Word count : 14 868/15 000

MSc Candidate, Student #1285644

Fractional exhaled nitric oxide non-suppression in asthma

Dissertation for the

MSc in Experimental and Translational Therapeutics

Supervised by

Professor Ian Pavord and Dr Timothy Hinks

University of Oxford

24 September 2021

Dissertation title: Fractional exhaled nitric oxide non-suppression in asthma

Degree: MSc in Experimental and Translational Therapeutics

Term submitted: Michaelmas, 24 September 2021

ABSTRACT

Background: Factional exhaled nitric oxide (FeNO) and blood eosinophils are biomarkers of the higher risk type-2 inflammatory phenotype in asthma.

Objectives: 1) To assess if patients who fail to suppress FeNO after monitored therapy exhibit corticosteroid resistance. 2) To translate non-suppression of type-2 biomarkers to inflammatory mediators in the airway and peripheral blood in severe asthma. 3) To develop a prototype risk scale predicting asthma attacks based on FeNO and blood eosinophils.

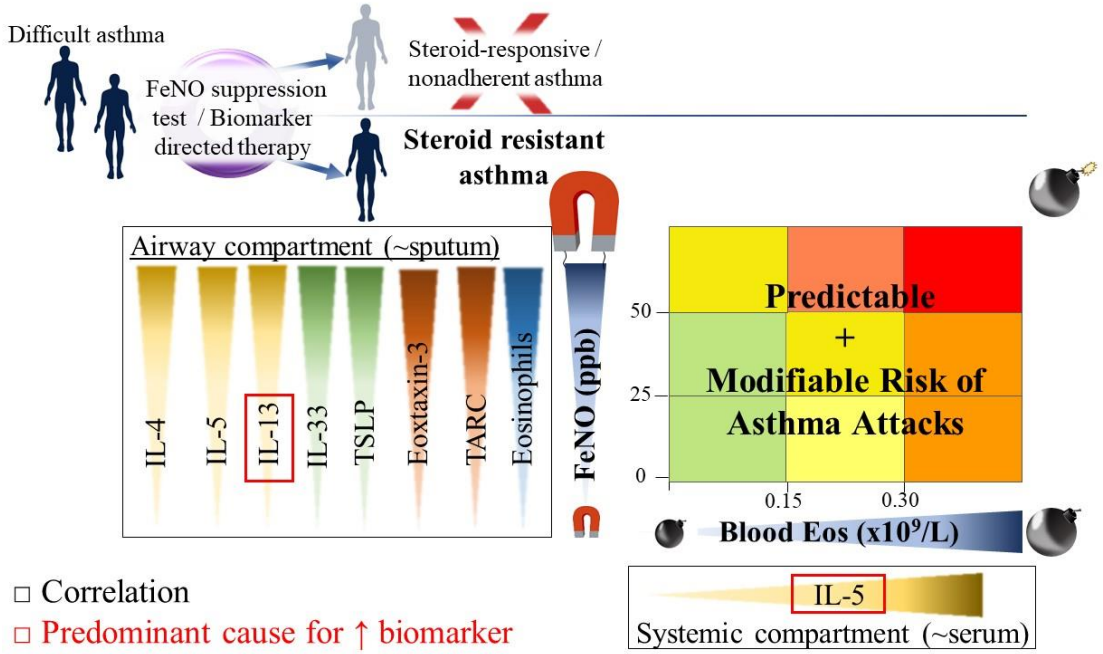
Methods: 1) Induced sputum eosinophils and 11 sputum supernatant plus 9 serum inflammatory proteins were analysed in a paired before/after analysis of FeNO suppression tests conducted in Oxford, compared on the basis of a positive/negative test (*i.e.:* ≥42/% decrease in FeNO following monitored high-intensity corticosteroid therapy). 2) These inflammatory mediators were also correlated to the FeNO and blood eosinophil levels at the point of maximum treatment intensity in a cross-sectional study pooling the Oxford FeNO suppression cohort and the RASP-UK trial cohort. 3) Biomarker-stratified trial-level attack rates were extracted and pooled from the control arms of 8 randomised clinical trials. These were used to derive rate ratios and the predicted asthma attack rate for different patient groups.

Results: 1) Thirty-four FeNO suppression tests were analysed. The 19 patients failing to suppress FeNO were older, more intensely treated, and had little or no trends for

improvement in clinical nor sputum/serum measurements compared to those who suppressed. 2) In 74 patients with severe asthma, FeNO correlated with airway type-2 cytokine, chemokine, alarmin and eosinophilia, whilst blood eosinophils correlated with serum interleukin-5. 3) The trial-derived (*n*=3051) prototype risk scale showed feasibility and potential to predict asthma attacks which can be prevented by anti-inflammatory therapy.

Conclusion: FeNO non-suppression carries significant translational, prognostic and theragnostic utility as a biomarker of type-2 airway inflammation in asthma – especially when used in combination with blood eosinophils.

Graphical abstract:



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This work is dedicated to the memory of my stepfather and friend R (1953-2006) who, like so many other asthma patients, succumbed to the complications of long-term oral corticosteroids.

ABBREVIATIONS

ACQ: asthma control questionnaire

ATS: American Thoracic Society

AUC: area under the curve

BRC: Biomedical Research Centre

BTS: British Thoracic Society

CCL17: C-C motif chemokine ligand 17 (also known as thymus activation regulated cytokine or TARC)

CCL26: C-C motif chemokine ligand 26 (also known as eotaxin-3)

CFC: chlorofluorocarbon

CI: confidence intervals

ERS: European Respiratory Society

FeNO: fractional exhaled nitric oxide

FEV1: forced expiratory volume in one second

FF: fluticasone furoate

FP: fluticasone propionate

FVC: forced vital capacity

GINA: Global Initiative for Asthma

ICS: inhaled corticosteroid

Ig: immunoglobulin

IL: interleukin

IM: intramuscular

INF: interferon

LABA: long-acting beta2-agonist

LLOD: lower limit of detection

LTE4: leukotriene E4

mAb: monoclonal antibody

MHC: major histocompatibility complex

NIHR: National Institute for Health Research

ORACLE: OxfoRd Asthma attaCk risk ScaLE

PGD2: prostaglandin D2

Rα: receptor alpha subunit

SABA: short-acting beta2-agonist

TARC: thymus activation regulated cytokine (also known as C-C Motif Chemokine Ligand 17 or CCL17)

TH: T-helper cell

TNF: tumour necrosis factor

TSLP: thymic stromal lymphopoietin

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INTRODUCTION

Asthma is a very prevalent chronic respiratory disease affecting approximately 400 million people in the world (To *et al.*, 2012). Most asthmatics have good control over their symptoms and experience no asthma attack on low to medium-dose inhaled corticosteroids (ICS)-containing treatment regimens. Nevertheless, 5 to 10% require high intensity treatment to maintain control, or have uncontrolled asthma despite treatment (Chung *et al.*, 2014; Hekking *et al.*, 2015). These patients have severe disease and comprise most of asthma-related spending, morbidity, and mortality (Barnes, 2012).

The heterogenous nature of asthma is now acknowledged across all disease severities (Haldar *et al.*, 2008; Wenzel, 2012). This heterogeneity is readily assessed by biomarkers. A biomarker is defined as a 'characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacological response to a therapeutic intervention' (Atkinson *et al.*, 2001). Fractional Exhaled Nitric Oxide (FeNO) and the peripheral blood eosinophil count are the most prominent and widely accessible biomarkers of type-2 inflammation in asthma (Pavord *et al.*, 2018; Pavord and Corren, 2020). The type-2 inflammatory phenotype can be identified in many but not all people with asthma (Woodruff *et al.*, 2009; Fahy, 2015; Heaney *et al.*, 2021) and carries important prognostic (*i.e.* predicting adverse outcomes) and theragnostic (*i.e.* predicting treatment responsiveness) information. Its recognition has had a profound impact on the management of type-2 high asthma, where the efficacy of anti-inflammatory therapies – ICS and/or monoclonal antibodies (mAbs) – are directed through the use of biomarkers (Green *et al.*, 2002; Pavord *et al.*, 2018; Couillard and Pavord, 2021a; Heaney *et al.*, 2021).

I herein review the mechanisms and clinical significance of FeNO elevation in asthma, with a particular focus on severe disease. To the latter point, I explore the evidence supporting

the FeNO suppression test to stratify uncontrolled asthma both in the published literature and by analysing tests conducted locally in Oxford (Couillard, Shrimanker, *et al.*, manuscript in preparation). My novel contribution to the field is to translate FeNO and blood eosinophil non-suppression in a cross-sectional analysis of sputum and serum inflammatory mediators in patients with severe asthma who are on high intensity corticosteroid therapy (Couillard, Shrimanker, *et al.*, 2021). Finally, the observation that FeNO provides complementary information to the peripheral blood eosinophil count both clinically and translationally has led me to derive a prototype scale based on biomarker-stratified randomised clinical trial (RCT) data (Couillard, Laugerud, *et al.*, 2021) which may predict treatment benefits of anti-inflammatory therapies (Couillard, Do, *et al.*, manuscript in preparation).

BACKGROUND

1. Diagnosis and stratification of severe asthma

1.1. What is asthma?

Asthma is a common chronic airways disease which affects 5 to 20% of adults in westernised countries (To *et al.*, 2012). It is better approached as a syndrome: a heterogenous group of conditions characterised by bronchial symptoms – wheezing, chest tightness, dyspnoea, cough and/or phlegm –, variable airflow obstruction, and airway inflammation/remodelling (Pavord *et al.*, 2018).

From a histopathological point of view, the hallmarks of asthma are inflammation and remodelling (Figure 1A). Accordingly, the discovery and widespread use of inhaled, anti-inflammatory corticosteroid therapy has had a major impact on clinical practice, ushering in the 'inflammation era' marked by very low asthma-related mortality rates (Figure 1B). Nevertheless, there exists a subgroup of patients with unrelenting symptoms, functional limitation, and frequent asthma attacks despite intensive therapy. Compared to patients with mild disease, these patients with severe asthma represent a five times greater financial burden (Cisternas *et al.*, 2003) and an eight times greater asthma death toll in the United-Kingdom (Royal College of Physicians, 2014). There is an unmet need to identify patients at high risk in a manner which translates both airway inflammation and treatment opportunities (Agusti *et al.*, 2016).

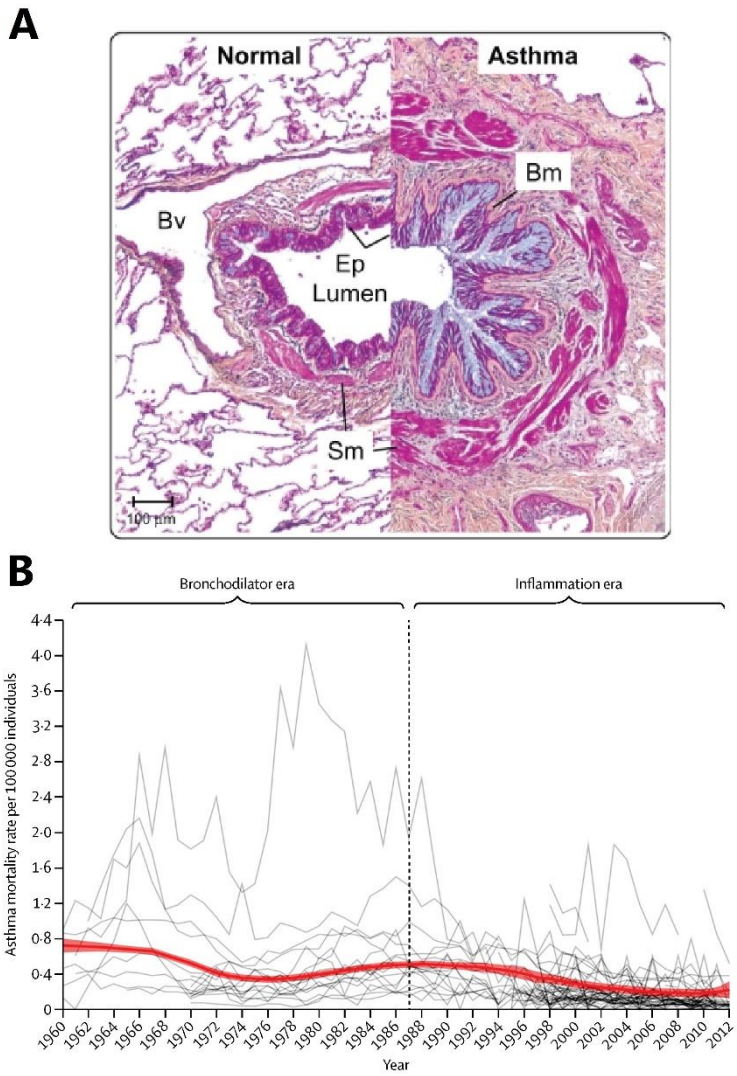

FIGURE 1. Asthma is an inflammatory pathology. Panel A shows the histopathology of medium-sized airways in health compared to asthma. Inflammation and remodelling are seen with goblet cell hyperplasia and mucus secretion (blue), basement membrane (bm) thickening, and smooth muscle hypertrophy (Sm). Reproduced from Wadsworth et al. (2011). Panel B shows asthma mortality rates between 1960 and 2012 in 46 countries for adults ages 5 to 34, with the weighted average mortality (red line ± shaded 90% confidence intervals) improving as anti-inflammatory therapy enters clinical practice. Reproduced from Pavord et al. (2018), itself adapted from Ebmeier et al. (2017).

1.2. Diagnosis of severe asthma

The definition of severe asthma relies heavily on uncontrolled symptoms and high-intensity corticosteroid treatment (Box 1). Distinguishing 'difficult' and 'severe' asthma is indeed difficult because some patients initially appear to have severe asthma until an in-depth assessment reveals one or more factors that contribute to ongoing symptoms and/or asthma attacks. The expression 'difficult asthma' describes this scenario and includes issues such as nonadherence to controller therapies and/or poor inhaler technique, as well as the incorrect attribution of symptoms to asthma rather than to comorbidities (Couillard, Jackson, *et al.*, 2021; Global Initiative for Asthma (GINA), 2021)(Figure 2). Of these factors, the assessment of treatment adherence is one of the most overlooked components: up to 80% of patients on high-intensity asthma treatment do not have optimal medication intake (Hekking *et al.*, 2015).

Asthma which requires treatment with high-dose ICS (*i.e.*: beclomethasone dipropionate chlorofluorocarbon (CFC) preparation-equivalent 2000µg/day or more) and long acting beta2-agonist or leukotriene modifier/theophylline for the previous year or systemic corticosteroids for >50% of the previous year to prevent it from becoming uncontrolled or which remains uncontrolled despite this therapy.'

Uncontrolled asthma is defined as at least one of the following:

1) Poor symptom control: Asthma Control Questionnaire (ACQ) consistently >1.5, Asthma Control Test <20, or ''not well controlled'' by GINA guidelines.
2) Frequent severe exacerbations: two or more bursts of systemic corticosteroids (>3 days each) in the previous year.
3) Serious exacerbations: at least one hospitalization, ICU stay or mechanical ventilation in the previous year.
4) Airflow limitation: after appropriate bronchodilator withheld, forced expiratory volume in 1 second (FEV_1) <80% predicted (in the face of reduced FEV_1/forced expiratory volume (FVC) defined as less than the lower limit of normal).
5) Controlled asthma that worsens on tapering of these high doses of ICS or systemic CS (or additional biologics

BOX 1. The definition of severe asthma. Reproduced from the American Thoracic Society (ATS)/European Respiratory Society (ERS)(Chung *et al.*, 2014).

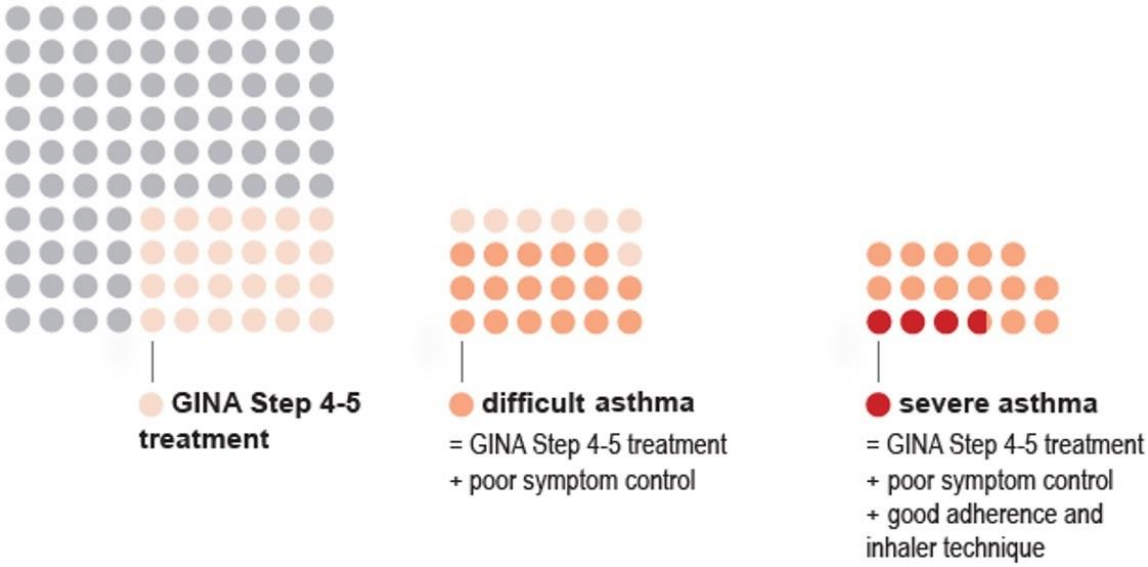

FIGURE 2. Distinguishing treatment intensity, symptoms, and severity in asthma. Proportion of adult asthmatics (●) with high-intensity treatment (● = Global Initiation for Asthma (GINA) step 4-5), difficult (●), and severe (●) asthma. Reproduced from GINA (2019).

1.3. The prevalence and role of type-2 inflammation in severe asthma

The label 'severe asthma', although stringently defined, often reflects different and dynamic biological processes. A key component of disease characterisation is to determine whether type-2 airway inflammation is present and active. Type-2, eosinophilic inflammation stems from the activity of interleukin (IL)-4, IL-5, and IL-13 (Pavord *et al.*, 2018). It is now estimated to be present in approximately half of adults with asthma (Fahy, 2015) and up to 95% of patients with severe disease (Heaney *et al.*, 2021).

Early evidence that asthma can be divided in two inflammatory subtypes with distinct physiologic and clinical characteristics was delivered at the turn of the millennium by two key publications. The first before/after corticosteroid study in patients stratified by sputum eosinophilia (sputum eosinophil count ≥3%), communicated by Pavord *et al.* (1999), highlighted the greater change in symptom scores, bronchial hyperresponsiveness and

airway inflammation in the ‘eosinophilic’ phenotype following two months of inhaled budesonide compared to the non-eosinophilic patients. Concurrently, Wenzel *et al.* (1999) published a cross-sectional clinical and translational characterisation of severe asthmatics with and without bronchial tissue eosinophilia: the analysis concluded that two distinct pathologic, physiologic, and clinical subtypes exist in severe asthma. These early studies were reproduced and dissected during the following decade (Green *et al.*, 2002; Berry *et al.*, 2007; Haldar *et al.*, 2008; Woodruff *et al.*, 2009). Type-2 airway inflammation is now acknowledged to represent a continuum reflecting specific clinical characteristics (Figure 3). Selected mediators associated with the type-2 response are shown in Table 1.

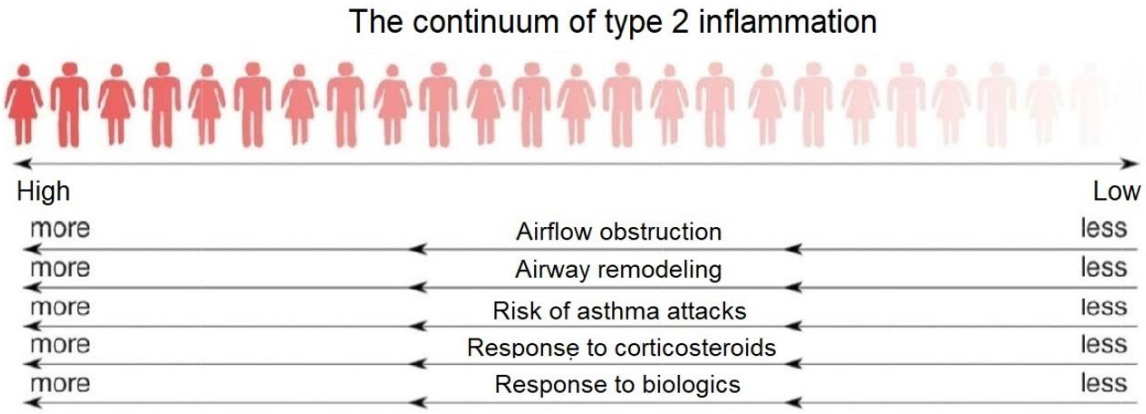

FIGURE 3. Type-2 airway inflammation is acknowledged as both a risk factor and a treatable trait. Figure modified from Murray and Nadel’s Textbook of Respiratory Medicine (2016, p. 728) and Couillard *et al.* (2020).

TABLE 1
Selected mediators of the epithelial type-2 response in asthma

Type	Name	Prominent roles
Alarmins	IL-33	Promote epithelium integrity and parasite expulsion by inducing type-2 cytokines
	TSLP	
Type-2* cytokines	IL-4	B-cell activation, IgE switch, Th0 differentiation into TH2 cells
	IL-5	Drives the differentiation, survival, and pathological activation of eosinophils
	IL-13	Promotes B-cell growth and differentiation, goblet cell hyperplasia and mucus production, smooth muscle contractility and hypertrophy
Chemokines	Eotaxin-3 (*i.e.*: CCL26)	Most potent eotaxin to induce the migration of eosinophils in humans
	TARC (*i.e.*: CCL17)	Induces migration of T cells (Th2 > Th1), immature dendritic cells, thymocytes, and regulatory T cells
Eicosanoids	LTE4	Leukotriene subtype; potent bronchoconstricting and proinflammatory properties
	PGD2	Prostaglandin subtype; activates ILC2, TH2 lymphocytes and eosinophils
Inflammatory cell types	ILC2	Produce large amounts of IL-4, IL-5, IL-13, and PGD2 in response to alarmin signalling
	Eosinophils	Main effector cell for the Th2 response; associated with airway hyperresponsiveness, tissue damage, mucus plugging, and Charcot-Leyden crystal formation. Sputum eosinophil counts ≥3% identify eosinophilic airway inflammation.
	Neutrophils	Important effector cells for the antimicrobial response; also associated with smoking; induce oxidative stress and tissue damage. Sputum neutrophils ≥61% identify neutrophilic airway inflammation.
Other non-type-2 mediators	IFN-γ	Increased antiviral Th1 response; suppresses Th2 response
	TNF	Promotes inflammation – including the type-2 response

*The term ‘type-2’ reflects production of interleukin (IL)-4, -5, and -13 by innate lymphoid cells (ILC)2 and T-helper (Th)2 lymphocytes. CCL, C-C Motif Chemokine Ligand; IFN, interferon; LTE, leukotriene E4; PGD2, prostaglandin D2; TARC, thymus and activation-regulated chemokine; TNF, tumour necrosis factor; TSLP, thymic stromal lymphopoietin. Adapted or sometimes reproduced from Kolmert et al. (2020); Lambrecht et al. (2019); Murphy and Weaver (2017).

