Novel biomarkers of inhaled corticosteroids adherence and response

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy (PhD) in the Faculty of Biology, Medicine and Health

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Contents

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List of Tables

List of Figures

List of abbreviations

ACQ	Asthma Control Questionnaire
AHR	Airway hyper-responsiveness
AQLQ	Asthma Quality of Life Questionnaire
ATS	American Thoracic Society
AUC	Area under the curve
AUROC	Area Under the Receiver Operating Characteristic curve
BALF	Bronco-alveolar lavage
BDP	Beclomethasone dipropionate
BMI	Body mass index
BMP	Beclomethasone monopropionate
BTS	British Thoracic Society
BUD	Budesonide
C_{base}	Baseline concentration
CFC	Chlorofluorocarbon
CI	Confidence interval
CIC	Ciclesonide
C_{max}	Maximum concentration
COPD	Chronic obstructive pulmonary disease
CV	Coefficient of variation
des-CIC	desisobutyryl-ciclesonide

DPI	Dry powder inhalers
EBT	Exhaled breath temperature
ELISA	Enzyme-Linked Immunosorbent Assay
FF	Fluticasone furorate
eNOS	endothelial NOS
eNose	Electronic nose
EOS	Blood eosinophils
ERS	European Respiratory Society
FDA	Food and Drug Administration
FDR	False discovery rate
FeNO	Fractional exhaled nitric oxide
FEV_1	Forced expiratory volume in one second
FF	Fluticasone furoate
FP	Fluticasone propionate
FVC	Forced vital capacity
GC-MS	Gas chromatography-mass spectrometry
GC-MS/MS	Gas chromatography-tandem mass spectrometry
GI	Gastrointestinal
GINA	The Global Initiative for Asthma
GP	General practitioner
HADS	Hospital Anxiety and Depression Scale
HFA	Hydrofluoroalkane
iAUC	incremental area under the curve

ICS	Inhaled corticosteroid(s)
IgE	Immunoglobulin E
INCA	Inhaler Compliance Assessment
iNOS	inducible NOS
IQR	Interquartile range
IV	Intravenous
LABA	Long-acting beta-agonist
LC-HMRC	Liquid chromatography coupled to high-resolution mass
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LoD	Limit of detection
LoQ	Limit of quantification
LTRA	Leukotriene receptor antagonist
MARS	Medication Adherence Report Scale
MDI	Metered-dose inhalers
MRM	Multiple reaction monitoring
ng/L	Nanogram per litre
nNOS	neuronal nitric oxide synthases
NO	Nitric Oxide
NOS	Nitric oxide synthases
OCS	Oral corticosteroids
***p*-MDI**	Pressured metered-dose inhalers
$PC2_0$	The provocative concentration of methacholine

PD	Pharmacodynamics
PEF	Peak expiratory flow
PEx	Particles in exhaled air
PExA	Particles in exhaled air method
PK	Pharmacokinetics
Qaw	Bronchial blood flow
QCs	Quality controls
RCT	Randomized clinical trial
ReCIVA	Respiration Collector for In Vitro Analysis
ROC	Receiver-operator characteristics
RTLF	Respiratory tract lining fluid
RV	Rhinovirus
SABA	Short-acting $beta_2$ agonists
SD	Standard deviation
SIGN	Scottish Intercollegiate Guidelines Network
SPA	Surfactant protein A
SPB	Surfactant protein B
SPC	Surfactant protein C
SPD	Surfactant protein D
SPSS	Statistical Package for the Social Sciences
TD	Thermal desorption
T_{max}	Time to maximum concentration

U-BIOPRED	The Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes
VOC	volatile organic compounds
WHO	The World Health Organization

Abstract

Backgrounds: Although steroid medications have proven effectiveness in controlling asthma and reducing the risk of exacerbation, poor adherence is common. Several methods have been investigated in assessing adherence; however, there is no validated gold standard. In this thesis, novel methods were tested to monitor adherence in serum and urine, and further candidate exhaled biomarkers were investigated.

Aims: The main aim was to objectively determine whether the level of detection of inhaled or oral corticosteroids in serum or urine could be used as a potential marker of medication use. We also investigated the response of inhaled steroid by using exhaled nitric oxide (FeNO), volatile organic compounds (VOC), exhaled breath temperature (EBT) and particles in exhaled air (PExA).

Methods: Firstly, we conducted a systematic review of the literature reporting biological methods; we included studies reporting direct measurement of exogenous corticosteroids in blood or the effect of adherence on exhaled nitric oxide. Next, we accessed data from the U-BIOPRED project and included severe asthma patients prescribed daily oral corticosteroids who completed the MARS adherence questionnaire and provided a urine sample for analysis of prednisolone and metabolites by liquid-chromatography mass spectrometry. Then, we assessed the feasibility of using liquid chromatography tandem mass spectrometry (LC-MS/MS) in detecting the most common ICS inhalers in serum blood over 8 hours post-dosing. Based on preliminary findings of these studies, we conducted a real-world study in severe asthma patients attending FeNO suppression clinic and compared their ICS levels in blood

with adherence rate using an electronic monitoring device over one week. Lastly, the same FeNO suppression patients were tested for an early response of ICS (within 2 hours of ICS administration) and one-week response using exploratory exhaled markers.

Results: We report poor adherence in around 40% of patients using oral steroids by using urinary prednisolone and MARS questionnaire. However, disagreement in adherence identification was identified between the methods in around 50% of cases.

After 8hrs of post-inhalation, all patients using budesonide (n=10) and beclomethasone dipropionate (n=15), and all but one using fluticasone propionate (FP, 28) had detectable serum drug levels. While fluticasone furoate was detected in two patients (of four), ciclesonide in none (of seven). Blood ICS levels correlated negatively with exacerbation rate, and (for FP only) positively with FEV_1 %predicted.

There was a significant increase in ICS concentration after one week of FP inhaler use. The ICS serum concentration at the second visit correlated with the number of inhalations taken over the week, and the time since the last dose taken, but not to the level of FeNO suppression.

As expected significant reduction in FeNO, was found following seven days of ICS treatment. This study also demonstrated for the first time, a rapid impact of ICS on EBT. For VOCs, we have shown that there was a clear variability in the pattern of some compounds following ICS use. In contrast, we do not observe any change any difference in all of the PExA parameters.

Conclusion: Poor adherence is a common problem in severe asthma, whether measured directly or self-reported and associated with poor asthma outcomes. The adherence method we developed in this thesis is potentially suitable to be implemented in clinical asthma

services. Furthermore, exhaled markers that are affected after ICS showed promising results and should be explored in other large clinical trials.

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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This thesis would not have been possible to stand without the valuable assistance of several individuals. First and foremost, I would like to thank the patients who put in their time and efforts to advance clinical research, your contribution is much appreciated.

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PEx proteins samples. I am extremely grateful to Dr Marcia Soares for her help and guidance in PExA sampling.

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Dedication

First and foremost, I would like to thank almighty Allah for answering my prayers and for giving me the strength and guidance.

I would like to dedicate this thesis to my beloved parents Homoud and Aisha Alahmadi, you made this possible, by teaching me the values necessary to overcome obstacles in my life and for your emotional support and your words of encouragements.

