effector cells such as blood eosinophils, CD4+ve T-cells and tissue mast cell populations, to look for the presence of mutations and associated phenotypes.

8.5 Future analysis plan for somatic non-driver variants (IDT panel)

Thus far, only somatic mutations consistent with known clonal haematopoietic driver mutations have been considered. Somatic mutation accumulation under non-malignant conditions are rarely considered. Savola et al.⁴²⁰ have demonstrated clonally expanded subsets of CD8⁺ T cells in newly diagnosed rheumatoid arthritis

patients containing multiple somatic mutations. A general population study looking at non-leukaemogenic somatic point mutations has found these to be associated with increased white blood cell counts.⁴⁹⁵

In future work I propose to examine the data for the presence of somatic mutations in genes of the IDT gene panel. I will use a conservative variant allele frequency threshold of $\geq 2\%$ (at which somatic mutations are rare) to look for somatic mutations that may or may not be passenger mutations but can contribute to LOE asthma disease. I will also be guided by whether these mutations have been associated with expanding cell populations in tissue (COSMIC)⁴⁹⁶.

9 . Summary discussion

The work presented here has examined three broad questions with regards to the role of IL-5 in the aetiology of the late onset endo-phenotype of eosinophilic asthma. The results of each are briefly summarised along with directions for future research

9.1 Differential effects of ICS and OCS on T2 inflammation

In my first chapter I compared the effects of initiating ICS in T2-high treatment naïve patients with the additional effects of OCS in T2-high patients via an observational trial of treatment. The additional benefits of OCS to patients established on ICS are not understood and the additive effects of OCS treatment had not been examined in this way previously.

The patients recruited to the OCS arm, who had high T2 biomarkers despite being established on ICS treatment had remarkably high levels of T2 mediator signaling in the airway at baseline. This re-capitulated the utility of the blood eosinophil count and FeNO in identifying refractory eosinophilic inflammation despite ICS treatment.

The introduction of ICS and the addition of OCS in these two groups of T2-high asthma patients resulted in similar clinical benefits although OCS had a bigger impact on small airways dysfunction. The pattern of the anti-inflammatory effects differed, however. OCS produced greater inhibition of blood eosinophils than ICS while ICS produced greater inhibition of FeNO than OCS. OCS had a broader suppressive effect on T2

mediators in sputum and serum than ICS, including IL-13 and eotaxin-3 (which can be suppressed in epithelial cells by blocking IL-13). It is hypothesized that the minor reduction of FeNO observed for our OCS group reflects either (i) inhibition of iNOS expression through reduction of IL-13 (this hypothesis expressed graphically in Figure 6-10) or (ii) the effect of OCS providing access to the small airways for ICS by reducing sputum eosinophilia and mucous plugging, or (iii) both.

This hypothesized effect of OCS on FeNO via suppression of IL-13 would align with the effects of FeNO observed in trials of anti-IL13 treatment such as lembrikizimab⁴⁹⁷⁴⁹⁸ and dupilumab.^{499 500} It would potentially fit with increased activity within the 'intrinsic' disease pathway in this population and with increased activity of ILC2s. ILC2s have been demonstrated in disease models to upregulate production of IL-13, in particular.¹¹⁸⁻¹²⁰

I aim to test this hypothesis using bronchial tissue from eosinophilic asthmatic patients established on moderate–high dose ICS, and healthy controls that I obtained by performing research bronchoscopies during the term of the work presented. The baseline parameters of these patients are presented in Table 9-1 at the bottom of this section. Using spatial transcriptomics (Xenium 10x Genomics in-situ platform) – for which data has already been acquired as part of a collaboration with colleagues – I will examine (i) if there is co-expression of iNOS with IL13 in epithelial cells (ii) if there is evidence that these cells have/have not been exposed to glucocorticoids with reference to expression of gluko-corticoid induced genes e.g., *FKBP5*, *SGK1*, *ZBTB16* and *PER1* and (iii) if positive, whether these epithelial cells expressing iNOS are closer / more frequently in proximity to cells expressing IL-13.

It is noted that 39% of the patients in the experimental OCS group had never received a course of oral corticosteroids prior to the study, and none were current candidates for biologic therapy based on current UK prescribing guidelines.⁵⁰¹ The finding of high baseline levels of T2 airway inflammation *and* a subsequent good clinical response to OCS suggests raised T2 biomarkers in patients taking ICS treatment may be an indicator of potential to benefit from biologic therapy before the present threshold of three clinically significant exacerbations requiring OCS treatment is met.

9.2 The role of soluble IL5 receptor α (sIL5R α) in eosinophilic asthma

In my second chapter I looked for evidence of a relationship between IL-5 and sIL-5R α in eosinophilic asthma *in vivo* which, had not been done previously. This was to examine whether the inhibitory effects of sIL-5R α on IL-5 seen *in vitro*²⁸⁸ were visible *in vivo* in eosinophilic asthma, with a view to whether sIL-5R α may be a therapeutic target.

This involved measuring IL-5 and sIL-5R α in plasma and in sputum of patients with eosinophilic asthma (at steady state) and healthy controls. No relationship between IL-5 and sIL-5R α in plasma for eosinophilic asthmatics or healthy controls was found but eosinophil expression of mIL-5R α was higher in blood than in sputum. The implication of this is that mIL-5R α expression is downregulated by IL-5 -induced cleavage of mIL-5R α in the tissue (and other cytokines such as eotaxin-3) as eosinophils transit from blood to sputum. It was hypothesised that levels of IL-5 in

the plasma of eosinophilic asthmatics are too low for mIL-5R α cleavage and for a relationship between IL-5 and sIL-5R α to be visible in plasma.

A small follow-up experiment to this work is planned to examine whether the high levels of IL-5 observed in patients taking the anti-IL-5R α antibody treatment (benralizumab) are due to neutralisation of sIL-5R α . This will be assessed indirectly using an eosinophil apoptosis assay and co-culture of serum from patients established / not established on benralizumab treatment.

9.3 Rare, high-impact mutations and late-onset eosinophilic asthma

In my third chapter I looked for rare genetic variants in genes that could influence IL-5 basal signalling such as *GATA3* and suppressor genes *ZFPM1* and *SOX4*. This was done by deep sequencing of a cohort of highly phenotyped LOE asthma patients and healthy controls using a targeted gene panel and a gene burden approach using a large publicly available control population dataset (gnomAD v4.1). I also looked for the presence of clonal populations secondary to somatic driver mutations which could potentially drive eosinophilic inflammation. These analyses had not been done before in asthma

The rare variant gene-burden analysis yielded six putative high-impact missense variants in *IL17RA* in LOE asthma (OR = 5.65, $p = 0.0009$). However, this did not reach the Bonferroni significance threshold. Because these were not known loss-of-function variants and because gain-of-function is rare for receptor mutations, and because one *IL17RA* mutation was seen in the small in-house age-matched healthy

control population the significance of these mutations is uncertain. Incidentally, one rare frameshift mutation and one rare missense mutation were identified within a portion of exon 10 of the thyroid receptor α gene *THRA*, that was sequenced due to overlap with another gene in the panel (OR = 32.0, $p < 0.002$). This result also did not reach Bonferroni significance. Because these are recognised loss-of-function mutations, because tissue thyroid disease is sub-clinical, affects Th2 differentiation *in vitro*, and because of epidemiological associations between hypothyroidism and asthma, thyroid genes may represent a fruitful follow-up target.

The clonal haematopoiesis analysis demonstrated that the odds of observing a driver mutation with a variant allele frequency $\geq 1\%$ were significantly higher within the LOE asthma group than in an aged-matched healthy control group (OR = 3.22, $p < 0.02$). This was also the case at the higher vAF threshold of $\geq 2\%$ (OR 6.43 $p < 0.05$). When counting individuals with mutations more LOE patients than healthy controls had a driver mutation compatible with clonal haematopoiesis (CHIP), but the difference did not reach statistical significance (e.g., at vAF $\geq 2\%$ (OR 5.23, $p = 0.13$). The statistical power of comparisons was limited due to a small control dataset with few events.

My plan to follow these analyses is to obtain a larger pool (pre-sequenced) of healthy controls for another clonal haematopoiesis analysis using the bioinformatic pipeline created. Should a statistically significant difference be evident I will compare LOE patients with CHIP vs those without CHIP with respect to treatment outcomes such as the likelihood of reaching remission-on-treatment with anti-inflammatory biologic therapy (note, patient samples were taken immediately *before* commencing biologic therapy). As a follow-up to the rare variant analysis I plan to examine 5'UTR variants.

9.4 Closing thoughts on late-onset eosinophilic asthma and the role of IL-5

Using the dataset from my second chapter I performed a *post-hoc* analysis to examine the strengths of the relationships between blood eosinophil counts, plasma IL-5 levels, sputum eosinophil counts and sputum IL-5 levels for LOE asthma patients and early onset allergic eosinophilic patients (Appendix section 11.3.3). There were no differences in the strengths of these relationships between the two sub-groups. Ratios of blood eosinophils to plasma IL-5 were also very consistent between the two phenotypes.

There are many aspects to the relationship between IL-5 and eosinophilic asthma yet to be understood. These include the reasons why anti-IL-5 therapies have been such a welcome success in this disease and for eosinophilic granulomatosis with polyangiitis (EGPA) yet have failed in eosinophilic oesophagitis trials, even failing to reduce eosinophilic infiltration.⁵⁰²

It is possible that the most important difference between the late onset eosinophilic asthma phenotype and the early onset allergic eosinophilic asthma phenotype is a quantitative difference in eosinophilic inflammation. This alone might end up explaining much of what appears to differentiate the phenotype, such as rapid lung function loss or greater response to biologic therapy. This would nonetheless leave open the question as to why later onset disease produces greater inflammation. Finding mechanistic answers to questions like this will likely be necessary if basal T2 signalling is to be ‘turned off’ and cure is to be achieved. In the current era of

biologic therapy however, the trait of eosinophilic inflammation is clinically more relevant to available treatment than mechanistic pathways.³⁵²

Table 9-1. Participant baseline parameters

This is an ongoing experiment for which I performed bronchoscopies to obtain bronchial tissue for the purpose of analysing effector cells in T2-high asthma using a spatial transcriptomic platform (Xenium 10x Genomics). Analysis is in progress. Project in collaboration with Raghad Yamani.

	Healthy controls n = 19	Eosinophilic asthmatics n= 27
Age (years)	33.1 (± 15.3)	49.8 (± 15.3)
Male : Female (n)	9 : 10	16 : 11
Age of onset symptoms (years)	n/a	21.7 (± 23.0)
Total IgE (IU/mL)†	20.0 (± 3.6)	203 (± 4.6)
BDP equivalent daily dose (mcg)‡	n/a	1050
Pre-BD FEV ₁ (% predicted)	99.9 (± 2.3)	68.4 (± 23.0)
Sputum eosinophils (%)‡	0.2 (0.2–0.3)	15.6 (1.0–24.3)
Blood eosinophils (x 10 ⁹ /L)	0.13 (± 0.01)	0.37 (± 0.04)
FeNO (ppb)	18.2 (± 2.3)	71.0 (± 12.8)
BAL eosinophils (%)	0.2 (0.2–0.2)	2.0 (0.6–12.2)

10 . References

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11 . Appendices

11.1 Key resources and supplementary data tables

Table 11-1. Commercial Assays

Reagent	Source	Identifier
QIAamp DNA Mini & Blood Mini Kit	QIAGEN	Cat#51106
Qubit dsDNA HS Assay Kit	Invitrogen	Cat# Q32854
Agilent High Sensitivity DNA Kit	Agilent	Cat# 5067-4626
KAPA Hyper Plus Kit	Roche	Cat# 07962428001
KAPA Dual-Indexed Adapter Set	Roche	Cat# 08278555702
HyperCap Target Enrichment Kit	Roche	Cat# 0828634500
HyperCap Bead Kit	Roche	Cat# 08286400001
AMPure XP Beads	Beckman Coulter	Cat# A63881
KAPA HyperChoice NGS Disc Probe Pool	Roche	
SeqCap EZ Prime Choice Library (custom)		Cat# 08247480001
NGS Discovery Probe Pool _Oxford_v6_ML	IDT	ID# 043492322
NGS Discovery Probe Pool _2x_v1_ML	IDT	ID# 043492322
xGen Hybridization and Wash Kit	IDT	Cat# 1080577

xGen Universal Blockers TS Mix	IDT	Cat# 1075474
xGen Library Amplification Primers Mix	IDT	Cat# 1077675
NextSeq 500/550 High Output Kit v2.5	Illumina	Cat# 20024907
NextSeq 500 High O'put Reagent C'ge v2	Illumina	Cat# 15057931
NextSeq High Output Flow Cell v2.5	Illumina	Cat# 20022408
NextSeq 500/550 Buffer Cartridge	Illumina	Cat# 15057941
NextSeq Accessory Box v2	Illumina	Cat# 15058251
Leukotriene E4 ELISA Kit	Cayman Chemicals	Cat# 501060
Prostaglandin D2 ELISA Kit	Cayman Chemicals	Cat# 512031
V-PLEX IL-5 kit	MSD	Cat# K151QSD
UPLEX Biomarker Group 1	MSD	Cat# K15067M-2
IL-5RA Human ELISA Kit	Abcam	Cat# ab155435
Wright-Giemsa Kit	Abcam	Cat# ab245888

Table 11-2. Specific chemicals, peptides and recombinant proteins not included in table 11-1.

Reagent	Source	Identifier
Buffer EB	QIAGEN	Cat# 19086
COT Human DNA	Illumina	Cat# PR-40-413503-00
UltraPure DNase/RNase-Free Water	Thermo Fisher	Cat# 10977015
HEPES buffer (1 M)	Thermo Fisher	Cat#15630-080
0.5 M EDTA, pH 8.0	Thermo Fisher	Cat# AM9260G
DPBS without calcium and magnesium	Thermo Fisher	Cat# 15326-239
RPMI 1640 w L-glutamine and phenol red	Thermo Fisher	Cat# 11879020
Lymphoprep™	Axis-Shield/Cytiva	Cat# 1114547
BD Pharmingen™ Stain Buffer (BSA)	BD Biosciences	Cat# 554657

Table 11-3. Antibodies and dyes

Reagent	Fluorochrome	Source	Identifier
anti-CD125	PE	Miltenyi Biotec,	Cat# 130-110-544
Anti-CD16	BV785	BioLegend	Cat# 302046
Anti-CD45	BV605	BioLegend	Cat# 304012
Anti-Siglec-8	PE-Cy7	BioLegend	Cat# 347112
Anti-CD123	FITC	Miltenyi Biotec	Cat# 130-113-325
Anti-CD62L	BV421	BioLegend	Cat# 304828
DRAQ7	Live/Dead	BioStatus	Cat# DR7-1000
FcR Blocking Reagent		Miltenyi Biotec	Cat# 130-059-901

Table 11-4. Software and algorithms

Software or algorithm	Source	Website
Burrows-Wheeler aligner (v0.7.18)	Li and Durbin ⁵⁰³	https://bio-bwa.sourceforge.net/
GATK (v4.3.0.0)	Broad Institute	https://software.broadinstitute.org/gatk/
Picard (v2.23.0)	Broad Institute	https://broadinstitute.github.io/picard/
VarDictJava (v1.8.3)	Lai et al. ⁵⁰⁴	https://github.com/AstraZeneca-NGS/VarDictJava
Bcftools (v1.17)	HTSlib	https://samtools.github.io/bcftools/
Annovar (v20191024)	Yang and Wang ⁵⁰⁵	https://annovar.openbioinformatics.org/
Integrative Genomics Viewer (IGV; v2.19.7)	Broad Institute	https://software.broadinstitute.org/software/igv/
Samtools v1.8	HTSlib	http://www.htslib.org/download/
FastQC (v0.12.1)	Bioconda	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
R (v4.3.0)	R-Project	https://www.r-project.org

ggplot v3.5.2	CRAN	https://ggplot2.tidyverse.org/
Graphpad Prism v 10.4.2	GraphPad Software, Inc	https://www.graphpad.com/
FlowJo (v10.8.1)	BD	https://www.bdbiosciences.com/en-gb/products/software/flowjo-v10
BD Biosciences Spectral Viewer	BD Biosciences	https://www.bdbiosciences.com/en-gb/resources/bd-spectrum-
BioRender	BioRender	https://biorender.com/
Hail v0.2.130	Hail	https://hail.is/
Variant effect predictor v112	Ensembl	https://www.ensembl.org/info/docs/tools/vep
CADD	Rentzsch et al. ⁴⁵²	https://cadd.gs.washington.edu
Variant effect predictor v112	McLaren et al ⁴⁴⁸	https://www.ensembl.org/info/docs/tools/vep
gnomAD v4.1.0 exomes	Broad Institute	https://gnomad.broadinstitute.org/
HapMap v3	Broad Institute	https://gnomad.broadinstitute.org/
Omni 2.5M array	Illumina	https://emea.illumina.com
1000 Genomes SNPs	1000 Genomes Project	https://www.internationalgenome.org

Datebase of Short Genetic Variants (dbSNP)	NCBI	https://www.ncbi.nlm.nih.gov/snp/?utm_source=chatgpt.com
Mills + 1000 Genomes GS Indels	Broad Institute	https://gnomad.broadinstitute.org/
SnpSift	Pablo Cingolani ⁴⁴⁹	https://pcingola.github.io/SnpEff/

Table 11-5. Baseline values of T2 mediators in sputum and serum for ICS, OCS and healthy control groups.

Comparison of baseline levels of mediators following log-normalization and using linear ANOVA with Bonferroni correction for multiple comparisons, $\alpha = 0.05$. Abbreviations; LLOD, lower limit of detection.