More recently, the development of mAbs which target type-2 inflammation has led to the recognition that the two biomarkers FeNO and blood eosinophils differentially predict responsiveness to specific biologics. The interplay between type-2 inflammation, biomarkers and therapeutic responsiveness to mAbs in asthma is shown in Figure 4.

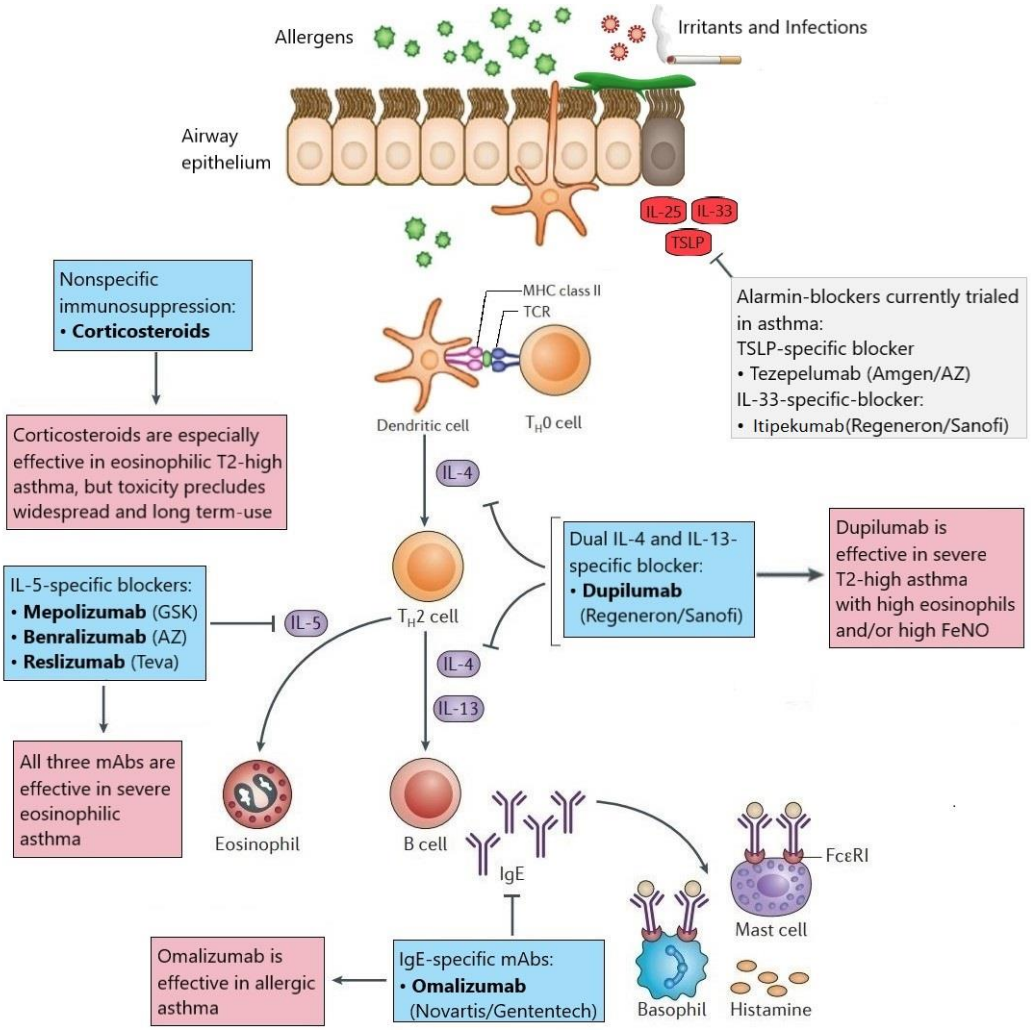

FIGURE 4. Targets and clinical efficacy of therapies in severe asthma. Corticosteroids have historically been the established treatment at the cost of associated toxicity. Targeting the end result of the allergic pathway – *e.g.*, immunoglobulin E (IgE) – has enjoyed modest success in asthma. The inhibition of more proximal drivers of inflammation such as interleukin (IL)-5, IL-4 and IL-13 has greater efficacy. Recent advances in targeting the most upstream inflammatory mediators (the alarmins thymic stromal lymphopoietin

(TSLP), IL-25 or IL-33) are promising but have yet to lead to a licensed biological therapy. AZ, AstraZeneca; FcεRI, high-affinity IgE receptor; GSK, GlaxoSmithKline; mAb, monoclonal antibody; MHC, major histocompatibility complex, T2; type-2; TCR, T cell receptor; TH, T helper 2. Figure reproduced and legend adapted from Couillard and Pavord (2021a), itself modified from Gandhi et al. (2016) and Lambrecht and Hammad (2015).

2. Pathobiology of fractional exhaled nitric oxide (FeNO)

2.1. Regulation of NO in the human airways

Nitric oxide (NO) is enzymatically produced in humans by the NO synthases (NOS). The enzyme exists in three distinct isoforms, all of which are found in the respiratory system. Two isoforms (NOS1 and NOS3) are constitutively active and produce minute quantities of NO in response to calcium and calmodulin. The third and most studied isoform – NOS2, also known as iNOS – can be induced following a variety of proinflammatory triggers. Induced NOS2 leads to the release of large amounts of NO for hours to days with positive and negative effects for the host (Figure 5)(Ricciardolo *et al.*, 2004). It is noteworthy that nonenzymatic NO formation and excretion also occurs in the salivary glands following uptake of plasma nitrate or oral exposure to nitrate (Zetterquist *et al.*, 1999).

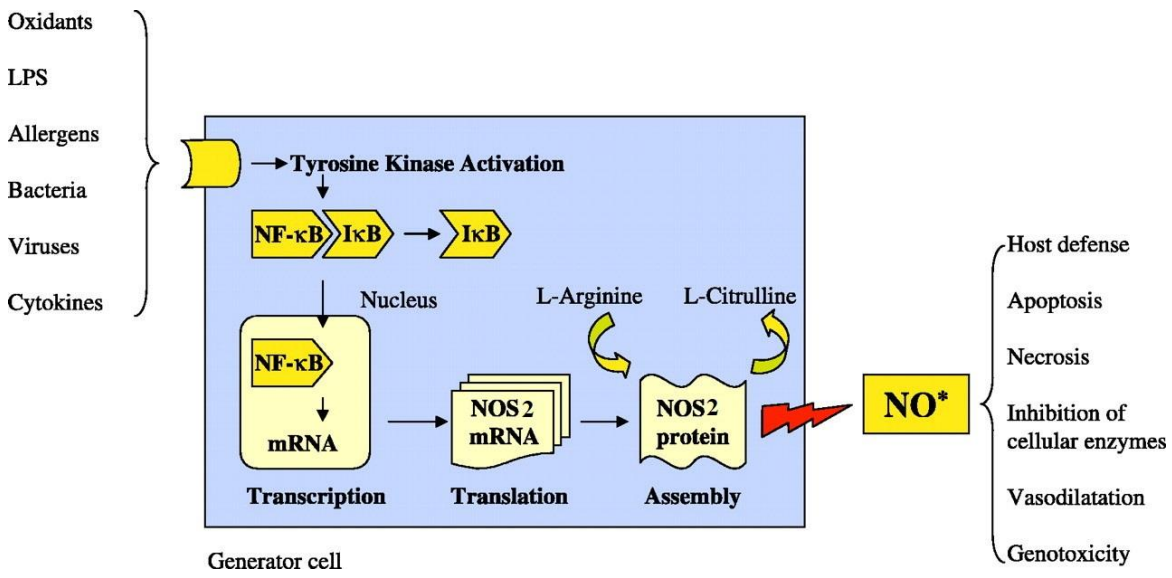

FIGURE 5. Signalling and subsequent expression of inducible nitric oxide synthase (NOS2). From left to right: a variety of triggers can lead to tyrosine kinase receptor signalling and nuclear transcription factor kappa B (NFkB) translocation to the nucleus, where the NOS2 gene is transcribed and NOS2 protein assembled. The NOS2 enzyme metabolises L-Arginine to L-Citrulline and NO; NO is involved in host defence but also has harmful effects. Figure and legend modified from Ricciardolo et al. (2004).

The fact that NOS2 is inducible makes it a prime candidate for the indirect measurement of inflammation. NOS2 is induced *in vitro* by the type-2 cytokines IL-4 (Guo *et al.*, 1997) and IL-13 (Suresh, Mih and George, 2007; Chibana *et al.*, 2008). Other cytokines reported to induce NOS2 synthesis are tumour necrosis factor (TNF), interferon-gamma (IFN-γ), and IL-1β (Alving and Malinovschi, 2010). Importantly, the proinflammatory upregulation of NOS2 can be inhibited by ICS *in vivo* (Guo *et al.*, 1995). In clinical trials, the predominant targeted strategy which decreases FeNO is IL-13 blockade (Hanania *et al.*, 2016; Castro *et al.*, 2018; Panettieri *et al.*, 2018). Thus, measuring the activity of NOS2 is an attractive method for identifying IL-13-induced airway inflammation which responds to anti-inflammatory therapy.

The first measurement of exhaled nitric oxide was reported in 1991 (Gustafsson *et al.*, 1991). Shortly after, Alving *et al.* observed greater FeNO levels in asthmatics than controls (1993). The pathological significance of FeNO elevation in asthma was confirmed by the observation that it is independent of the NO content from the nose and sinus cavities (Massaro *et al.*, 1996). It predominantly reflects NOS2 expression in bronchial epithelial cells (Lane *et al.*, 2004). A schematic overview of production of NO in the human airways in health and asthma is shown in Figure 6.

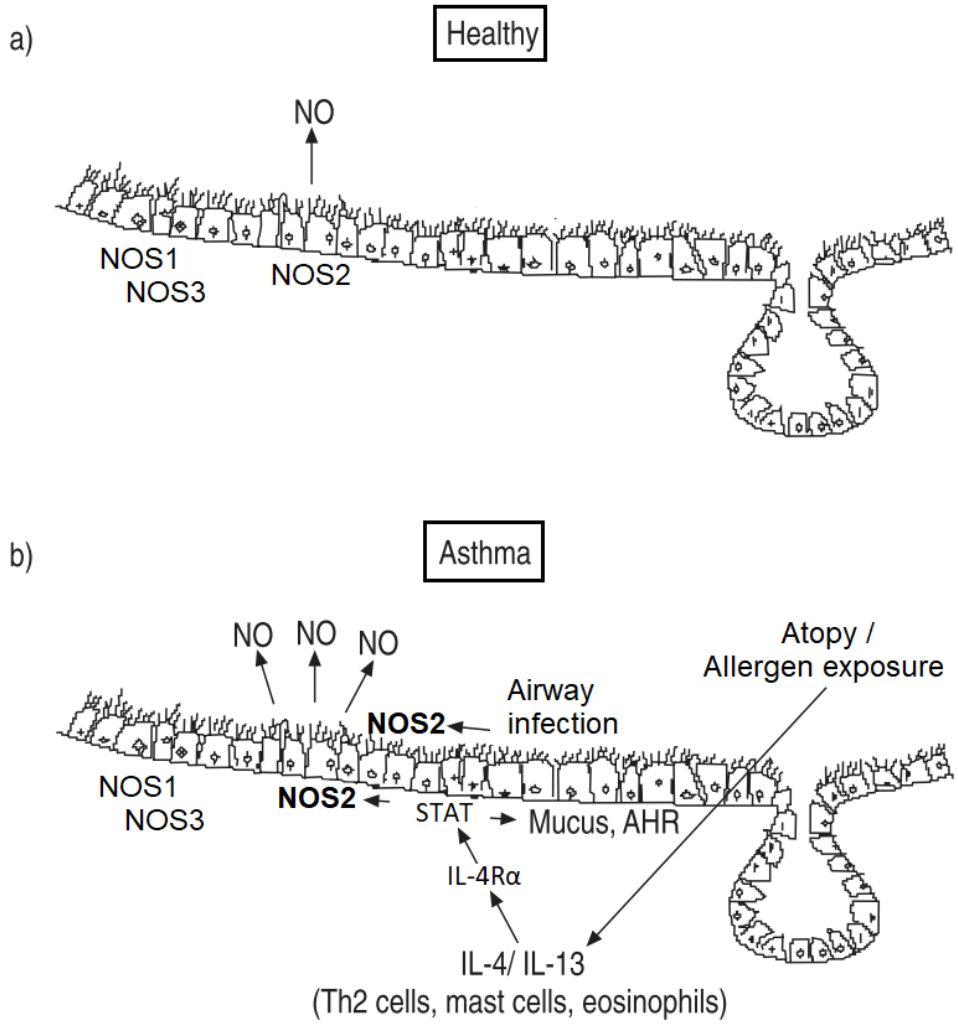

FIGURE 6. Nitric oxide (NO) production in the human airways. Panel A: In health, small quantities of NO are measured in exhaled air are due to the basal expression of constitutive and inducible nitric oxide synthase (NOS) enzymes NOS1-3. Panel B: In

people with asthma, intrinsic characteristics and environmental triggers increase levels of exhaled NO. Both interleukin (IL)-4 and IL-13 interact with a common dimeric receptor subunit – the IL-4 receptor alpha (IL-4Rα) – to promote mucus production, airway hyperresponsiveness (AHR), and NOS2 protein expression in the airway epithelium. STAT: signal transducer and activator of transcription; Th2: T-helper cell type 2. Figure and legend adapted from Alving and Malinovschi (2010); Gandhi *et al.* (2016).

2.2. Measurement of FeNO

The first descriptions of exhaled NO measurement were based on a range of sophisticated, expensive, and stationary methods (Gustafsson *et al.*, 1991; Alving, Weitzberg and Lundberg, 1993). Fortunately, technological progress has made it possible to measure FeNO with increasingly simple, affordable, and miniature devices (Figure 7). These do not require calibration, weigh 1 kg, are battery-powered, and cost less than 10 GBP per use (Maniscalco *et al.*, 2016; National Institute for Health and Care Excellence, 2019; Tanabe *et al.*, 2019).

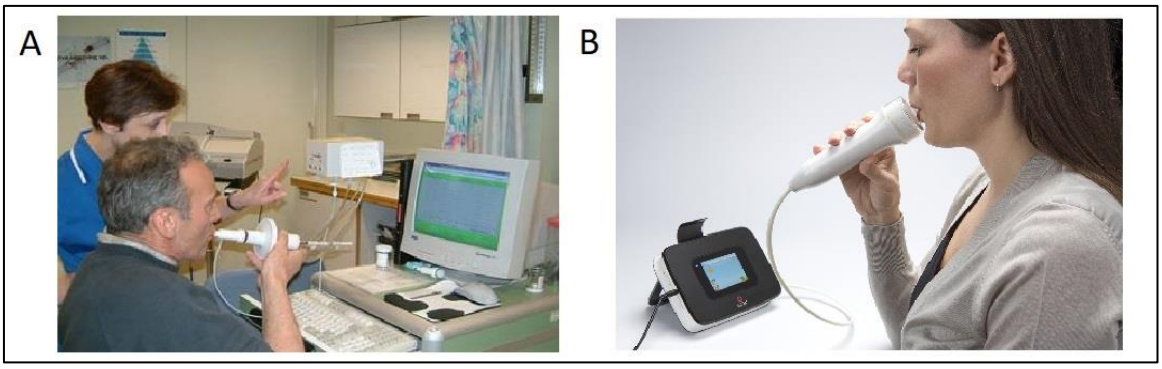

FIGURE 7. Progress in FeNO measurement technologies. A) Chemiluminescence analyser: model LR2000 (Logan Research Ltd, Rochester, UK; photography dated late 1990s, courtesy of Prof. Ian Pavord). B) Electrochemical analyser: model NIOX VERO® (Aerocrine AB, Solna, Sweden; 2013)

Standardised device specifications, measurement units and techniques have been published by the ATS and ERS (2005). The applied physiology for a FeNO measurement using a single-breath electrochemical analyser is hereafter described (Figure 8). First, to avoid contamination by pathogens or NO from the ambient air, inhalation to total lung capacity occurs through an antimicrobial filter and a NO scrubber (Smith and Taylor, 2005). Second, to minimise NO contamination from the nasal and sinus cavities by promoting upper airway closure, a counter pressure of 5 cm H_2O is applied during oral exhalation (Kharitonov and Barnes, 1997). Third, to keep the expiratory flow rate constant and thus avoid variability in the FeNO measurement (Silkoff *et al.*, 1997), the patient is instructed to exhale for 10 seconds at a constant flow rate (*i.e.* 50 mL/s) by following visual feedback queues on the device screen. Fourth, to allow FeNO measurement during the end-tidal NO-plateau, a 10-second exhalation time is required. Finally, the electrochemical technology is based on the amperometry principle which states that the sensor signal is directly proportional to the partial NO pressure in the sample. The partial pressure is then converted to a concentration (*i.e.* FeNO, expressed in ppb)(Cao, Buttner and Stetter, 1992). The modern electrochemical analyser yields a FeNO measurements within 60 seconds and requires a single manoeuvre.

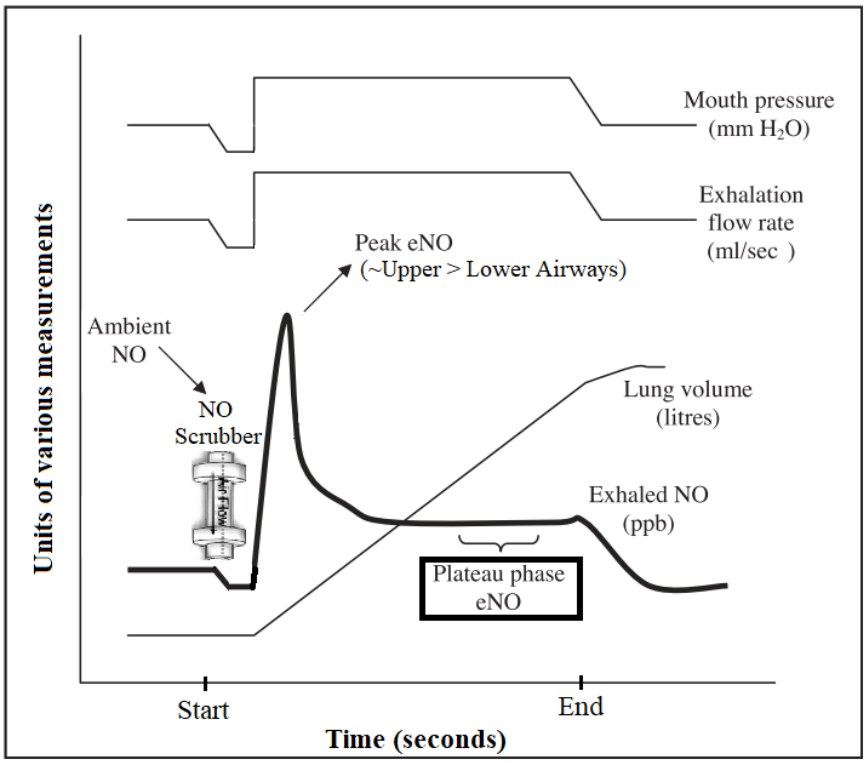

FIGURE 8. Mouth pressure, exhalation flow rate, lung volume, and measured exhaled nitric oxide (eNO) levels during a fractional exhaled NO (FeNO) manoeuvre. Initially, environmental air is inhaled via a NO scrubber. Peak eNO occurs early due to upper airway washout. Sampling for the FeNO measurement occurs when the end-tidal plateau is observed. Figure and legend adapted from ATS/ERS (2005); Smith and Taylor (2005)

2.3. FeNO interpretation

The ATS has published guidelines for the interpretation of FeNO measurements (Dweik *et al.*, 2011). Low, intermediate, and high FeNO measurements are defined as <25, 25-<50, and ≥50 ppb, respectively. In effect, a FeNO ≥50 ppb suggests uncontrolled airway inflammation and corticosteroid responsiveness. Measurements in the intermediate zone (25-<50 ppb) require cautious interpretation, especially considering the list of factors that affect FeNO independently of asthma and type-2 inflammation (Table 2). The minimal

clinically important difference for a rise or fall of FeNO is ≥10 ppb or ≥ 20% (Dweik *et al.*, 2011).

TABLE 2
Non-asthmatic factors which influence exhaled nitric oxide results

Decreasing values	Variable effect	Increasing values
◦ Current > past smoking	◦ Age	◦ Male gender
◦ Bronchial provocation	◦ Body mass index	◦ Height
◦ Electrochemical (*vs* chemiluminescence) analyser	◦ Diurnal variation	◦ Atopy
	◦ Forced spirometry	◦ Rhinitis
◦ Cystic fibrosis	◦ Exercise	◦ Recent respiratory viral infection
◦ Primary ciliary dyskinesia	◦ Menstrual cycle	◦ Recent oral nitrate intake
		◦ Air pollution
		◦ HIV infection

HIV, human immunodeficiency virus. Adapted from Alving and Malinovschi (2010); Dweik et al. (2011); Thudium et al., (2020).

3. Clinical utility of FeNO in asthma

When assessing the utility of FeNO as a surrogate measurement of airway inflammation in asthma, it is important to consider that it is the only biomarker currently endorsed by the ATS, ERS and British Thoracic Society (BTS) which produces a point-of-care measurement, *i.e.* a result immediately available for interpretation at the bedside (ATS/ERS, 2005; SIGN-BTS, 2019). Furthermore, although FeNO has been identified as a useful biomarker for diagnosing asthma, I herein focus on its utility in managing patients with established asthma.

3.1. FeNO to predict corticosteroid-responsiveness

Considering the risks of short-acting beta2-agonist (SABA) overreliance and monotherapy – which range from increased asthma morbidity (Nwaru *et al.*, 2020) to asthma-related fatality (Speizer *et al.*, 1968; Ernst *et al.*, 1992; Suissa *et al.*, 2000) –, it is important to

recognise which patients would especially benefit from ICS exposure despite an apparently mild asthma. Furthermore, a symptom-based approach to tailor ICS doses can be problematic at more advanced stages of the disease, where symptoms are not aligned with airway inflammation (Haldar *et al.*, 2008; Shaw *et al.*, 2012) and where biologics targeting type-2 cytokines can lead to an important improvement in disease burden in patients with the right inflammatory phenotype (Pavord *et al.*, 2018).

In ICS-naïve patients, raised FeNO predicts improvement in lung function, airway hyperresponsiveness, and asthma control following ICS initiation (Smith *et al.*, 2005; Knuffman *et al.*, 2009; Martin *et al.*, 2016). The theragnostic value of FeNO for these patients appears to exceed that of the sputum eosinophil count (Cowan *et al.*, 2015). Likewise, initiation of ICS leads to a decrease in FeNO levels (Kharitonov, Yates and Barnes, 1996).

In patients already established on ICS, elevated FeNO identifies patients at risk of asthma loss of control during step-down in ICS dose. Indeed, a meta-analysis of 348 patients with mild-to-moderate asthma who underwent FeNO measurement before reducing ICS showed that a FeNO ≥50 ppb at baseline was associated with a three-fold increase in the risk of exacerbations (Wang *et al.*, 2020). Similarly, in the negative trial of the inhaled AZD1419 compound for moderate-to-severe asthma, daily FeNO measurements during treatment withdrawal revealed that a two-fold relative increase of FeNO over 3 weeks was associated with a three-fold increased risk of subsequent loss of control (Psallidas *et al.*, 2021). Finally, in severe asthma, the RASP-UK trial compared composite biomarker (FeNO, blood eosinophils and periostin)-guided therapy adjustments with a symptom-risk-based strategy algorithm in patients with a FeNO <45 ppb at baseline. In the prespecified per-protocol analysis, the proportion of patients with lower ICS doses at the end of the 48-week follow-

up period in those randomised to the biomarker-guided management arm was six-fold that of patients randomised to symptom-risk management (31 *vs* 5% of patients)(Heaney *et al.*, 2021). The reverse – *i.e.*, increased FeNO suggesting the need for an increase in ICS dose – is not necessarily true, as there appears to be no reliable benefit from this step-up strategy (Lehtimäki *et al.*, 2016). Collectively, these data support the use of FeNO in the chronic management of moderate-to-severe asthma, insofar as the safe reduction of ICS dosage is an important goal of asthma management.