I further dedicate this thesis to my wife, Alaa, who has shown me a tremendous amount of support during my studies. Alaa, you have always been beside me in good or bad, and you have guided me to the right path many times by making me look at the bright side, and for that, I am eternally grateful to you. To my children, Nada and Malik, coming home to you two every day and seeing how you filled it with laughter and joy have been my source of inspiration. I love you all.

I would like to express also special gratitude to my sisters, brothers and friends for believing in me, standing by me and for their continuous support and words of advice during these past years.

About the author

I graduated with BSc in Respiratory Therapy from Prince Sultan medical college of health sciences, (Dahran, Saudi Arabia) in 2011. Following that, I was recruited by Taibah University to work as a clinical instructor. After a one year of working, in 2013, I moved to the USA and started pursuing my master's degree at Georgia State University in health sciences with a concentration in respiratory therapy (Atlanta, Georgia). My master thesis was focused on senior respiratory therapy students' and clinical instructors' perceptions of effective teaching characteristics in clinical education. In April 2016, I joined with the University of Manchester to study four-years PhD degree working with severe asthma team that extended for four years.

Statement of work

All of the studies explained in this thesis were done under the supervision of Dr Stephen Fowler, Dr Robert Niven and Professor Brian Keevil.

Chapter 2: Assessment of adherence to corticosteroids in asthma by drug monitoring or fractional exhaled nitric oxide: a systematic review.

I performed all of the systemic literature searches, extracted all relevant studies and scored those papers quality by a specific recommended tool. Quality assessment scoring only was checked and repeated by Adam Peel and Dr Stephen Fowler.

Chapter 3: Medication adherence in patients with severe asthma prescribed oral corticosteroids in the U-BIOPRED cohort.

In this project, the study design was conceived by Dr Stephen Fowler, Fahad Alahmadi. I did the data extraction, statistical analysis and the initial draft of the paper.

Chapter 4: Serum inhaled corticosteroid detection for monitoring adherence in severe asthma.

In this project, the protocol design was conceived by Dr Stephen Fowler, Dr Rob Niven, Professor Brian Keevil and Fahad Alahmadi. Patient recruitment and spirometry test, FeNO and peripheral blood cannulation were all done by Fahad Alahmadi. I performed all the statistical data analysis with valuable input from Dr Stephen Fowler and Dr John Belcher. Blood assays and samples analysis were carried out by Professor Brian Keevil.

Chapter 5: Serum inhaled corticosteroids detection and electronic monitoring of adherence

In this project, the protocol design was conceived by Dr Stephen Fowler, Dr Rob Niven, Professor Brian Keevil and Fahad Alahmadi. Patients' recruitment and spirometry test, FeNO and peripheral blood samples were all done by Fahad Alahmadi, Dr Katie Bayfield and Rebecca Logan. INCA inhalers were collected and checked by asthma nurse and Fahad Alahmadi. Blood samples analysis were carried out by Professor Brian Keevil. I performed all the statistical data analysis and initial manuscript draft with valuable input from Dr Stephen Fowler.

Chapter 6: The effect of inhaled corticosteroids on exhaled breath biomarkers in severe asthma

In this project, the protocol design was conceived by Dr Stephen Fowler, Dr Rob Niven, Professor Brian Keevil and Fahad Alahmadi. Patient recruitment and spirometry test, VOCs FeNO, PExA samples and peripheral blood samples were all done by Fahad Alahmadi, Dr Katie Bayfield and Rebecca Logan. Dr Max Wilkinson have done the VOCs samples analysis and extracted the data, and Fahad Alahmadi cleaned the data. The PExA samples were shipped to the Institute of Medicine at Sahlgrenska Academy, at the University of Gothenburg, Sweden and analysed by Hatice Katia. I performed all the statistical data analysis and initial manuscript draft with valuable input from Dr Stephen Fowler.

List of abstracts

- **Alahmadi F,** Simpson A, Gomez C, Wheelock C, Shaw D, Fleming L, et al. Measures of adherence in patients with severe asthma prescribed systemic steroids in the U-BIOPRED cohort. European Respiratory Journal. 2018;52(suppl 62):PA3992.
- **Alahmadi F,** Niven R, Elsey L, Keevil B, George K, Fowler S. P15 Detection of inhaled corticosteroids in the serum–relationship to adherence and markers of asthma severity. BMJ Publishing Group Ltd; 2019.
- Wang R, **Alahmadi F**, Niven R, Fowler S. Within-day repeatability of fractional exhaled nitric oxide in severe asthma. (submitted, ERS 2020)

Publications

- **Alahmadi F,** Peel A, Keevil B, Niven R, Fowler S. Assessment of adherence to corticosteroids in asthma by drug monitoring or fractional exhaled nitric oxide: a systematic review. (Manuscript under review, *Clinical & Experimental Allergy*, March 2020)

- **Alahmadi F,** R. Niven, L. Elsey, B. Keevil, K. George, and S. Fowler. Serum Inhaled Corticosteroid Detection for Monitoring Adherence in Severe Asthma. (Manuscript under review, *Journal of Allergy and Clinical Immunology*, March 2020)
- **Alahmadi F,** Simpson A, Gomez C, Wheelock C, Shaw D, Fleming L, Roberts G et al. Medication adherence in patients with severe asthma prescribed oral corticosteroids in the U-BIOPRED cohort. (Manuscript under review *Journal of Allergy and Clinical Immunology: In Practice,* November 2019).

1 Introduction

1.1 ASTHMA

Asthma is one of the most common chronic inflammatory diseases. In western countries around 300 million individuals have asthma, and children are affected more than adults. Its prevalence is continuing to increase and is estimated to reach 400 million by 2025 (1). The United Kingdom has the highest number of asthma patients in the world, with almost 18% of the population affected (2, 3). The Global Initiative for Asthma (GINA) strategy report has defined asthma as "a heterogeneous disease usually characterised by chronic airway inflammation. It is defined by a history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and intensity, together with variable expiratory airflow limitation" (4). However, according to the British Thoracic Society (BTS), applying such definitions in the clinical setting is controversial due to the evidence of high variability of asthma symptoms, and thus there is no clear recommendation in asthma diagnosis (5).

1.1.1. Aetiology and risk factors

The exact causes of asthma are complicated and poorly understood. A wide range of risk factors is reported to be related to causing asthma; these are categorised into host and environmental risk factors. Host factors associated with asthma include genetic predisposition, atopy, airway hyper-responsiveness, gender, race and obesity. Environmental factors include viral infection, exposure to air pollution, a multifactorial parental factor which occurred during maternity or the childhood period, and tobacco smoking. Moreover, factors related to the place of work (occupational asthma) are considered a common cause in adult asthmatics (6). Asthma can be exacerbated due to viral infections, which are present in 80%

of children with asthma and 75% of adult asthmatics (7). Rhinovirus (RV) in particular accounts for most of the infective exacerbation (8).