Analyte (pg/ml) LLOD	Sample type n per group	H. Controls Geomean ($\pm$ log SE) pg/ml	ICS Group Geomean ($\pm$ log SE) pg/ml	OCS Group Geomean ($\pm$ log SE) pg/ml	3-way ANOVA F value	3-way ANOVA P value	Post-hoc HC v ICS P value	Post-hoc HC v OCS P value	Post-hoc ICS v OCS P value
IL-1 β 0.12	Sputum HC=18 ICS=13 OCS=15	88.6 [$\pm$ 0.11]	116 [$\pm$ 0.25]	112 [$\pm$ 0.13]	0.28	ns	ns	ns	ns
	Serum HC=23 ICS=20 OCS=18	0.28 [$\pm$ 0.09]	0.26 [$\pm$ 0.09]	0.22 [$\pm$ 0.09]	0.18	ns	ns	ns	ns
IL-4 0.00	Sputum HC=18 ICS=13 OCS=15	0.17 [$\pm$ 0.11]	0.41 [$\pm$ 0.20]	1.09 [$\pm$ 0.12]	9.28	<0.001	ns	<0.001	ns
	Serum HC=23 ICS=20 OCS=18	0.13 [$\pm$ 0.09]	0.11 [$\pm$ 0.06]	0.15 [$\pm$ 0.06]	0.69	ns	ns	ns	ns
IL-5 0.11	Sputum HC=18 ICS=13 OCS=15	0.73 [$\pm$ 0.08]	9.54 [$\pm$ 0.22]	22.4 [$\pm$ 0.17]	27.27	<0.001	<0.001	<0.001	ns
	Serum HC=23 ICS=20 OCS=18	0.52 [$\pm$ 0.07]	0.79 [$\pm$ 0.07]	0.89 [$\pm$ 0.10]	2.38	.10	ns	ns	ns
IL-13 0.26	Sputum HC=18 ICS=13 OCS=15	2.37 [$\pm$ 0.10]	6.58 [$\pm$ 0.17]	16.2 [$\pm$ 0.13]	11.27	<0.001	0.06	<0.001	ns
	Serum HC=23 ICS=20 OCS=18	2.71 [$\pm$ 0.04]	2.61 [$\pm$ 0.03]	2.89 [$\pm$ 0.04]	0.28	ns	ns	ns	ns
TARC 0.08	Sputum HC=18 ICS=13 OCS=15	21 [$\pm$ 0.21]	144 [$\pm$ 0.31]	373 [$\pm$ 0.18]	8.20	0.001	<0.05	0.001	ns
	Serum HC=23 ICS=20 OCS=18	208 [$\pm$ 0.05]	239 [$\pm$ 0.07]	289 [$\pm$ 0.07]	1.28	ns	ns	ns	ns

Eotaxin-1 7.25	Sputum	71.6 [±0.12]	225 [±0.18]	577 [±0.15]	23.34	<0.001	<0.001	<0.001	0.09
	HC=18 ICS=13 OCS=15								
Eotaxin-2 0.20	Serum	317 [±0.05]	235 [±0.09]	237 [±0.09]	1.03	ns	ns	ns	ns
	HC=23 ICS=20 OCS=18								
Eotaxin-3 0.48	Sputum	128 [±0.18]	1013 [±0.43]	2959 [±0.17]	7.70	0.001	0.06	0.001	ns
	HC=18 ICS=13 OCS=15								
IL-33 0.29	Serum	1358 [±0.07]	908 [±0.08]	904 [±0.07]	1.97	ns	ns	ns	ns
	HC=23 ICS=20 OCS=18								
TSLP 0.10	Sputum	16.0 [±0.19]	378 [±0.29]	1265 [±0.18]	21.70	<0.001	<0.001	<0.001	ns
	HC=18 ICS=13 OCS=15								
LTE4 19.50	Serum	22.5 [±0.04]	38.7 [±0.04]	48.7 [±0.07]	12.33	<0.001	<0.01	<0.001	ns
	HC=23 ICS=20 OCS=18								
PGD2 16.25	Sputum	1.29 [±0.05]	1.53 [±0.07]	3.75 [±0.16]	6.00	<0.01	ns	<0.01	<0.05
	HC=18 ICS=13 OCS=15								
	Serum	1.08 [±0.13]	0.70 [±0.10]	0.75 [±0.13]	0.73	ns	ns	ns	ns
	HC=23 ICS=20 OCS=18								
	Sputum	3.36 [±0.15]	17.3 [±0.23]	58.8 [±0.14]	14.17	<0.001	<0.05	<0.001	ns
	HC=18 ICS=13 OCS=15								
	Serum	3.18 [±0.03]	3.65 [±0.03]	4.28 [±0.04]	3.59	<0.05	ns	<0.05	ns
	HC=23 ICS=20 OCS=18								
	Sputum	192 [±0.14]	420 [±0.19]	1529 [±0.21]	15.75	<0.001	0.08	<0.01	<0.001
	HC=15 ICS=12 OCS=11								
	Sputum	465 [±0.17]	1160 [±0.13]	2327 [±0.07]	6.54	<0.01	ns	<0.01	ns
	HC=14 ICS=12 OCS=11								

Table 11-6. DNA library preparation of samples

Performed using KAPA Unique Dual-Indexed Adapters (UDIs) (Cat# 08278555702) to permit accurate de-multiplexing following sequencing. Libraries remain stored at -80 °C

Sample	DNA (ng/μl)	Library (ng/μl)	ID	P7 sequence	P5 sequence
A01	32	176	UDI-P 97	TGTACCTG	ACATGGAC
A02	16	162	UDI-P 98	CACGTTCA	GTGCAAGT
A03	28	138	UDI-P 99	GGCCTTAT	CCGGAATA
A04	28	163	UDI-P 100	AATTCCGC	TTAAGGCG
A05	22	160	UDI-P 101	TGAGTTGC	ACTCAACG
A06	20	161	UDI-P 102	CAGACCAT	GTCTGGTA
A07	34	191	UDI-P 103	GGTCCTAA	CCAGGATT
A08	38	190	UDI-P 104	AACTTCGG	TTGAAGCC
A09	26	181	UDI-P 105	GCTCCATA	CGAGGTAT
A10	24	160	UDI-P 106	ATCTTGCG	TAGAACGC
A11	37	151	UDI-P 107	TGGCAGAA	ACCGTCTT
A12	43	140	UDI-P 108	CAATGAGG	GTTACTCC
A13	30	127	UDI-P 109	GTGTGACT	CACACTGA
A14	20	164	UDI-P 110	ACACAGTC	TGTGTCAG
A15	34	172	UDI-P 111	TACACCGT	ATGTGGCA
A16	11	178	UDI-P 112	CGTGTTAC	GCACAATG

Table 11-6 continued

Sample	DNA (ng/μl)	Library (ng/μl)	ID	P7 sequence	P5 sequence
A17	21	118	UDI-P 113	GTGATCCT	CACTAGGA
A18	11	133	UDI-P 114	ACAGCTTC	TGTCGAAG
A19	19	110	UDI-P 115	GATGAGCT	CTACTCGA
A20	12	126	UDI-P 116	AGCAGATC	TCGTCTAG
A21	20	122	UDI-P 117	TGGTATGC	ACCATACG
A22	38	128	UDI-P 118	CAACGCAT	GTTGCGTA
A23	23	129	UDI-P 119	TCAAGGCA	AGTTCCGT
A24	51	151	UDI-P 120	CTGGAATG	GACCTTAC
A25	40	118	UDI-P 121	TGATGCCT	ACTACGGA
A26	37	124	UDI-P 122	CAGCATTTC	GTCGTAAG
A27	26	113	UDI-P 123	GAGAACTG	CTCTTGAC
A28	15	150	UDI-P 124	AGAGGTCA	TCTCCAGT
A29	35	136	UDI-P 125	GCTTCTCA	CGAAGAGT
A30	50	128	UDI-P 126	ATCCTCTG	TAGGAGAC
A31	36	124	UDI-P 127	GGCATGAA	CCGTACTT
A33	26	167	UDI-P 128	AATGCAGG	TTACGTCC
A34	48	120	UDI-P 129	GCTCTTAC	CGAGAATG
A35	36	146	UDI-P 130	ATCTCCGT	TAGAGGCA
A36	39	121	UDI-P 131	TGGTCTCA	ACCAGAGT

Table 11-6 continued

Sample	DNA (ng/μl)	Library (ng/μl)	ID	P7 sequence	P5 sequence
A37	34	140	UDI-P 132	CAACTCTG	GTTGAGAC
A38	26	121	UDI-P 133	TGACAGTG	ACTGTCAC
A39	34	59	UDI-P 134	CAGTGACA	GTCACTGT
A41	26	142	UDI-P 135	GTTGTCAC	CAACAGTG
A42	28	142	UDI-P 136	ACCACTGT	TGGTGACA
A43	32	172	UDI-P 137	GACTCACT	CTGAGTGA
A44	50	148	UDI-P 138	AGTCTGTC	TCAGACAG
A45	34	138	UDI-P 139	GCTGGTAT	CGACCATA
A46	33	129	UDI-P 140	ATCAACGC	TAGTTGCG
A47	21	120	UDI-P 141	TATCTCGC	ATAGAGCG
A48	38	73	UDI-P 142	CGCTCTAT	GCGAGATA
A49	25	159	UDI-P 143	GTCACACA	CAGTGTGT
A50	25	161	UDI-P 144	ACTGTGTG	TGACACAC
A51	23	124	UDI-P 97	TGTACCTG	ACATGGAC
A52	32	111	UDI-P 98	CACGTTCA	GTGCAAGT
A53	34	100	UDI-P 145	TCACTTGG	AGTGAACC
A54	32	109	UDI-P 146	CTGTCCAA	GACAGGTT
A55	31	115	UDI-P 147	GTCTCGAA	CAGAGCTT
A56	56	107	UDI-P 148	ACTCTAGG	TGAGATCC

Table 11-6 continued

Sample	DNA (ng/μl)	Library (ng/μl)	ID	P7 sequence	P5 sequence
A57	63	101	UDI-P 149	GTGTACGT	CACATGCA
A58	56	108	UDI-P 150	ACACGTAC	TGTGCATG
A59	51	114	UDI-P 151	GAGTTAGC	CTCAATCG
A60	29	112	UDI-P 152	AGACCGAT	TCTGGCTA
A61	27	70	UDI-P 153	TGTCCAGT	ACAGGTCA
A62	42	56	UDI-P 154	CACTTGAC	GTGAACTG
A63	50	68	UDI-P 155	TGTTCGAC	ACAAGCTG
A64	31	76	UDI-P 156	CACCTAGT	GTGGATCA
A65	25	62	UDI-P 157	GTATGTGC	CATACACG
A66	32	64	UDI-P 158	ACGCACAT	TGCGTGTA
A67	33	54	UDI-P 159	TGGAACCT	ACCTTGGA
A68	104	69	UDI-P 160	CAAGGTTC	GTTCCAAG
A69	55	61	UDI-P 161	TCCGAGAA	AGGCTCTT
A70	46	75	UDI-P 162	CTTAGAGG	GAATCTCC
A71	59	73	UDI-P 163	GTA CTGGA	CATGACCT
A72	42	66	UDI-P 164	ACGTCAAG	TGCAGTTC
A73	58	77	UDI-P 165	TGGTTCAC	ACCAAGTG
A74	28	69	UDI-P 166	CAACCTGT	GTTGGACA
A75	29	52	UDI-P 167	TGCTGGAT	ACGACCTA

Table 11-6 continued

Sample	DNA (ng/μl)	Library (ng/μl)	ID	P7 sequence	P5 sequence
A76	57	60	UDI-P 168	CATCAAGC	GTAGTTCG
A77	57	105	UDI-P 169	GCCAAGAT	CGGTTCTA
A78	19	47	UDI-P 170	ATTGGAGC	TAACCTCG
A79	46	88	UDI-P 171	GTACGTTG	CATGCAAC
A80	53	94	UDI-P 172	ACGTACCA	TGCATGGT
A81	50	123	UDI-P 173	GTGGATTC	CACCTAAG
A82	34	120	UDI-P 174	ACAAGCCT	TGTTCCGA
A83	39	107	UDI-P 175	TAGTTCCG	ATCAAGGC
A84	10	74	UDI-P 176	CGACCTTA	GCTGGAAT
A85	32	123	UDI-P 140	ATCAACGC	TAGTTGCG
A86	54	111	UDI-P 178	CTTGGCAA	GAACCGTT
A87	38	69	UDI-P 144	ACTGTGTG	TGACACAC
B01	32	129	UDI-P 99	GGCCTTAT	CCGGAATA
B02	30	122	UDI-P 100	AATTCCGC	TTAAGGCG
B03	49	130	UDI-P 101	TGAGTTGC	ACTCAACG
B04	20	132	UDI-P 102	CAGACCAT	GTCTGGTA
B05	10	148	UDI-P 103	GGTCCTAA	CCAGGATT
B06	21	115	UDI-P 104	AACTTCGG	TTGAAGCC
B07	31	138	UDI-P 105	GCTCCATA	CGAGGTAT

Table 11-6 continued

Sample	DNA (ng/μl)	Library (ng/μl)	ID	P7 sequence	P5 sequence
B08	20	160	UDI-P 106	ATCTTGCG	TAGAACGC
B09	42	150	UDI-P 107	TGGCAGAA	ACCGTCTT
B10	48	160	UDI-P 108	CAATGAGG	GTTACTCC
B11	50	133	UDI-P 109	GTGTGACT	CACACTGA
B12	22	154	UDI-P 110	ACACAGTC	TGTGTCAG
B13	21	134	UDI-P 111	TACACCGT	ATGTGGCA
B14	77	109	UDI-P 179	GTGTCAAC	CACAGTTG
B15	88	64	UDI-P 180	ACACTGGT	TGTGACCA
C01	13	157	UDI-P 112	CGTGTTAC	GCACAATG
C02	28	104	UDI-P 113	GTGATCCT	CACTAGGA
C03	17	99	UDI-P 114	ACAGCTTC	TGTCGAAG
C04	14	81	UDI-P 115	GATGAGCT	CTACTCGA
C05	33	77	UDI-P 09	ATTATCAA	GAGAGTCG
C06	17	61	UDI-P 10	CCAACATT	AAGAGAGC
C07	27	64	UDI-P 116	AGCAGATC	TCGTCTAG
C08	17	89	UDI-P 117	TGGTATGC	ACCATACG
C09	31	92	UDI-P 118	CAACGCAT	GTTGCGTA
C10	29	144	UDI-P 119	TCAAGGCA	AGTTCCGT
C11	21	96	UDI-P 120	CTGGAATG	GACCTTAC

Table 11-6 continued

Sample	DNA (ng/μl)	Library (ng/μl)	ID	P7 sequence	P5 sequence
C12	25	105	UDI-P 121	TGATGCCT	ACTACGGA
C13	20	72	UDI-P 122	CAGCATTC	GTCGTAAG
C14	18	83	UDI-P 123	GAGAACTG	CTCTTGAC
C15	22	110	UDI-P 124	AGAGGTCA	TCTCCAGT
C16	23	112	UDI-P 125	GCTTCTCA	CGAAGAGT
C17	24	93	UDI-P 126	ATCCTCTG	TAGGAGAC
C18	32	112	UDI-P 127	GGCATGAA	CCGTACTT
C19	15	110	UDI-P 128	AATGCAGG	TTACGTCC
C20	38	90	UDI-P 181	TAGCGATC	ATCGCTAG
C21	23	84	UDI-P 182	CGATAGCT	GCTATCGA
C22	36	74	UDI-P 183	GTGCAGTA	CACGTCAT
C23	35	74	UDI-P 184	ACATGACG	TGTACTGC
C24	42	139	UDI-P 185	TCGAAGTG	AGCTTCAC
C25	16	115	UDI-P 186	CTAGGACA	GATCCTGT
C26	25	70	UDI-P 187	TACCACAG	ATGGTGTC
C27	40	160	UDI-P 188	CGTTGTGA	GCAACACT
C28	33	110	UDI-P 189	GCGACTAT	CGCTGATA
C29	30	54.8	UDI-P 141	TATCTCGC	ATAGAGCG
C30	24	119	UDI-P 142	CGCTCTAT	GCGAGATA

Table 11-6 continued

Sample	DNA (ng/μl)	Library (ng/μl)	ID	P7 sequence	P5 sequence
C31	23	70	UDI-P 143	GTCACACA	CAGTGTGT
Z01	34	134	UDI-P 129	GCTCTTAC	CGAGAATG
Z02	46	100	UDI-P 130	ATCTCCGT	TAGAGGCA
Z03	27	145	UDI-P 131	TGGTCTCA	ACCAGAGT
Z04	21	77	UDI-P 11	GCCTAGCC	CAGACCAA
Z05	38	82	UDI-P 132	CAACTCTG	GTTGAGAC
Z06	20	156	UDI-P 133	TGACAGTG	ACTGTCAC
Z07	22	72	UDI-P 134	CAGTGACA	GTCACTGT
Z08	35	152	UDI-P 135	GTTGTCAC	CAACAGTG
Z09	38	128	UDI-P 136	ACCACTGT	TGGTGACA
Z10	12	90	UDI-P 137	GACTCACT	CTGAGTGA
Z11	19	98	UDI-P 138	AGTCTGTC	TCAGACAG
Z12	73	87	UDI-P 139	GCTGGTAT	CGACCATA
Z13	56	139	UDI-P 01	GTAACATC	AATCGCTG
Z14	36	83	UDI-P 02	AGGTAAGG	GAATCGTG
Z15	44	118	UDI-P 03	ACAGGTAT	ATGGTGGC
Z16	68	86	UDI-P 04	AATGTTCT	AGGTGACT
Z17	39	109	UDI-P 05	TCTGCAAG	AAGGTGAA
Z18	43	125	UDI-P 06	CAGCGGTA	CCAAGTCA