Unfortunately, the subtilties of the published literature on the utility of FeNO to initiate anti-inflammatory therapy in ICS-naïve patients and to monitor ICS dose-reduction in more advanced disease is lost in the Cochrane review on the subject (Mulhem, 2018). Indeed, the authors did not detail nor segment their results according to the patient characteristics and methodologies of the included trials. Perhaps because of this Cochrane review but also because of its overall unreliability when used in isolation, FeNO-guided management has not entered clinical practice.

3.2. The FeNO suppression test to verify adherence

The fact that raised FeNO identifies corticosteroid responsiveness provides a convenient framework to assess ICS treatment adherence in a manner which also provides objective proof of clinical benefit to the physician and patient. This rationale has led to the development and validation of the FeNO suppression test for difficult, uncontrolled, and/or severe asthma with persistently high FeNO readings.

Since its first description (McNicholl *et al.*, 2012), the FeNO suppression test methodology has been adapted to different clinical settings, protocols and technologies. In essence, it involves the concomitant objective monitoring of corticosteroid usage (either via directly observed intake, a chipped inhaler, and/or a nurse-administered intramuscular

triamcinolone injection), FeNO, and asthma control parameters (symptom scores, lung function, and blood/sputum eosinophils) over 4 to 35 days. The target population is patients with persistent symptoms and/or asthma attacks despite GINA treatment step 4-5 therapy, associated with FeNO ≥40 ppb on two occasions. (McNicholl *et al.*, 2012; Faruqi *et al.*, 2019; Heaney *et al.*, 2019; Boddy *et al.*, 2020)

The protocol and criteria for FeNO suppression interpretation were initially developed by McNicholl *et al.* (2012) in a small sample of patients (*n*=22) thoroughly investigated for treatment adherence (prescription ICS refills, prednisolone/cortisol blood levels, interviews) and directly observed to use their inhaler. The objective was to identify previous nonadherence with the best receiver-operating characteristics possible using the degree of FeNO suppression observed with monitored ICS therapy. The criteria with the best area under the curve (AUC) for identifying nonadherence (ICS prescription filling <80% in past 6 months) was Lg10ΔFeNO ≥0.24, where Lg10ΔFeNO was defined as: {mean (Lg10FeNO Day 0, Lg10 FeNO Day 1)} - {mean (Lg10 FeNO Day 6, Lg10 FeNO Day 7)} (AUC 0.88, 95% confidence intervals [CI]: 0.72-1.04). However, the definition retained for practical reasons was based on a simulated 5-day test, *i.e.*: replacing FeNO measurements at days 6 and 7 in the above formula by those of days 4 and 5 (AUC 0.86, 95%CI: 0.68-1.00). In effect, a ≥42% fall in FeNO between the mean of days 0-1 and that of days 6-7 (or 4-5) implied nonadherence. The 5-day test was validated in an independent sample of 40 patients with modest test sensitivity (67%) and excellent specificity (95%). Interestingly, in the proof-of-concept phase of this study, the investigators also reported that administering intramuscular triamcinolone on day 7 can further suppress FeNO in nonadherent patients but they did not include this component in their test definition.

Heaney *et al.* (2019) later reported on a 7-day FeNO suppression test using remotely monitored ICS via a chipped inhaler in addition to the inhalers used at baseline. In this larger cohort of patients (*n*=201 completed tests), 65% suppressed their FeNO according to the 5-day definition put forth by McNicholl et al. (2012). The FeNO suppressors were found to have significantly greater improvements in forced expiratory volume in 1 second (FEV1), FEV1/forced vital capacity (FVC) ratio, and 5-item Asthma Control Questionnaire (ACQ-5) values after a 1-month post-test monitoring period. FeNO suppression to a FeNO ≤35 ppb after 1-month of monitored therapy was associated with the greatest predictive value for good adherence (≥70% of chipped inhaler doses taken) during this period, with an AUC of 0.81 (95%CI 0.72-0.91). In summary, it became possible and valid to perform the FeNO suppression test using remote monitoring.

The studies by McNicholl *et al.* (2012) and Heaney *et al.* (2019) are important in three respects. First, they show feasibility and utility for the FeNO suppression test to check treatment adherence and responsiveness. Second, they characterise patients who suppress FeNO and derive clinical benefit following increased ICS exposure: these are predominantly younger females with less severe, more allergy-driven disease. Last and most importantly, the studies highlight that a third of patients with difficult asthma have severe and presumably corticosteroid-resistant disease. This group of 'FeNO non-suppressors' has not been further investigated and may provide an ideal model to study corticosteroid resistance and FeNO-related mechanisms in a cohort adequately controlled for treatment adherence.

3.3. FeNO and blood eosinophils as modifiable risk factors of asthma attacks

Reduction of the risk of asthma attacks is an important objective of current management guidelines (GINA, 2021). However, our ability to do this is limited because the independent

risk associated with clinical risk factors has not been defined (*e.g.*, the type-2 inflammatory phenotype is associated with lower lung function), some are difficult to identify and/or modify (*e.g.*, nonadherence and obesity), and others can be modified independently of an effect on asthma attacks (*e.g.*, bronchodilator monotherapy improves symptoms but not attack rates)(Lazarus *et al.*, 2001). Furthermore, as reviewed above, FeNO taken in isolation is not a reliable prognostic and theragnostic biomarker. These issues mean that a useful estimation of the risk of asthma attacks and the likely benefit of treatment has not been possible.

Five recent analyses of the control arms of RCTs across the range of asthma severities have shown that the combination of FeNO and the blood eosinophil count identifies the risk of asthma attacks (Shrimanker *et al.*, 2019; Pavord *et al.*, 2020; Busse *et al.*, 2021; Kraft *et al.*, 2021; Lee *et al.*, 2021). This relationship appears to be similar in strength and additive to that seen with other risk factors such as a history of an attack in the last year and GINA treatment step (Suruki *et al.*, 2017). Importantly, the excess risk associated with raised type-2 biomarkers can be removed by exposure to any ICS in mild asthma (Beasley *et al.*, 2019), higher dose ICS in moderate asthma (Lee *et al.*, 2021), and biologics targeting type-2 inflammation in severe asthma (Pavord *et al.*, 2012; Castro *et al.*, 2014, 2018; Corren *et al.*, 2017).

Collectively, these findings suggest that FeNO and blood eosinophils are important prognostic biomarkers across the range of asthma severities. They not only provide strong and independent prognostic information, but also identify a specific treatment opportunity; much in the same way blood pressure and serum cholesterol predict heart attacks which are prevented by antihypertensive and statin medications. In cardiovascular medicine, a common and effective approach is to tabulate the risk associated with blood pressure and

cholesterol on the background of risk due to unmodifiable risk factors (age and gender) and less modifiable risk factors (smoking)(Conroy *et al.*, 2003). It is possible that similar framework can be applied to predict the risk of asthma attacks which can be modified by anti-inflammatory treatment.

RESEARCH QUESTIONS, HYPOTHESES AND AIMS

1. Research questions

1.1. Is FeNO non-suppression a clinically relevant model to study corticosteroid resistance?

1.2. Does the failure of the type-2 biomarkers FeNO and the peripheral blood eosinophil count to respond to high-intensity corticosteroid therapy reflect underlying unchecked type-2 inflammation?

1.3. Can FeNO and blood eosinophils form the basis for a personalised asthma attack risk stratification system which also identifies anti-inflammatory treatment opportunities?

2. Hypotheses

2.1. FeNO non-suppression provides a good *in vivo* model to study corticosteroid resistance.

2.2. FeNO and blood eosinophil non-suppression reflect airway and systemic inflammation, respectively.

2.3. The availability of biomarker-stratified RCTs across the spectrum of asthma severity indicates feasibility for a risk stratification system based on FeNO and blood eosinophils, analogous to those produced in cardiovascular medicine.

3. Aims

3.1. To compare changes in clinical and airway/systemic inflammatory mediators before/after a FeNO suppression test between patients who do and do not suppress FeNO.

3.2. To investigate potential mechanisms underlying the relationship of FeNO and blood eosinophils to inflammatory mediators in the airway and peripheral blood in severe asthma.

3.3. To develop a prototype risk scale predicting asthma attacks centred on FeNO and the blood eosinophil count.

METHODS

1. Longitudinal analysis of FeNO suppression tests conducted in Oxford

1.1. Study objective

To compare changes in clinical and airway/systemic inflammatory mediators before/after a FeNO suppression test between patients who do and do not suppress FeNO.

1.2. Study design

An observational, longitudinal, clinical, and translational analysis of FeNO suppression tests conducted in the Oxford Special Airways Clinic was performed.

1.3. Patient population

Patients were recruited for FeNO suppression testing after multidisciplinary evaluation in the Oxford Special Airways Clinic. Tests were ordered to assess adherence and corticosteroid-resistance prior to referring patients for biological therapies. Patients

included had consented to the research protocol and underwent testing between January 2015 and February 2020. Testing and sputum induction was stopped in March 2020 due to the pandemic.

To be included in this analysis, patients had to be ≥18 years old, have an established diagnosis of GINA-defined asthma (GINA, 2021) under high dosage ICS *(i.e.*: CFC-propelled beclomethasone dipropionate equivalent dose >1000µg/day) plus at least one other controller medication, and have a persistently high FeNO (FeNO > 40 ppb on two occasions). Exclusion criteria were the lack of consent to research, concurrent diagnosis of a confounding pulmonary disease (including physician-diagnosed chronic obstructive pulmonary disease) or inability to perform/understand the study procedures.

1.4. FeNO suppression test protocol

The FeNO suppression test was conducted according to a local adaptation of the protocol described by McNicholl *et al.* (2012)(Figure 9). Briefly, patients with asthma underwent 7 to 35 days of additional inhaled and/or systemic corticosteroids (+1000µg inhaled fluticasone propionate per day and, if FeNO did not suppress on day 7, +80mg intramuscular triamcinolone with follow-up 28 days later). Treatment adherence was monitored via a chipped inhaler (INCA™ device) and/or the nurse-administered triamcinolone injection. In addition to clinical assessments and FeNO measurement in clinic on days 0, 7, and/or 35, patients were instructed to perform daily FeNO measurements at home for days 1-6. Some FeNO suppression tests were limited to 7 days due to patient availability or physician decision.

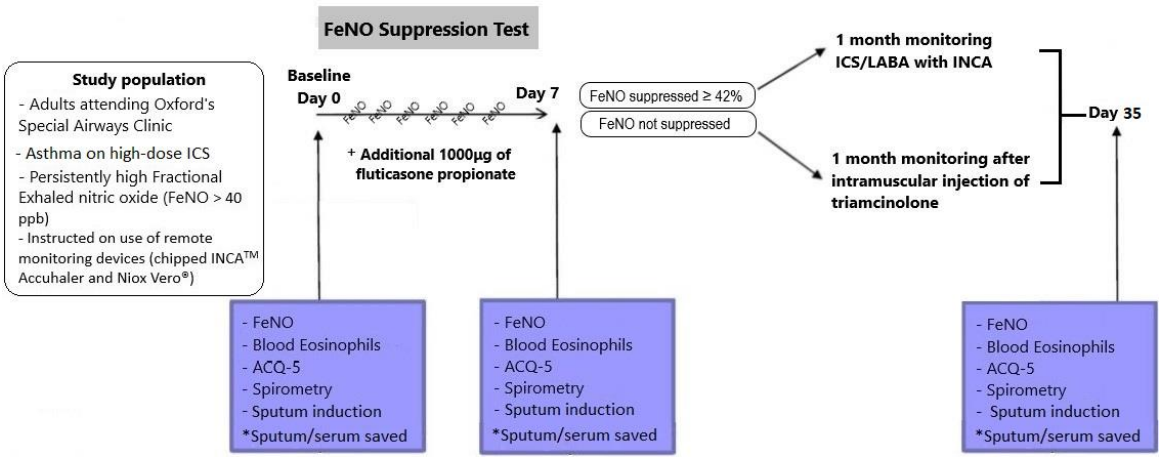

FIGURE 9. The FeNO suppression test, as performed by the Oxford Special Airways Clinic. ACQ-5, 5-item asthma control questionnaire; FeNO, fractional exhaled nitric oxide; GINA, global initiative for asthma; ICS, inhaled corticosteroid; LABA, long-acting beta2-agonist. Figure adapted from Heaney et al. (2019).

A positive FeNO suppression test was defined as a Lg10ΔFeNO ≥0.24, where Lg10ΔFeNO is calculated as: {mean (Lg10FeNO Day 0, Lg10 FeNO Day 1)} - {Lg10FeNO Last Day measured (*i.e.* FeNO at day 35 or, if unavailable, day 7)}. In effect, a ≥42% decrease in FeNO between these time points equates to a positive FeNO suppression test. The flexible 35-or-7-day-based equation was retained to increase study power.

Medical notes and research forms completed on day 0, 7, and 35 were reviewed to assess whether nonadherence issues had been observed or discussed with the patient. Indeed, whilst inappropriately performed tests (*i.e.*: <50% additional ICS doses administered and no triamcinolone injected) were excluded, the FeNO suppression clinical encounters often resulted in inhaler technique corrections and/or patients admitting to pre-existing nonadherence.

1.5. Additional study procedures

Patients underwent same-day detailed clinical assessment, ACQ-5 (Juniper *et al.*, 2005), spirometry, FeNO measurement (NIOX VERO, Circassia Ltd., Oxford, UK) at 50 mL/s, phlebotomy and sputum induction by hypertonic saline nebulization. Induced sputum was processed and Dulbecco's phosphate-buffered saline (DPBS)-eluted sputum supernatant frozen (-80°C) prior to differential cell counting as previously described (Bafadhel *et al.*, 2012). Sputum eosinophil values were added the arbitrary value of 0.25% to allow analysis of zero-values. Serum was aliquoted and frozen (-80°C). Patients underwent these procedures on days 0, 7 and, when performed, day 35, but for the purpose of the aim 1 analysis only paired samples were analysed for before/after changes.

1.6. Inflammatory protein measurements

Sputum supernatant and serum eotaxin-3, IL-4,-5,-13,-33, IFN-γ, LTE4, PGD2, TNF, TARC and TSLP were measured in duplicates using multiplex electrochemiluminescent assays (Meso Scale Discovery, Rockville, Maryland, USA: #K15067L) or single enzyme-linked immunosorbent assays (ELISA: for LTE4 and PGD2) (Cayman Chemical, Ann Arbor, Michigan, USA: #501060 and #512031, samples diluted 2-fold). These inflammatory mediators were selected following a review of the literature which focused on FeNO-related inflammation and mediator responsiveness to biological therapies which also lower FeNO (Alving and Malinovschi, 2010; Corren *et al.*, 2017; Castro *et al.*, 2018; Regeneron/Sanofi, 2019; Kolmert *et al.*, 2020). Cytokine levels that were not quantified were assigned the arbitrary value of 0.5×the lower limit of detection (LLOD) to allow analysis.

1.7. Ethics and funding

All subjects were adults who provided written informed consent. Study protocols were approved by local UK ethics committees (references 08/H0406/189 and 18/SC/0361). The study was funded by a non-restricted Type-2 innovation grant from Sanofi-Genzyme; Oxford's Biomedical Research Centre (BRC) grant of the National Institute of Health and Research (NIHR); and by the Wellcome Trust.

1.8. Statistics

Normal distribution was assessed by a combination of Shapiro-Wilk testing for normality (*p*-value greater than 0.05 identifying normality) and visual inspection of frequency histograms, with $\log_{10}$-transformation of data acknowledged to be log-normally distributed in larger populations, *i.e.*: FeNO, blood eosinophil counts, and induced sputum differential cell percentages (Dasgupta *et al.*, 2013). All sputum and serum cytokine data were approached with nonparametric tests due to agglomeration of values around the 0.5×LLOD level.

Demographics were compared between FeNO suppressors and non-suppressors using unpaired *t*-tests for parametric variables, Mann-Whitney tests for nonparametric variables, and Fisher's exact test or Chi-Square for categorical variables.

Before *vs* after changes in paired clinical parameters (FEV1, FEV1/FVC ratio, ACQ-5 score) and airway/serum analytes were analysed in FeNO suppressors and non-suppressors, respectively, using paired *t*-tests for normally and log-normally distributed variables, or Wilcoxon signed-rank test for nonparametric variables.

Differences between FeNO suppressors *vs* non-suppressors were analysed by comparing individual before/after changes between groups using unpaired *t*-tests for normally and log-

normally distributed variables, or Mann-Whitney tests for nonparametric variables. The 'before/after changes' in these analyses were differences (calculated as individual [after - before] values) for normally distributed variables, or fold-changes (calculated as individual [after ÷ before] values) for lognormally distributed variables (Dasgupta *et al.*, 2013) and nonparametric variables.

Sensitivity analyses of positive findings were conducted to assess whether the final optimisation method for FeNO suppression (*i.e.*: +1000μg inhaled fluticasone propionate or +80mg triamcinolone intramuscularly) resulted in significantly different before/after changes. Furthermore, AUC were computed for the log-transformed FeNO values (dependent variable) *vs* time course of FeNO suppression testing (independent variable; segmented in days 0 to 7 and days 7 to 35), with their 95% CI analysed for differences.

Statistical analyses were performed using SPSS statistical package version 27 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism version 9.1 (GraphPad Software, San Diego, CA, USA). The before/after changes were controlled for multiplicity of testing by applying the Benjamini-Hochberg procedure with a false discovery rate (FDR)<0.05 (Benjamini and Hochberg, 1995). All other statistics were analysed with a two-sided α of 0.05.

2. Translating non-suppression of type-2 biomarkers in severe asthma

2.1. Study objective

To investigate potential mechanisms underlying the relationship of FeNO and blood eosinophils to inflammatory mediators in the airway and peripheral blood in severe asthma.

2.2. Study design

Induced sputum eosinophils and sputum/serum inflammatory protein levels were analysed in a pooled cross-sectional analysis of patients with severe asthma and healthy controls. Inflammatory mediators and clinical parameters were correlated to FeNO or blood eosinophils.

2.3. Study population

To increase confidence in optimal treatment adherence, included patients had severe asthma (Chung *et al.*, 2014) confirmed to be on high-dose inhaled corticosteroids (ICS)(beclomethasone dipropionate equivalent dose ≥2000μg/day) who had sputum analysed after biomarker-guided management studies: FeNO suppression or participation in the Refractory Asthma Stratification Program UK (RASP-UK) clinical trial (Heaney *et al.*, 2021).

Patients and methods for the Oxford FeNO-suppression cohort were previously described in the Methods sections 1.3-1.4. Samples analysed were those performed on the last day of the FeNO suppression test available for analysis, *i.e.*: day 35 or, if unavailable, day 7. For the aim 2 analysis, all samples were included (paired and unpaired).

Patients included after completion of the RASP-UK trial (NCT02717689) had received 8-weekly biomarker or clinically-guided treatment advisories for 1 year (Heaney *et al.*, 2021) and had objective adherence measurements (prescription refills, cortisol and prednisolone levels when appropriate) prior to being recruited for the associated bronchoscopy study (NCT02883530). Importantly, patients with elevated FeNO in the RASP-UK cohort also undertook a FeNO suppression test prior to trial enrolment (McNicholl *et al.*, 2012).

Samples analysed were those performed on the day of screening/enrolment for the associated Bronchoscopy Study, *i.e.*: after completion of the 12-month clinical trial.

Healthy controls, recruited in Oxford, were never or ex-smokers ages 18 and over, reported no atopy or lung disease, and had normal lung function.

2.4. Study procedures

Patients and controls underwent same-day detailed clinical assessment, sputum induction and phlebotomy described in Methods section 1.5; only the FeNO suppression and healthy control cohorts had serum saved due to differences between the Oxford and RASP-UK protocols.

2.5. Inflammatory protein measurements

Sputum and serum inflammatory proteins were quantified as described in the Methods section 1.6.

2.6. Ethics and funding

All subjects were adults who provided written informed consent. Study protocols were approved by local ethics committees (Oxford cohort, references 08/H0406/189 and 18/SC/0361; RASP-UK cohort, reference 16/EM/0260). Funding was provided by the non-restricted Type-2 innovation grant from Sanofi-Genzyme; the RASP-UK Medical Research Council grant; Oxford and Leicester's NIHR BRC grants; and by the Wellcome Trust.

2.7. Statistics

Normality testing and demographics were analysed as described in the methods section 1.8.

The main analyses were Spearman correlations computed between same-day FeNO, blood eosinophil counts and analytes, controlled for multiplicity of testing (FDR<0.05). Retained correlations were then translated in median-fold differences across biomarker categories (FeNO: <25, 25<50, ≥50 ppb; blood eosinophils: <0.15, 0.15-<0.30, ≥0.30×10^9/L) with Jonckheere-Terpstra tests used to assess the ordinal trends. Analyte levels for healthy controls were compared to severe asthma stratified according to each of the 6 biomarker categories mentioned above with a Kruskall-Wallis test adjusted for 6 comparisons using Dunn's multiple comparison test.

Sensitivity analyses were conducted to assess the effect of removing patients on systemic corticosteroids and separating the RASP-UK and FeNO suppression cohorts.

To assess if FeNO and blood eosinophils had additive effect on underlying inflammation levels, exploratory multiple linear regressions for log_{10}-transformed FeNO and blood eosinophils (dependent variable) versus each log-transformed analyte (independent variable) were performed.

Statistical analyses were performed using SPSS statistical package version 27 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism version 9.1 (GraphPad Software, San Diego, CA, USA). Statistical significance was ascertained by the Benjamini-Hochberg procedure for Spearman correlations (FDR<0.05)(Benjamini and Hochberg, 1995) and otherwise with a two-sided α of 0.05.

3. Derivation of a prototype asthma attack risk scale centred on biomarkers

3.1. Study objective

To develop a prototype risk scale predicting asthma attacks centred on FeNO and the blood eosinophil count.

3.2. Prototype scale design

A 3×3 biomarker-stratified risk matrix was derived to predict annual asthma attack rates in patients at different GINA steps according to a recent history (≤1 year) of a severe asthma attack and the presence of ≥2 GINA-defined clinical risk factors (Table 3)(GINA, 2021). The grid used established cut points for blood eosinophil counts (<0.15, 0.15-<0.30, ≥0.30×10^9 cells/L) and FeNO (<25, 25-<50, ≥50 ppb)(Dweik et al., 2011; GINA, 2021; Pavord et al., 2018) (Figure 10).

3.3. Outcome to predict

The outcome to predict was the absolute number of severe asthma attacks to occur in the following 12 months if treatment is not changed. Severe asthma attacks are defined as acute asthma episodes requiring treatment with systemic steroids for 3 or more days and/or hospitalisation (Reddel *et al.*, 2009).