1.1.2. Asthmatic lung:

Asthma is an inflammatory condition associated with structural changes within the airway. As illustrated in Figure 1.1, a comparison between healthy and asthmatic airways, the lumen area changes in asthmatic individuals, becoming narrowed and blocked, limiting the airflow in and out of the lung. Due to obstruction of airways and increased levels of mucus production, many changes to the airway will occur, including mucous cell hyperplasia (9), epithelial layer thickening (10), smooth muscle hyperplasia and hypertrophy (11), submucosal gland hyperplasia and excessive mucus production and deposition to the airway (12). Parallel to the airway remodelling is the inflammatory response, and this joint interaction will lead to oedema, bronchial wall thickening, and bronchoconstriction will occur by smooth muscle contraction. All of these interactions are responsible for the airflow obstruction. Various factors can trigger the bronchoconstriction response, such as exposure to irritants or exercise. It is suggested to measure airway hyper-responsiveness (AHR), e.g. histamine or methacholine challenge tests, in order to measure the response of bronchoconstriction.

The cycle of airway inflammation plays a significant role in asthma pathophysiology as a result of the interaction of different types of inflammatory cells and their mediators. Key inflammatory cells in asthma include mast cells and eosinophils. Inflammatory mediators which are produced by those inflammatory cells include chemokines, cytokines, cysteinyl-leukotrienes and nitric oxide and are the effectors of long-term inflammation (13). In allergic

asthma, Immunoglobulin E (IgE) antibody is responsible for interacting with several leukocytes.

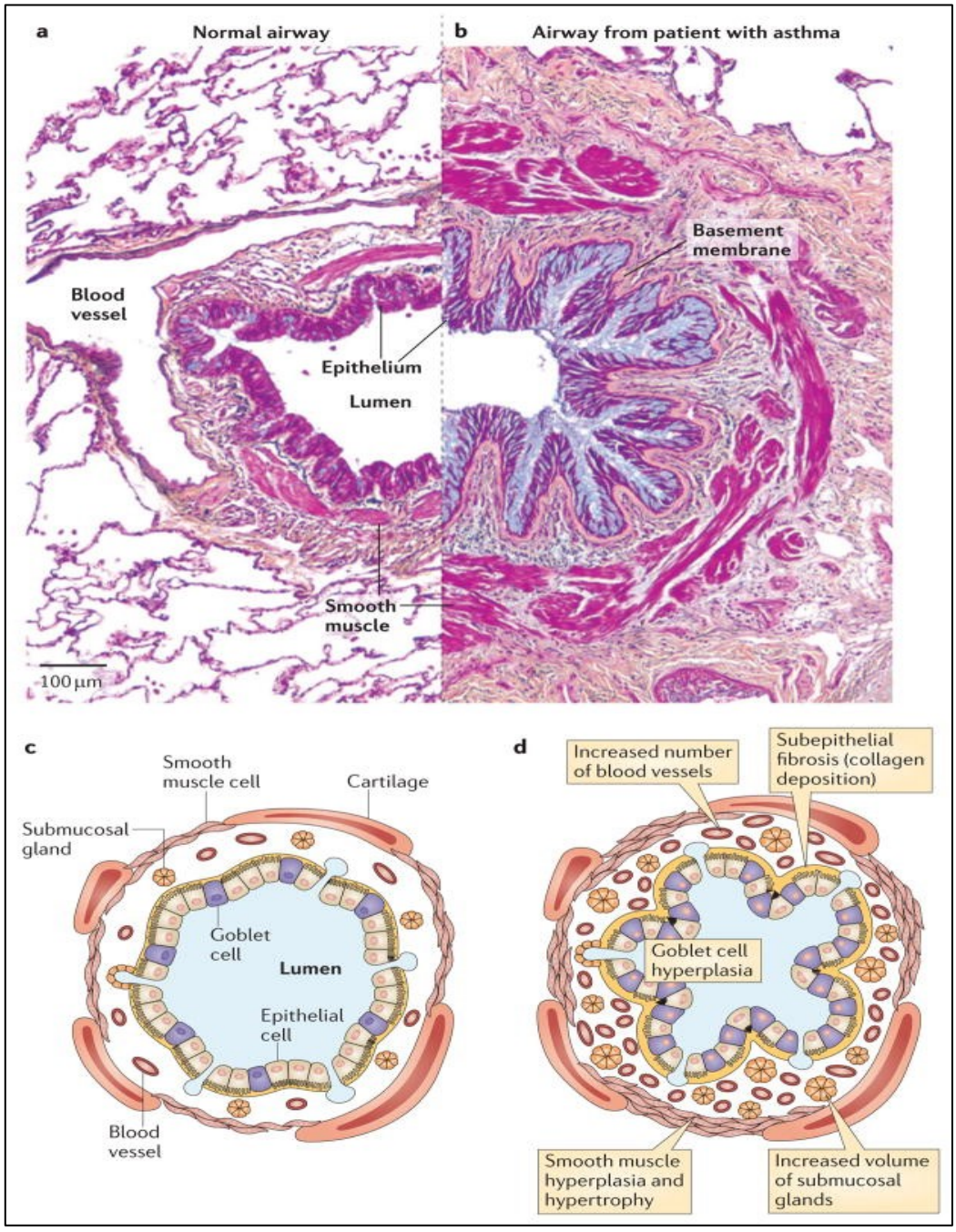


Figure 1. 1. Airway structures in a healthy lung and an asthmatic lung (part b; a schematic representation is depicted in part d).

Reproduced from Wadsworth et al. 2011, (14) with permission of the rights holder, Dovepress.

1.1.3. Diagnosis

Asthma diagnosis can be challenging to health care practitioners as most of the signs and symptoms are non-specific, and potentially overlap with other primary pulmonary or cardiac conditions. Moreover, the variable and episodic nature of asthma symptoms indicate that patients should be evaluated during the presence of symptoms (symptomatic); otherwise, it possibly leads to a false negative test. In addition, the clinical manifestation alone cannot confirm an asthma diagnosis; thus, objective tests are needed to avoid mis-diagnosis. In this regard, asthma has remained a clinical diagnosis that is investigated by most primary care providers. These diagnostic processes were implemented into different national or international asthma guidelines such as the BTS and the Scottish Intercollegiate Guidelines Network (SIGN) British Guidelines on the Management of Asthma. Currently, most healthcare providers follow national and international asthma guidelines as a rule of thumb.

There is no established "gold standard" test that can confirm the asthma diagnosis. However, some approaches and assessments are used to determine the probability of asthma. First, a full detailed medical history that includes variability of symptoms during the day and at night, atopy, family or personal history of sensitivity to a specific allergen, sound of wheezing, sputum eosinophils. In addition to the medical history, there are objective measures of changes in lung function after bronchial provocation challenge or asthma treatment that will help to diagnose asthma; these include:

a. Variation of at least 20% in peak expiratory flow (PEF) over at least two weeks.

b. An increase in Forced Expiratory Volume in 1 second (FEV1) of >12% and 200ml, 15-30 minutes after the inhalation of 200-400mcg salbutamol.

c. An increase of 15% or more in FEV1 after administration of inhaled corticosteroid 400 mcg beclomethasone for six to eight weeks.

d. Methacholine challenge test, where PC_{20} which is less than or equal to 1 mg/ml indicates severe bronchial hyper-responsiveness

However, the above tests have some disadvantages. They depend on patient performance and effort, and may show an improvement in values after many attempts due to improved technique, not because of the lung's response. Some studies which measured tidal breathing found that measurements of FEV_1 may improve following a deep breath (15, 16). Furthermore, some asthma patients have normal lung volumes before administering any medications, and it is challenging to show a reaction. Due to all these drawbacks, a pre-bronchodilator peak flow test as a diagnostic tool in asthma disease was found to have low sensitivities (53%) and negative predictive value (38%), while for post-bronchodilator the values were 6% and 16% respectively (17). In this context, using these tests to exclude asthma is challenging; however, in the case of a positive test, it can be used in symptomatic patients to diagnose asthma. These tests are relatively inexpensive and can be easily implemented in a primary care setting.