Table 11-6 continued

Sample	DNA (ng/μl)	Library (ng/μl)	ID	P7 sequence	P5 sequence
Z19	23	128	UDI-P 07	CGCCTTCC	AAGTCCGC
Z20	28	71	UDI-P 08	CAATAGTC	GTGAGCTG
Z21	102	113	UDI-P 190	ATAGTCGC	TATCAGCG
Z22	69	98	UDI-P 191	GAGCTCAT	CTCGAGTA
Z23	46	134	UDI-P 192	AGATCTGC	TCTAGACG

Abbreviations: ID, unique dual-indexed (UDI) adapter identity number; P7 sequence, the i7 index sequence on the P7 adapter; P5 sequence, the i5 index sequence on the P5 adapter

Table 11-7. Coordinates of exonic coverage

Probes used in IDT then Roche targeted panels. A breakdown per probe is available on request to the author. Abbreviations: Chr, chromosome

IDT Panel

Chr	Start	End	Gene
1	67307364	67307967	IL12RB2
1	159289714	159289753	FCER1A
1	161215234	161215370	FCER1G
1	206774330	206774541	IL10
2	8678845	8679317	ID2
2	102175478	102179874	IL1R1
2	136118046	136118149	CXCR4
2	191029576	191029866	STAT4
3	3119401	3128844	IL5RA Promotor
3	3126560	3126613	IL5RA
3	3126933	3126990	TRNT1
3	12158702	12158912	TIMP4
3	32993066	32993182	CCR4
3	46208282	46208313	CCR1
3	46265128	46266706	CCR3
3	46357477	46360940	CCR2
3	46370854	46370988	CCR5
3	53846568	53846648	IL17RB
3	159988835	159989174	IL12A
4	53459302	53460862	FIP1L1
4	141636583	141637224	IL15
5	35875983	35879603	IL7R
5	55851357	55851633	IL31RA
5	111075946	111078026	TSLP

Table 11-7 continued

Chr	Start	End	Gene
5	132060655	132060868	IL3
5	132069001	132739074	IL5 Promotor
5	132073789	132073982	CSF2
5	132556674	132556838	IL5
5	132658173	132659471	IL13
5	132682486	132682678	IL4
5	135895703	135895841	IL9
5	139475533	139476454	STING1
5	148826611	148828623	ADRB2
5	157575603	157575775	ADAM19
5	159330432	159330487	IL12B
6	21593751	21598619	SOX4
6	31575565	31575927	TNF
6	52186375	52186458	IL17A
6	137881035	137883312	TNFAIP3
7	22725884	22726258	IL6
7	38249243	38249572	TRGC2
7	38265349	38265678	TRGC1
7	75772104	75772211	CCL26
7	75813643	75813806	CCL24
7	142498725	142499111	TRBC2
8	39103607	39105445	ADAM9
8	56009908	56014169	LYN
8	58581686	58582859	SDCBP
8	88327910	88328025	MMP16
9	6255968	6257983	IL33
9	35609979	35610300	CD72
10	87965287	87971930	PTEN

Table 11-7 continued

Chr	Start	End	Gene
11	33266367	33266514	IL10RB
11	47354860	47355546	SPI1
11	57390483	57390650	PRG2
11	60855869	60855950	PTGDR2
11	117986391	117986534	IL10RA
12	57095408	57096761	STAT6
12	122174157	122174221	IL31
13	118727602	118727726	IL13RA
14	20891399	20891451	RNASE3
14	20955487	20955536	RNASE2
14	22462932	22463210	TRDC
14	22547506	22547778	TRAC
14	22848947	22849041	MMP14
14	23118582	23119255	CEBPE
14	105601419	105601728	IGHE
15	58749480	58749791	ADAM10
16	11256079	11256204	SOCS1
16	27362252	27364778	IL4RA
16	55505339	55506691	MMP2
16	57392684	57392801	CCL22
16	57438679	57438748	CCL17
16	58045007	58046901	MMP15
16	85899162	85899223	IRF8
16	88453280	88453678	ZFPM1
17	34285742	34285884	CCL11
17	35880729	35880793	CCL5
17	40100064	40100589	NR1D1
17	58192726	58192922	EPX

Table 11-7 continued

Chr	Start	End	Gene
17	78924959	78925387	TIMP2
19	7688758	7689430	FCER2
19	17244943	17245919	NR2F6
19	18058995	18059613	IL12RB1
19	39737938	39738029	CLC
19	41301587	41301746	TGFB1
20	31605283	31606515	ID1
20	35226690	35226984	MMP24
20	46008908	46009064	MMP9
22	17084954	17085229	IL17RA
22	28794555	28795732	XBP1
22	32801705	32802122	TIMP3
22	36859554	36916201	CSF2RB Promotor
22	36913628	36913677	CSF2RB
X	1212556	1212723	CRLF2
X	1336616	1336926	IL3RA
X	47586521	47586789	TIMP1
X	155997581	155997787	IL9R
1	1789053	1789269	GNB1
1	36466099	36466200	CSF3R
1	43337839	43337938	MPL
1	43545072	43545171	PTPRF
1	64834565	64834657	JAK1
1	114708538	114708654	NRAS
1	150926778	150926808	SETDB1
1	155900391	155900618	RIT1
1	190098021	190099134	BRINP3
1	241511952	241512051	rs199822819

Table 11-7 continued

Chr	Start	End	Gene
2	25234282	25234420	DNMT3A
2	25742032	25744476	ASXL2
2	197392306	197392461	SF3B1
2	208237076	208237175	IDH1
3	38138662	38139028	MYD88
3	47017096	47017254	SETD2
3	105658973	105659229	CBLB
3	128481022	128481318	GATA2
3	136338218	136338460	STAG1
3	160462721	160462735	rs1210060191
4	24527927	24528041	DHX15
4	54258738	54258837	PDGFRA
4	54657999	54658098	KIT
4	105190429	105190528	TET2
4	152322884	152323149	FBXW7
5	1253731	1253831	TERT
5	36953679	36953778	NIPBL
5	150054072	150054430	CSF1R
5	171387928	171388027	NPM1
5	177511794	177511927	DDX41
6	106086554	106086595	PRDM1
7	50319032	50319131	IKZF1
7	101816013	101816112	CUX1
7	105040939	105041038	KMT2E
7	139360243	139360342	LUC7L2
7	140726457	140726556	BRAF
7	148807628	148807727	EZH2
7	152136830	152136929	KMT2C

Table 11-7 continued

Chr	Start	End	Gene
8	89971093	89971313	rs1187082186_rs777460725
8	116847503	116847691	RAD21
8	127736559	127736658	MYC
9	5021988	5022213	JAK2
9	21968178	21968277	CDKN2A
9	83969317	83969475	HNRNPK
9	136496074	136497558	NOTCH1
10	8055656	8055896	GATA3
10	86437528	86437627	WAPAL
10	87864460	87864559	PTEN
10	110567775	110567874	SMC3
11	532639	532755	HRAS
11	32389061	32389179	WT1
11	62593878	62594040	MTA2
11	64765392	64765518	SF1
11	86245230	86245343	EED
11	94456274	94456373	rs587781384
11	108227611	108227888	ATM
11	118436513	118436944	KMT2A
11	119206418	119206612	CBL
12	285459	285662	KDM5A
12	11650095	11650194	ETV6
12	22625164	22625586	ETNK1
12	25209798	25209911	KRAS
12	49021778	49021877	KMT2D
12	49623543	49623642	PRPF40B
12	111418146	111418877	SH2B3
12	112419069	112419168	PTPN11

Table 11-7 continued

Chr	Start	End	Gene
13	28004055	28004174	FLT3
13	32648773	32648880	PDS5B
13	48303913	48304049	RB1
13	72759798	72759878	DIS3
14	95711758	95711802	TCL1A
15	90084262	90084361	IDH2
15	101642447	101642646	rs754640606
16	3727721	3729874	CREBBP
16	18809560	18809659	SMG1
16	30700825	30700878	SRCAP
16	67610833	67611613	CTCF
17	1655350	1655543	PRPF8
17	7666177	7666276	TP53
17	31095208	31095369	NF1
17	31937247	31937520	SUZ12
17	39765793	39766493	IKZF3
17	42201741	42201864	STAT5B
17	42315748	42315800	STAT3
17	46031479	46031703	KANSL
17	60600415	60600886	PPM1D
17	76736164	76736464	SRSF2
18	44701347	44701832	SETBP1
19	12938680	12938770	CALR
19	17826746	17826910	JAK3
19	19320860	19321135	MAU2
19	33301341	33302414	CEBPA
19	55655080	55655179	U2AF2
20	32358755	32358854	ASXL1

Table 11-7 continued

Chr	Start	End	Gene
20	58840107	58840841	GNAS
21	34792138	34792610	RUNX1
21	43094468	43094788	U2AF1
22	28687900	28687986	CHEK2
22	30334597	30334696	SF3A1
22	41093002	41093101	EP300
X	15321509	15321772	PIGA
X	15790467	15790566	ZRSR2
X	40049906	40050005	BCOR
X	41334253	41334297	DDX3X
X	44873552	44873712	KDM6A
X	48791110	48791329	GATA1
X	53380097	53380196	SMC1A
X	71118755	71118854	MED12
X	77508354	77508629	ATRX
X	124022600	124022699	STAG2
X	130005225	130005324	BCORL1
X	134377618	134377755	PHF6
X	155071528	155071658	BRCC

Table 11-8. List of all clonal haematopoiesis driver mutations identified

Chr	Pos	Ref	Alt	Type	RefD	AltD	vAF	Class	Gene	Protein Ch'	Interpro	COS	CHIP
1	1815790	T	C	SNV	768	20	0.025	Missense	GNB1	p.K57E	Yes	12	21
1	150943967	C	T	SNV	618	11	0.018	Missense	SETDB1	p.S308L	Yes		
1	190265008	G	A	SNV	897	10	0.011	Stop_gained	BRINP3	p.R159X	Yes	4	
2	25234373	C	T	SNV	749	9	0.012	Missense	DNMT3A	p.R882H	Yes	935	94
2	25234373	C	T	SNV	618	14	0.022	Missense	DNMT3A	p.R882H	Yes	935	94
2	25239171	G	C	SNV	530	7	0.013	Nonsynony'	DNMT3A	p.H789Q	Yes		
2	25240313	G	A	SNV	1195	16	0.013	Stop_gained	DNMT3A	p.R771X	Yes	15	23
2	25240354	T	G	SNV	1350	21	0.015	Nonsynony'	DNMT3A	p.N757T	Yes		
2	25240360	A	G	SNV	1230	18	0.014	Missense	DNMT3A	p.F755S	Yes	4	8
2	25240418	G	A	SNV	735	125	0.145	Missense	DNMT3A	p.R736C	Yes	18	15

Table 11-8 continued

Chr	Pos	Ref	Alt	Type	Ref D	Alt D	vAF	Class	Gene	Protein Ch'	Interpro	COS	CHIP
2	25240420	T	C	SNV	1470	59	0.039	Missense	DNMT3A	p.Y735C	Yes		24
2	25240639	C	T	SNV	643	15	0.023	Splice_donor	DNMT3A			5	6
2	25240639	C	A	SNV	484	18	0.036	Splice_donor	DNMT3A				
2	25241686	A	G	SNV	736	320	0.303	Nonsynony'	DNMT3A	p.L653S	Yes	1	
2	25244214	G	A	SNV	1279	17	0.013	Stop_gained	DNMT3A	p.R598X	Yes	12	10
2	25244338	C	G	SNV	872	98	0.101	Missense	DNMT3A	p.R556S	Yes		
2	25244543	CAG*	C	Del	783	12	0.015	Frameshift	DNMT3A	p.R544Afs*96			
2	25244595	T	G	SNV	848	11	0.013	Nonsynony'	DNMT3A	p.T538P	Yes		
2	25246670	G	A	SNV	1211	18	0.015	Missense	DNMT3A	p.A410V		1	1
2 ^A	25247070	G	A	SNV	744	17	0.022	Missense	DNMT3A	p.A368V	Yes		2

* Full deletion: CAGTTGTTGTTTCCGCACATGAGCACCTCACG

Table 11-8 continued

Chr	Pos	Ref	Alt	Type	Ref D	Alt D	vAF	Class	Gene	Protein Ch'	Interpro	COS	CHIP
2 ^J	25247079	TAC	T	Del	1303	23	0.016	Frameshift	DNMT3A	p.M364Ifs*28			
2 ^D	25247666	C	T	SNV	637	7	0.011	Stop_gained	DNMT3A	p.W313X	Yes	5	5
2 ^H	197402110	T	C	SNV	595	217	0.267	Missense	SF3B1	p.K700E	Yes	714	33
2	197402635	C	G	SNV	983	28	0.028	Missense	SF3B1	p.K666N	Yes	53	7
4 ^B	105234763	TC	T	Del	1066	18	0.017	Frameshift	TET2	p.N275Ifs*18		25	1
4	105235590	C	T	SNV	1312	36	0.027	Stop_gained	TET2	p.R550X		57	8
4 ^G	105237346	G	A	SNV	870	28	0.031	Missense	TET2	p.C1135Y		4	1
4	105243591	G	T	SNV	1014	69	0.064	Stop_gained	TET2	p.E1206X			
4	105243757	G	A	SNV	1001	22	0.022	Missense	TET2	p.R1261H		17	1

Table 11-8 continued

Chr	Pos	Ref	Alt	Type	Ref D	Alt D	vAF	Class	Gene	Protein Ch'	Interpro	COS	CHIP
4	105272571	C	T	SNV	918	11	0.012	Missense	TET2	p.T1397I	Yes		
4 ^I	105276248	G	T	SNV	1261	18	0.014	Nonsynony'	TET2	p.G1913V		2	
5 ^E	177513369	C	T	SNV	481	7	0.014	Missense	DDX41	p.S405N	Yes		
7 ^B	148815508	T	C	SNV	660	9	0.013	Missense	EZH2	p.K515R	Yes	5	
7	148815508	T	C	SNV	839	12	0.014	Missense	EZH2	p.K515R	Yes	5	
7	152185587	C	A	SNV	899	143	0.123	Missense	KMT2C	p.A1685S	Yes	38	
7 ^K	152230009	C	A	SNV	1120	75	0.063	Missense	KMT2C	p.G964C	Yes		
7 ^A	152247946	T	C	SNV	1647	34	0.020	Missense	KMT2C	p.I830V		1	
7 ^E	152265083	C	A	SNV	1985	27	0.012	Missense	KMT2C	p.R380L	Yes	131	
7	152273799	A	C	SNV	2154	96	0.043	Stop_gained	KMT2C	p.Y306X	Yes	5	1

Table 11-8 continued

Chr	Pos	Ref	Alt	Type	Ref D	Alt D	vAF	Class	Gene	Protein Ch'	Interpro	COS	CHIP
8	116851985	C	T	SNV	1104	112	0.092	Missense	RAD21	p.R478Q			
8	116863244	G	A	SNV	459	6	0.013	Missense	RAD21	p.R54W	Yes	2	
8 ^F	127738361	G	C	SNV	964	10	0.010	Missense	MYC	p.Q48H	Yes	3	
8 ^I	127738809	G	A	SNV	1312	18	0.014	Nonsynony'	MYC	p.A198T	Yes	1	
11 ^L	62596773	A	C	SNV	1159	17	0.014	Nonsynony'	MTA2	p.M249R	Yes	1	
11 ^C	108284314	C	A	SNV	894	11	0.012	Missense	ATM	p.D1278E	Yes		
11 ^B	119278265	C	G	SNV	491	6	0.012	Missense	CBL	p.L399V	Yes	2	
12 ^K	11869562	T	C	SNV	645	220	0.264	Missense	ETV6	p.L201P		12	
12	25245326	G	A	SNV	325	218	0.400	Nonsynony'	KRAS	p.T20M	Yes	7	
12 ^J	49032991	T	A	SNV	1088	18	0.016	Missense	KMT2D	p.Q3905L		3	
12 ^D	49037437	C	T	SNV	1040	29	0.027	Missense	KMT2D	p.A3307T			

Table 11-8 continued

Chr	Pos	Ref	Alt	Type	Ref D	Alt D	vAF	Class	Gene	Protein Ch'	Interpro	COS	CHIP
12 ^F	112455966	G	A	SNV	675	8	0.012	Missense	PTPN11	p.R220H	Yes	3	
13 ^C	72781305	G	A	SNV	487	5	0.010	Stop_gained	DIS3	p.R94X	Yes		
16	18811771	A	G	SNV	878	13	0.015	Missense	SMG1	p.V3633A	Yes		
17	1659994	T	A	SNV	845	55	0.061	Missense	PRPF8	p.D1598V	Yes	1	
17	7673802	C	T	SNV	456	133	0.225	Missense	TP53	p.R273H	Yes	1266	2
17	39765845	C	T	SNV	924	11	0.012	Missense	IKZF3	p.R492Q	Yes	1	
17	60600711	CT	C	Del	952	15	0.016	Frameshift	PPM1D	p.F101Sfs*64			
19	17834702	T	C	SNV	863	9	0.010	Missense	JAK3	p.D740G	Yes		
19 ^L	17843141	G	C	SNV	725	410	0.360	Missense	JAK3	p.P151R	Yes	13	
21	34886871	C	T	SNV	1167	23	0.019	Missense	RUNX1	p.C108Y	Yes	1	

Table 11-8 continued

Chr	Pos	Ref	Alt	Type	Ref D	Alt D	vAF	Class	Gene	Protein Ch'	Interpro	COS	CHIP
22	28687925	C	T	SNV	527	6	0.011	Missense	CHEK2	p.R578H	Yes	15	
22 ^G	28694101	C	A	SNV	645	9	0.014	Missense	CHEK2	p.K507N	Yes	1	
22	41176401	G	A	SNV	1528	25	0.016	Nonsynony'	EP300	p.R1645Q	Yes	5	
X ^G	45083507	G	C	SNV	396	12	0.029	Missense	KDM6A	p.R1163P	Yes	6	
X	53394872	T	C	SNV	435	5	0.011	Nonsynony'	SMC1A	p.E960G	Yes	3	
X	53405077	G	A	SNV	503	7	0.014	Missense	SMC1A	p.R711W	Yes	6	
X	71140816	C	T	SNV	579	6	0.010	Stop_gained	MED12	p.Q2076X	Yes	4	
X	155071546	C	T	SNV	598	8	0.013	Stop_gained	BRCC3	p.Q7X	Yes	1	

Abbreviations: Chr, chromosome; Pos, position; Ref, reference allele, Alt, alternate allele; RefD, number of reads supporting reference allele; AltD, number of reads supporting alternate allele; vAF, variant allele frequency; Protein Ch', protein change; Interpro, interpro domain, COS, number of occurrences in all tissues identified on 'cosmic97' list of somatic mutations in cancer; CHIP, number of occurrences driver mutations identified in list of clonal haematopoiesis-associated mutations compiled from five large studies. Superscript letters on Chr denote individuals with ≥ 2 mutations.