TABLE 3
Clinical Risk Factors for Asthma Exacerbations

Risk factors	Value (if pertinent)
Poor control of asthma symptoms	mean ACQ score ≥ 1.5
Limited lung function:	
low FEV1	< 60-80% predicted
high postbronchodilator reversibility	>12% change in FEV1
Adherence poor (inadequate technique or inhaler use)	
Reliever use excessive	> one 200-dose canister/month
Intubation or ICU admission for asthma on history	
Comorbidities:	
chronic rhinosinusitis	
obesity	body mass index ≥ 35 kg/m²
psychiatric disease	psychosis, substance abuse
Environmental exposure:	
smoking	
allergen exposure in sensitised patient	
air pollution	especially high O_3 and/or NO_3

ACQ, asthma control questionnaire; FEV1, forced expiratory volume in 1 second; ICU, intensive care unit; **PoLAR ICE,** mnemonic. Adapted from GINA (2021)

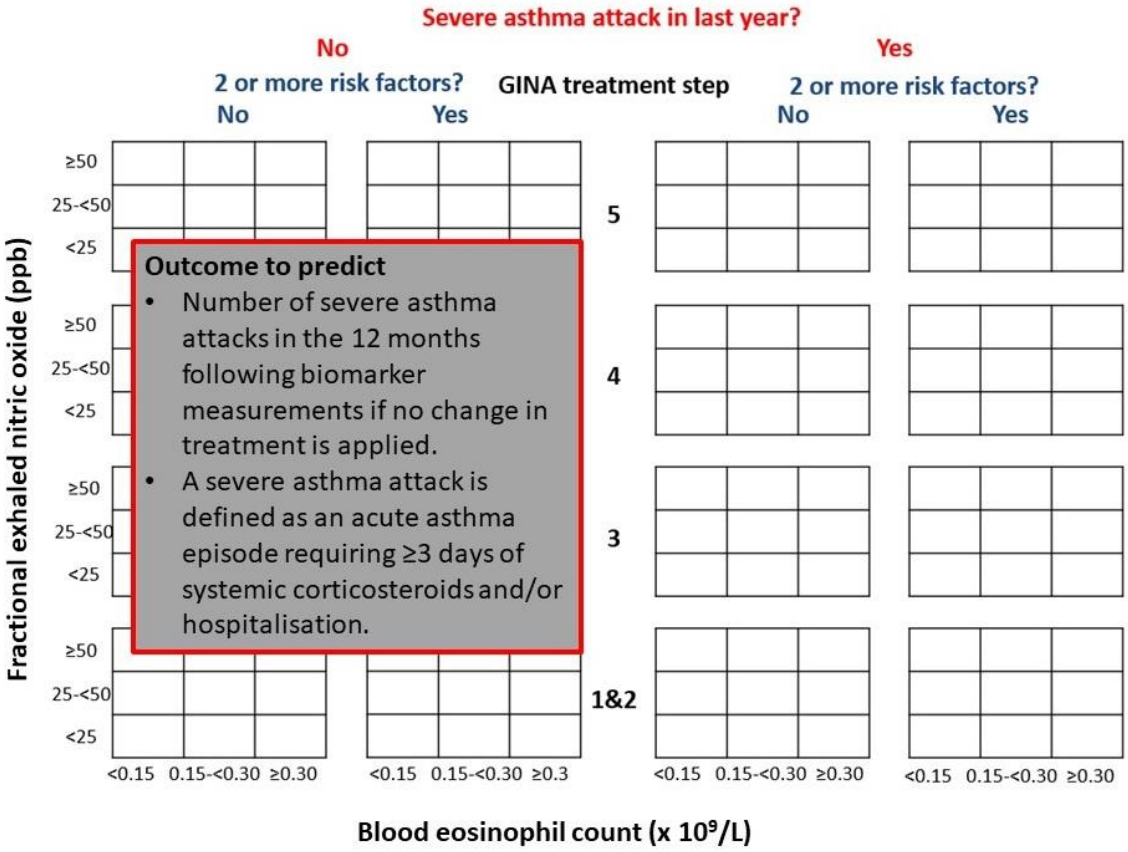

FIGURE 10. Prototype asthma attack risk scale blueprint. GINA, global initiative for asthma.

3.4. Sources of data

Trial-level data were extracted from biomarker-stratified analyses (Shrimanker *et al.*, 2019; Pavord *et al.*, 2020; Busse *et al.*, 2021; Kraft *et al.*, 2021; Lee *et al.*, 2021) of randomised control trials in mild GINA step 1 asthma (Beasley *et al.*, 2019), moderate GINA step 4 asthma (Lee *et al.*, 2021) and moderate to severe GINA step 4-5 asthma (Pavord *et al.*, 2012; Castro *et al.*, 2014, 2018; Corren *et al.*, 2017; Panettieri *et al.*, 2018). The derivation trials characteristics are described in Appendix 1.

3.5. Data collection for prototype derivation

Individual trials' control arm biomarker-stratified rate ratios. Attack rates of patients randomised to reliever-only, equivalent inhaled corticosteroid-dose or placebo arms (*i.e.*: with no change to their maintenance anti-inflammatory therapy) were grouped according to composite biomarker categories defined by the baseline blood eosinophil count (<0.15, 0.15-<0.3, ≥0.3×10^9/L) and FeNO (<15, 25-<50, ≥50 ppb). These data were used to derive biomarker-stratified rate ratios for each trial, calculated as the absolute asthma attack rate for the selected subgroup divided by the weighted mean for the remainder of the group, *e.g.:* [(absolute asthma attack rate for subgroup 1)×(frequency n1)] ÷ [(frequency-weighted mean for the remaining subgroups 2 to 9)×(Σ(n2 to 9))].

Several assumptions were made during data collection for the derivation of rate ratios. For both the Novel START (Pavord *et al.*, 2020) and CAPTAIN trials (Lee *et al.*, 2021), only the percentage of patients with one or more severe attacks(s) in the 52-weeks of follow-up was reported so an annualised rate was imputed as -log10(1 - %incidence). For Novel START (Pavord *et al.*, 2020) and the pooled AstraZeneca (AZ) trials (Kraft *et al.*, 2021), the data of patients with a baseline FeNO of 20-<50 ppb were regrouped into the 25-<50 ppb categories, as the difference of 5 ppb in FeNO is not clinically relevant (Dweik *et al.*, 2011). For the DREAM trial, biomarker stratified attack rates were available for some but not all of the pre-specified 9 combinations of biomarkers, as the published analysis stratified using only two cut points for fractional exhaled nitric oxide (FeNO <25 or ≥25 ppb)(Shrimanker *et al.*, 2019). Although the attack data from all the placebo-treated patients were used to derive the rate ratios for DREAM, only those for the scale's prespecified categories were included in the pooled analysis.

Pooling of biomarker-stratified rate ratios. Finding that the individual trials' biomarker-stratified asthma attack rate ratios were similar across the range of disease severities, an aggregate frequency-weighted mean rate ratio was calculated for each composite biomarker category. In effect, the aggregate biomarker-stratified rate ratio is a pooled fold-change in asthma attack rates for that biomarker group compared to others; it assumes that the risk is generalisable across all included trials and asthma severities.

Reference annual attack rates for GINA treatment steps. Annual severe asthma attack rates per GINA treatment steps were extracted from a large U.S. claims-based database of 222,817 patients (Suruki *et al.*, 2017). The reported rates were assumed to reflect low-risk patients. Steps 1 and 2, which did not differ statistically, were combined to better reflect the newer GINA 2021 guidelines for treatment steps 1 and 2 (GINA, 2021). The rates were corrected using the proportionate number of patients with and without an attack in the 12 months prior to the study's index date.

Risk associated with a recent asthma attack. The risk conferred by a recent asthma attack was derived from the same dataset as the annual attack rates for GINA treatment steps (Suruki *et al.*, 2017).

Risk associated with clinical profiles. The biomarker-independent risk conferred by the GINA-specified clinical factors listed in Table 3 is unknown but was estimated for patients with <2 and ≥ 2 risk factors by comparing low-risk and high-risk populations in the derivation cohorts, *i.e.* the Novel START (Pavord *et al.*, 2020) and CAPTAIN (Lee *et al.*, 2021) trials, respectively.

3.6. Data collection for prototype validation

The resultant scale was validated by comparing observed versus predicted biomarker-stratified asthma attack rates in the derivation trials. The predicted rates were calculated by applying the risk scale parameters in proportion to the reported clinical characteristics of each individual trial's control arm population. This baseline predicted rate was then multiplied by the aggregate biomarker-stratified rate ratio to define the stratified prediction. For example, a trial composed of 49% Step 4, 51% Step 5, frequently exacerbating patients with high-risk clinical profiles had a predicted baseline asthma attack rate of: {[(0.49×Step 4 attack rate) + (0.51×Step 5 attack rate)] × (rate ratio for ≥1 asthma attack in prior 12 months) × (rate ratio for ≥2 risk factors)}. This baseline predicted rate was then multiplied by the aggregate biomarker-stratified rate ratio for the subgroup of interest.

The reliability, agreement, and association between the frequency-weighted predicted and observed rates were measured by the intraclass correlation coefficient, a Bland-Altman analysis for fixed bias, and a weighted least squares regression.

3.7. Data collection to assess the modifiable risk identified by biomarkers

Observed biomarker-stratified treatment effect. To inform on the extent to which the excess risk identified by raised biomarkers is equal to the biomarker-stratified efficacy of anti-inflammatory treatment, the stratified attack rates of patients randomised to control and active treatment arms were extracted from the Novel START, CAPTAIN, QUEST, and DREAM trial analyses (Shrimanker *et al.*, 2019; Pavord *et al.*, 2020; Busse *et al.*, 2021; Lee *et al.*, 2021). The control *vs* active arm definitions for observed rates are shown in Table 4.

TABLE 4
Scheme for calculation of predicted rates for control and active treatment arms

Included study (reference(s) used)	**Group(s) analysed**	
	Control arm	**Active arm**
Novel START (Pavord *et al.*, 2020)	Albuterol as-needed only	Any ICS-containing regimen (~low-dose ICS)
CAPTAIN (Lee *et al.*, 2021)	Any FF 100µg-containing arm (~ low-dose ICS)	Any FF 200µg-containing arm (~ high-dose ICS)
QUEST (Shrimanker *et al.*, 2019; Busse *et al.*, 2021)	Placebo (any volume)	Dupilumab 200mg subcutaneously fortnightly (anti-IL-4Rα)
DREAM (Shrimanker *et al.*, 2019)	Placebo	Mepolizumab (any dose) (anti-IL-5)

FF, fluticasone furoate; ICS, inhaled corticosteroid; IL, interleukin; Rα, receptor alpha subunit.

The assumptions made during this data collection process are similar to those listed in the Methods section 3.5, except that the sponsor of the CAPTAIN trial (GlaxoSmithKline) provided the annualised severe asthma attack rates for composite biomarker groups using a FeNO cut point of 20 ppb. Hence, similar to the Novel START study, data of patients with a baseline FeNO of <20 ppb were regrouped into the <25 ppb group, as the difference of 5 ppb in FeNO is not clinically relevant (Dweik *et al.*, 2011).

Rate ratios were calculated between the control arm and active arms' annualised asthma attack rates in two groups of patients: a) patients with any raised type-2 biomarker at baseline (blood eosinophils $\geq 0.15\times10^9$ cells/L or a FeNO ≥25 ppb) and b) those with low type-2 biomarkers at baseline (blood eosinophils $<0.15\times10^9$/L and FeNO ≥25 ppb.

Predicted biomarker-stratified treatment effect. To explore the implied hypotheses that a) type-2 high asthma has an anti-inflammatory treatment effect equal to moving from any biomarker high stratum's predicted risk to the biomarker low stratum's predicted risk and b) type-2 low asthma does not respond to anti-inflammatory therapy (*i.e.*: rate ratio of 1.00: equal to staying in a similar type-2 low biomarker stratum), predicted control *vs* active treatment arm asthma attack rates were calculated for the Novel START, CAPTAIN,

QUEST, and DREAM trials. The predicted rates were calculated according to the scheme shown in Table 4 based on the trials' control arm patients' clinical circumstances as described in Methods section 3.6. In effect, the relative predicted treatment effects are the same across trials.

TABLE 5
Scheme for calculation of the predicted rates for control and active treatment arms

	Baseline stratified group assessed for predictions	**Predicted biomarker-stratified rate used in the prototype risk scale**	
		Control arm	**Active arm**
T2-High	Blood Eos ≥0.15 **or** FeNO ≥25	Blood Eos ≥0.15 **or** FeNO ≥25 ppb*	Blood Eos <0.15 **and** FeNO <25
T2-Low	Blood Eos <0.15 **and** FeNO <25	Blood Eos <0.15 **and** FeNO <25	Blood Eos <0.15 **and** FeNO <25

*An aggregate frequency-weighted rate ratio for this composite subgroup was computed using the derivation dataset. Blood Eos, peripheral blood eosinophil count (in cells×10^9 cells/L); FeNO, fractional exhaled nitric oxide (in ppb).

Comparing the aggregate observed versus predicted impact of anti-inflammatory treatments. The control *vs* treatment arm rate ratios calculated for the observed and predicted biomarker-stratified data were tabulated across individual trials. The main outcome of this analysis was the comparison of the frequency-weighted mean percentage reduction in asthma attack rates for all observed *vs* predicted asthma attack rates. It is noteworthy that the minimal clinically important difference for annual asthma attack rates has been suggested to be a 20 to 40% relative change (Bonini *et al.*, 2020).

3.8. Ethics and funding

This work was supported by the Oxford Respiratory NIHR BRC and by the Wellcome Trust.

The studies described were conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonisation of Technical Requirements for Registration

of Pharmaceuticals for Human Use Good Clinical Practice guidelines. Study protocols received independent ethics review and approval. All patients provided written informed consent in the included studies.

3.9. Statistics

The reliability, agreement, and association between the predicted and observed rates were measured by the intraclass correlation coefficient (two-ways mixed model for absolute agreement, single measures), a Bland-Altman analysis for fixed bias (Y= Difference (observed rate – predicted rate), X=average), and a weighted least squares regression (no fixed intercept). All analyses were weighted by frequency and computed using SPSS statistical package version 27 (SPSS Inc, Chicago, IL). Limits of statistical significance were defined by 95% CI whenever possible.

RESULTS

1. Longitudinal analysis of FeNO suppression tests conducted in Oxford

Several of these results will be reported in Couillard, Shrimanker, *et al*. (manuscript in preparation).

1.1. Demographics

Eighty-seven FeNO suppression tests were planned between 2015 and 2020; 34 completed tests were retained after applying inclusion/exclusion criteria (Figure 11).

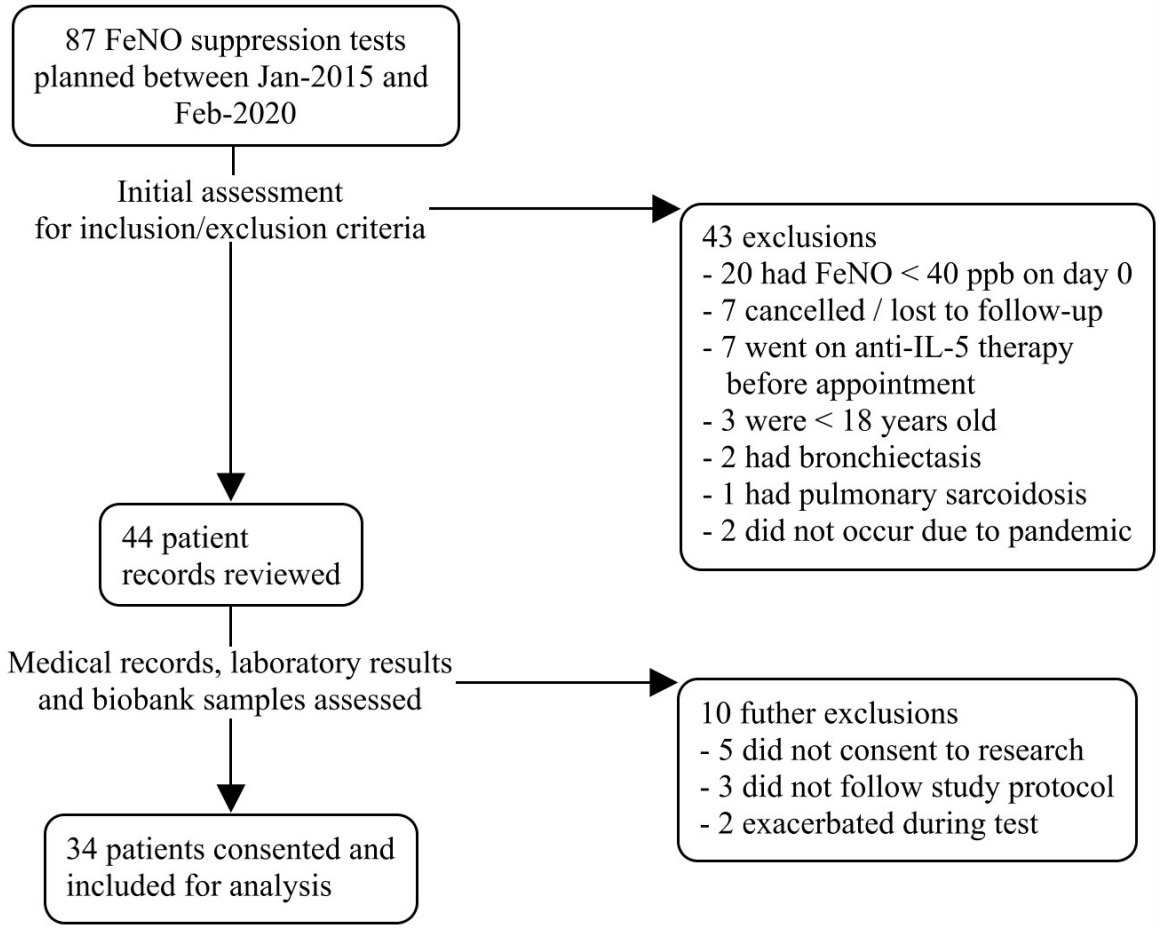

FIGURE 11. Flowchart for fractional exhaled nitric oxide (FeNO) suppression study inclusion. IL-5, interleukin 5.

Fifteen patients suppressed FeNO and 19 did not. FeNO non-suppressors were older, on higher background ICS dosage, had lower baseline blood eosinophil count, and had little or no adherence/inhaler technique issues noted (Table 6). Overall paired sample availability was low, especially for sputum differential cell counts.

TABLE 6
Baseline subject Characteristics

Parameter	FeNO Suppressed n= 15		Not Suppressed n= 19		*p*
Age, y	42	± 13	57	± 16	0.006
Male	5	(33)	10	(53)	ns
BMI, kg/m²	26	± 4	28	± 5	ns
Comorbidities: Atopy	12	(80)	12	(63)	ns
Nasal Polyps	7	(47)	7	(37)	ns
Gastro-oesophageal reflux	2	(13)	3	(16)	ns
Cardiovascular disease	2	(13)	1	(5)	ns
Smoking status: Never-Smoker	11	(58)	12	(80)	
Ex-Smoker	7	(37)	2	(13)	ns
Current smoker	1	(5)	1	(7)	
ACQ-5 score at baseline	2.8	± 1.4	2.5	± 1.5	ns
Asthma attacks in past year*	1	[0-3]	4	[0-5]	ns
ICS, BDP-CFC eq., µg/d	1561	± 502	1921	± 344	0.02
On maintenance OCS	3	(20)	9	(47)	ns
FEV_1, % predicted	89	± 19	78	± 17	ns
FEV_1/FVC ratio, % observed	75	± 17	67	± 11	ns
FeNO ppb	119	[75-190]	94	[60-136]	ns
Blood eosinophils, cells $\times 10^9$/L	0.54	[0.50-0.83]	0.46	[0.36-0.59]	0.03
Total IgE levels, kU/L	545	[35-1551]	229	[77-359]	ns
Sputum eosinophils, %	29	[7-41]	13	[3-39]	ns
Final optimisation method:					
+FP 1000µg inhaled-only	12	(80)	13	(68)	ns
+Triamcinolone 80mg IM	3	(20)	6	(32)	
Adherence issues noted	8	(53)	2	(11)	0.007
Number of paired before/after samples					
Sputum differential cell count	4	(27)	5	(26)	ns
Sputum supernatant	7	(47)	12	(63)	ns
Serum	9	(60)	16	(84)	ns

Data are presented as no. (%), mean ± SD, or median [interquartile range]. *Asthma attacks are defined as acute asthma episodes requiring 3 days or more of systemic corticosteroids. *p*-values reported are unpaired *t*-tests for parametric variables, Mann-Whitney tests for nonparametric variables, Fisher's exact test or Chi-Square for categorical variables. ACQ-5, asthma control questionnaire; BDP-CFC eq., beclomethasone dipropionate with CFC propellant equivalent; BMI, body mass index; FeNO, fractional exhaled nitric oxide; FEV_1, forced expiratory volume in 1 second (post-bronchodilator); FP, fluticasone propionate; FVC, forced vital capacity; ICS, inhaled corticosteroid; IM, intramuscular; ns, $p \geq 0.05$. Reproduced from Couillard *et al*. (manuscript in preparation)

1.2. Comparison of FeNO suppression test before/after responses

The clinical, biomarker, and sputum/serum inflammatory responses during the FeNO suppression tests are detailed in Table 7, and selected analytes are plotted in Figure 12.

After correction for multiplicity of testing, only the before/after change in FeNO was significantly different between FeNO suppressors and non-suppressors (3.4 *vs* 1.5-fold, $p<0.0001$). Furthermore, ACQ-5 scores were found to significantly improve before *vs* after the test in FeNO suppressors alone (2.8 before, 1.4 after; $p=0.005$). The difference in ACQ-5 for FeNO suppressors was not significantly different than non-suppressors. Exploration of these results did not show any difference in the proportion of suppressors *vs* non-suppressors with an ACQ-5 improvement greater than 0.5 (*i.e.* the minimal clinically important difference) nor in the proportion of those who reached an ACQ-5 < 1.5 at test termination (*i.e.* the threshold for good symptom control)(Juniper *et al.*, 2005).

All other differences detailed in Table 7 were not retained after controlling for multiplicity of testing. Nevertheless, it is noteworthy that more sputum/serum inflammatory mediators decreased in FeNO suppressors than non-suppressors (15/22 *vs* 8/22 mediator values numerically decreasing, chi-square test $p=0.03$). This trend was especially striking for sputum eosinophils, which decreased 5.4-fold in FeNO suppressors while increasing 1.3-fold in non-suppressors ($p=0.06$). Exploration of the latter results showed that, in the 9 patients with paired sputum eosinophil cell counts, those whose FeNO suppressed had significantly lower FeNO values at test termination than non-suppressors (median: 27 *vs* 54 ppb, unpaired *t*-test on $\log_{10}$-transformed FeNO values $p=0.02$). Although these findings were further explored (Figure 13), correlation and regression analyses lacked power and thus statistical significance. On that basis a pooled cross-sectional analysis of optimised severe asthma was performed (see Results section 2).