Further, the examination of the upper part of the airways can help to identify allergic rhinitis, which will appear like mucosal swelling or nasal polyps. The presence of atopic eczema will support the diagnosis of disease (18, 19). Several signs and symptoms that may suggest an alternative diagnosis to asthma include localised wheeze, crackles, stridor, clubbing, or heart murmurs (20). Thus, clinicians need to consider the differential diagnosis before starting the management plan for asthma.

1.1.4. Asthma control and severity

Previous versions of several international asthma guidelines were based on the asthma level of severity, which was categorised as intermittent, mild, moderate and severe (21). However, some recent studies have revealed that asthma can be controlled regardless of the level of the severity. Further, the majority of asthmatics are well-controlled. Therefore, the latest versions of asthma guidelines are currently based on the level of asthma control instead of asthma severity, although some clinicians do not differentiate between severity and control of asthma or use them conversely. For this reason, we will discuss the difference between asthma severity and control.

Asthma control is defined as the ability to prevent asthma symptoms by treatment, and reducing the usage of reliever treatment to no more than twice per 14 days (22). The primary aim of controlling asthma is to achieve a good quality of life during both day and night by controlling the symptoms, lung function decline, and also preventing exacerbations. On the other hand, asthma severity refers to either the degree of asthma symptoms or the effect of asthma disease on overall patient condition and health status (23). The concept of asthma severity is based on the intensity of treatment needed for achieving reasonable asthma control (5). The degree of asthma severity may be affected by unrelated disease and patients' physical behaviour, and these indicators are alternative measurements for future risk.

Uncontrolled asthma is characterised by the presence of frequent symptoms or exacerbations and reduced lung function in asthma patients regardless of the dose of medications (24). Many uncontrolled asthmatics are likely to have mild asthma and can be controlled with a minimum daily dose of ICS (4). On the other hand, 'difficult to treat' asthma patients are

characterised by uncontrolled asthma despite the usage of a high dose of ICS, with or without oral steroids. Several potential contributory factors associated with difficult asthma patients include poor inhaler technique, non-adherence, false diagnosis or number of comorbidities (GINA).

1.1.5. Severe asthma

The American Thoracic Society (ATS) and the European Respiratory Society (ERS) defined severe asthma as "asthma which requires treatment with high dose inhaled corticosteroids (ICS) plus a second controller (and/or systemic corticosteroids) to prevent it from becoming 'uncontrolled', or which remains 'uncontrolled' despite this therapy." (25). The majority of patients with asthma can achieve reasonable symptom control using the appropriate inhaled medication. The 5 - 10% of asthmatics at the severe end of the spectrum are still symptomatic despite high dose inhaled corticosteroids and require frequent courses, or maintenance regimes, of oral steroids (26). The cost of treating severe asthma in the United Kingdom is estimated to be up to 50% of the total healthcare budget for asthma (27).

1.1.6. Management

There is no available cure or prevention treatment for asthma, as with most of the chronic pulmonary diseases. Therefore, the goal of all current asthma treatment is to maintain and control the symptoms of asthma and to avoid any exacerbation in the future, or reduction in lung volume. Most cases of asthma can be controlled by using rescue medications including bronchodilators, at the acute onset of asthma, or regular preventer drugs such as ICS, leukotriene receptor antagonist (28) for controlling the long-term phase or their combination. However, with the increase in asthma severity, controlling asthma becomes complicated, and additional phenotype-specific treatment is indicated.

Several evidence-based guidelines have been developed to guide health care practitioners in treating asthma, and these guidelines categorised management as non-pharmacological and pharmacological interventions. According to BTS guidelines in treating asthma, it is essential to assess or prevent each patient's triggers, such as environmental or dietary factors. This can minimise the need for pharmacological asthma therapies (4). Moreover, it is recommended to discuss the patients' concern about environmental burdens, to avoid failure of compliance with the prescribed asthma treatment. However, most of the non-pharmacological studies are observational, with weak evidence of the effectiveness of this approach; further interventional clinical trials are needed.

The Global Strategy for Asthma Management and Prevention (GINA) designed an evidence-based step by step approach in asthma management (Figure 1.2). GINA recommended following cyclical management to control asthma, which includes continuous patient assessment, pharmacological or non-pharmacological treatment adjustment, and a review of the response. If the disease is not well-controlled, treatment can be escalated to the next

step; however, inhaler technique and adherence must be checked before stepping up. If asthma is well-controlled for three months with the current medication, then this medication can be “stepped-down” to the previous step. Pharmacological interventions in asthma treatment are categorised into relievers, controllers and add-on therapies, which will be explained in the figure below (1.2).