11.2 Supplementary figures

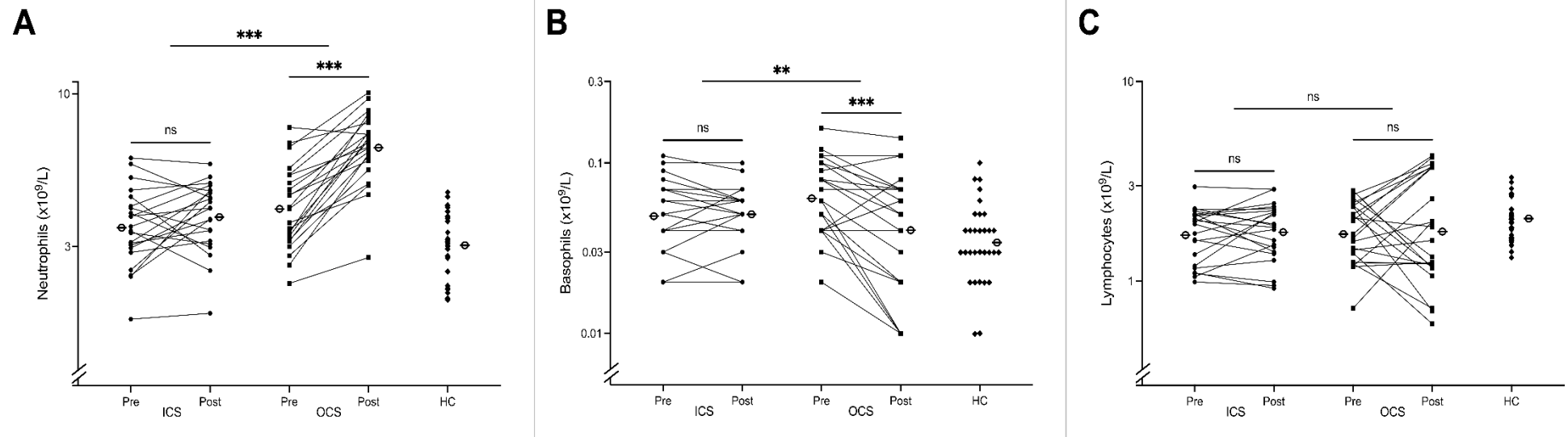


Figure 11-1. The effect of ICS and OCS treatment on other white cell counts in blood. A. Neutrophils B. Basophils C. Lymphocytes. Statistical comparisons performed via ANOVA * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

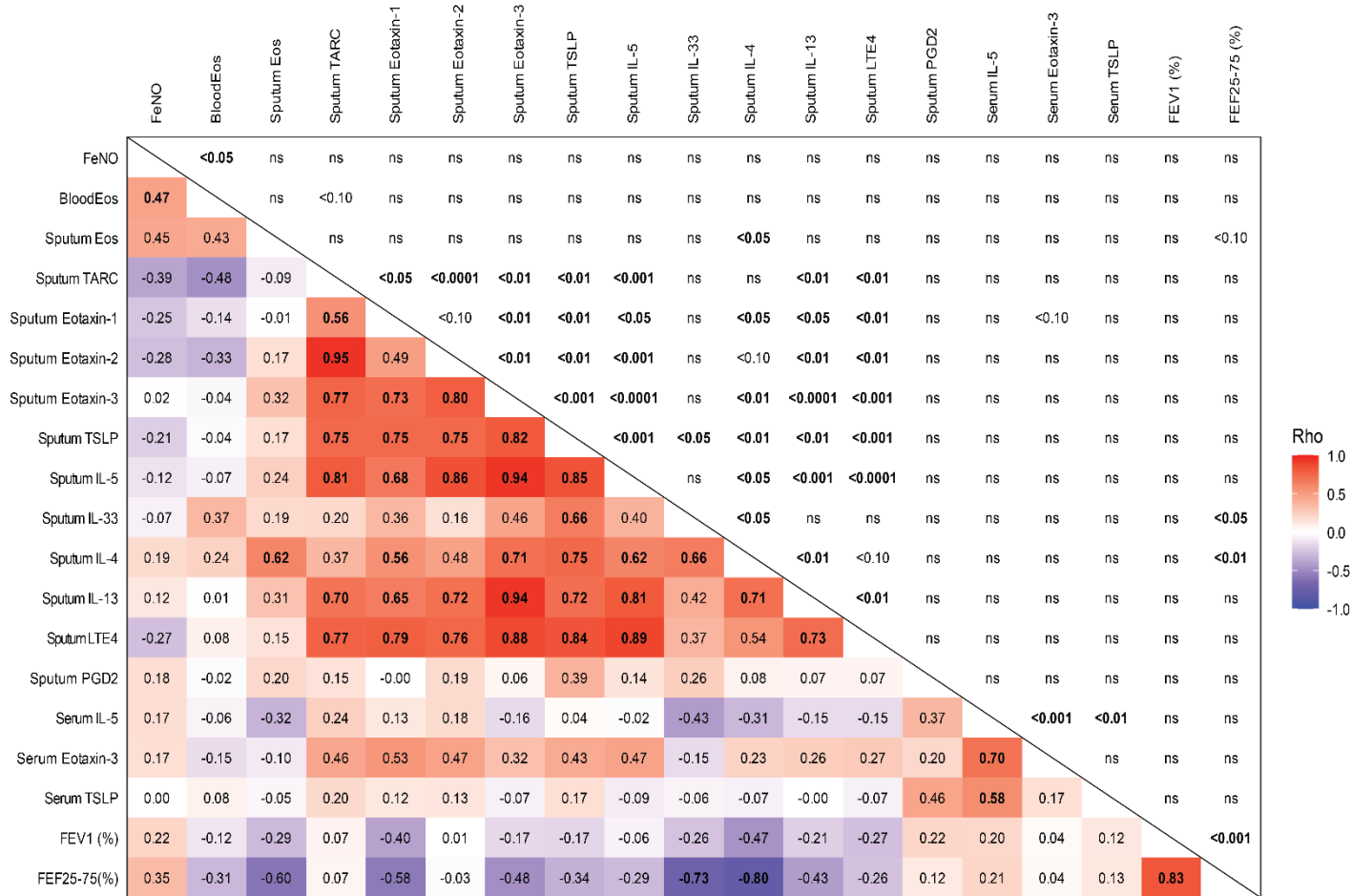


Figure 11-2. Baseline correlation matrix of parameters for the ICS group. Spearman's r and uncorrected p values provided. Significant values in bold

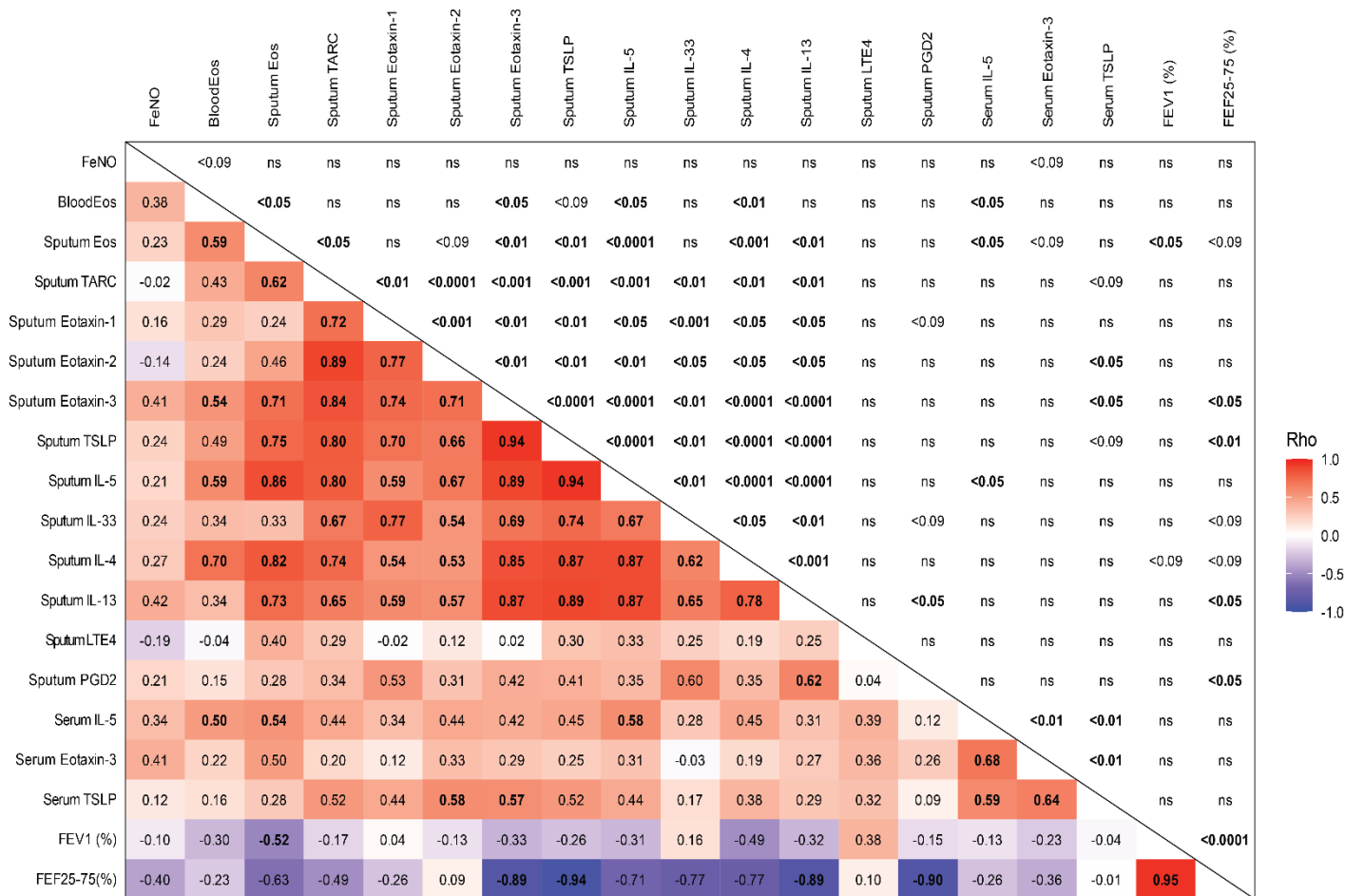


Figure 11-3. Baseline correlation matrix of parameters for the OCS group. Spearman's r and uncorrected p values provided. Significant values in bold

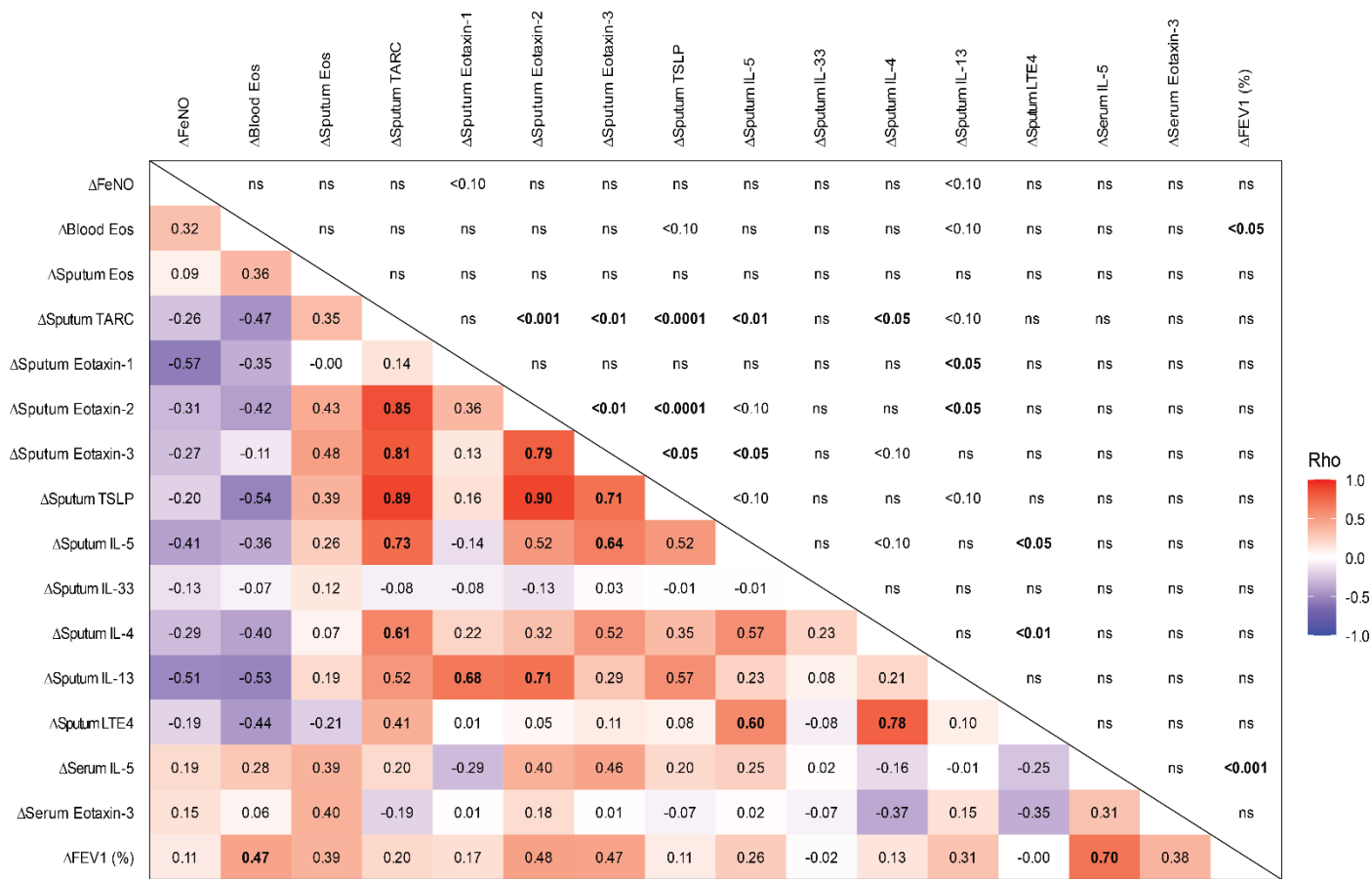


Figure 11-4. Associations in parameter changes following ICS treatment. Correlation matrix showing the relationship between changes (Δ) following ICS treatment. Spearman's r and uncorrected p values provided. Significant values in bold

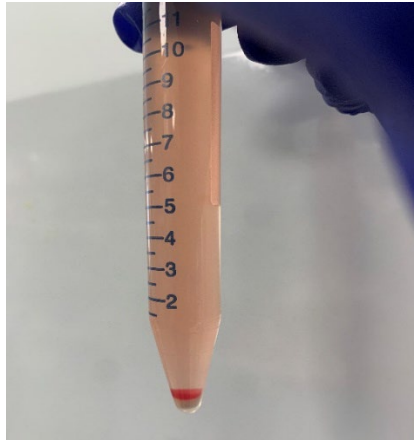


Figure 11-5. Photograph of a layer of red cell casts overlying leucocytes in RPMI media following the second round of red cell lysis using a customised hypotonic red-cell lysis protocol, and after centrifugation (see Methods section 7.2.6). Red cell casts were aspirated from above the cell pellet and cells were re-suspended in 2 ml of RPMI 1640 with L-Glutamine and 10% FCS and 20mM HEPES and then placed on ice ready for Fc receptor blocking and staining

Various pilot experiments were performed to optimize leucocyte extraction from sputum. These included using CD16 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany, cat: 130-045-701) and MACSxpress® Whole Blood Eosinophil Isolation Kit (Miltenyi, cat: 130-104-446) which, both performed well in blood but caused unacceptable eosinophil depletion in sputum samples. Different fluorochrome combinations were trialled before arriving at the final panel including different live/dead dyes. The addition of EDTA to dPBS was helpful in reducing sputum cell adhesion for cell sorting fluidics. A large volume high quality sputum sample processed promptly was important.