TABLE 7
Comparison of before/after clinical and inflammatory changes according to FeNO suppression test result

	Analyte (pg/mL or stated) LLOD*	FeNO suppressed (n=15) Before	After	Change†	*p* for before *vs* after	FeNO not suppressed (n=19) Before	After	Change†	*p* for before *vs* after	*p* for change in FeNO suppressed *vs* Not suppressed
Clinical	**ACQ-5 score§**	2.8 ±1.4	1.4 ±0.9	-1.4	**0.0005**	2.5 ±1.5	1.9 ±1.3	-0.5	0.04	0.04
	FEV_1 (L)§	2.79 ±0.86	3.05 ±0.96	+0.25	0.009	2.39 ±0.92	2.56 ±0.89	+0.17	ns	ns
	FEV_1 (% pred)§	89 ±19	98 ±19	+8	0.01	78 ±17	83 ±18	+6	ns	ns
	FEV_1/FVC (%)§	75 ±17	78 ±12	+3	ns	67 ±11	70 ±10	+2	ns	ns
Biomarker	**FeNO (ppb)§§**	119 [75-190]	35 [20-55]	0.3-fold	**<0.0001**	94 [60-136]	56 [43-123]	0.7-fold	**<0.0001**	**<0.0001**
	Blood Eos ($\times 10^9$/L) §§	0.54 [0.5-0.83]	0.42 [0.1-0.6]	0.8-fold	0.01	0.46 [0.26-0.58]	0.25 [0.19-0.38]	0.7-fold	0.049	ns
Sputum	**Eosinophils (%)§§**	32.9 [12.2-40.8]	3.9 [1.6-17]	0.2-fold	0.02	13.5 [5.5-63.3]	16.8 [0.9-72.8]	1.3-fold	ns	ns
	Neutrophils (%)§§	40.9 [13.7-60]	45.1 [14.7-74.8]	1.3-fold	ns	69.3 [15.1-76.9]	18.3 [5.8-76.6]	1.4-fold	ns	ns
	IL-4 0.2	0.4 [0.1-1]	0.1 [0.1-0.9]	0.9-fold	ns	0.8 [0.3-1]	0.5 [0.1-1]	1-fold	ns	ns
	IL-5 0.5	3.7 [1-21.6]	1.4 [0.5-7.4]	0.5-fold	0.02	7.5 [1.9-16.8]	3.9 [2-7.4]	1.1-fold	ns	ns
	IL-13 4.2	6.6 [5.7-20.1]	9.2 [5.4-19.6]	1-fold	ns	7.5 [4.9-9.5]	7.7 [6.3-10.8]	0.9-fold	ns	ns
	IL-33 0.6	1.4 [0.3-1.4]	0.3 [0.3-0.7]	0.7-fold	ns	1.6 [1.4-1.9]	1.4 [0.4-1.7]	1-fold	ns	ns
	TSLP 0.9	3.9 [0.8-14.4]	3.0 [1.1-9.4]	0.8-fold	0.046	7.2 [4.8-14.4]	6.9 [4.1-10.3]	1.2-fold	ns	ns

(Table 7 continues next page)

… Table 7

	…Analyte LLOD†	…FeNO suppressed Before	After	Change*	*p*	…FeNO not suppressed Before	After	Change*	*p*	…*p* for suppressed *vs* not suppressed
…Sputum	**Eotaxin-3** 4.2	134 [22-511]	143 [2-346]	0.7-fold	ns	361 [29-670]	211 [19-496]	1.4-fold	ns	ns
	TARC 0.4	43 [6-82]	16 [6-60]	0.8-fold	ns	36 [11-252]	31 [7-241]	1.2-fold	ns	ns
	LTE4 7.8	305 [71-1000]	64 [42-247]	0.4-fold	0.046	163 [50-910]	80 [47-677]	0.9-fold	ns	ns
	PGD2 19.5	291 [79-1342]	93 [45-196]	0.3-fold	0.03	251 [143-344]	176 [119-320]	0.9-fold	ns	0.045
	IFN-γ 0.3	0.8 [0.2-3.4]	0.2 [0.2-2]	0.7-fold	ns	0.2 [0.2-0.3]	0.4 [0.2-0.5]	2.3-fold	ns	ns
	TNF 0.4	1.5 [0.2-6.1]	0.9 [0.2-5.9]	1-fold	ns	1.4 [0.7-2.1]	0.6 [0.2-3]	1-fold	ns	ns
Serum	**IL-4** 0.1	0.1 [0.1-0.1]	0.1 [0.1-0.1]	1-fold	ns	0.1 [0.1-0.1]	0.1 [0.1-0.1]	1-fold	ns	ns
	IL-5 0.4	1.4 [0.8-3.5]	0.6 [0.5-1.4]	0.4-fold	ns	1.6 [0.4-2.5]	0.6 [0.5-1.6]	0.8-fold	0.047	ns
	IL-13 6.7	7 [3-15]	3 [3-12]	0.9-fold	0.04	4 [3-13]	6 [3-10]	1-fold	ns	ns
	IL-33 0.4	0.4 [0.2-0.8]	0.2 [0.2-0.8]	1-fold	ns	0.5 [0.2-0.8]	0.3 [0.2-0.8]	1-fold	ns	ns
	TSLP 0.5	2 [1.3-3.4]	1.7 [0.8-2.3]	0.6-fold	ns	2.7 [1.7-3.6]	2.3 [1.3-3.6]	0.8-fold	ns	ns

(Table 7 continues next page)

…**Table 7**

	…Analyte LLOD†	…FeNO suppressed Before	After	Change*	*p*	…FeNO not suppressed Before	After	Change*	*p*	…*P* for suppressed *vs* not suppressed
…Serum	**Eotaxin-3** 4.2	14 [9-30]	15 [11-30]	1-fold	ns	23 [11-36]	15 [6-31]	0.8-fold	ns	ns
	TARC 0.2	205 [159-861]	195 [147-478]	0.8-fold	0.03	251 [141-399]	225 [91-369]	0.7-fold	ns	ns
	IFN-γ 0.3	0.7 [0.2-1.2]	0.6 [0.2-1.2]	1-fold	ns	0.3 [0.2-1.1]	0.3 [0.2-0.9]	1-fold	ns	ns
	TNF 0.4	0.9 [0.5-1.4]	0.8 [0.3-1.5]	0.9-fold	ns	1.2 [0.2-2.1]	0.8 [0.2-1.7]	0.9-fold	ns	ns

Data are presented as mean ± SD or median [interquartile range]; units of measured are in pg/mL unless otherwise stated. *Cytokine levels that were not quantified were assigned the arbitrary value of 0.5×the lower limit of detection (LLOD) to allow analysis. †Change refers to the mean difference (calculated as the mean of individual [after - before] differences) for normally distributed variables (indicated by §), or the median fold-change (calculated as the median of individual [after ÷ before] fold-changes) for lognormally distributed variables (indicated by §§) and nonparametric variables (all cytokine data). **Bold** p-values are those retained after controlling for multiplicity of testing (false discovery rate <0.05). *p*-values reported for before/after changes are paired *t*-tests for normally and lognormally distributed variables, Wilcoxon signed-rank tests for nonparametric variables. *p*-values reported for FeNO suppressed *vs* not suppressed patients are unpaired *t*-test for normally and lognormally distributed variables, Mann-Whitney tests for nonparametric variables. ACQ-5, 5-item asthma control questionnaire; Eos, eosinophils; FeNO, fractional exhaled nitric oxide; FEV1, forced expiratory volume in 1 second (post-bronchodilator); FVC, forced vital capacity; IFN, interferon; IL, interleukin; LTE4, leukotriene E4; ns, $p \geq 0.05$; PGD2, prostaglandin D2; TNF, tumour necrosis factor; TARC, thymus activation regulated cytokine (CCL17); TSLP, thymic stromal lymphopoietin. Reproduced from Couillard, Shrimanker, *et al*. (manuscript in preparation)

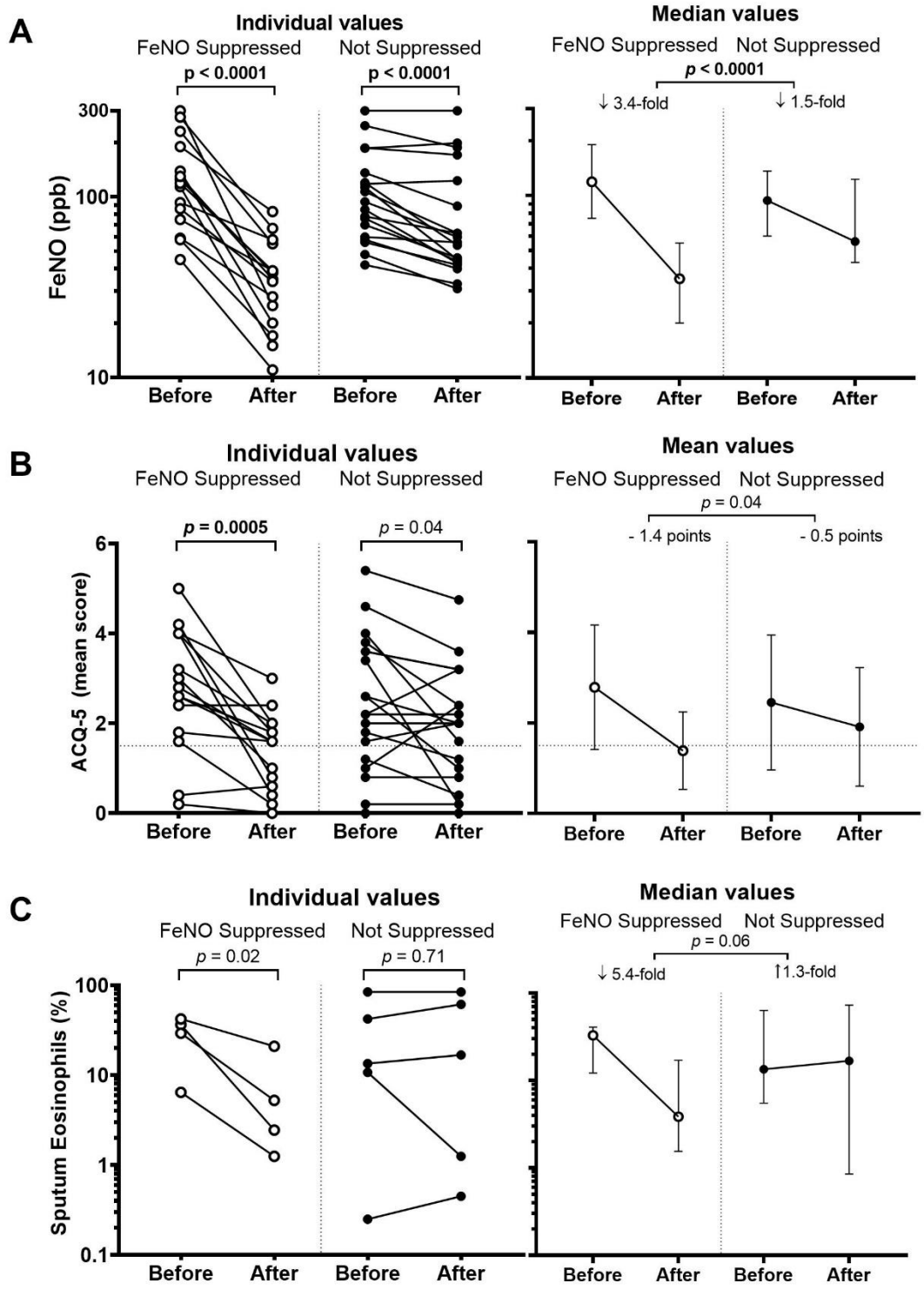

FIGURE 12. Before and after changes in selected analytes following a FeNO suppression test according to its results. Panel A: FeNO, Panel B: 5-item asthma control questionnaire (ACQ-5), with the 1.5-point threshold for good symptom control delimited by the dotted line; Panel C: Sputum eosinophils. Right-sided plot values are median [IQR] or mean ± SD. **Bold** *p*-values are those retained after controlling for a false discovery rate <0.05; see Table 7 for full results.

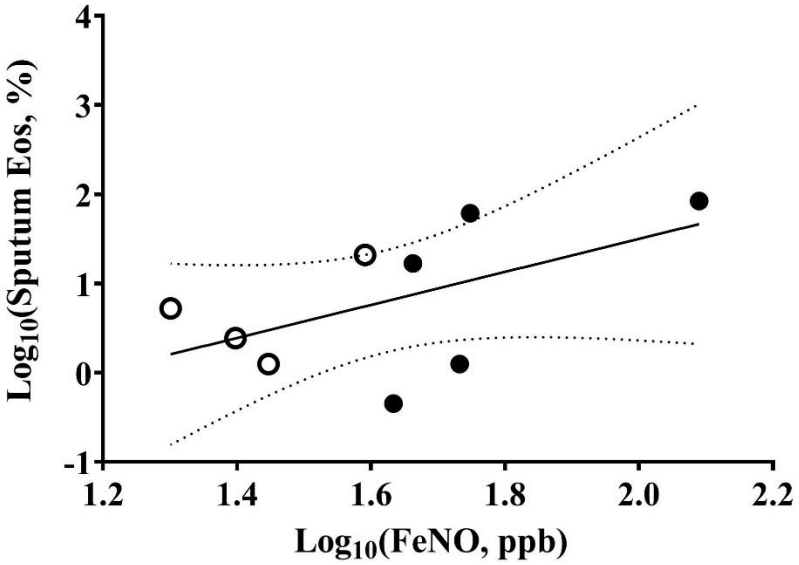

FIGURE 13. Log_{10}-transformed optimised ('after') sputum eosinophils (Eos) and FeNO values. Linear regression line shown with 95%CI indicated by dotted lines; equation $Log_{10}(Y) = 1.85*Log_{10}(X) - 2.2$ (p=0.14 for non-null slope). Spearman correlation, r=0.54 (p=0.14). ○, FeNO suppressed; ●, FeNO not suppressed.

1.3. Sensitivity analyses on the optimisation method

Both patients who did and did not suppress FeNO received triamcinolone 80mg intramuscularly on day 7 (3/15 *vs* 6/19; see Table 6). As this decision was taken when FeNO did not suppress by more than 42% with the additional fluticasone propionate 1000µg inhaled per day alone (see Methods, Figure 9), a sensitivity analysis was conducted to assess whether the final degree of FeNO suppression or ACQ-5 improvement varied according to the optimisation method. The results of these analyses are shown in Figure 14 and suggest that, although both optimisation methods were used in different circumstances to ensure optimal FeNO suppression, the magnitude of change did not differ significantly between methods. These considerations were further explored in a time course plot (Figure 15), with AUC analyses showing similar degrees of log_{10}-FeNO suppression for both optimisation methods (Table 8).

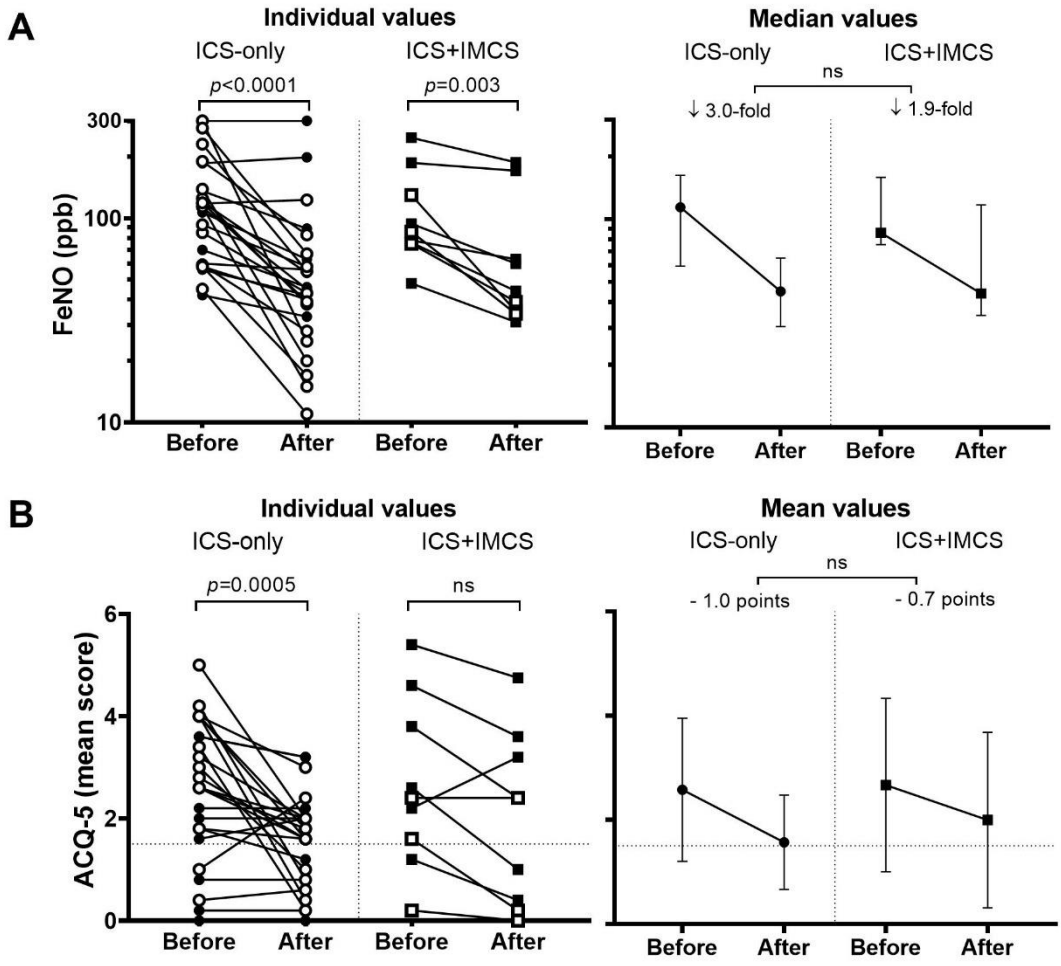

FIGURE 14. Before and after changes in selected analytes following a FeNO suppression test according to the optimisation method. Panel A**:** FeNO, Panel B: 5-item asthma control questionnaire (ACQ-5), with the 1.5-point threshold for good symptom control delimited by the dotted line. ICS, inhaled corticosteroid (*i.e.*: additional fluticasone propionate 1000µg inhaled daily throughout); IMCS, intramuscular corticosteroid (*i.e.*: triamcinolone 80mg intramuscularly on day 7); ○□, FeNO suppressed; ●■, FeNO not suppressed.

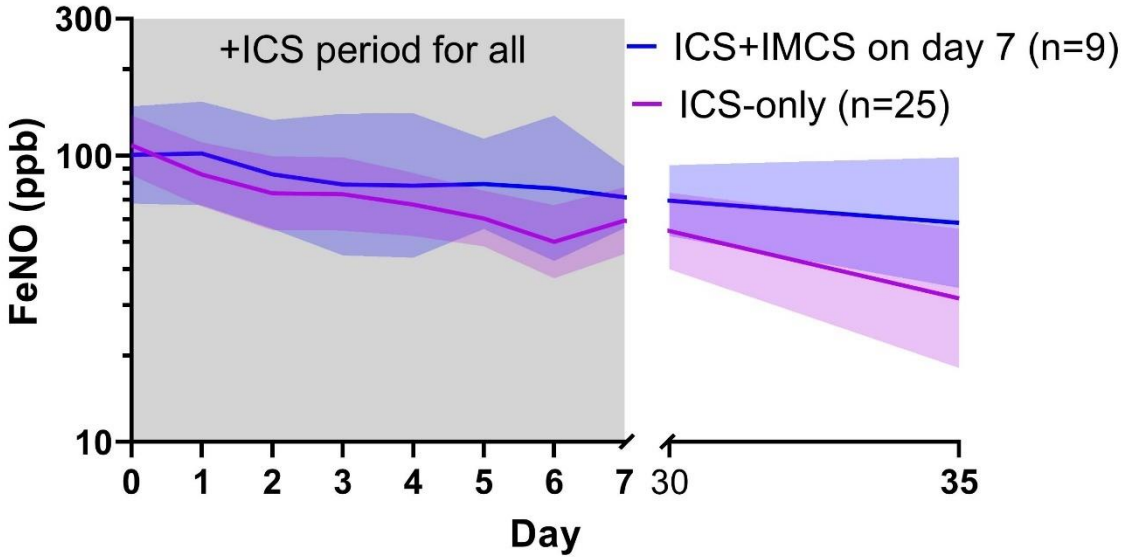

FIGURE 15. FeNO suppression time course according to optimisation method. Full lines connect the geometric mean values at each day of measurement (**bold** day numbers), with shaded coloured areas in the plot corresponding to the 95% CI.

TABLE 8
Area under the curve per segment of the FeNO suppression test according to optimisation method.

Optimisation method (n)	**Total area under the curve per segment**					
	Days 0 to 7		**Days 7 to 35**		**Days 0 to 35**	
ICS+IMCS on day 7 (n=9)	13.5	(12.7-14.2)	50.7	(41.7-59.7)	63.0	(54.4-73.9)
ICS-only (n=25)	12.9	(11.9-13.9)	45.8	(33.7-58.0)	56.9	(45.6-71.8)
p	ns		ns		ns	

Areas under the curve (95% confidence intervals) shown are computed for mean log_{10}-transformed FeNO values according to time in each subgroup. ICS, inhaled corticosteroid; IMCS, intramuscular corticosteroid; ns, $p \geq 0.05$.

1.4. Summary of aim 1 results

- The study power was limited by the halt of FeNO suppression tests in March 2020 due to the pandemic.
- Patients who did not suppress FeNO have distinct phenotypic characteristics. These are similar to those reported previously, *i.e.*: older age, greater background

corticosteroid therapy, less improvement in FeNO and symptom scores following the test, and less likelihood of finding adherence/inhaler technique issues.

- Although the before/after analyses for sputum and serum mediators were underpowered, trends were observed which suggest that FeNO suppressors have greater overall decreases in sputum/blood eosinophilia, type-2 cytokines, chemokines and alarmins following FeNO suppression. In contrast, FeNO non-suppressors had significantly higher end-test FeNO values associated with numerically higher sputum eosinophil counts and other inflammatory protein levels.
- The differences between FeNO suppressors and non-suppressors are not explained by the method of optimisation used, as ICS and intramuscular corticosteroids were employed with similar effectiveness to suppress FeNO.
- Although these results are underpowered and observational in nature, they suggest that the assessment of corticosteroid resistance can be based on the failure to suppress FeNO during a high-intensity corticosteroid therapy. Nevertheless, it is important to emphasise that the definition for FeNO suppression was derived primarily to identify pre-existing nonadherence – not to assess corticosteroid-resistant type-2 inflammation (McNicholl *et al.*, 2012; Faruqi *et al.*, 2019; Heaney *et al.*, 2019; Boddy *et al.*, 2020). It is possible that the absolute value of biomarkers reached following high-intensity corticosteroid therapy, notwithstanding the optimisation method used to get there, is the most important factor in determining the persistence of corticosteroid-refractory type-2 inflammation. Hence, a cross-sectional analysis of the absolute levels of biomarkers achieved at the optimally treated state may be more useful to translate corticosteroid-resistant type-2 inflammation in severe asthma.

2. Translating non-suppression of type-2 biomarkers in severe asthma

2.1. Demographics

We included 74 patients with severe asthma and 10 healthy controls. Patients included from the Oxford FeNO-suppression cohort (*n*=34) and RASP-UK bronchoscopy study cohort (*n*=40) were similar except for a higher BMI, lower ICS dose and lower FEV1 in the RASP-UK group (Table 9). There were 60 sputum supernatants and 30 serum samples available for analysis in asthma.