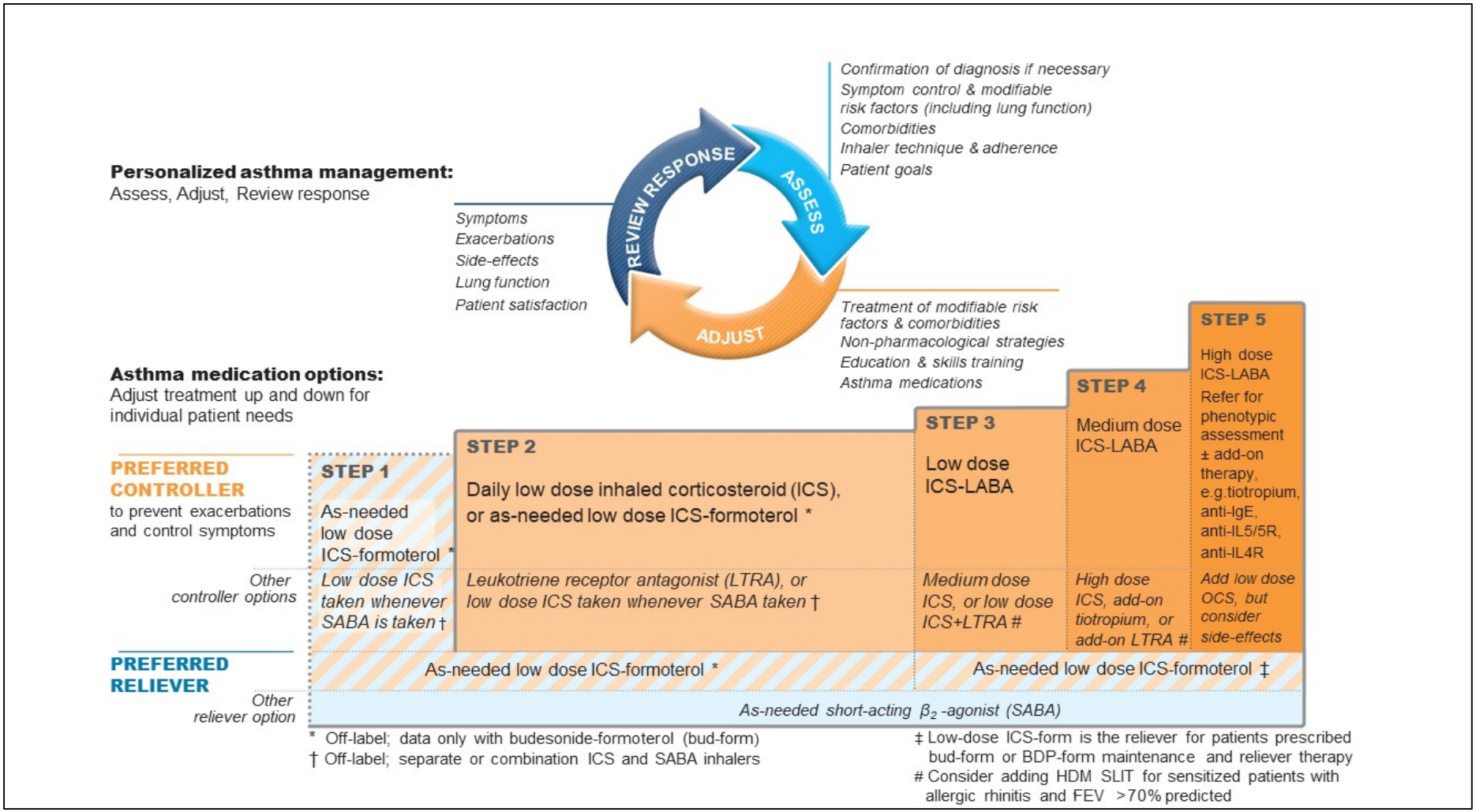


Figure 1. 2. Stepwise management for adult asthma. ICS: Inhaled corticosteroids; SABA, short-acting beta$_2$ agonists; LABA, long-acting beta-agonist; OCS, oral corticosteroids; anti-IgE, anti-immunoglobin E therapy; LTRA, leukotriene receptor antagonist; anti-IL, anti-leukotriene. Adapted from GINA 2019, (4).

1.1.7. Corticosteroids

After the exploration of adrenal steroid hormones, arthritis was the first disease that was treated with cortisone, in 1949 (29). The effect of cortisone on treating arthritis was noticeable, and that discovery won the Nobel Prize in the following year. Since that time, researchers have carried out many experiments to test the feasibility of corticosteroids in different inflammatory conditions. Cortisone treatment of asthma was reported for the first time in 1950 (30). Six years later, a controlled clinical trial showed a high efficacy of corticosteroid in treating asthma exacerbations (31). After that, prednisolone and hydrocortisone medications were developed and introduced into clinical practice as systemic corticosteroid therapy. However, despite their efficacy, it has been found that if oral corticosteroids treatments are used for a long period, several severe side effects occur such as hypertension, osteoporosis, diabetes and obesity. Therefore, this led Clark et al. to develop he inhaled corticosteroids (ICS), particularly in early 1972 the beclomethasone inhaler, which has a safer administration with minimal adverse effects compared to systemic steroids (32). Within a few years of ICS development, a considerable number of controlled trials had shown good clinical outcomes of ICS medications in treating asthma (33-35). The ICS can be delivered through different systems, and each device has its advantages and limitations. The most frequently used devices are pressurised metered-dose inhaler (36) and dry powder inhalers (DPI), and less commonly through a nebuliser (liquid capsules).

In the last three decades, many ICS compounds have been approved to be used for asthma treatment in different countries. The ICS compounds most commonly used in the United Kingdom (UK) include beclomethasone dipropionate (BDP), budesonide (BUD), ciclesonide

(CIC), fluticasone furoate (FF), and fluticasone propionate (FP). These compounds vary considerably as to their efficacy and safety properties. They can produce an effect on the respiratory system that provides long-term control by reducing or suppressing airway inflammation. The medication's effects on the lungs varies depending on the pharmacokinetics (PK) and pharmacodynamics (PD) profile of each inhaler. Pharmacokinetics is defined as the movement of the drug to the fluid in the body (blood or plasma). Pharmacodynamics is simply defined as "what the drug does to the body". Both PK/PD features should be studied to explore or evaluate the outcome of the drug over time. The ideal PK/PD of ICS characteristics is to predict a high pulmonary efficacy, with minimal oropharyngeal and systemic side effects. The higher efficacy of ICS into lungs can be achieved by maximising the percentage of the of the inhaled particles that reaches the lungs and stays for a maximum time (37). The side effects of oropharyngeal and systemic exposure can be minimised by reducing the size of the inhaled particles: the larger their size, the more their deposition in the oropharynx area, and by decreasing the proportion of oral bioavailability and increasing the protein binding (37).

Pharmacokinetic and pharmacodynamics of inhaled corticosteroids

Before explaining the PK and PD characteristics of each available ICS inhaler, it is useful to understand the fate of ICS in the body, and this is briefly illustrated schematically in Figure 1.3 (37). After ICS inhalation, a small portion of the dose is deposited in the lungs, producing an effect on the inflamed tissue. This portion is estimated to be between 10 to 20% of the dose in the old ICS devices, whereas the new generation of ICSs can deliver between 40% to 60% of the nominal dose (38). Also, very tiny particles (< 1µcg) are exhaled without interacting with the body. After a while, side effects may occur due to the drugs being absorbed into the

systemic circulation (pulmonary bioavailability) via the pulmonary blood. The other inhaled particles of the drug that do not enter the lungs, estimated to be between 40% and 90%, are deposited in the oropharyngeal area can enter the systemic circulation if not completely rinsed and cleared. The swallowed particles are subsequently absorbed by the gastrointestinal (GI) tract and enter into the systemic circulation (oral bioavailability). In the liver, the first-pass metabolism absorbs the swallowed ICS portion and escaped portion and inactivates most of the particles by the first-pass metabolism (39). The combination of the pulmonary-based particles, and particles from GI absorption that are distributed into the systemic circulation, may cause several side effects. In addition, the ICS concentration in the blood depends on the level of pulmonary and oral bioavailability absorption.