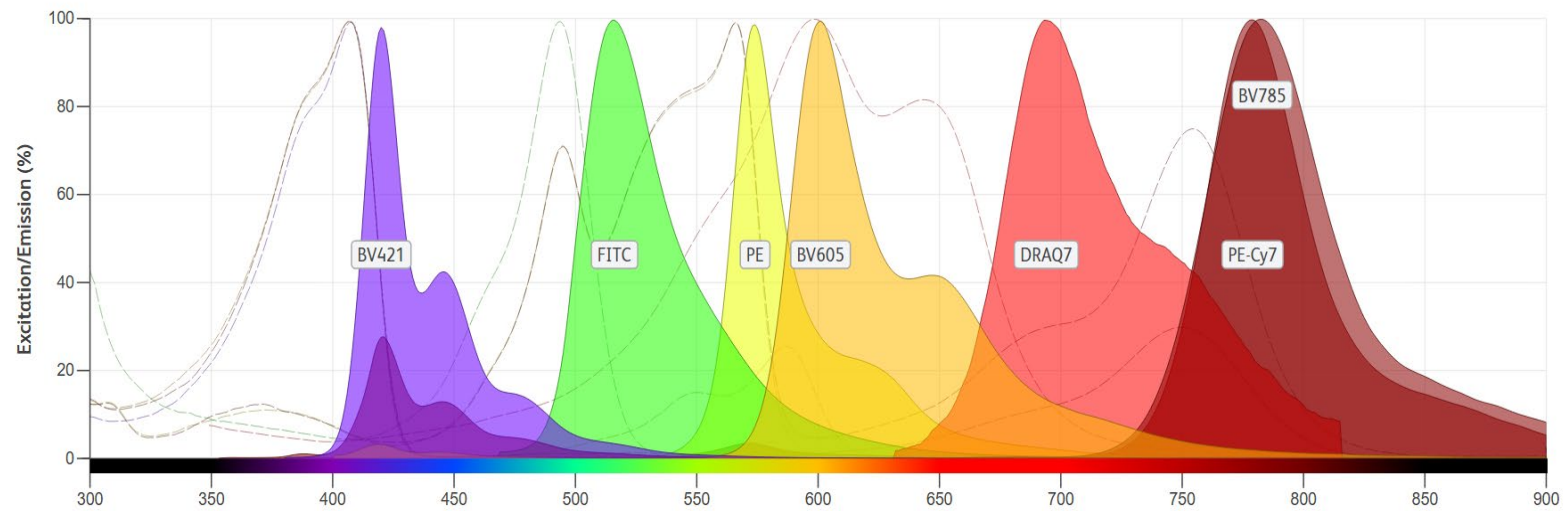


Figure 11-6. Excitation and emission spectrum of fluorophores used in the experiment. It was possible to deconflict PE-Cy7 and BV785 using different lasers. Image from BD spectrum viewer <https://www.bdbiosciences.com/en-gb/resources/bd-spectrum-viewer>

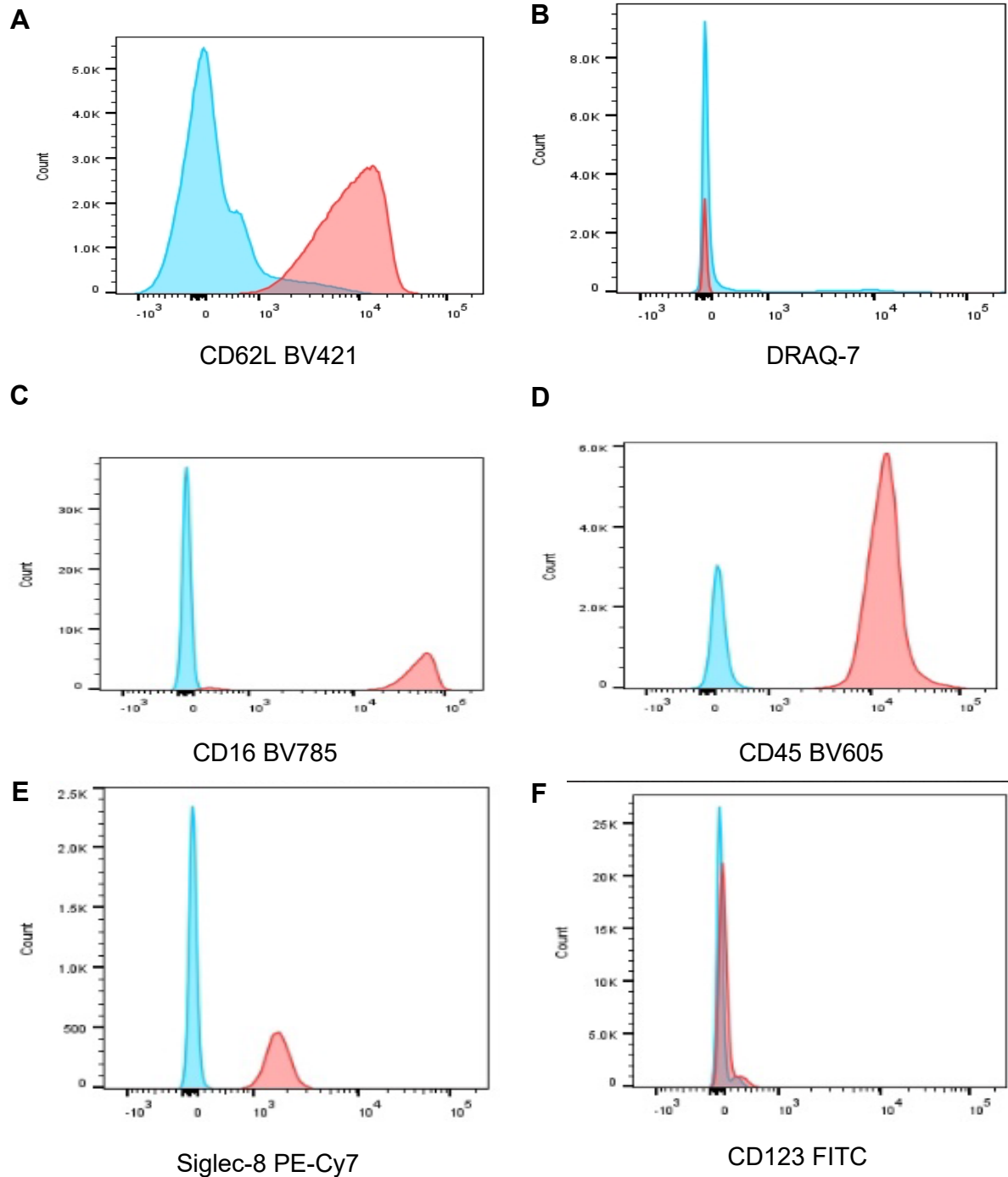


Figure 11-7. Histograms comparing fluorescence minus one (FMO) conditions (Blue) and fully stained samples (Red) for all fluorophores and dye. CD125 (IL-5R α) is presented in the Results section Figure 2.12. These data are from one participant and were acquired as a calibration experiment prior to the study. (A) CD62L BV421 (B) DRAQ-7 viability dye (C) CD16 BV785 (D) CD45 BV605 (E) Siglec-8 PE-Cy7 (F) CD123 FITC. Samples have been

gated as per the study proper; Granulocytes > Single cells > Live cells > CD45⁺ cells. Draq-7 viability is gated on Granulocytes > Single cells. CD45 BV605 is gated on Granulocytes > Single cells > Live cells. Siglec-8 PE-Cy7 is gated on Granulocytes > Single cells > Live cells > CD45⁺ cells > CD16⁻ cells. Note, CD123 stains faintly in the granulocyte population. Viability of blood leukocytes was >96% in this pilot: In retrospect, it would have been desirable to lyse some cells during the pilot for the purposes of demonstrating a larger DRAQ-7 positive population (a small population with variable staining is visible distributed around 10⁴ a.u).

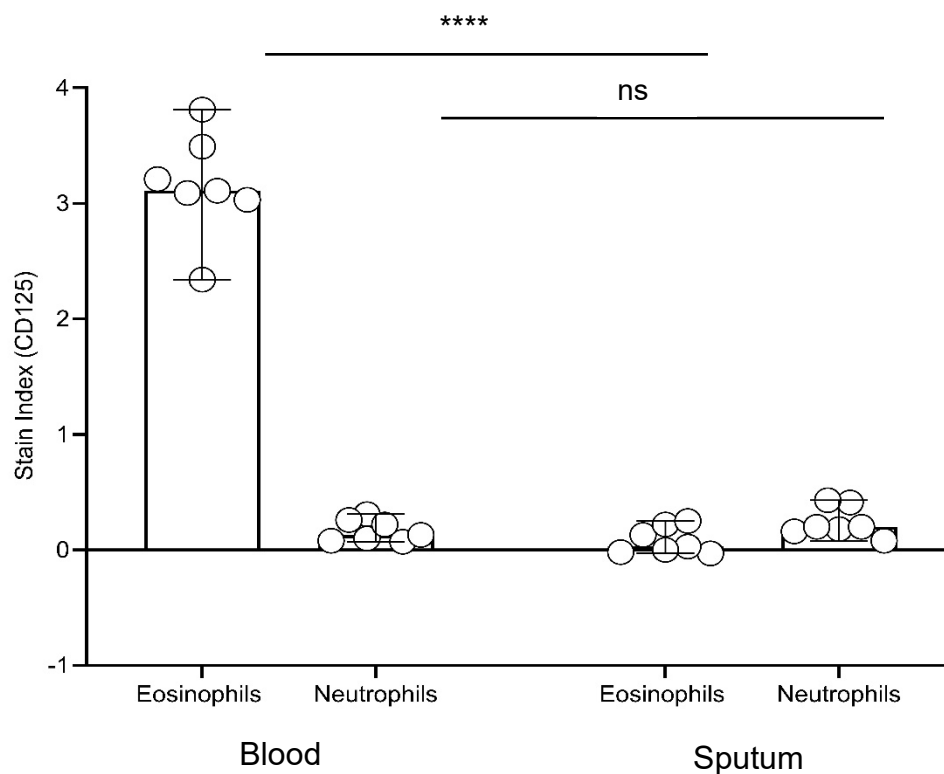


Figure 11-8. Comparison of stain index for eosinophils and neutrophils in blood and sputum, n=7. *** = $p < 0.0001$. This result is consistent with other data showing CD125 expression on resting neutrophils is generally weak. ⁵⁰⁶

Assay Class: High Sensitivity DNA Assay
 Data Path: H:\...gh Sensitivity DNA Assay_DE20901391_2022-06-10_15-52-49.xad

Created: 10/06/2022 15:52:49
 Modified: 10/06/2022 16:45:40

Electropherogram Summary Continued ...

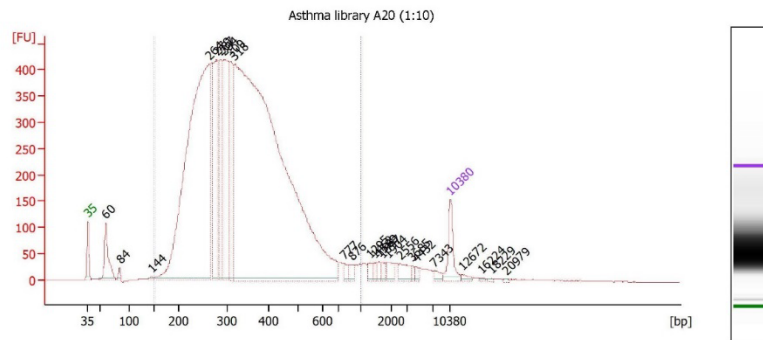


Figure 11-9. Example electropherogram trace (Agilent Bioanalyzer) from one sample library. There is a dominant fragment population centred at approximately 300 bp and the majority of fragments are within the ~250–450 bp size range. There is minimal signal in the low molecular weight region (<150 bp) indicating low adapter or primer dimer concentration. Library concentration is 8.6 ng / μ l

Assay Class: High Sensitivity DNA Assay
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Created: 23/06/2022 15:39:39
 Modified: 27/06/2022 11:14:39

Electropherogram Summary Continued ...

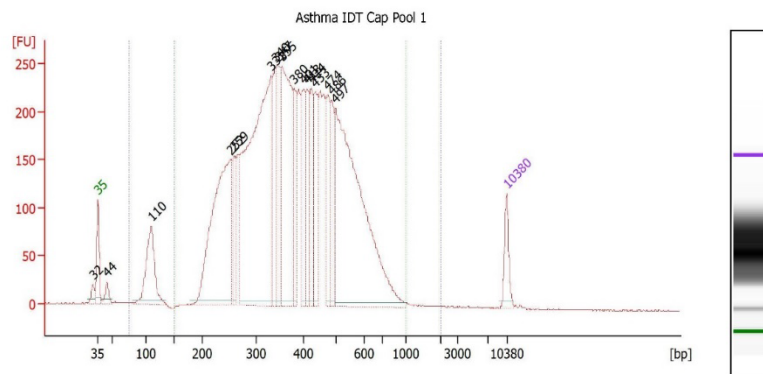


Figure 11-10. Example electropherogram trace from a pool of libraries. There is a dominant fragment distribution spanning approximately 250–550 bp with a peak centred around 350 bp. Baseline fluorescence is <150 bp. Pooled library concentration was 37.9nM which was diluted to a final concentration of 4nM for sequencing.



Figure 11-11. Example of a somatic driver mutation call accepted following satisfactory visual inspection of BAM file in integrative genomics viewer (IGV). The mutation is a single base pair CT>C deletion with frameshift effect at location chr 17:60600711 in the PPM1D gene (denoted by black arrow ▼). The deletion is represented by the white space containing a black bar [-]. The two reads containing the mutation are represented on the track above. The reads do not contain other 'noise' relative to the mutation 'signal' such as other divergent variant calls across the two reads. Sample of total reads presented.

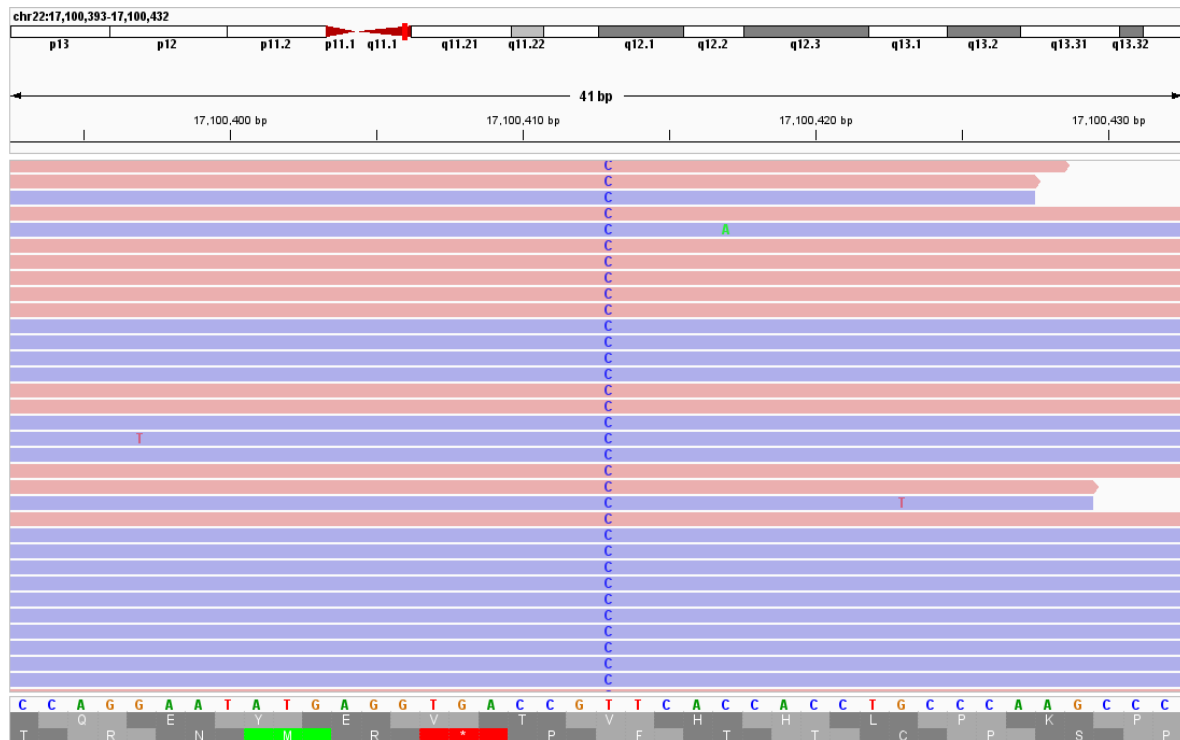


Figure 11-12. Example of a putative germline missense mutation call accepted following satisfactory visual inspection of BAM file in IGV. This is a T>C missense mutation at chr 22:17100413 in the *IL17RA* gene. The variant allele (blue C) is represented approximately equally on forward and reverse strands and there are few other divergent mutations across these strands. Sample of total reads presented.