TABLE 9
Subject characteristics

	All subjects			Severe asthma patients		
Parameter	**Healthy controls (n=10)**	**Severe Asthma (n=74)**	***p***	**FeNO suppression cohort (n=34)**	**RASP-UK cohort (n=40)**	***p***
Age, y	35 ± 15	53 ± 13	0.0002	50 ± 16	55 ± 9	ns
Male, *n*	5 (50)	41 (55)	ns	15 (44)	26 (65)	ns
BMI, kg/m²	22 ± 4	30 ± 6	0.0008	27 ± 5	31 ± 7	0.006
Atopy	0 (0)	55 (74)	<0.0001	24 (71)	31 (78)	ns
Smoking status Never-Smoker	8 (80)	55 (74)	ns	23 (68)	32 (80)	ns
Ex-Smoker	2 (20)	17 (23)		9 (27)	8 (20)	
Current smoker	0 (0)	2 (3)		2 (6)	0 (0)	
ACQ-5 score		1.6 ± 1.2		1.7 ± 1.1	1.5 ± 1.3	ns
Asthma attacks in past year*		1 [0-4]		1 [0-3]	1 [0-4]	ns
ICS dose, BDP-CFC eq.,µg/d		2391 ± 1084		2792 ± 1235	2050 ± 806	0.003
Any systemic corticosteroid		39 (53)		22 (65)	17 (43)	ns
FEV_1, % predicted	99 ± 10	85 ± 19	0.03	90 ± 20	82 ± 17	ns
FEV_1/FVC ratio, %	84 ± 5	70 ± 11	0.0002	73 ± 12	67 ± 10	0.02
FeNO, ppb	19 [11-28]	42 [27-64]	0.0008	45 [34-64]	37 [19-66]	ns
Blood eosinophils, $\times 10^9$/L	0.14 [0.09-0.18]	0.25 [0.13-0.50]	0.02	0.25 [0.19-0.51]	0.26 [0.1-0.47]	ns
Sputum Eosinophils, %	0.0 [0.0-0.1]	2.8 [0.5-18.2]	0.0001	2.2 [0.9-18.6]	3 [0.3-18.8]	ns
Sputum supernatant available	6 (60)	60 (81)	ns	21 (62)	40 (100)	ns
Serum available	10 (100)	30 (41)	0.0003	30 (88)	0 (0)	<0.0001

Data are presented as no. (%), mean ± SD, or median [interquartile range]. *Asthma attacks are defined as acute asthma episodes requiring 3 days or more of systemic corticosteroids. *P*-values reported are unpaired *t*-tests for parametric variables, Mann-Whitney tests for nonparametric variables, Fisher's exact test or Chi-Square for categorical variables. ACQ-5, asthma control questionnaire; BDP-CFC eq., beclomethasone dipropionate with CFC propellant equivalent; BMI, body mass index; FeNO, fractional exhaled nitric oxide; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; ICS, inhaled corticosteroid; ns, $p \geq 0.05$.

2.2. Inflammatory mediators and other analytes according to type-2 biomarkers

The distribution of sputum, serum, and clinical analytes according to FeNO and blood eosinophils is detailed in Table 10. In the 74 patients with severe asthma, FeNO correlated significantly with sputum eosinophils (r=0.51), IL-4 (r=0.48), IL-5 (r=0.47), IL-33 (r=0.35), TSLP (r=0.41), eotaxin-3 (r=0.55), TARC (r=0.32), and asthma attacks in the previous year (r=0.25). Conversely, blood eosinophils correlated with serum IL-5 (r=0.41) only. The extended correlation matrix based on the significant correlations stated above is shown in Figure 16. Among the exploratory inter-analyte correlations, the strongest correlations were between sputum IL-5 and eotaxin-3 (r=0.89); sputum TSLP and TARC (r=0.85); and sputum TSLP and eotaxin-3 (r=0.84). Symptom scores did not correlate with any measured analyte nor biomarker.

Stratification by FeNO (<25, 25-<50, ≥50 ppb) of serum/sputum analytes in patients with asthma showed significant positive ordinal trends in values across categories for sputum eosinophils (fold-change in medians, FeNO <25 to ≥50 ppb: 17-fold, p=0.001), IL-4 (7.6-fold, p=0.0006), IL-5 (8.9-fold, p=0.0006), IL-33 (1.8-fold, p=0.02), TSLP (5-fold, p=0.002), eotaxin-3 (10-fold, p<0.0001), and TARC (3.5-fold, p=0.005).

Stratification by blood eosinophils (<0.15, 0.15-<0.3, ≥0.3×10^9/L) showed increasing median values across categories for serum IL-5 (1.9-fold, p=0.04).

Stratified sputum and serum analyte levels are shown in Figure 17 for biomarkers with a Spearman correlation coefficient greater than 0.4.

TABLE 10
Analytes according to FeNO and blood eosinophil-based stratification strategies, with corresponding correlation coeffecients

	Analyte (pg/mL or stated) LLOD†	FeNO (ppb) <25 (n=17)	25-<50 (n=30)	≥50 (n=27)	r (p)	Blood Eos (×10⁹/L) <0.15 (n=21)	0.15-<0.30 (n=22)	≥0.30 (n=31)	r (p)	Healthy controls (n=10)
Biomarker	**FeNO (ppb)**	16 [13-20]	39 [32-42]	83 [60-123] *		38 [23-55]	38 [26-74] *	45 [25-89] *	0.24 (0.04)	19 [11-28]
Biomarker	**Blood Eos (×10⁹/L)**	0.17 [0.1-0.54]	0.24 [0.1-0.35]	0.26 [0.19-0.55] *	0.24 (0.04)	0.09 [0.05-0.12]	0.23 [0.19-0.25]	0.54 [0.36-0.66] *		0.14 [0.09-0.18]
Sputum	**Eos (%)**	0.8 [0.4-5.3]	2.7 [1.1-17.8] *	12.8 [3.3-35.5] *	**0.51 (0.0002)**	2.7 [0.7-6.1]	5.1 [0.5-30.5] *	4.3 [1-21] *	ns	0.3 [0.3-0.4]
Sputum	**IL-4** 0.2	0.1 [0.1-0.3]	0.4 [0.1-1.1] *	0.8 [0.2-1.2] *	**0.48 (<0.0001)**	0.3 [0.1-1] *	0.4 [0.1-0.9]	0.3 [0.1-1] *	ns	0.1 [0.1-0.1]
Sputum	**IL-5** 0.5	1.2 [0.4-4.6]	4.6 [1.9-7.8]	10.9 [2.9-29.8] *	**0.47 (0.0002)**	2.3 [1.1-9.7]	5.3 [1.5-15.1] *	4.7 [1.8-10.8] *	ns	0.3 [0.2-2.7]
Sputum	**IL-13** 4.2	6.4 [2.1-8.8]	7 [5.8-14.2] *	8.4 [6.4-13.9] *	0.26 (0.04)	7 [5.1-11.5] *	8.3 [4-12.5] *	7.6 [6-12.2] *	ns	2.1 [2.1-2.1]
Sputum	**IL-33** 0.6	0.9 [0.3-1.3]	0.9 [0.3-2.1] *	1.7 [0.7-2.9] *	**0.35 (0.006)**	0.9 [0.3-1.9]	1.4 [0.5-2.6] *	1 [0.3-2.3] *	ns	0.3 [0.3-0.3]
Sputum	**TSLP** 0.9	2.4 [1-9.3]	6.4 [2.3-10.7] *	11.9 [5-20.7] *	**0.41 (0.001)**	4.9 [1.5-16.9]	9.1 [1.9-2.6]	7.1 [2.5-15] *	ns	0.9 [0.5-1.8]
Sputum	**Eotaxin-3** 4.2	34 [2-71]	133 [23-369] *	353 [245-804] *	**0.55 (<0.0001)**	76 [23-264]	215 [9-418] *	191 [29-390] *	ns	2 [2-26]
Sputum	**TARC** 0.4	17 [9-89]	27 [18-77]	58 [38-301] *	**0.32 (0.02)**	35 [19-107] *	41 [9-101]	36 [17-88]	ns	6 [2-21]
Sputum	**LTE4** 7.8	59 [23-114]	138 [42-465] *	133 [42-730] *	ns	64 [23-139]	94 [48-343]	163 [49-676] *	ns	7 [4-70]

(Table 10 continues next page)

… Table 10

	…Analyte LLOD	…FeNO (ppb) <25	 25-<50	 ≥50	*r* (*p*)	…Blood Eos (×10^9/L) <0.15	 0.15-<0.30	 ≥0.30	*r* (*p*)	…Healthy controls
…Sputum	**PGD2** 19.5	241 [173-384]	217 [119-354]	209 [135-439]	ns	213 [133-505]	219 [183-389]	222 [117-439]	ns	89 [43-200]
	IFN-γ 0.3	0.3 [0.2-0.5]	0.4 [0.2-1.8]	0.6 [0.2-1.5]	ns	0.5 [0.2-1.7]	0.4 [0.2-2.6]	0.3 [0.2-0.8]	ns	0.2 [0.2-2.1]
	TNF 0.4	1.5 [0.4-10.2]	2 [0.8-7.5]	3.3 [1.5-6.7]	ns	2.5 [1-6.7]	3.2 [0.5-8.5]	2 [0.7-8.6]	ns	2.9 [0.4-16.7]
Serum	**IL-4** 0.1	0.1 [0.1-0.1]	0.1 [0.1-0.1]	0.1 [0.1-0.1]	ns	0.1 [0.1-0.1]	0.1 [0.1-0.1]	0.1 [0.1-0.1]	ns	0.1 [0.1-0.1]
	IL-5 0.4	1.1 * [1-1.2]	0.6 * [0.5-0.9]	0.6 * [0.4-1.6]	ns	0.4 [0.4-0.6]	0.6 * [0.6-1.6]	0.8 * [0.6-1.5]	**0.41** **(0.03)**	0.2 [0.2-0.4]
	IL-13 6.7	3.3 [3.3-3.3]	3.3 [3.3-9.9]	3.3 [3.3-14.1]	ns	3.3 [3.3-3.3]	8.8 [3.3-13.3]	3.3 [3.3-10]	ns	9.2 [7.8-9.8]
	IL-33 0.4	0.2 [0.2-0.3]	0.8 [0.2-0.8]	0.2 [0.2-0.8]	ns	0.2 [0.2-0.8]	0.4 [0.2-0.8]	 [0.2-0.8]	ns	0.4 [0.4-0.4]
	TSLP 0.5	1.3 [1-2.1]	1.8 [0.6-2.5]	2.7 * [1.8-4.5]	ns	1.7 [1.2-3.6]	2.3 * [1.9-4.4]	1.8 [0.8-2.7]	ns	1.4 [0.9-1.5]
	Eotaxin-3 4.2	9 [6-30]	18 [7-32]	16 [13-29]	ns	18 [14-31]	29 * [12-34]	13 [7-18]	ns	8 [4-13]
	TARC 0.2	427 [108-571]	252 [147-463]	226 [89-430	ns	314 [195-664]	190 [92-252]	344 [146-480]	ns	206 [124-285]
	IFN-γ 0.3	0.4 [0.4-0.5]	0.5 [0.2-0.7]	0.3 [0.2-1.2]	ns	0.3 [0.2-0.6]	0.7 [0.2-2.3]	0.2 [0.2-0.6]	ns	0.4 [0.2-0.7]
	TNF 0.4	1.8 * [0.9-3.8]	0.6 [0.2-2]	1.2 * [0.2-4.2]	ns	1.7 * [0.2-2.3]	0.6 [0.2-2]	0.9 * [0.3-1.9]	ns	0.2 [0.2-0.3]

(Table 10 continues next page)

… Table 10

	…Analyte	…FeNO (ppb) <25	25-<50	≥50	*r* (*p*)	…Blood Eos (×10^9/L) <0.15	0.15-<0.30	≥0.30	*r* (*p*)	…Healthy controls
Clinical	**ACQ-5 score**	1.2 [0.5-1.8]	1.6 [0.2-2.2]	2 [0.8-3]	ns	1.6 [0.5-2.1]	1.7 [0.7-2.9]	1.2 [0.6-2.2]	ns	
	FEV1 (% predicted)	88 [78-103]	85 [75-98]	81 [72-96]	ns	81 [77-94]	83 [74-97]	85 [76-99]	ns	
	FEV_1/FVC (%)	72 [64-82]	68 [61-79]	72 [60-77]	ns	71 [62-82]	68 [61-77]	72 [61-80]	ns	
	Asthma attacks (past year)	1 [0-3]	1 [0-4]	3 [0-5]	**0.25 (0.03)**	1 [0-5]	1.5 [0-4]	1 [0-4]	ns	

Data are presented as median [interquartile range] in pg/mL unless stated otherwise. Spearman correlation coefficients (*r*) and associated *p*-values are **in bold** if retained after controlling for a false discovery rate <0.05 across the 52 computed correlations. *adjusted *p*-value <0.05 compared to healthy controls on Kruskal-Wallis test adjusted for 6 comparisons. †Cytokine levels that were not quantified were assigned the arbitrary value of 0.5×the lower limit of detection (LLOD) to allow analysis. ACQ-5, 5-item asthma control questionnaire; Eos, eosinophils; FeNO, fractional exhaled nitric oxide; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; IFN, interferon; IL, interleukin; LTE4, leukotriene E4; ns, *p*≥0.05; PGD2, prostaglandin D2; TNF, tumour necrosis factor; TARC, thymus activation regulated cytokine (CCL17); TSLP, thymic stromal lymphopoietin. Reproduced from Couillard, Shrimanker, *et al.* (2021).

p / *r*	FeNO	Blood Eos	Sputum Eos	Sputum IL-4	Sputum IL-5	Sputum IL-13	Sputum IL-33	Sputum TSLP	Sputum Eotaxin-3	Sputum TARC	Serum IL-5	ACQ-5 score	FEV1	FEV1/FVC ratio	Asthma attacks (past yr)
FeNO		0.04	**0.0002**	**<0.0001**	**0.0002**	0.04	**0.006**	**0.001**	**<0.0001**	**0.02**	ns	ns	ns	ns	**0.03**
Blood Eos	0.24		ns	ns	ns	ns	ns	ns	ns	ns	**0.03**	ns	ns	ns	ns
Sputum Eos	**0.51**	0.25		0.0005	<0.0001	0.02	ns	0.002	0.001	0.005	ns	ns	0.04	0.02	ns
Sputum IL-4	**0.48**	0.06	0.49		<0.0001	0.0004	<0.0001	<0.0001	<0.0001	<0.0001	ns	ns	ns	ns	ns
Sputum IL-5	**0.47**	0.14	0.55	0.71		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.006	ns	ns	0.01	ns
Sputum IL-13	0.26	0.05	0.33	0.44	0.60		<0.0001	<0.0001	<0.0001	<0.0001	ns	ns	ns	ns	ns
Sputum IL-33	**0.35**	0.03	0.25	0.81	0.65	0.48		<0.0001	<0.0001	<0.0001	ns	ns	ns	ns	ns
Sputum TSLP	**0.41**	0.14	0.44	0.65	0.84	0.57	0.61		<0.0001	<0.0001	ns	ns	ns	ns	0.049
Sputum Eotaxin-3	**0.55**	0.15	0.51	0.79	0.89	0.63	0.70	0.83		<0.0001	ns	ns	ns	ns	ns
Sputum TARC	**0.32**	0.02	0.42	0.63	0.83	0.51	0.56	0.85	0.70		0.04	ns	ns	0.04	ns
Serum IL-5	0.03	**0.41**	0.27	0.14	0.62	0.36	0.17	0.40	0.83	0.85		ns	ns	0.01	ns
ACQ-5 score	0.19	0.00	0.22	0.04	0.08	0.09	-0.11	0.07	0.06	0.13	0.00		ns	ns	ns
FEV1	-0.17	0.04	-0.29	-0.16	-0.21	0.05	-0.06	-0.09	-0.14	-0.14	-0.06	-0.05		<0.0001	ns
FEV1/FVC ratio	-0.14	0.01	-0.34	-0.18	-0.31	-0.07	-0.08	-0.23	-0.22	-0.27	-0.48	-0.03	0.69		ns
Asthma attacks	**0.25**	0.00	0.17	0.21	0.18	0.11	0.16	0.26	0.25	0.09	0.11	-0.01	-0.15	0.02	

r
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4

FIGURE 16. Correlation matrix for fractional exhaled nitric oxide (FeNO), blood eosinophils (Eos), and selected analytes in severe asthma. **Bold** Spearman coefficient of correlations (*r*) and *p*-values indicate statistically significant values in the primary analysis which controlled for a false discovery rate <0.05; the rest of the matrix is exploratory. Asthma attacks are defined as acute events requiring ≥3 days of systemic corticosteroids in the past year. ACQ-5, 5-item asthma control questionnaire; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; IL, interleukin; ns, $p \geq 0.05$; TARC, thymus activation regulated cytokine (CCL17); TSLP, thymic stromal lymphopoietin. Reproduced from Couillard, Shrimanker, *et al.* (2021).

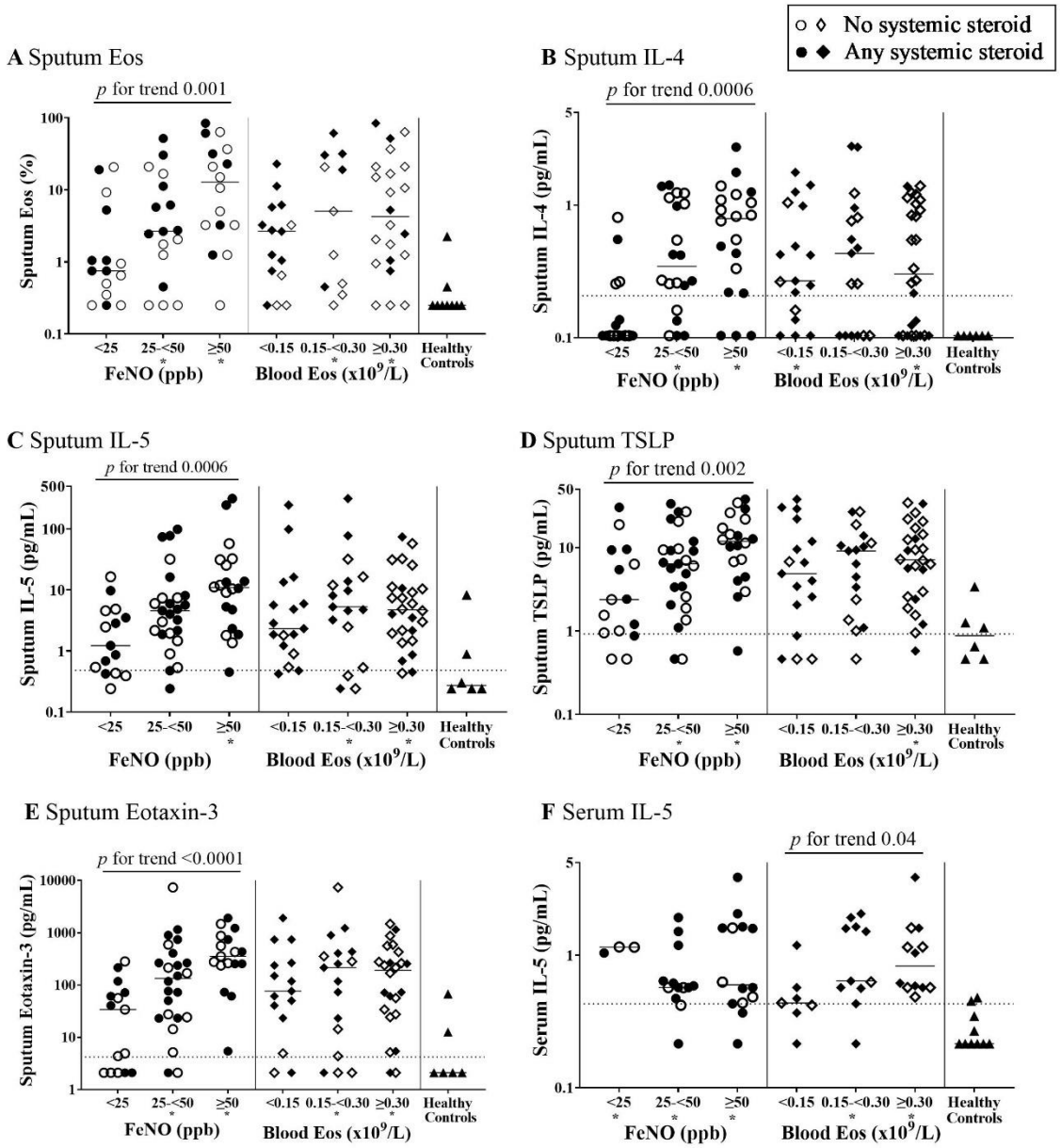

FIGURE 17. Selected inflammatory mediators translated according to different stratification strategies. Analytes presented are those with Spearman correlation *r*>0.4 and retained after controlling for the false discovery rate <0.05. *P* for trend is a Jonckheere-Terpstra test across biomarker categories. Bars indicate medians and dotted lines the lower limit of detection. *adjusted *p*-value <0.05 compared with healthy controls on Kruskal-Wallis adjusted for 6 comparisons with Dunn's multiple comparison test. Eos, eosinophils; FeNO, fractional exhaled nitric oxide; IL, interleukin; TSLP, thymic stromal lymphopoietin. ○◊, no systemic corticosteroid; ●♦, any systemic corticosteroid.

2.3. Sensitivity and exploratory analyses

The directions of trends were consistent when removing systemic corticosteroid-treated patients or when separating the RASP-UK and Oxford FeNO suppression cohorts (not shown).

Exploratory multiple regression of continuous log-transformed inflammatory mediator values did not demonstrate an additive effect for blood eosinophils on FeNO in identifying inflammation levels (not shown).

2.4. Summary of aim 2 results

- In a cross-sectional analysis of patients with severe asthma and proven adherence to high-intensity corticosteroid therapy, FeNO and blood eosinophils identified distinct components and compartments of type-2 inflammation. FeNO levels reflected airway type-2 cytokines (IL-4, IL-5), alarmins (IL-33, TSLP), chemokines (eotaxin-3, TARC) and sputum eosinophilia. In contrast, blood eosinophils correlated with serum IL-5 and not with airway inflammation.
- These findings translate beautifully to cut points used in clinic, where FeNO <25 ppb *vs* ≥ 50 ppb and blood eosinophils <0.15 *vs* ≥ 0.30×10^9/L are commonly used to differentiate type-2 low and type-2 high asthma, respectively.
- The highest FeNO and blood eosinophil categories generally had greater sputum eosinophils, sputum/serum type-2 cytokine, chemokine and alarmin levels than healthy controls.
- There was no significant correlation between symptom scores and objective measurements of inflammation, lung function or asthma attack history.

- The results were similar when removing patients on systemic steroids and when separating the two patient populations (Oxford and RASP-UK cohorts).

3. Derivation of a prototype asthma attack risk scale centred on biomarkers

3.1. Derivation cohort and parameters

For biomarker-stratified multipliers. Of the 8476 patients included in the eight derivation trials (Pavord *et al.*, 2012; Castro *et al.*, 2014, 2018; Corren *et al.*, 2017; Panettieri *et al.*, 2018; Beasley *et al.*, 2019; Lee *et al.*, 2021)(detailed in Appendix 1), 3313 patients were randomised to a control arm; 262 had missing data in the biomarker-stratified analyses published in the literature (Shrimanker *et al.*, 2019; Pavord *et al.*, 2020; Busse *et al.*, 2021; Kraft *et al.*, 2021; Lee *et al.*, 2021), resulting in the inclusion of 3051 patients' biomarker-stratified asthma attack rates. The individual RCT's main characteristics, biomarker-stratified rate ratios and their aggregate values are shown in Table 11. The aggregate asthma attack rate ratios included for derivation of the prototype scale were 0.65 in the lowest (blood eosinophils <0.15 x 10^9/L and FeNO <25 ppb) to 2.29 in the highest (blood eosinophils ≥0.3×10^9/L and FeNO) type-2 biomarker combination group; a 3.5-fold difference in the risk of severe asthma attacks between these two groups. Blood eosinophils and FeNO provided independent prognostic information and their impact was additive in 4 of the 5 biomarkers-stratified analyses (Shrimanker *et al.*, 2019; Busse *et al.*, 2021; Kraft *et al.*, 2021; Lee *et al.*, 2021).

Reference annual attack rates for GINA treatment steps. Publication-level data from the 222,817 patients with asthma reported in the U.S. database (Suruki *et al.*, 2017) were included. The GINA treatment step-specific annual attack rates are shown in Table 12.