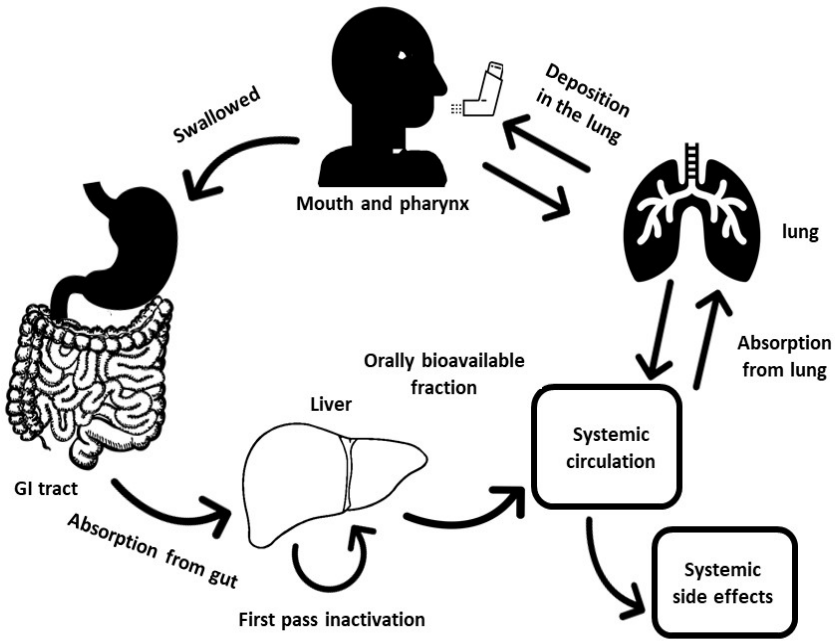


Figure 1. 3. Diagram represents the path of inhaled corticosteroid through different body systems.
The figure is adapted from Derendrof et al. 2006, (40).

Mechanism of action

All of the ICS agents have the same mechanism of action to produce the anti-inflammatory effect. Corticosteroids enter the phospholipids' pulmonary membrane to link with glucocorticosteroid receptors located in most of the body cells. After the subsequent binding with glucocorticosteroid receptors, the receptor will be activated and translocated to enter the nucleus layer and bind with a specific DNA. Hence, this will activate or suppress gene sequence transception or protein synthesis (41). Inhaled corticosteroids inhibit the production of several mediators such as eosinophils, lymphocytes, macrophages and cell adhesion molecules (42) that minimise the level of airway inflammation and improve asthma symptoms (43). In contrast the nongenomic effects, which is not influenced by the gene expression and characterized by rapid and short effect (usually between 60-90min) (44). The nongenomic effect are initiated by nonspecific interactions with corticosteroids receptors with the cellular membrane or by cytoplasmic glucocorticosteroid receptors (45). Another mode of action of nongenomic effect is the inhibition of the extraneuronal uptake of norepinephrine, which is may increase the time period of the vasoconstriction and that may reflect on reduction of the blood flow (46, 47).

The PK and PD characteristic factors include glucocorticoid receptor bindings, half-life clearance, bioavailability, protein binding, residence time and distribution volume, which contribute to the efficacy and safety of ICS (48, 49). For that reason, safety and efficacy parameters should be studied as a delicate balance in the development of ICS for asthma. For instance, a high dose of ICS with high deposition particles in the lung (pulmonary bioavailability) will increase the efficacy of the drug; however, serious potential systemic side effects could occur due to the large portion of ICS which enter the systemic circulation.

Moreover, the potential systemic side effects are positively correlated with high efficacy, with higher receptor-binding affinities and longer half-lives (49).

Inhaled corticosteroid medications can be administered as either active or inactive metabolites, with pulmonary esterase activity responsible for activation of pro-drugs. Three ICS inhalers: FP, BUD, and FF enter the lungs as an active compound, while BDP) and CIC get activated and metabolised in the body and enter the lung as a form of beclomethasone-17-monopropionate (BMP) and desisobutyryl-ciclesonide (des-CIC) respectively. The advantage of these parent prodrugs (BDP and CIC) is that they have less local side effects in the oropharynx deposition, as these compounds do not have a pharmacological response and also do not exert the same effect as the other active drugs. A comparison of the PD and PK of the five most common ICS been used in the UK market (FP, BUD, BDP, CIC, FF) are described below in Table 1.1.

Table 1. 1. Pharmacokinetic properties of beclomethasone dipropionate, fluticasone propionate, budesonide, ciclesonide and fluticasone furoate.

From (37, 38, 40).

Drug	**Beclometasone dipropionate (BDP) / 17-monopropionate (BMP) MDI**	**Fluticasone propionate (FP) DPI**	**Budesonide (BUD) DPI**	**Ciclesonide (CIC) / desisobutyryl-CIC (des-CIC) MDI**	**Fluticasone furoate (FF) DPI**
Relative affinity for the glucocorticoid receptor	53/1345	1800	935	12/1200	2989
Oral bioavailability (%)	15/41	<1	11	<1/<1	<1
Relative protein binding (%)	87/13	90	88	99/99	99
Systemic clearance (L/h)	15/120	90	84	152/228	65
Half-life IV (h)	0.5/2.7	7.8	2.8	0.36/3.4	15
Particle size (μm)	<2	6	2.5	<2	<2

Drug affinity (receptor-binding)

Drug efficacy is defined as the ability to exert or produce a pharmacological effect. The ICS drug effect is mediated on glucocorticosteroid receptor affinities, and this affinity varies by ICS. The binding affinity is compared with dexamethasone (binding affinity of dexamethasone ~100), signifying the affinity of ICS. The higher the binding affinity to glucocorticoid receptors, the lower the concentration required to produce the same effect. From Table 1.1 above, FF, FP, the active prodrug of both BDP and CIC agents have the highest binding affinity, estimated at 2989, 1800, 1345 and 1200 respectively. Budesonide has an affinity of 935, while the inactive compound of BDP and CIC has a very low receptor affinity. The higher affinity does not mean more desired therapeutic effects, as it may lead to potential systemic side effects as these are induced via the same receptor. Therefore, it is not necessarily the case that a high receptor-binding affinity is an advantage (50).

Pulmonary and oral bioavailability

Most of the inhaled particles absorbed in systemic circulation by the GI tract and the other particles are delivered subsequently to the systemic circulation via the lungs. Thus, the concentration of the ICS in the blood is a combination of the inhaled fraction that is absorbed into systemic circulation and the oral fraction (51). Inhaled medications with a high percentage of oral bioavailability mean limited therapeutic effects, with high potential of local side effects, so it is useful for ICS medications to have a lower oral bioavailability rate. The highest ICS oral bioavailability from Table 1.1 above showed the active metabolite of BDP having 41% and below 1% for des(CIC), FP and FF, but 11% for BUD (52).

The bioavailability has an impact on the clinical safety of the medication profile. As previously mentioned, to receive the required therapeutic effects on the lungs, it is recommended to have inhaled the drugs with a good pulmonary deposition. However, the implication of the increase in pulmonary deposition may lead to inducing unwanted side effects due to higher systemic exposure. Thus, a high rate of bioavailability (oral and pulmonary) will affect the safety of the ICS and increase systemic side effects.

Protein binding

The therapeutic effect on the lungs depends on free drug that is not bound with protein. Common proteins that drugs can bind to include serum albumin, lipoprotein, and glycoprotein. If the drug has a low protein-binding rate, there is a higher chance of free drug that can bind to corticosteroid receptors and vice versa. However, higher protein binding could be an advantage, as it may lead to minimal systemic side effects because it lowers the unbound fraction in the systemic cycle. Protein binding of BDP has been reported as 87%, BUD is 88% and FP is 90%. A high protein binding for CIC, des-CIC and FF was reported (see Table 1.1), with the highest protein binding occurring with ciclesonide (CIC) and mometasone. Padden et al. provide a good example of the extent to which the protein binding would affect the systemically bioavailable results at different ICS doses (37). Firstly, they assume that if a patient uses FP at a dose of 400 µcg, the protein binding is 90% and the unbound free drug is 10%. Around 296 µcg (74%) of the dose would be ingested through the GI tract and subsequently goes through first-pass metabolism (99% of 296 µcg) and the remaining 3 µcg will end up in the systemic circulation. On the other hand, the other 104 µcg are deposited in the lungs and then will be absorbed in the systemic cycle. In total after the protein bindings,

only 10% of 107 μcg, in other words, 10 μcg will be unbound and become systemically available.