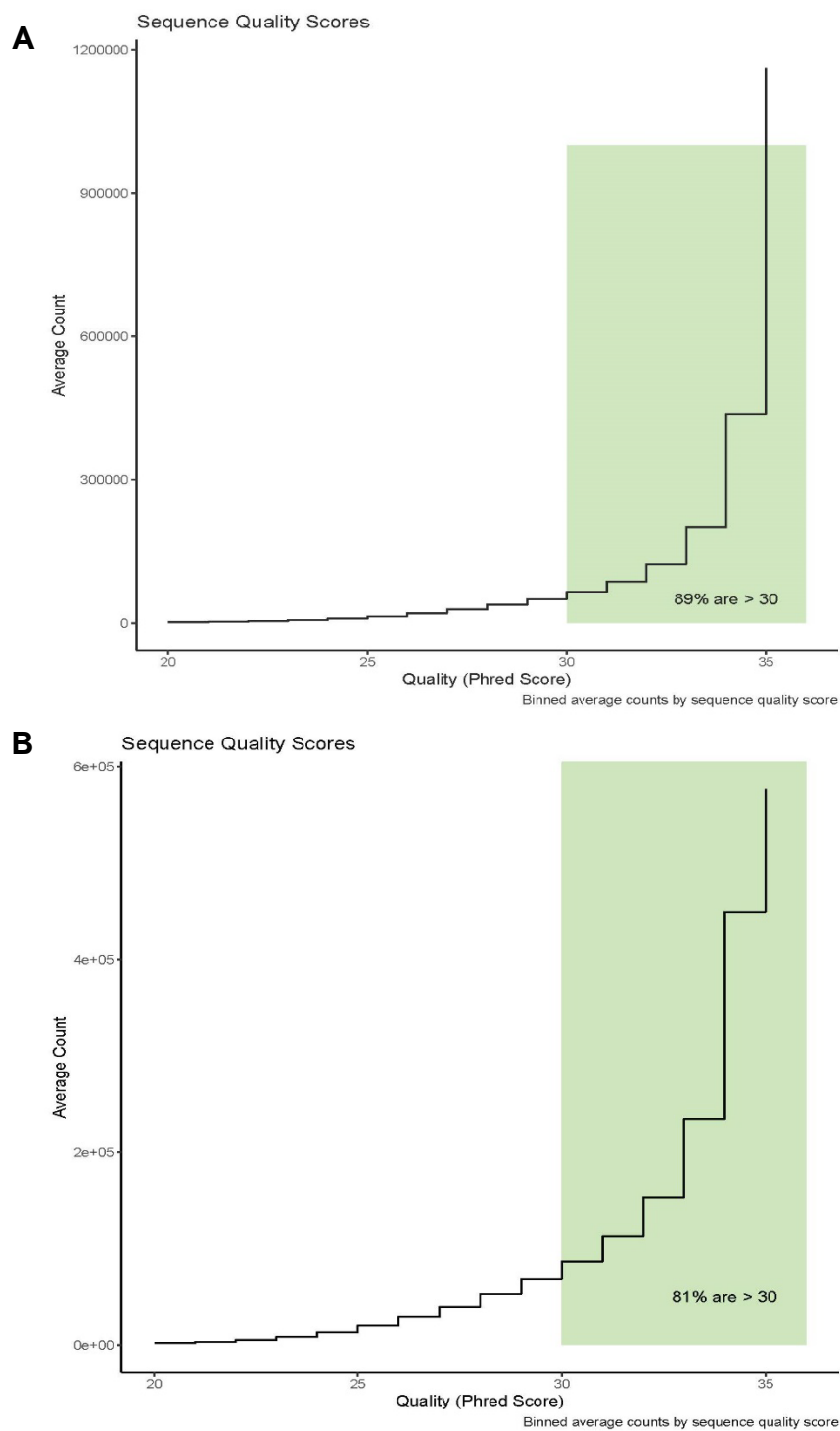


Figure 11-13. Distribution of base quality calls for (A) IDT and (B) Roche sequencing panels. A Phred score of 30 approximates an accuracy of 99.9%.

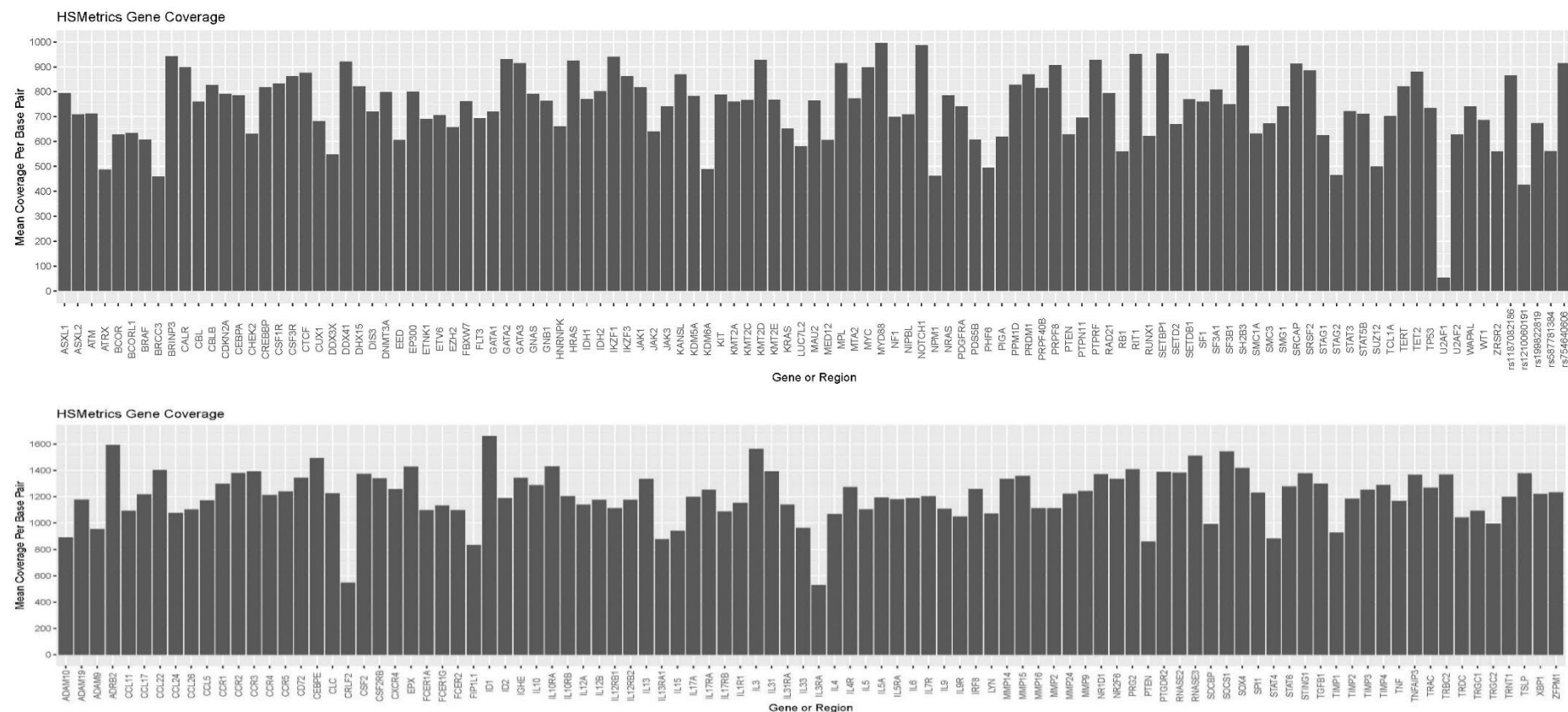


Figure 11-14. Mean coverage per base pair across genes in the Roche (top) and IDT (bottom) panels. Note, coverage is calculated on base pairs covered by probes which ‘overhang’ off-target areas, including for example some intronic areas between exons and is therefore lower than mean coverage per base pair within exons. Poorer coverage for the gene U2AF1 is anticipated due to duplication of the U2AF1 gene on chromosome 21 in hg38 called U2AF1L5, which results in reads being flagged as multi-mapped

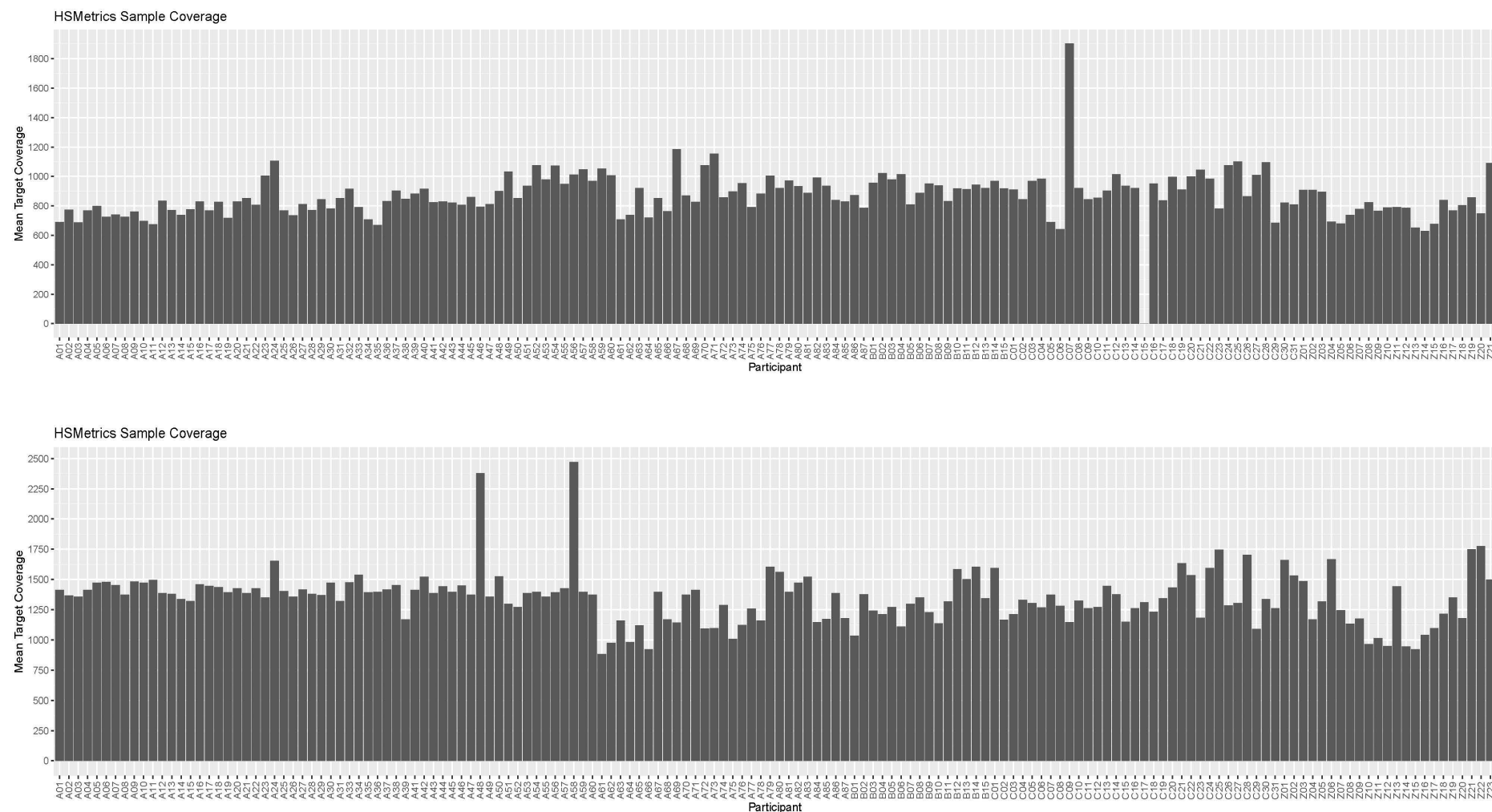


Figure 11-15. Mean coverage per base pair across individual samples in the Roche (top) and IDT (bottom) panels. Sample 'C16' was removed from analyses on the Roche panel.

Gene burden of high-impact variants for LOE asthma versus recruited healthy controls

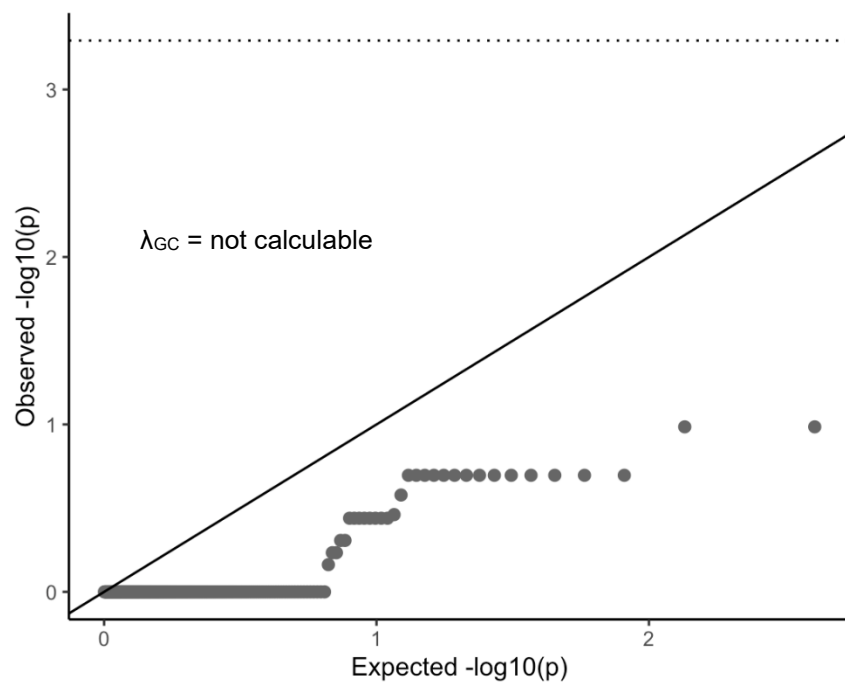


Figure 11-16. QQ plot comparing the gene burden of the most protein-deleterious classes of variants (including transcript ablation, splice donor or acceptor variants, stop gained, frameshift variants, stop lost, and start lost variants) between LOE asthma patients (n= 123) and recruited healthy controls (n= 30). No significant associations were observed. The λ_{GC} could not be reliably calculated due to the extreme rarity of alleles in the above classes with variant allele frequency < 0.001.

11.3 Pilot experiments

11.3.1 Serum IL-5 levels of eosinophilic asthma patients on anti-IL-5R α therapy

A pilot experiment was performed to compare serum IL-5 levels between (i) eosinophilic asthmatic patients established on anti-IL-5R α therapy benralizumab (Fasenra ® AstraZeneca) and (ii) eosinophilic asthmatic patients immediately before the introduction of anti-IL-5R α /IL-5 therapy. Patients were at steady state and were excluded if they had taken any systemic corticosteroids within the preceding 2 months. A small number of participants were recruited among group (i) due to the infrequency of clinic visits of this patient group, particularly during the coronavirus pandemic. IL-5 levels were measured by ELISA (Meso Scale Discovery, USA, Cat: K151QSD) and corrected for 1:2 dilution during the assay (see Methods, Chapter 2). Participant baseline parameters are presented in Table 11-11 below and results are presented in Figure 7-7.

Table 11-9. Participant baseline parameters

	Established on IL-5R α (n=4)	Pre- IL-5R α /IL-5 (n=15)
Age (years)	47.0 (25–64)	59.4 (39–77)
Male : Female (n)	2 : 2	9: 6
Age of onset symptoms (years)	19.0 (5–45)	36.3 (15–64)
Chronic rhinosinusitis +/- polyps (n)	11 : 4	2 : 2
Total IgE (IU/mL)†	410 (153%)	230 (125%)
BDP equivalent daily dose (mcg)‡	800 (700–920)	1600 (800–1960)
Pre-BD FEV ₁ (% predicted)	81.5 ($\pm$ 23.4)	60.0 ($\pm$ 20.7)
Blood eosinophils (x10 ⁹ /L)†	0.00 (0%)	0.71 (53%)
Past peak blood eosinophils (x10 ⁹ /L)†	0.98 (39%)	0.94 (39%)
FeNO (ppb)†	54.6 (56%)	70.1 (69%)
Days on Benralizumab	439 (102–704)	n/a

Values given as mean $\pm$ SE except † = geomean (coefficient of variation, %) and Age/Age of onset and Days on Benralizumab are given as mean (range) ‡ = median (IQR).

Abbreviations: BDP, beclometasone dipropionate; FeNO, fractional exhaled nitric oxide; Pre-BD FEV₁, pre-bronchodilator forced expiratory volume in 1 second; IgE, immunoglobulin E.

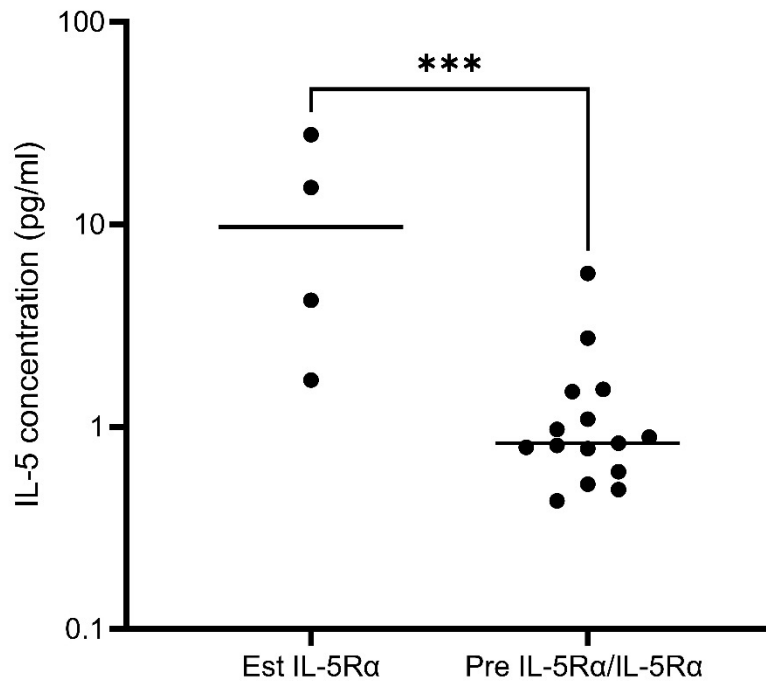


Figure 7-7 (repeated). Comparison of serum IL-5 levels between patients with eosinophilic asthma before initiation of anti-IL-5Rα/IL-5 monoclonal antibody therapy and those established on anti-IL-5Rα treatment. Bars represent the median. Data points are unpaired. Geomean serum IL-5 levels were 7.42 pg/ml (95%CI: 1.00–55.00) for those patients established on IL-5Rα and 1.00 pg/ml (95%CI: 0.68–1.45) for patients about to start anti-IL-5Rα/IL-5 therapy. *** = $p < 0.001$. Abbreviations; Est, established on IL-5Rα biologic therapy; Pre, prior to anti-IL-5Rα/IL-5 biologic therapy.