TABLE 11
Biomarker-stratified data and rate ratios derived from included trials

Blood Eos ($\times 10^9$/L)	FeNO (ppb)	**Novel START** (Pavord *et al.*, 2020) **Step 1 asthma; low-risk; 9% with attack in past 12m**			**CAPTAIN** (Lee *et al.*, 2021) **Step 4 asthma; high-risk; 62% with attack in past 12m**			**Benralizumab 2b, PATHWAY, STRATOS 1-2** (Kraft *et al.*, 2021) **1% step 3, 50% step 4, 49% step 5; high-risk; with attack in past 12m;**			**QUEST** (Busse *et al.*, 2021) **47% step 4, 53% step 5 asthma; high-risk; with attack in past 12m**			**DREAM** (Shrimanker *et al.*, 2019) **Step 5; high-risk; with attack in past 12m**			**Aggregate data for the prototype risk scale**	
		n*	***Attack rate***†	***Rate ratio***	***n***	***Attack rate***†	***Rate ratio***	***n****	***Attack rate***	***Rate ratio***	***n***	***Attack rate***	***Rate ratio***	***n***	***Attack rate***	***Rate ratio***	***N***	***Rate ratio***
<0.15	<25	18	0.05	0.98	228	0.85	0.54	199	0.58	0.81	106	0.56	0.52	23	1.98	0.76	574	**0.65**
	25-<50	23	0.00	0.00	40	0.10	1.11	82	0.46	0.64	35	0.62	0.61	(9)	(1.78)	(0.71)	180	**0.66**
	≥50	8	0.00	0.00	17	0.15	1.74	23	0.57	0.81	21	0.53	0.53				69	**0.86**
0.15-<0.30	<25	19	0.07	1.50	240	0.07	0.82	191	0.56	0.76	96	0.82	0.80	12	1.54	0.59	558	**0.81**
	25-<50	42	0.02	0.36	87	0.07	0.79	173	0.67	0.96	53	1.14	1.17	(23)	(2.70)	(1.07)	355	**0.88**
	≥50	32	0.01	0.24	24	0.12	1.43	52	1.29	1.93	25	0.48	0.47				133	**1.16**
≥0.30	<25	4	0.30	6.35	248	0.11	1.29	102	0.58	0.82	89	0.84	0.84	18	1.95	0.75	461	**1.12**
	25-<50	22	0.00	0.00	147	0.09	1.00	133	0.87	1.30	97	1.24	1.31	(66)	(3.08)	(1.22)	399	**1.12**
	≥50	51	0.13	4.40	66	0.18	2.14	107	1.01	1.53	98	1.78	2.12				322	**2.29**
Analysed		219	0.05	1.00	1097	0.09	1.00	1062	0.70	1.00	620	0.99	1.00	151	2.52	1.00	3051	1.00
Missing[#]		4			121			120			14			4			262	
Total		223			1218			1182			634			155			3313	

Aggregate ratios in bold (rightmost column) are those included to derive the prototype risk scale: in effect, an aggregate rate ratio is a mean fold-change in the asthma attack rate for patients with that biomarker combination. Numbers between brackets were extracted to calculate frequency-weighted rate ratios but were not used to derive the scale, as this analysis stratified using only two cut points for fractional exhaled nitric oxide (FeNO <25 or ≥25 ppb). *For Novel START and the pooled AstraZeneca (AZ) trials, data of patients with a baseline FeNO of 20-<50 ppb were regrouped into the 25-<50 ppb group, as the difference of 5 ppb in FeNO is not clinically relevant. †For both the Novel START and CAPTAIN, only the percentage of patients with one or more severe attacks(s) in the 52-weeks of follow-up was reported so a rate was imputed as $-\log_{10}(1 - \%\text{incidence})$. # Missing data were excluded from analyses. Blood Eos = peripheral blood eosinophil count. Reproduced from Couillard, Laugerud, *et al.* (2021).

TABLE 12
Asthma attack rates according to GINA treatment step and asthma attack history

GINA treatment step	*n* (%)	Annual severe asthma attack rate for whole sample (95% CI)	Annual severe asthma rate for patients with no attack in prior 12 months
1	103,415 (46.4)	0.139 (0.136-0.142)	**0.096**
2	36,616 (16.4)	0.143 (0.138-0.147)	
3	38,497 (17.3)	0.153 (0.148-0.158)	**0.105**
4	36,039 (16.2)	0.186 (0.181-0.191)	**0.128**
5	7,736 (3.5)	0.455 (0.434-0.478)	**0.312**
Overall*	222,817 (100)	0.161 (0.153-0.163)	0.110 (0.109-0.112)

Numbers in bold (rightmost column) are those used to derive the prototype risk scale: these were calculated in proportion to the overall rate of patients with no exacerbation in the 12 months before the index date. The index date was defined as the date of the first recorded asthma medical code during the study. We were unable to obtain exact annual rates per GINA step for patients with no prior exacerbation. *The *n*=514 'unclassifiable' patients are not shown in this table but are reported in the original publication and accounted for in our calculations. GINA = Global Initiative for Asthma, CI = confidence intervals. Data from Suruki et al. (2017).

Risk associated with a recent asthma attack. If there was a history of a severe attack in the previous year, the baseline risk was multiplied by 2.8. This rate ratio was calculated from the annual severe attack rates reported by Suruki *et al.* (2017). It similar to that calculated in the LAVOLTA studies (Hanania *et al.*, 2016), which showed that the excess risk of asthma attacks associated with a history of an attack in the last year was independent of blood eosinophils and FeNO.

Risk associated with ≥ 2 clinical risk factors. If two or more risk factors listed in Table 3 were present, the baseline rate was independently multiplied by 1.3.

3.2. Development of the prototype OxfoRd Asthma attaCk risk ScaLE

The result from applying biomarker-stratified and clinically stratified rate ratios to the GINA treatment step rates is shown in Figure 18. To populate each cell of the prototype OxfoRd Asthma attaCk risk ScaLE (ORACLE), the reference rate for GINA treatment steps 1&2, 3, 4 and 5 was multiplied by the appropriate risk pertaining to that group, *e.g.*: the rightmost column's rates are calculated as [Biomarker-stratified rate ratio as per rightmost column of Table 11] × [GINA treatment step-specific attack rate as per rightmost column of Table 12] ×2.8 ×1.3. In effect, each cell of the scale represents the predicted annual asthma attack rate for a patient with a given set of GINA treatment step, biomarker measurements, asthma attack history, and clinical circumstances.

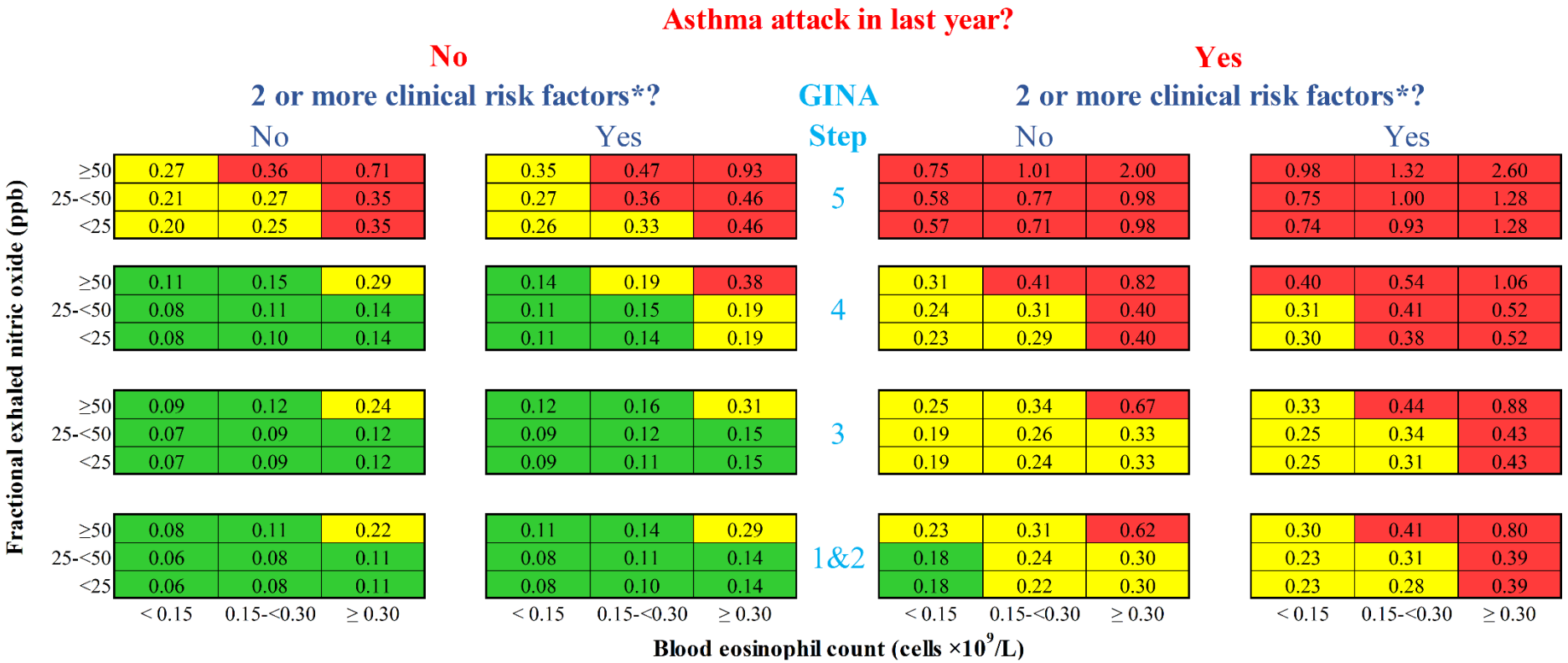

FIGURE 18. The prototype OxfoRd Asthma attaCk risk ScaLE (ORACLE). Numbers in each cell are predicted annual asthma attack rates for patients over the age of 12 if treatment is not changed. An asthma attack is an episode of acute asthma requiring treatment with systemic steroids ≥ 3 days and/or hospitalisation. Blood eosinophil count is contemporaneous or the highest result in last 12 months; fractional exhaled nitric oxide level (FeNO) is contemporaneous. *Risk factors are defined by the Global Initiative for Asthma (GINA) guidelines (2021) : poor symptom control (ACQ score ≥1.5), low lung function (FEV1 <80% predicted), adherence issues, reliever over-use (>200-dose salbutamol cannister/month), intubation or intensive care unit admission for asthma previously, comorbidities (one of: chronic rhinosinusitis, obesity, psychiatric disease), environmental exposures (one of: smoking, allergen, pollution). Reproduced from Couillard, Laugerud, *et al.* (2021).

3.3. Validation of the prototype ORACLE

There was close reliability, agreement and association between frequency-weighted observed and predicted asthma attack rates.

Reliability. The intraclass correlation coefficient [95%CI] was 0.83 [0.78–0.86]. This signifies 'good' reliability (Koo and Li, 2016).

Agreement. The Bland-Altman fixed bias was -0.08 [-0.58–0.41] (Figure 19). This is a reasonably low fixed bias estimate but the large confidence intervals exceed the minimal clinically important difference for asthma attack rates (Bonini *et al.*, 2020) in groups with a predicted rate below ~1 event per year.

Agreement and association. The weighted least squares regression equation was y=0.94x - 0.08 (slope [0.92–0.96], constant [-0.09–-0.07]) with R^2=0.79 (Figure 20). Although not a classical calibration plot, the regression equation was close to the ideal equation y=x (Steyerberg, 2019, p.297).

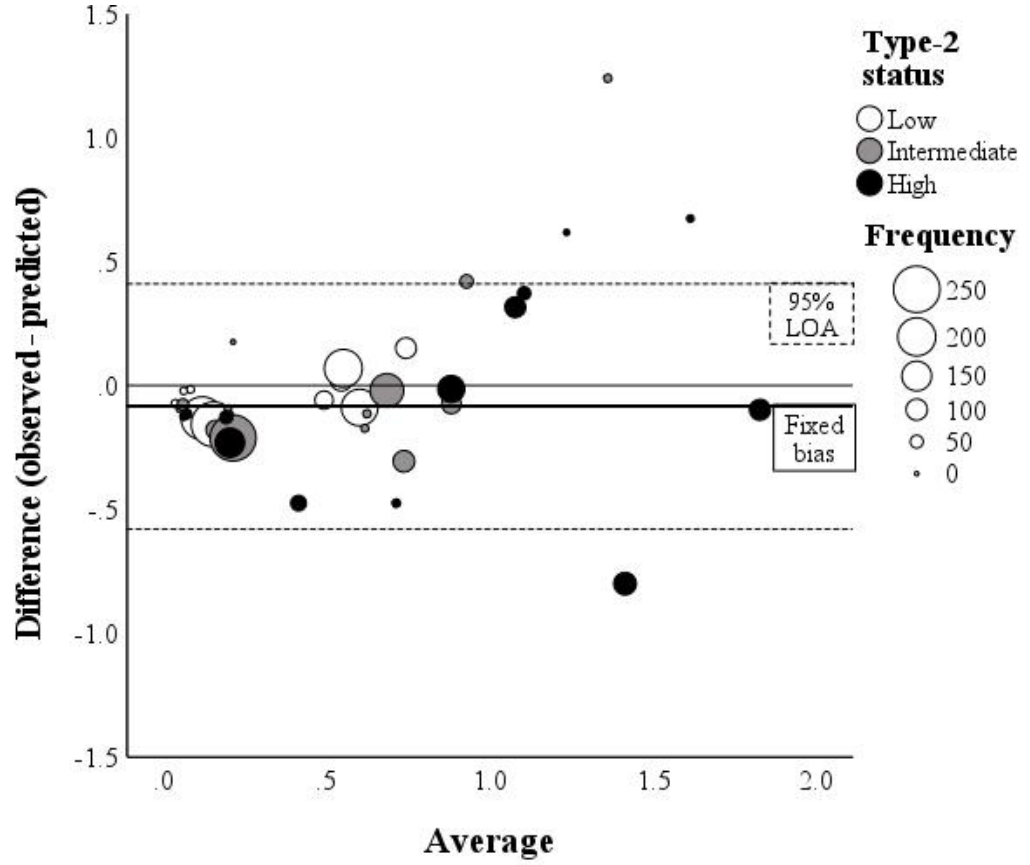

FIGURE 19. Bland-Altman plot. LOA, limits of agreement.

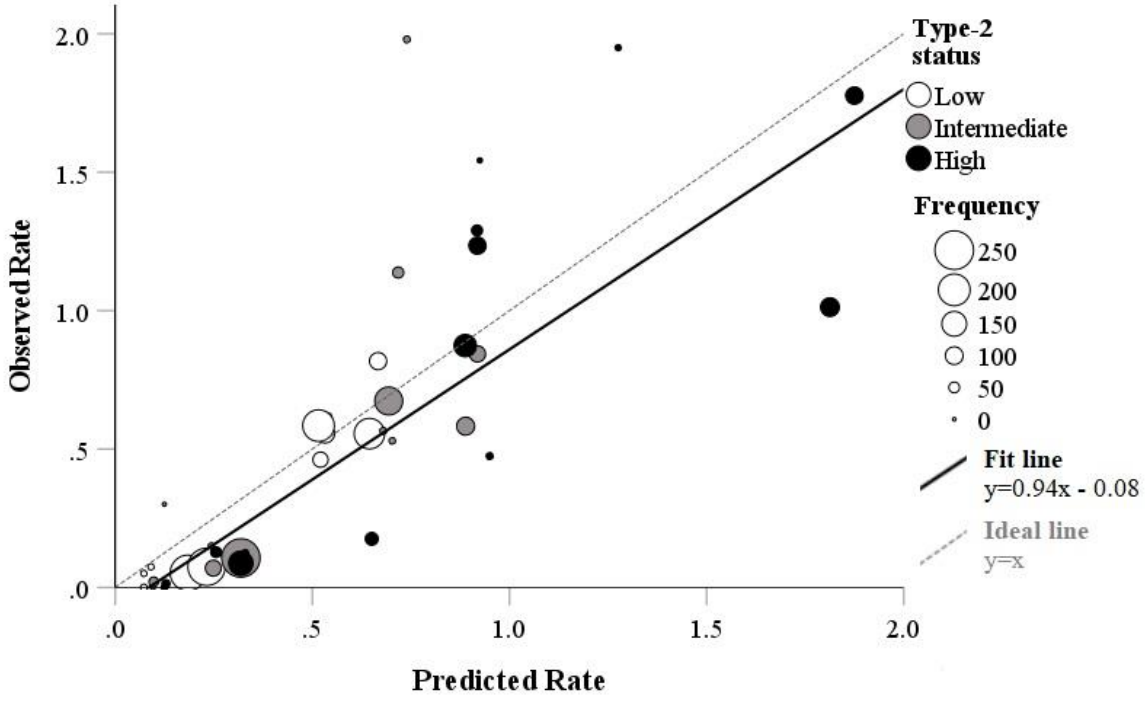

FIGURE 20. 'Calibration plot' of observed versus predicted rates.

3.4. Modifiable risk observed and predicted by raised type-2 biomarkers

The observed *vs* ORACLE-predicted biomarker-stratified annual asthma attack rates and rate ratios for the Novel START (Pavord *et al.*, 2020), CAPTAIN (Lee *et al.*, 2021), QUEST (Shrimanker *et al.*, 2019; Busse *et al.*, 2021) and DREAM (Shrimanker *et al.*, 2019) trials' control *vs* treatment arms are shown in Table 13 (to be reported in Couillard, Do, *et al.*, manuscript in preparation).

There were 3925 patients with at least one type-2 biomarker high at baseline (*i.e.*: blood eosinophils $\geq 0.15 \times 10^9$ cells/L or FeNO ≥25 ppb). The observed *vs* predicted frequency-weighted mean rate ratios were 0.59 *vs* 0.58; the corresponding percentages reduction in asthma attacks were 41% and 42%, respectively. Although not possible to calculate 95%CIs for this analysis, the rate ratios were all clinically and statistically significant in the biomarker-stratified publications and thus the mean estimate is overwhelmingly likely to be significant (Shrimanker *et al.*, 2019; Pavord *et al.*, 2020; Lee *et al.*, 2021).

For the 814 patients with low type-2 biomarkers at baseline (*i.e.:* blood eosinophils $<0.15 \times 10^9$ cells/L and FeNO <25 ppb), the observed *vs* predicted rate ratios of 0.86 vs 1.00; the corresponding percentages reduction in asthma attacks were 14% and 0%, respectively. Although not possible to calculate 95%CIs for this analysis, these were reported as large and not significant in the biomarker-stratified publications and thus the mean estimate likely overlaps with the no-effect rate ratio (1.00)(Shrimanker *et al.*, 2019; Pavord *et al.*, 2020; Lee *et al.*, 2021) and is not clinically significant (Bonini *et al.*, 2020).

A demonstration-only web application has been developed to show how ORACLE quantifies the excess risk conferred by raised type-2 biomarkers which is removed by anti-inflammatory therapy: www.oraclescore.com (Couillard, Do, *et al.*, 2021)

TABLE 13

Predicted *vs* observed impact of anti-inflammatory treatments according to baseline biomarkers

Baseline biomarkers	Annual severe asthma attack rate		Included trial: control *vs* active interventions (n)				Weighted mean % reduction
			Novel START Salbutamol *vs* Any ICS*†	**CAPTAIN** FF100 *vs* FF200*	**QUEST** Placebo *vs* Dupi 200	**DREAM** Placebo *vs* Any Mepo	Control *vs* Active
Type-2 High Blood Eos ≥0.15×10^9 cells/L or FeNO ≥25 ppb			(n=201 *vs* 377)	(n=903 *vs* 909)	(n=514 *vs* 484)	(n=145 *vs* 392)	(n=1763 *vs* 2162)
	Observed	Control arm	0.05	0.40	1.07	2.46	
		Active arm	0.03	0.26	0.41	1.19	
		% Reduction	35%	35%	62%	52%	**41%**
	Predicted	Control arm	0.13	0.32	0.93	1.28	
		Active arm	0.07	0.19	0.53	0.74	
		% Reduction	42%	42%	42%	42%	**42%**
Type-2 Low Blood Eos <0.15×10^9 cells/L and FeNO <25 ppb			(n=18 *vs* 60)	(n=194 *vs* 211)	(n=106 *vs* 139)	(n=23 *vs* 63)	(n=341 *vs* 473)
	Observed	Control arm	0.05	0.27	0.56	1.98	
		Active arm	0.03	0.22	0.58	1.71	
		% Reduction	41%	20%	-4%	14%	**14%**
	Predicted	Control arm	0.07	0.19	0.53	0.74	
		Active arm	0.07	0.19	0.53	0.74	
		% Reduction	0%	0%	0%	0%	**0%**

*For both the Novel START and CAPTAIN studies, data of patients with a baseline FeNO of <20 ppb were regrouped into the <25 ppb group, as the difference of 5 ppb in FeNO is not clinically relevant. †For Novel START, only the percentage of patients with one or more severe attacks(s) in the 52-weeks of follow-up was reported so a rate was imputed as -log10(1 - %incidence). Dupi 200, dupilumab 200 mg/2w ; FF100-200, fluticasone furoate 100 or 200µg/d-containing inhaler; ICS, inhaled corticosteroid; Mepo, mepolizumab. Data from Shrimanker *et al.* (2019); Pavord *et al.* (2020); Busse *et al.* (2021); Lee *et al.*, (2021). Table reproduced in Couillard, Do, *et al.* (manuscript in preparation).

3.5. Summary of aim 3 results

- The prototype asthma attack risk scale (ORACLE) was developed using trial-level data while making several pragmatic assumptions to effectively tabulate the excess risk conferred by two biomarkers of type-2 inflammation – the blood eosinophil count and FeNO. The resultant prototype shows feasibility and potential to predict asthma attacks which can be prevented by treatment.
- Although the relative risk conferred by high biomarkers was consistent at all stages of the disease, the absolute difference in asthma attack rates depended primarily on the background risk.
- There was good correlation, reliability, and agreement between the observed and predicted rates in the derivation trials.
- The excess risk of asthma attacks predicted by ORACLE for patients with high type-2 biomarkers at baseline is similar to the treatment effect of anti-inflammatory strategies in type-2 high trial populations. These strategies include exposure to any low-dose ICS in mild asthma (Pavord *et al.*, 2020), increased ICS dosage in moderate asthma (Lee *et al.*, 2021), and type-2 targeting biologics in severe asthma (Shrimanker *et al.*, 2019).
- Although promising, the prototype ORACLE is indeed a prototype and is thus not validated for clinical practice. It is possible that a refined version based on individual patient data could prove clinically useful.

DISCUSSION

In answer to the research questions, the main findings of this dissertation are three-fold (Figure 21). First, patients with uncontrolled asthma who fail to suppress FeNO despite monitored high-intensity corticosteroid therapy have distinct clinical, biomarker, and inflammatory mediator responses before and after a FeNO suppression test which suggest corticosteroid resistance (Couillard *et al*., manuscript in preparation). Second, FeNO and blood eosinophil non-suppression provide complementary mechanistic information on two different immune compartments in severe asthma: FeNO reflects type-2 cytokine, chemokine, alarmin and eosinophilic inflammation in the airways, whilst blood eosinophils reflect the systemic pool of effector cells and circulating IL-5 (Couillard, Shrimanker, *et al.*, 2021). Third, the prognostic and theragnostic values of these type-2 biomarkers in RCTs allow the tabulation of the risk of asthma attacks in a manner which also predicts treatment benefits across the range of disease severities (Couillard, Laugerud, *et al.*, 2021; Couillard, Do, *et al.*, manuscript in preparation).