Secondly, they compare it with a CIC inhaler drug which has more than 99% of protein binding. Again, if 400 μcg particles were inhaled, half of the dose (200 μcg) would be digested in the GI tract and the other half (200 μcg) would go to the lungs (53). After the first-pass metabolism (98%), a very low concentration (4 μcg) will enter the systemic circulation, assuming all of the 200 μcg that is deposited in the lungs will be available in the systematic circulation. The 99% protein binding will interact with 204 μcg, leaving around 2 μcg of the dose to enter the systemic circulation. As a result, this medication would have low cortisol suppression (54). Moreover, CIC would have a low detection level in the blood due to systematic bioavailability of the active CIC being below 1% (55).

Clearance and half-life

The hepatic clearance or the body clearance, defined by the amount of drug eliminated most often by the liver, is expressed by volume (L) per unit of time (h). Clearance is usually a fixed value if systematically absorbed and cleared by the liver, and around 90 L/h^{-1} is the highest rate that can be cleared by the liver (hepatic blood flow) (48). The clearance rate for most ICS drugs showed similar hepatic blood flow. BUD was reported to be 84L/h (56), FP range between 66-90 L/h (57) and FF was reported below 65 L/h. The active parent of BDP (beclomethasone 17-monopropionate) and CIC (des-CIC) have the highest clearance rate, detected at 120 and 228L/h, and both metabolites go to extrahepatic absorption (38).

The half-life of the drug is defined by the time required for the concentration of the drug in the plasma to be reduced by 50%, and it is determined by the hepatic clearance rate and the volume of distribution. The elimination of the half-life of a drug, when the drug is administered by IV, is significantly lower than inhalation type administration, which results in a longer residency time in the lungs (52). The highest inhaled half-life was reported with FP and FF inhalers (14.4 and 23 h respectively), whereas BUD and the active prodrug of BDP have a similar inhalation half-life when compared to their IV half-life (58, 59).

ICS lung deposition

Several factors can highly influence particle deposition, for example, delivery device properties, the velocity of the drugs, patient techniques or the particle size. Optimum particle sizes that can be deposited in the lungs range between 1 and 5 μm. If the particles are greater than 5 μm, they would be deposited in the oropharyngeal area and in the upper part of the trachea, which may cause local side effects. Some of the larger particles can pass through to the bronchi or bronchioles due to their low density. In theory, small targeted particles (<0.3 μm) can cross the small airway with a diameter < 2 μm to produce a therapeutic effect, while particles less than 1μm are expected to enter the peripheral airways and alveoli. Based on some studies, ideal ICS formation should have a smaller particle size, as it is associated with better outcomes (60, 61). Beclomethasone and CIC inhalers have the highest level of particles (more than 50%) deposited in the lungs. The inhaled particle deposition is highly dependent on patients' inhaler technique.

ICS delivery methods

The evolution of many inhaler devices and equipment makes it easier for clinicians to identify the appropriate device based on the patient's condition. To attain excellent delivery of inhaled medication, knowledge of the specific technique and breathing pattern is required for each device. However, it has been reported that patients are transferred to different types of inhalers to minimise their drug cost (62). Different inhalers are now available in the market and can be used as either pressured metered-dose inhalers (*p*-MDI) or dry powder inhalers (DPI). MDIs are pressure-based devices composed of surfactants and lubricants. Most of the MDIs work with HFA (hydrofluoroalkane) propellants, and the medication component is stocked in a solution. Other drugs in the market used chlorofluorocarbon (CFC) propellants, but in 2008 the Food and Drug Administration (FDA) announced that drugs using CFCs should no longer be manufactured or prescribed to patients due to environmental concerns, particularly because of the Montreal protocol agreement in 1987, which is about substances that deplete the ozone layer (63).

In MDIs, it is estimated that the lung deposition is around 10-15%, and this increases to around 20% if a spacer is attached to the inhaler. Dry powder inhalers' effectiveness is patient dependant; however, drug deposition may also be affected if lung function is poor.

As mentioned previously, inhaler technique is one of the factors that may affect the delivery of the drug to the lungs. Before starting to use MDI and DPI, patients should exhale to the point of functional residual capacity. Both methods can be performed based on a specific technique, a slow and deep inhalation for MDI and a high inspiratory flow for DPI, besides coordination. In other words, both MDIs and DPIs need a full inspiratory vital capacity.

The ability to coordinate the timing of inhalation and activation for some patients when using MDIs is challenging, but it is less important than doing the proper technique (slow and deep inhalation) (60, 64, 65). Ideally, at the beginning of inhalation patients should push the actuator at the same time; but if the actuator is pushed after starting the slow inhalation that is less important (66, 67). Furthermore, holding the breath at the end of inhalation to help sedimentation in slow inhalation is relatively unimportant (66). With fast inhalation in MDI, the possibility of particle deposition in the mouth and pharynx increases (68). Larger particles will mainly be deposited in the upper part of the oropharyngeal area, but on the other hand, the smaller particles (around 1.5μm), either in slow or fast inhalation, are deposited in the lung and oropharyngeal area (68).

One of the reasons for designing DPIs inhalers is to eliminate any coordination problems identified in MDIs (69). There are many types of DPI devices on the market, starting from single-dose that patients can fill, such as Aerolizer, Rotahaler to multi-dose devices (e.g. Accuhaler and Turbuhaler). Not surprisingly with the many types of DPI devices, lung deposition varies between these types. Around 12% - 40% of the released drugs reach the lungs; however, 20% - 25% of the dose remains in the device (70-72). If drug deposition from DPIs is weak, it is most likely due to drug particles from coarser carrier lactose pieces or drug pellets (69). Some factors affect the delivery in DPIs into the pulmonary system, which include: slow inspiratory flow rate, high humidity, and changes in the temperature (73). The DPIs' delivery also depends on fast inhalation from the patient. The rate of delivered drug to the lungs improves on fast inhalation in most DPI devices. Borgstrom et al. identified with Turbuhaler (14.8%) that if the inspiratory flow rate increases, the total lung distribution will

increase (27.7%) (74). This is different from MDIs that need breath-holding with slow inhalation to improve lung deposition of the drug.

Inhaled corticosteroids and beta2 (β2) agonists

Inhaled corticosteroids are usually combined with reliever medications. Formoterol and salmeterol are the most regularly used long-acting inhaled β2-agonists (LABA); they are often prescribed twice daily and can be utilised for longer action (24 hours). LABA inhalers should be not used by themselves as the only treatment for asthma because of the absence of anti-inflammatory effect, and their use as monotherapy may cause an increase in serious events related to mortality (75). Several beneficial outcomes reported when ICS is combined with LABA medications include: improvements in symptoms such as night-time asthma, lung volumes, reducing the use of inhaled rescue medication (short-acting B2-agonists), reduce asthma exacerbations, and the ability to control asthma in more patients in a short time at a lower dose of ICS (76). Some side effects are associated with the use of LABA, for example tachycardia, tremor, headaches, muscle cramps, and sometimes hypokalaemia. However, these side effects can probably be avoided if the LABA is used regularly in association with an ICS.