11.3.2 Calibration experiments for IL-5 and anti-IL-5Rα assays

A series of experiments were performed to understand potential effects on results for the IL-5 assay (Meso Scale Diagnostics, Rockville, MD, USA cat: K151QSD) and particularly for the sIL-5Rα assay (Abcam, Cambridge, UK, cat: ab155435) which, has few reports

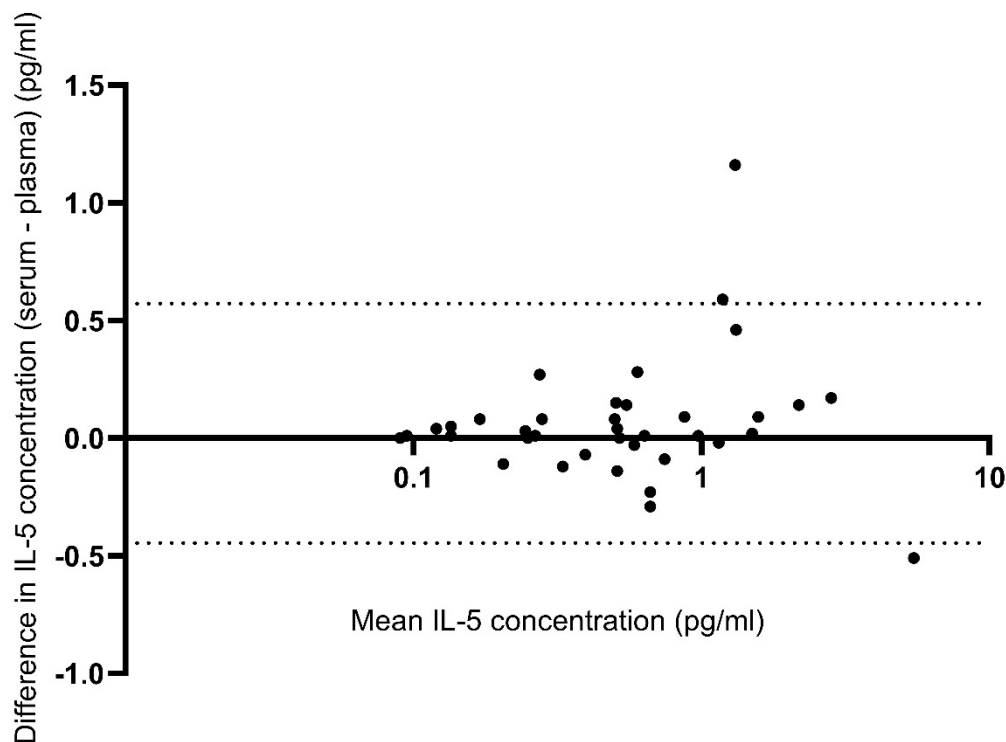


Figure 11-17. Bland–Altman plot showing the difference between measurements of IL-5 (serum – plasma) plotted against their mean. The solid line represents the mean bias, and dotted lines indicate the 95% limits of agreement. A Bland–Altman analysis showed a mean bias (serum – plasma) of -0.06 with 95% limits of agreement from -0.57 to 0.45 ($n = 38$), indicating minimal systematic bias but moderate variability between measurements.

in the literature. These included choosing biological samples to use for the assays (e.g., serum vs plasma or PBS vs DTT sputum supernatant). 38 paired serum and plasma samples taken simultaneously from eosinophilic asthmatic patients were tested. All patients were at steady state and were excluded if they had taken any systemic corticosteroids within the preceding 2 months. Bland Altman analyses were performed to compare serum vs plasma for both IL-5 and sIL-5R α (Figures 11-17 and 11-18). There were acceptable agreement levels between serum and plasma for

IL-5 and sIL-5R α assays and plasma was selected for further testing given the greater availability of samples.

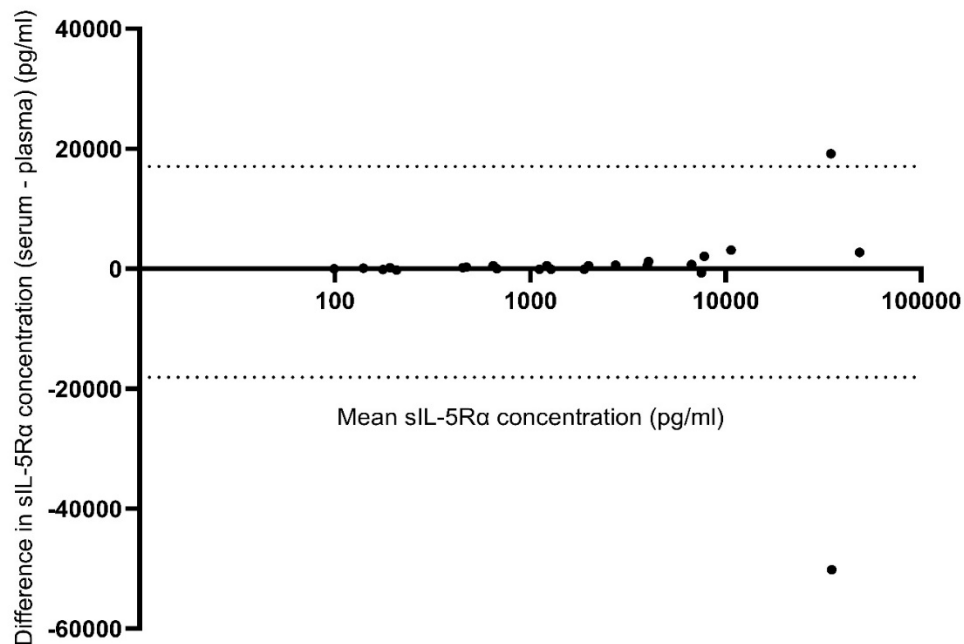


Figure 11-18. Bland–Altman plot showing the difference between measurements of sIL-5R α (serum – plasma) plotted against their mean. The solid line indicates the mean ratio, and dotted lines indicate the 95% limits of agreement. Given the wide dynamic range of sIL-5R α values a log-transformed Bland–Altman analysis was performed showed a mean ratio (Serum/Plasma) of 1.05, with 95% ratio limits of agreement from 0.40 to 2.78 ($n = 37$), indicating minimal proportional bias but substantial variability between individual measurements.

Comparisons of PBS vs DTT for the sIL-5R α assay were performed with 1:2 and 1:20 dilutions ($\geq 1:2$ dilution with proprietary diluent advised by the manufacturer) 4 paired samples from induced sputum were from eosinophilic asthmatic patients at steady state and not taking any systemic corticosteroids were used. No sIL-5R α

was detectable above the lower limit of detection (98.9 pg/ml) in any DTT sample and only one sample was above the LLOD in undiluted PBS (1113 pg/ml) which, was not measurable in 1:2 or 1:20 dilutions for the same sample. This is considered due to the increased dilution involved in producing DTT samples during sputum induction. Given this result, and the wider use of PBS for measuring IL-5 in sputum in the literature, undiluted PBS was used for sIL-5R α assays.

The effect of incubation on sIL-5R α detection was also trialled using plasma incubated at 37C for one hour compared with samples maintained at room temperature for the same period. There was no effect on measurable sIL-5R α concentrations of incubation (Wilcoxon signed-rank test, $p = 1.00$; $n = 4$).

Heterologous spike-in experiments were also performed for both the IL-5 and sIL-5R α ELISA assays. This was done using recombinant human IL-5R α protein (Biotechne, Minneapolis, MN, USA cat: 130-093-927) and recombinant human IL-5 (Miltenyi Biotec, Bergisch Gladbach, Germany, cat: 130-093-927). Both these reagents were those used in the experiments performed by Jian Luo that are discussed in section 7.1. The results of the IL-5 spike into the sIL-5R α assay are shown in Figure 11-19 while results of the sIL-5R α spike into the IL-5 assay are shown in Figure 11-20.

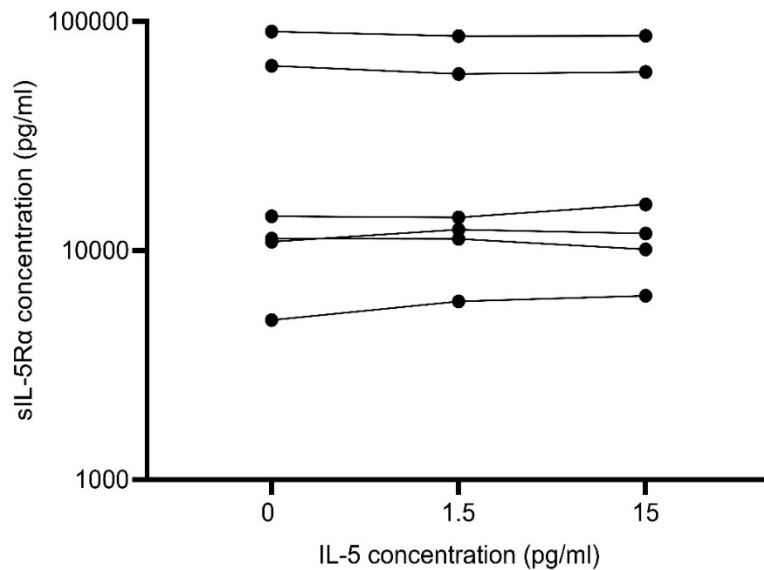


Figure 11-19. IL-5 spike into IL-5R α experiment. Individual cytokine concentrations measured in matched samples at baseline (0 pg/mL) and after spike-in of 1.5 pg/mL and 15 pg/mL. No significant differences were observed across conditions.

There was no effect of spiking IL-5 into the sIL-5R α ELISA assay with no significant differences observed across spike-in concentrations (Friedman test, $\chi^2(2) = 1.00$, $p = 0.61$; $n = 6$). There was an observed small increase in IL-5 levels from spiking sIL-5R α into the IL-5 assay. Cytokine concentrations differed significantly across conditions (Friedman test, $\chi^2(2) = 10.57$, $p = 0.005$; $n = 7$) with an augmentation of measurable IL-5 (~ 20-25%) from 200 $\rightarrow$ 2000 pg/ml sIL-5R α spike-in but no significant increase between 2000 pg/ml and 40,000 pg/ml. The well layout on this ELISA plate was suboptimal for this experiment (a consistent left-to-right formation of low $\rightarrow$ medium $\rightarrow$ high spike-in concentrations). It is suspected that that well allocation on this ELISA plate may have contributed to this unusual finding and the experiment will be repeated.

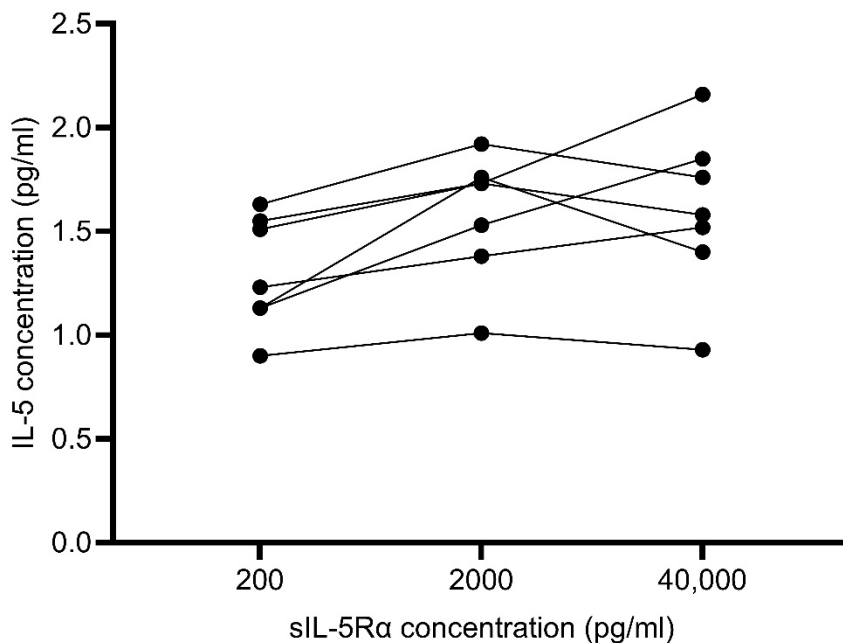


Figure 11-20. IL-5Rα spike into IL-5 experiment. Individual cytokine concentrations were measured in matched samples after spike-in of 200 pg/mL, 2000 pg/ml and 40,000 pg/mL. Cytokine concentrations differed significantly across conditions with a suggestion of mild augmentation of measurable IL-5 (~ 20-25%) between the 200 → 2000 pg/ml sIL-5Rα concentrations. It is suspected that well allocation may have contributed to this unusual finding.

11.3.3 Comparing plasma IL-5 levels between late-onset eosinophilic asthma patients and early onset atopic eosinophilic asthmatics

A *post-hoc* comparison from section 7 was undertaken of the relationships between sputum and plasma IL-5 levels and blood and eosinophil counts between patients with an identifiable late onset eosinophilic asthma phenotype and those with an

identifiable early onset atopic phenotype. Patients were at steady state and were excluded if they had taken any systemic corticosteroids within the preceding 2 months. Participants were assigned to the late onset non-atopic group if there was a clear age of onset of respiratory symptoms ≥ 30 years of age and no clinical history of eczema or atopy. Patients were assigned to the early onset atopic group if onset ≤ 13 years of age and a clinical history of either eczema or atopy. Participants who did not meet these criteria were omitted from this analysis. IL-5 levels were measured by ELISA (MSD, cat: K151QSD). Participant baseline parameters are presented in Table A5.2 and results are presented in Figure A5.6.

There were no statistically significant differences between the two groups in correlations between the 4 variables. The relationships between sputum and blood eosinophil counts (Figure A5.5) and between blood eosinophil counts and plasma IL-5 (Figure A5.6) are presented.

Table 11-10. Participant baseline parameters

	Early-onset eos asthma (n=14)	Late-onset eos asthma (n=25)
Age (years)	52.8 (19–78)	62.9 (37–77)
Male : Female (n)	2 : 12	15 : 10
Age of onset symptoms (years)	6.6 (1– 13)	51.4 (30–75)
Chronic rhinosinusitis +/- polyps (n)	4 : 10	6 : 19
Total IgE (IU/mL)†	177 (199%)	118 (137%)
Sensitization to aeroallergens (from 5) ‡	3 (1.3–4)	0 (0–1)
IgE to House Dust Mite (IU/mL)†	1.38 (103%)	0.04 (111%)
BDP equivalent daily dose (mcg)‡	800 (800–1600)	800 (400–1000)
Pre-BD FEV ₁ (% predicted)	71.7 (±26.2)	68.4 (±23.0)
Sputum eosinophils (%)‡	27.2 (3.5–44)	34.2 (11–68.5)
Blood eosinophils (x10 ⁹ /L)†	0.42 (59%)	0.67 (36%)
FeNO (ppb)†	44.8 (83%)	65.4 (62%)
Plasma IL-5 (pg/ml) †	0.51 (120%)	0.79 (79%)

Values given as mean ± SE except † = geomean (coefficient of variation, %) and Age/Age of onset and ‡ = median (IQR). Abbreviations: BDP, beclometasone dipropionate; FeNO, fractional exhaled nitric oxide; Pre-BD FEV₁, pre-bronchodilator forced expiratory volume in 1 second; IgE, immunoglobulin E.

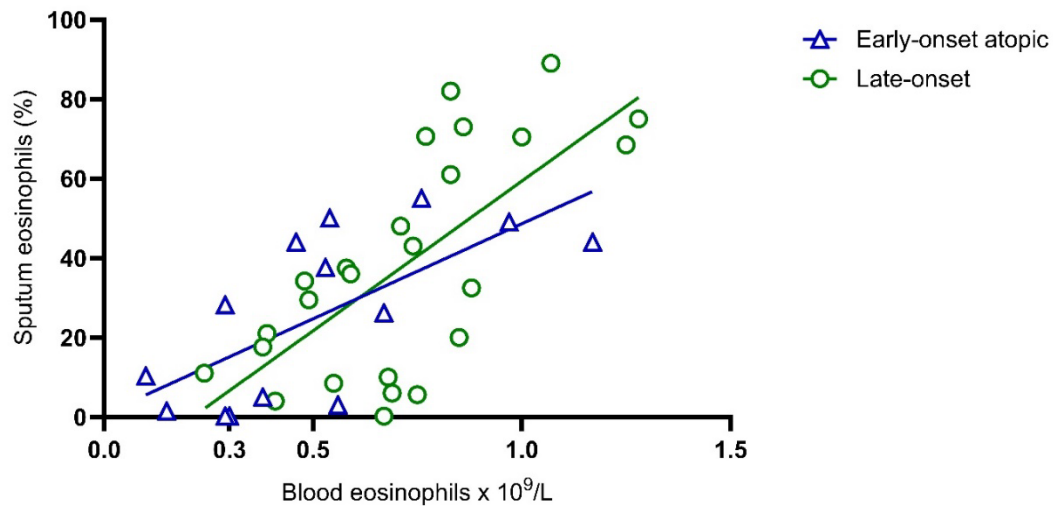


Figure 11-21. Comparison of the relationship between blood eosinophils and sputum eosinophils for early-onset atopic eosinophilic asthmatic patients and late-onset eosinophilic asthmatics. Blood eosinophil counts and sputum eosinophil counts were strongly associated for both early-onset group ($r_s = 0.64$, $p < 0.01$, $n = 14$) and the late-onset group ($r_s = 0.65$, $p = <0.001$, $n = 25$). A Fisher r-to-z comparison following log transformation indicated no significant difference between the correlations ($z = -1.05$, $p = 0.29$ two tailed). Linear trend line is presented on figure.