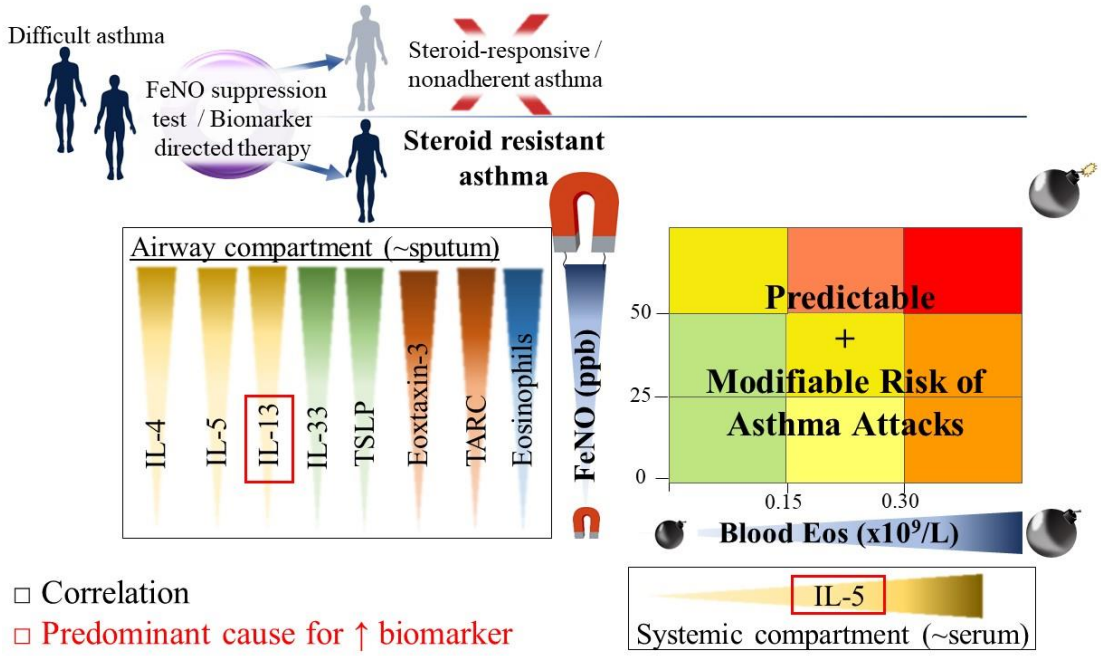

FIGURE 21. Graphical abstract. Fractional exhaled nitric oxide (FeNO) non-suppression carries significant translational, prognostic and theragnostic utility as a biomarker of type-2 airway inflammation in asthma – especially when used in combination with the peripheral blood eosinophil count (blood Eos). FeNO identifies type-2 activity and the chemotactic pull to the airways (analogous to a magnet), whilst blood Eos reflect the systemic pool of potential effector cells and circulating interleukin (IL)-5 (analogous to unlit explosives). The two biomarkers have additive value in predicting asthma attacks which can be prevented by anti-inflammatory therapy. TARC, thymus activation regulated cytokine; TSLP, thymic stromal lymphopoietin.

1. Comparing the findings to the previous literature

1.1. Characterisation of FeNO non-suppressors

The FeNO suppression test has been clinically described in four publications (McNicholl *et al.*, 2012; Faruqi *et al.*, 2019; Heaney *et al.*, 2019; Boddy *et al.*, 2020). These have consistently shown FeNO non-suppressors to be older males with higher baseline asthma morbidity and lesser before/after improvements in symptom scores, lung function, and FeNO. In this regard, the characteristics of the Oxford FeNO suppression cohort closely reflect the current literature. However, none of the published analyses included sputum/serum inflammatory mediators.

Despite an underpowered analysis curtailed by a pandemic complicating sputum induction and respiratory assessments, the longitudinal analysis of sputum and serum mediators in Oxford yielded multiple trends which extend the existing knowledge on FeNO suppression testing (Couillard *et al.*, manuscript in preparation). First, although no inflammatory mediator before/after difference was retained after controlling for multiplicity of testing, the number of numerical decreases in inflammatory mediators observed in FeNO

suppressors was greater than in non-suppressors. Conversely, patients who failed to suppress FeNO had significant residual levels of inflammation – sometimes higher than at the start of the test, as noted for sputum eosinophils. Second, although the magnitude of FeNO suppression was again useful to identify pre-existing nonadherence, there were interesting trends at the optimised stated which suggested that the end-of-test biomarker value may be a more useful indicator of residual type-2 inflammation than the 'FeNO non-suppressor' label. Third and last, the sensitivity analysis conducted on different therapeutic optimisation methods (*i.e.*: additional ICS and/or intramuscular corticosteroids) showed no tangible impact on the outcome of the FeNO suppression test.

In summary, these results imply that, although the answer to the question: 'Can you suppress FeNO, yes/no' investigates pre-existing nonadherence and suitability for biological therapies, the question: 'How low can your biomarkers go' may better relate to corticosteroid resistance. Importantly, the 'how low can you go' question can be explored using a combination of optimisation methods.

1.2. Translating FeNO and blood eosinophil non-suppression in severe asthma

To my knowledge, this was the first study to show that raised FeNO, an airway IL-13-induced biomarker (Chibana *et al.*, 2008), identifies persistent eosinophilic chemokines in the sputum in addition to other type-2 cytokines and TSLP (Couillard, Shrimanker, *et al.*, 2021). The greatest correlations between FeNO and sputum mediators were with sputum eosinophils and eotaxin-3. This finding is consistent with the recent demonstration that higher FeNO effectively predicts greater airway mucosal expression of eotaxin-3: the most highly-expressed and specific IL-13-induced gene in the ADEPT bronchoscopy-based study (Silkoff *et al.*, 2017). It is also concordant with other studies showing that eotaxin-3 is the most potent eosinophil chemokine *in vitro* and in asthma (Provost *et al.*, 2013; Larose

et al., 2015). Finally, the novel finding that a raised FeNO identifies higher sputum IL-4, eotaxin-3, TARC and TSLP levels is supported by high-quality data in the controlled clinical trial setting where the two FeNO-lowering biologics dupilumab (anti-IL-4/-13) and tezepelumab (anti-TSLP) also reduce serum levels of these mediators (Corren *et al.*, 2017; Castro *et al.*, 2018; Menzies-Gow *et al.*, 2021). To summarise, this study's results concord with current knowledge and clinical trial data. Importantly, the conclusion that raised FeNO in the setting of high-intensity corticosteroid therapy identifies persistent airway inflammation is based on a cohort with proven adherence to their controller therapies – a limitation noted in the ADEPT study (Silkoff *et al.*, 2017) and a major issue in the FeNO-related literature up to now.

The weak correlation observed between sputum IL-13 and FeNO was not retained after correction for multiplicity of testing. Although it is probable that a larger study would have shown significance for sputum IL-13, sputum IL-4 was found to correlate significantly with FeNO. Taken together, these results may reflect the complex dimeric receptor system which signals both IL-4 and IL-13 (Guo *et al.*, 1997; Khurana Hershey, 2003).

Blood eosinophils better reflected systemic IL-5 production which might involve autocrine secretion by eosinophils (Dubucquoi *et al.*, 1994). This finding is supported by the strong correlations between these analytes in our cohort and the clinical efficacy of biologics targeting IL-5, despite a lack of effect on FeNO in clinical trials (Pavord *et al.*, 2012).

An important negative finding was the lack of an additive inflammatory signal for blood eosinophils on FeNO, contrary to what is observed for the risk of asthma attacks (Shrimanker *et al.*, 2019; Busse *et al.*, 2021; Kraft *et al.*, 2021). Indeed, there was no additive signal in the multiple linear regression of mediators versus both biomarkers. This negative result supports a model where stable asthma is an equilibrium to which FeNO and

blood Eos contribute different yet complementary information on the type-2 inflammatory response; analogous to a magnet (FeNO-related airway chemokines) pulling systemic effector cells (blood eosinophils) to the airways. Under this model, it is possible that a trigger (*e.g.*, a virus or allergen)(Wang *et al.*, 2018; Muehling *et al.*, 2020) leads to a multiplicative event – an asthma attack. This model is further supported by data showing 30-fold greater endobronchial eosinophils at the time of an acute exacerbation (Saetta *et al.*, 1994), and by the two-fold increase of FeNO observed in the days preceding a period of loss of asthma control (Psallidas *et al.*, 2021). It is also partly supported by the MEX study (McDowell *et al.*, 2020), where FeNO and to a lesser extent blood eosinophils retained their ability to identify sputum eosinophilia at the time of an asthma attack in mepolizumab-treated patients, despite significant suppression of blood eosinophils.

1.3. Modelling the risk of asthma attacks using biomarkers

To my knowledge, the prototype ORACLE represents the first attempt to tabulate the risk of asthma attacks using FeNO and blood eosinophils as key treatable traits in a manner analogous to cardiovascular risk scores which use blood pressure and serum cholesterol levels (Couillard, Laugerud, *et al.*, 2021; Couillard, Do, *et al.*, manuscript in preparation).

Of the several risk stratification systems that have been developed for asthma (Ellman *et al.*, 1997; Grana *et al.*, 1997; Lieu *et al.*, 1999; Schatz *et al.*, 2003; Yurk *et al.*, 2004; Miller *et al.*, 2006; Peters *et al.*, 2006; Osborne *et al.*, 2007; Sato *et al.*, 2009; Eisner, Yegin and Trzaskoma, 2012; Bateman *et al.*, 2015; Loymans *et al.*, 2016), only two include FeNO as a parameter (Sato *et al.*, 2009; Loymans *et al.*, 2016) and none have assessed the blood eosinophil count. Loymans *et al.* found that the addition of FeNO over a combination of history and spirometry parameters only marginally increased the accuracy of their model to predict asthma attacks (2016), while Sato *et al*. showed that FeNO could not accurately

predict this outcome (2009). Important limitations of these analyses are that they did not focus on modifiable risk; the patients' treatment and assessment during the observation period was not necessarily standardised; and the authors did not consider sufficiently the relationship between different potential risk predictors and the mechanism of asthma attacks. This last limitation is particularly important. For example, although measures such as lung function and symptom scores are associated with the risk of severe asthma attacks, they can be improved independently of the risk of severe asthma attacks (*i.e.* following treatment with long acting beta2 agonist monotherapy)(Lazarus *et al.*, 2001) indicating that they are not necessarily on the same causal pathway. The prototype ORACLE has none of these limitations. It proposes to derive a scale from clinical trial data, where there is a high degree of standardisation of treatment and assessment of key outcomes (*i.e.* asthma attacks). Furthermore, the prototype is based on two biomarkers that are not only closely associated with the mechanism of asthma attacks but are easily modified with treatment. Finally, there is existing and consistent evidence that the prognostic and theragnostic value of these biomarkers is independent of other risk factors (Pavord *et al.*, 2018).

The fact that blood eosinophils and FeNO provide additive predictive information is biologically plausible considering the 'two-compartment, two-hit theory' (proposed in Couillard, Jackson, *et al.*, 2021): FeNO reflects tupe-2 inflammation in the airway compartment whereas blood eosinophils are related to the systemic pool of effectors cells and IL-5 activity (data from Couillard, Shrimanker, *et al.*, 2021). Interestingly, ORACLE's biomarker-stratified aggregate rate ratios showed that FeNO added relatively little prognostic information to blood eosinophils when the blood eosinophil count was low. One potential explanation is that IL-13 mediated chemoattraction of eosinophils to the airway does not occur when the circulating pool of eosinophils in depleted. This would explain why the beneficial effects of depleting circulating eosinophils with monoclonal antibodies

targeting IL-5 are relatively independent of FeNO (Pavord *et al.*, 2012). It also suggests greater utility for measuring and interpreting FeNO when this is combined to the blood eosinophil count.

Finally, the demonstration that the excess risk identified by raised biomarkers in ORACLE was equivalent to the benefits of specific anti-inflammatory treatment in RCT populations warrants discussion. This analysis was performed using data from four of the eight RCTs included in the prototype derivation . It was not possible to extend this analysis to the other derivation RCTs (Castro *et al.*, 2014; Corren *et al.*, 2017; Panettieri *et al.*, 2018) on the basis that the associated biomarker-stratified analysis (Kraft *et al.*, 2021) strictly reported on the placebo arm attack rates, and because the original trial publications did not report on the composite biomarker definitions of interest (Castro *et al.*, 2014; Corren *et al.*, 2017; Panettieri *et al.*, 2018). Likewise, despite a systematic review of the literature (Couillard and Pavord, 2021b), it was not possible to find external trials reporting on the appropriate composite biomarker-stratified subgroups' control and active treatment attack rates in a manner which allows ORACLE-predicted rates to be calculated (Sorkness *et al.*, 2007; Corren *et al.*, 2011; Brusselle *et al.*, 2013; Hanania *et al.*, 2013, 2015, 2016; Wenzel *et al.*, 2016; Harris *et al.*, 2016; Park *et al.*, 2016; Hardy *et al.*, 2019; Brightling *et al.*, 2021; Menzies-Gow *et al.*, 2021). Nevertheless, the demonstration that the ORACLE prototype closely predicted treatment benefits in four RCTs comprising mild (Beasley *et al.*, 2019), moderate (Lee *et al.*, 2021), and moderate-to-severe (Pavord *et al.*, 2012; Castro *et al.*, 2018) asthma populations seems sufficient to prove the concept of 'predictable and modifiable risk' (Figure 21).

2. Study limitations

2.1. Characterisation of FeNO non-suppressors

This analysis was significantly underpowered; not so much because of the low number of FeNO suppression tests completed prior to the pandemic disruption, but especially due to the weak number of paired valid sputum samples available for analysis. The published literature reports on sputum induction and processing success rates of up to 92% (Hunter *et al.*, 1999); the rate for the day 0 of the Oxford FeNO suppression cohort was 44%. In consequence, there were 26% paired before/after sputum differential cell counts to analyse. The paired sputum supernatant availability was better at 56%. Although there may have been technical issues relating to cell viability during a period when sputum induction was carried out in a separate clinical site than the respiratory laboratory, the significant difference between the number of sputum supernatants harvested (signifying a sputum plug had been isolated and processing occurred) and valid sputum differential cell counts reported (signifying the cytospin slide was valid for interpretation)(Pizzichini *et al.*, 1996; Pavord *et al.*, 1997) is difficult to explain by a single factor. Serum samples were certainly available in greater quantities but, as expected, were less useful to assess FeNO-related mechanisms.

It was, in part, to improve the study power that a second analysis was conducted combining all the 'optimised' Oxford samples (paired and unpaired) with external RASP-UK samples thought to be equivalent in 'patient optimisation' methodology.

2.2. Translating FeNO and blood eosinophil non-suppression in severe asthma

Despite better study power than the above analysis, the pooled cross-sectional study was also relatively underpowered in certain respects. Indeed, considering the availability of

clinical measurements (*n*=74/74: ACQ-5, lung function, number of asthma attacks), sputum supernatants (*n*=60/74) and serum samples (*n*=30/74, the RASP-UK cohort lacking serum) for 52 (*m*) correlations controlled for a false discovery rate (*q*) <0.05, the power to detect a correlation coefficient of *r*=0.40 with a raw two-sided *p*-value <0.041 (*i.e.* below the highest retained p-value's (i/m)*q threshold – its rank (i) was 43) equals 94, 87 and 57%, respectively (Benjamini and Hochberg, 1995). Consequently, there was likely a type-II error for serum mediator correlations.

The study was cross-sectional in design and assessed correlation, not causality. Furthermore, the inflammatory mediators assessed were selected on the basis of a review of the literature (Alving and Malinovschi, 2010; Corren *et al.*, 2017; Castro *et al.*, 2018; Regeneron/Sanofi, 2019; Kolmert *et al.*, 2020) and the analysis was thus biased towards type-2 inflammatory mechanisms. An unbiased proteome-wide analysis may have yielded different results (Li *et al.*, 2018).

The study pooled two different cohorts which employed different methods to asthma optimisation and adherence checking. However, the sensitivity analysis conducted on both subsets independently was concordant with the pooled results.

2.3. Modelling the risk of asthma attacks using biomarkers

The prototype risk scale is indeed a prototype and several assumptions were made in its development, validation and modifiable risk assessment. These assumptions have been disclosed transparently and are herein summarised. First, the pooling of biomarker-stratified rate ratios across asthma severities assumes that FeNO and blood eosinophil predict asthma attacks independently of disease severity, but there were inconsistencies in this principle for mild asthma (Pavord *et al.*, 2020) – the latter data possibly reflecting low sample numbers. Whether mild disease is associated with different mechanisms of asthma

attacks or a lesser prognostic importance of FeNO are other possibilities. Second, the categorisation of clinical profiles as 'high-risk' on the basis of having ≥2 clinical risk factors (which independently multiplied the asthma attack rate by 1.3) and ≥1 attack in the previous 12 months (which independently multiplied the rate by 2.8) was somewhat arbitrary but supported by the derivation datasets. Third, the scale tabulates the risk of asthma attacks according to categories of blood eosinophils, FeNO and clinical circumstances. This was because the biomarker-stratified analyses on which the prototype is based reported data categorically. This also allowed for a format which emulates the very successful cardiovascular risk scales (Conroy *et al.*, 2003). There may be more effective ways of displaying the continuous risk associated with these variables. Fourth and last, although the biomarker-stratified rate ratios were adjusted for each other, the scale's other variables were not.

Despite these assumptions, the prototype scale proved the feasibility for a biomarker-centred approach to identify and reduce the risk of asthma attacks in a useful manner. Of course, the prototype is not validated for clinical practice; it is important that a refined version of ORACLE be derived and validated using a systematic review and meta-analysis of individual participant data (Couillard and Pavord, 2021b). This is a tall order, but increasingly possible thanks to the interest and credibility generated by the prototype.

CONCLUSION

To conclude, FeNO non-suppression carries significant translational, prognostic and theragnostic utility as a biomarker of type-2 airway inflammation – especially when used in combination with the peripheral blood eosinophil count. Indeed, the enclosed studies show that FeNO and blood eosinophils relate to different immune compartments and components of type-2 inflammation. The complementary, independent, and additive values of FeNO and blood eosinophils support a two-compartment, two-hit model. Under this model, both biomarkers should be part of routine asthma assessments to reduce the risk of asthma attacks in much the same way as doctors measure blood pressure and cholesterol to assess and reduce the risk of heart attacks. This concept has been explored in the prototype asthma attack risk scale ORACLE, which shows feasibility and potential for a treatable trait-based approach reflecting the underlying pathobiological mechanisms.

The current work explored biomarker measurements performed at the stable state. As discussed, it is likely that this state represents an equilibrium where airway type-2 activity and the chemotactic pull to the airways (identified by FeNO) are balanced with the systemic pool of available effector cells and circulating IL-5 (identified by blood eosinophils). How these factors interact at the time of an asthma attack with regards to cellular trafficking, eosinophil priming/oxidative activity, mucus production, and bronchial hyperreactivity need to be investigated. Finally, more work is underway to derive and validate a definitive ORACLE scale suitable for use across the range of clinical practice and capable of delivering an improvement in key outcomes.

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APPENDIX 1:
Description of the trials used to derive the prototype risk scale

Trial name [registration number](Ref) **Study design**	**Arm included (*n* / total *N*)**	**GINA Step: No (%)**	**ACQ mean score**	**FEV1 % predicted**	**PostBD change in FEV1 (%)**	**Blood Eos (x10^9/L)**	**FeNO (ppb)**
Novel START [ACTRN12615000999538] (Beasley *et al.*, 2019) 52-week, randomised, open-label, parallel-group, controlled trial	Salbutamol as needed (223/668)	Step 1: 219 (100)	1.1 (0.7)	89 (14)	nd	0.3 (0.2)	40 (5-235)*
CAPTAIN [NCT02924688] (Lee *et al.*, 2021) 52-week, phase IIIA, randomised, double-blind, active-controlled, parallel-group double versus triple inhaler trial	Fluticasone furoate/ vilanterol 100/25µg, with or without umeclidinium (1218/2439)	Step 4: 1097 (100)	2.5 (0.6)	58 (13)	30 (18)	0.23 (0.91)†	20.0 (0.7)†
Benralizumab 2b trial [NCT01238861] (Castro *et al.*, 2014) 52-week, randomized, controlled, double-blind, dose-ranging, Phase IIb clinical trial	Placebo +Maintenance therapy with moderate to high dose ICS and LABA (222/606)	Step 4: 122 (55) Step 5 100 (45)	Eos High: 2.7 (1.0) Eos Low: 2.5 (0.8)	Eos High: 65 (15) Eos Low: 69 (15)	Eos High: 18 (15) Eos Low: 13 (13)	Eos High: 0.53 (30) Eos Low: 0.16 (0.09)	Eos High: 37.9 (31.9) Eos Low: 20.7 (13.9)
PATHWAY [NCT02054130] (Corren *et al.*, 2017) 52-week, randomized, double-blind, placebo-controlled, Phase II clinical trial	Placebo +Maintenance therapy with a moderate to high dose ICS and LABA (138/550)	Step 4: 73 (53) Step 5: 65 (47)	2.7 (0.7)	60 (14)	nd	0.38 (0.33)	37.8 (39.7)
STRATOS 1 [NCT02161757] (Panettieri *et al.*, 2018) 52-week, randomized, double-blind, parallel-group, placebo-controlled, tralokinumab phase III clinical trial	Placebo +Maintenance therapy with moderate to high dose ICS and LABA (400/798)	Step 3: 3 (1) Step 4: 194 (49) Step 5: 203 (51)	2.6 (0·9)	62 (13)	23 (24)	0.25 (0.20)	29·6 (28·2)

…Trial name	Arm included	GINA	ACQ	FEV1	PostBD Δ	Blood Eos	FeNO
STRATOS 2 [NCT02194699] (Panettieri *et al.*, 2018) 52-week, randomized, double-blind, parallel group, placebo-controlled, tralokinumab phase III clinical trial	Placebo +Maintenance therapy with moderate to high dose ICS and LABA (422/837)	Step 3: 14 (3) Step 4: 196 (47) Step 5: 207 (50)	2.6 (0.9)	61 (15)	26 (25)	0.27 (0.23)	31.7 (27.2)
LIBERTY ASTHMA QUEST [NCT02414854] (Castro *et al.*, 2018) 52-week randomised, double-blind, placebo-controlled, parallel-group trial	Placebo (1.14 mL and 2 mL) +Maintenance therapy with moderate to high dose ICS and ≤ 2 additional controllers (634/1902)	Step 4: 293 (49) Step 5: 327 (51)	2.7 (0.7) and 2.8 (0.8)	58 (13) and 58 (14)	25 (19) and 26 (18)	0.37 (0.34) and 0.39 (0.42)	34.5 (28.5) and 38.4 (38.0)
DREAM [NCT01000506] (Pavord *et al.*, 2012) 52-week, multicentre, randomised, double-blind, placebo-controlled mepolizumab trial	Placebo +Maintenance therapy with high-dose ICS and LABA (155/616)	Step 5: 151 (100)	2.5 (1.1)	59 (15)	21 (nd)	0.28 (1.01)††	33.7 (0.8)††

Data are mean (SD) unless otherwise indicated; *median (range); † geometric mean (SD of log); †† geometric mean on loge scale (SD). ACQ, asthma control questionnaire; Blood Eos, peripheral blood eosinophil count ($\times 10^9$ cells/L), FeNO, fractional exhaled nitric oxide (ppb), FEV1, forced expiratory volume in 1 second; ICS = inhaled corticosteroid; LABA = long-acting beta2-agonist; *n* = number of patients in the control arm; *N* = overall number of patients enrolled in trial; nd = not disclosed; postBD, postbronchodilator