Ratios between blood eosinophils and plasma IL-5 levels were analysed using log-transformed values [$\log(\text{Blood eos}) - \log(\text{plasma IL-5})$]. Mean log-ratios did not differ between early-onset atopic eosinophilic asthmatics (-0.18 , $n = 14$) and late-onset eosinophilic asthmatics (-0.16 , $n = 25$; Welch's $t = -0.05$, $p = 0.96$), corresponding to a ratio of ratios of 0.98 (95%CI $0.62-1.62$), meaning the average blood eos/plasma IL-5 ratio in the early onset atopic eosinophilic asthma group is about 98% of that in the late onset eosinophilic group.

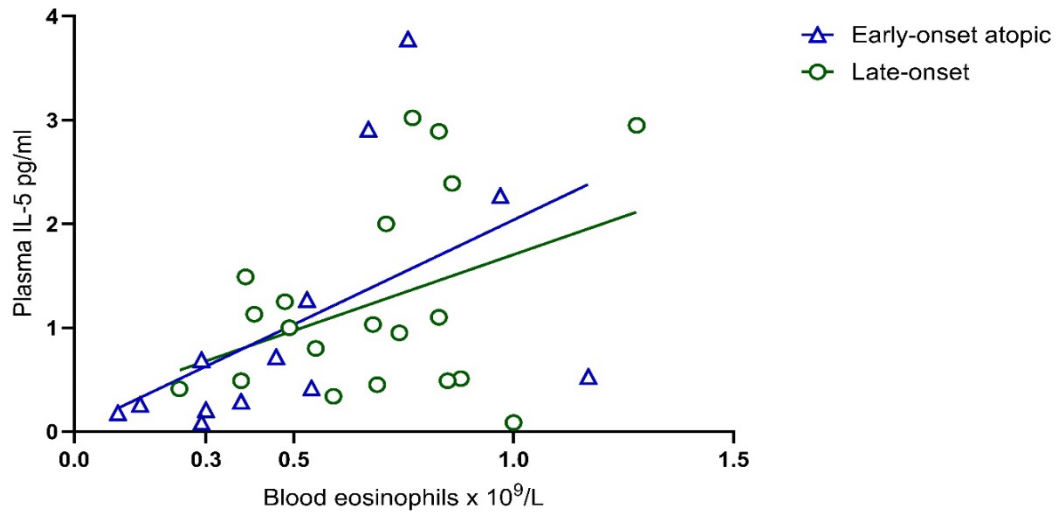


Figure 11-22. Comparison of the relationship between blood eosinophils and plasma IL-5 for the early-onset and late-onset groups. Blood eosinophil counts and plasma IL-5 levels were moderately associated in the early-onset group ($r_s = 0.59$, $p < 0.05$, $n = 14$) but not for the late onset group ($r_s = 0.03$, $p = 0.88$, $n = 25$). A Fisher r-to-z comparison following log transformation indicated that the difference between correlations approached statistical significance ($z = 1.74$, $p = 0.083$ two-tailed). Linear trend line is presented on figure.

11.4 Sputum induction protocol

Sputum induction protocol created to standardise the process and for Oxford University Hospitals NHS Trust use

Induced Sputum Testing for the Non-invasive Measurement of Sputum Inflammation in Adult Patients

Background

Sputum induction is a technique which allows the non-invasive measurement of airway inflammation; which is the number of inflammatory cells in the airways. Studies have demonstrated that measuring the cells in the airways via sputum can inform clinical decisions and identify treatment responses for patients with airways disease including asthma and COPD.

For example, using sputum induction we can measure the number of eosinophils a patient has. This cell has been found to be increased during exacerbations in some patients and correlated to responses to corticosteroid therapy. New treatments of asthma and COPD can only be used in patients that have elevated eosinophils, so measurement is crucial in management of these conditions.

The procedure is simple and safe. A working group on the safety of sputum induction in 2002 did not identify cases of death or need for hospital admission in patients undergoing sputum induction for the assessment of airway inflammation. Inhalation of hypertonic or isotonic saline causes airway constriction in asthmatic subjects and in COPD, particularly in those with airway hyperresponsiveness (AHR). Excessive broncho-constriction is an important safety consideration. This can be limited by pre-treatment with a short acting β -2 agonist but does not completely prevent airway constriction in all subjects. In a review of 692 asthmatic subjects undergoing sputum

induction after pre-treatment with β -2 agonist the average fall in FEV1 was 4.1% – 10.3% Airway constriction is usually quickly reversed by treatment with a short acting β -2 agonist.

Our protocol includes the addition of some salbutamol to the induced saline solution adopting the trial procedure of Delvaux et al, 2004 which reduced bronchoconstriction without affecting the laboratory analyses of sputum.

Factors that may affect the safety of sputum induction include:

- Pre-treatment with short acting β -2 agonist
- Degree of airflow limitation before sputum induction
- Overuse of short acting β -2 agonist
- Degree of asthma control
- Nebuliser output
- Concentration and duration of inhaled saline
- Frequency and timing of safety assessment by FEV1 or PEF

Higher risk* for airway constriction during sputum induction includes the following: highly reactive airways, exacerbation visit, recent increased use of β -2 agonist, as well as baseline FEV1 <60% predicted.

Equipment needed

- DevilBiss ultrasonic nebuliser
- Nebulising chamber and lid
- Mouthpiece, connector and non-reusable plastic tubing
- Blunt dispensing needle and 10ml syringe
- Bacterial Filter
- 0.9% Normal Saline and 4% Hypertonic Saline
- 2.5mg/2.5mls Salbutamol
- Purified water

- Spirometer
- Sputum collection pots
- Tissues
- Glass of water
- Timer
- Gloves
- Apron
- Salbutamol MDI plus Aerochamber
- Induced Sputum form



Figure 1. Devilbiss nebuliser. Please note disposable filters and mouthpieces are used in place of those illustrated

Inform the laboratory technician that a sputum sample will be produced within the next hour

- Prompt processing of samples increases the likelihood of a successful sample

Preparation of the Patient Prior to the Test

Patients should be informed about spirometry to aid adequate preparation for the test and to enable the patient to give informed consent. Many variables may affect the test – before it, the patient should avoid:

- Smoking for 24 hours;
- Drinking alcohol for at least four hours;
- Eating a large meal for at least two hours;
- Taking short-acting bronchodilators for four hours;

Sputum Induction Method

The aim of sputum induction is to obtain satisfactory samples of sputum originating from the small airways. A small plug can be enough to measure cell counts.

Prior to commencing the induction it is essential to explain the procedure fully to the patient:

- Instruction on how to breathe during nebulisation. Tidal breathing is used throughout.
- Instruction about blowing the nose and rinsing the mouth and swallowing water prior to trying to expectorate sputum. (It is important that the subject move quickly through this procedure to prevent loss of sputum due to swallowing).
- Instruction on how to expectorate effectively. It is necessary to explain and demonstrate the technique for coughing up sputum and moving sputum from the back of the throat, forward to the specimen container.

- Advice to spit out saliva before coughing up sputum which can improve the quality of samples
- A reminder not to swallow the sputum as it comes up the bronchial tree.
- Guidance on posture: sitting straight upright during nebulisation, and leaning forward during expectoration.
-

Initial test procedure: All patients

1. Perform baseline spirometry following ARTP/BTS 1994 guidelines and record the best FEV₁ (out of three recordings the patient produces).
2. Administer 400micrograms (4 puffs) Salbutamol MDI via aero-chamber prior to starting the following procedure
3. Repeat spirometry after 15 minutes and record the best FEV₁ (out of three recordings the patient produces). This is the postbronchodilator baseline value.
4. If baseline FEV₁ is $\geq 60\%$ predicted sputum induction should proceed using 5 minute cycles of ~4% hypertonic saline solution (Protocol 1).
5. If baseline FEV₁ is $< 60\%$ predicted or the patient is deemed higher risk* of broncho-constriction the induction should begin using ~0.9% normal saline solution (Protocol 2) and then switch to ~4% hypertonic saline solution if the FEV₁ has not dropped by $>10\%$ after the first 5 minute cycle. An FEV₁ of less than 1.0L is a contraindication to proceeding on with sputum induction.
6. If baseline FEV₁ is $< 1\text{L}$ sputum induction should not be performed.
7. The operator should wear protective clothing (gloves and apron).
8. Connect the filter and tubing to the nebuliser, fill the canister with purified water between the minimum and maximum markings and put the cup into position.

Protocol 1: Patients with FEV1 $\geq$ 60% predicted

1. Create ~4% hypertonic saline solution in the nebuliser cup.

Using a sterile field add:

- (i) 15mls of 0.9% normal saline
- (ii) 16mls of 7% hypertonic saline (4 x 4ml vials)
- (iii) 1ml of 2.5mg/2.5mls salbutamol

2. Put the lid on the cup and the metal bar into position which keeps the lid in place. Connect the tubing and attach the mouthpiece using the connector. The nebuliser must be set at a rate of 1ml/min
3. Ask the patient to breathe tidally through the mouthpiece for 5 minutes, or until the nebuliser turns itself off. The nebuliser should be kept upright. Instruct the patient to discontinue inhalations if troublesome symptoms develop.
4. If during the inhalation the patient coughs and produces any sputum ask the patient to expectorate this into a sputum pot.

5. After 5 minutes of inhalation, the patient will be asked to rinse their mouth and throat with water (swallowing the water) and blow their nose in order to reduce squamous cell contamination and post-nasal drip.
6. Ask the patient to cough using a deep cough to expectorate sputum. The effort of coughing may loosen the sputum to help the patient expectorate. Several attempts at coughing should be made until the sound of the cough becomes dry and unproductive.
7. If the patient manages to expectorate visually inspect the sputum to ensure there are a number of mucus plugs seen. If so discontinue sputum induction.
8. Measure the patients FEV₁ after completion of one procedure cycle (5 mins).
 - If FEV₁ has fallen by <20% compared with the postbronchodilator [baseline] value continue with the next induction cycle.
 - If FEV₁ has fallen by ≥20% compared with the postbronchodilator [baseline] value (or if symptoms occur) stop the induction.
9. If a satisfactory sputum sample achieved stop procedure.
10. If little or no sputum is achieved repeat cycles
11. Total duration of inhalations must not exceed 20 minutes

Protocol 2: Patients with FEV₁ < 60% predicted and/or deemed higher risk*

1. Create ~0.9% hypertonic saline solution in the nebuliser cup.

Using a sterile field add:

- (i) 31mls of 0.9% normal saline
- (ii) 1ml of 2.5mg/2.5mls salbutamol

2. Put the lid on the cup and the metal bar into position which keeps the lid in place. Connect the tubing and attach the mouthpiece using the connector. The nebuliser must be set at a rate of 1ml/min
3. Ask the patient to breathe tidally through the mouthpiece for 2 minutes, or until the nebuliser turns itself off. The nebuliser should be kept upright. Instruct the patient to discontinue inhalations if troublesome symptoms develop.
4. If during the inhalation the patient coughs and produces any sputum ask the patient to expectorate this into a sputum pot.
5. Measure the patients FEV₁ after one minute.

- If FEV₁ has fallen by <20% compared with the postbronchodilator [baseline] value complete the cycle.
 - If FEV₁ has fallen by ≥20% compared with the postbronchodilator [baseline] value (or if symptoms occur) stop the induction.
6. After completion of the first 5 minutes of inhalation, the patient will be asked to rinse their mouth and throat with water (swallowing the water) and blow their nose in order to reduce squamous cell contamination and post-nasal drip.
 7. Ask the patient to cough using a deep cough to expectorate sputum. The effort of coughing may loosen the sputum to help the patient expectorate. Several attempts at coughing should be made until the sound of the cough becomes dry and unproductive.
 8. If the patient manages to expectorate visually inspect the sputum to ensure there are a number of mucus plugs seen. If so discontinue sputum induction.
 9. Measure the patients FEV₁ after one procedure cycle (5 minutes).
- If FEV₁ is stable (less than 10% fall from postbronchodilator baseline value) empty the cup and replace the 0.9% normal saline solution with ~4% hypertonic saline solution (per Protocol 1) and proceed with further inhalation cycles.

- If FEV₁ has fallen by more than 10% but not more than 20% of postbronchodilator baseline value proceed with the next induction cycle with normal saline i.e., **do not increase the concentration**
- If FEV₁ has fallen by $\geq 20\%$ compared with the postbronchodilator [baseline] value (or if symptoms occur) stop the induction.

10. If a satisfactory sputum sample achieved stop procedure.

11. If little or no sputum is achieved repeat 5 minute cycles

12. Total duration of inhalations must not exceed 20 minutes

Safety Procedures During the Test: All patients

Inhaled hypertonic saline is a bronchoconstrictor so sputum induction using ultrasonically nebulised hypertonic saline should be carried out with care. The usual laboratory resuscitation apparatus plus nebulised salbutamol and ipratropium should be readily available. A doctor must be nearby during each procedure. Sputum induction should only be undertaken with trained medical personnel within easy access.

In the event that a patient becomes dyspnoeic and/or wheezy and/or develops chest discomfort, the inhalation of saline should be stopped, and 2.5mg nebulised salbutamol (or inhaled salbutamol if unavailable) +/- 400mcg nebulised ipratropium should be given. The procedure is designed to ensure that patients will not

experience undue bronchoconstriction. Patients must remain at the site until their FEV₁ has returned to within 95% of pre-bronchodilator baseline value

Post procedure

1. Ensure that all sputum samples that are collected are refrigerated and collected by the lab within 2 hours of expectoration.
2. Ensure that the patient's spirometry has returned to within 95% of pre-bronchodilator baseline value before allowing the patient to leave the department.
3. All consumables (tubing and filter) are disposed of into a clinical waste bag, and mouthpiece and connector are sent to CSSD to be ethylene oxidized.
4. At the end of the day the purified water can be discarded and the removable white plastic connector, at the base of the yellow nebuliser chamber should be removed and the yellow chamber should be washed in warm soapy water and allowed to dry.

Results

Complete the sputum processing form (Appendix 1). If no sputum is obtained document this on the sputum form in the appropriate section.

References

- Chanez P, Holz O, Ind P W et al. Sputum induction, Report of Working Group 1, Eur Resp J, 2002; 37, 3s-8s
- Delvaux M, Henket M, Lau L et al. Nebulised salbutamol administered during sputum induction improves bronchoprotection in patients with asthma, Thorax, 2004, 59, 111-115
- Pizzichini M M M, Leigh R, Djukanović R and Sterk P J. Safety of sputum induction, Eur Resp J, 2002; 37, 9s-18s

Training

It is the responsibility of the practitioner following this document to ensure they have the relevant training and competence to complete the task safely.

Review

This document will be reviewed every three years, or before this date, particularly if national guidance or local arrangements change.

Document History

Category:	Guideline
Summary:	
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Related Documents:	
Author(s):	James Melhorn
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This Document replaces:	

Date of revision	Version number	Reason for review or update

Appendix 1

Sputum Processing Form – Patient Information

OAS number

Patient Addressograph

Date:

Time:

Has the patient given consent to this procedure?
Yes / No

Prior to the procedure, has the patient avoided:

Processes Avoided	Yes	No	N/A
Smoking for 24 hours;			
Drinking alcohol for at least four hours;			
Wearing any tight clothing;			
Eating a large meal for at least two hours;			

Prior to commencing the induction it is essential to explain the procedure fully to the patient:

- Have you given instruction on how to breathe during nebulisation?

Yes / No

- Have you explained about blowing their nose and rinsing their mouth and swallowing the water prior to trying to expectorate sputum?

Yes / No

- Have you explained how to expectorate effectively?

Yes / No

- Have you reminded the patient not to swallow the sputum as it comes up the bronchial tree?

Yes / No

- Have you explained the importance of posture during the procedure: sitting straight upright during nebulisation, and leaning forward during expectoration?
Yes / No

Baseline FEV₁ Values

Pre bronchodilator:

	•		

 L

(15 min interval)

Post bronchodilator:

	•		

 L

Protocol 1: 4% Hypertonic Saline solution, 5 minute cycles

Nb.

- (i) 15mls of 0.9% normal saline
- (ii) 16mls of 7% hypertonic saline (4 x 4ml vials)
- (iii) 1ml of 2.5mg/2.5mls salbutamol

Protocol 2: Starting with 0.9% Normal Saline for those patients with baseline FEV1<60% predicted or at higher risk, increasing to 4% Hypertonic saline after one cycle if no excessive bronchoconstriction. 5 minute cycles.

Nb.

- (i) 31mls of 0.9% normal saline
- (ii) 1ml of 2.5mg/2.5mls salbutamol

Protocol 1:

Protocol 1 : 4% Hypertonic saline FEV1 (end cycle 1):

	•		

 L

Protocol 1 : 4% Hypertonic saline FEV1 (end cycle 2):

	•		

 L

Protocol 1 : 4% Hypertonic saline FEV1 (end cycle 3):

	•		

 L

Protocol 1 : 4% Hypertonic saline FEV1 (end cycle 4):

	•		

 L

Post procedure best FEV1

	•		

 L

Protocol 2:

Protocol 2 : 0.9% saline FEV1 (one minute)

	•		

L

Protocol 2 : 0.9% saline FEV1 (end cycle 1):

	•		

L

Protocol 2 : 0.9% or 4% saline FEV1 (end cycle 2):

	•		

L

Protocol 2 : 0.9% or 4% saline FEV1 (end cycle 3):

	•		

L

Protocol 1 : 0.9% or 4% saline FEV1 (end cycle 4):

	•		

L

Post procedure best FEV1

	•		

L

Post Nebulisation

Sputum Obtained? Yes ☐ No ☐

Inspection of sputum : Mucoid ☐ Mucopurulent ☐ Purulent ☐

Procedure carried out by:

Signature :

Position:
.

Place these sheets in OAS CRF

Note: airway hyper-responsiveness to hypertonic saline cannot be assessed using this sputum induction protocol (given concurrent administration of short acting β -agonist). However, please document FEV1 values on EPR for those with an FEV1 drop of $\geq 15\%$ if participant not known to have AHR.