Some of the advantages of salmeterol and formoterol compounds are that they can produce a prolonged period of bronchodilation while avoiding bronchoconstriction; moreover, the effect of salmeterol is slightly slower than that of formoterol. In addition, adherence level to ICS inhalers increased when LABA was prescribed (77). Also, a clinical trial found that there was a significant reduction in cost if LABA was combined with ICS agents, compared to separate ICS and LABA (78). LABA and ICS inhalers are accessible in the form of fluticasone

propionate and salmeterol (Seretide) or budesonide and formoterol (Symbicort). Other examples include small particle beclomethasone with formoterol (Fostair) and fluticasone furoate with vilanterol prescribed as a single daily dose (Relvar).

Oral corticosteroids

A short course of oral corticosteroids (OCS) treatment in addition to ICS has shown clinical effectiveness in the event of acute asthma exacerbation. Despite the benefits of ICS inhalers and new biological therapies which reduce the use of OCS in many asthma patients, OCS retain a role in managing asthma exacerbation regardless of side effects. According to BTS/SIGN guidelines in asthma management, daily use of OCS are still considered for patients who were not previously able to control their asthma with high dose inhaled therapies (4, 5). However, patients who use oral corticosteroids for more than three months, or use more than four courses yearly, will be potentially at high risk of serious side effects. Therefore, patients should be closely monitored for weight, blood pressure, glucose and bone density in these specific periods (25). Prednisolone is the most common OCS, and most often prescribed as a single dose to be taken in the morning, due to the circadian rhythm of cortisol.

Side effects of corticosteroids

Many systemic side effects result from using OCS; the main ones include an increase in body weight, osteoporosis, growth reduction in children, skin thinning, cataracts, and glaucoma (79-81). By way of contrast, as they mainly interact in the airway, ICS medications have fewer and more localised side effects such as taste and voice changes, and thrush which may have an impact on the quality of life of asthmatic patients (40).

Boulet et al. have studied patients' perceptions regarding potential side effects of inhaled corticosteroids, and found that weight gain is the major concern of asthma patients (82). Also, corticosteroids medications have a significant impact on health, especially in the presence or development of other chronic diseases such as diabetes or adrenal insufficiency. As a consequence of such local and systemic side effects, there is a potentially high risk of nonadherence which may lead to uncontrolled asthma symptoms and significant hospitalisation stay (83).

1.2 ADHERENCE

Over 2000 years ago, Hippocrates was the first who noticed patients not taking their medication regimens, and finding those patients later complaining about the drugs not being helpful for them (84). Since that time, many clinicians and researchers have become interested in the area of medication adherence.

Different terms have been used in describing the taking of medication for various health disorders. Historically, this was recognised as 'compliance', which is defined as the level to which patients take their prescribed medications as instructed by health care providers. This implies that patients are passive and do not have any role in their medical plan, but should simply follow what their health provider tells them to do. The term 'adherence' has superseded the term 'compliance', and adherence is defined by The World Health Organisation (WHO) as "The extent to which a person's behaviour – taking medication, following a diet, and/or executing lifestyle changes, corresponds with agreed recommendations from a health care provider" (85). 'Concordance' is a third term; concordance is when "doctor and patient agree on the therapeutic decisions that incorporate their respective views". Generally, the differences between these terms is as follows: the term 'compliance' suggests that the patient has a one-sided relationship with their health care providers, because the patient had no interaction when the decision about the treatment was made. 'Adherence', however, implies patient involvement and self-decision, whereas 'concordance' is a broader concept which pertains to a full communication between both doctors and patients to reach the best-shared treatment decision (86).

Non-adherence to medications is a global healthcare issue. It is estimated that half of the patients who have a chronic condition do not use their medication as prescribed (87). The annual cost of non-adherence in the US is between $100 and $290 billion (88), while it costs the National Health Services (NHS) more than £500 million per year (89). The consequences of this have resulted in the health care systems worldwide trying to understand the root of the causes of non-adherence, applying frequent assessments, and developing new interventions and measurements that could potentially prevent this high cost.

1.2.1 Non-adherence in severe asthma

Non-adherence to corticosteroids is prevalent, and identified in all different asthma severities. It ranges from 30% to 70 % in adults (90-92) and 50% in children (93), and is more common in severe asthma patients (25). A study that monitored adherence to ICS by patients referred to the Northern Ireland severe asthma service found that 32% of the patients had filled less than 50% of their prescriptions in the preceding six months, and 88% of these denied poor adherence before checking their prescription records (94). Similarly, a study in Leicester studied adherence level in difficult asthma patients, and around 65 % (n = 75 / 115) were identified as filling less than 80% of the prescribed ICS (95). Additionally, Jochman et al. recently monitored adherence of paediatric asthmatic patients by using a daily electronic monitoring device for 8 - 16 weeks, and found that only 42% showed good adherence by taking at least 80% of prescribed ICS (96).

The rate of non-adherence of patients using OCS is similar. However, most of the studies assessed adherence to OCS by a direct method, particularly by measuring the level of prednisolone in the blood. In the Ireland study, amongst new referrals, 50% were found to

have undetectable prednisolone with a detectable cortisol level (94). Patients in this study were confronted with the objective measurements when they denied their low adherence. The same methodology was carried out in the Royal Brompton Hospital in London, where low adherence level to OCS was identified in (33%) difficult asthmatics using at least 15 mg of prednisolone daily . However, three had detectable prednisolone, but cortisol was normal, which should have been suppressed after using OCS. In the Leicester study poor adherence to OCS was found in 26% of the participants, who were assessed only by their prescription refill records (95).

In the Belfast or London studies most of the patients were referred to tertiary asthma centres from other physicians' clinics due to asthma severities and poor adherence, which was not identified before referral. This indicates adherence was not routinely checked by their clinic. Also, the high rate of poor adherence in those studies suggests that an objective assessment, such as measuring prednisolone assays, should be regularly available in several tertiary hospitals for routinely assessing patients' adherence.

There are many implications of non-adherence to patients and health care systems. Low adherence levels will cause a decrease in disease control that leads to poor health outcomes. As a consequence, non-adherence was associated with many factors including increased hospital admission leading to high mortality rate and cost, decreased quality of life (QoL), and low predicted lung function (97). These poor outcomes imply that non-adherence is a significant issue which should be avoidable by educating and encouraging patients to take their prescribed medication regularly.

1.2.2 Types and causes of non-adherence

In order to understand and analyse the underlying mechanism of non-adherence, I will first explain the nature of the forms of asthma non-adherence: erratic, intelligent and unwitting (88). Other commonly used terms to categorise non-adherence are intentional and unintentional (98). The potential causes that have been studied in the literature relate to non-adherence in chronic diseases.

Erratic non-adherence is most commonly associated with patient forgetfulness, which is the most common type of non-adherence. In this form, patients fully understand their medication regimen and tries to adhere to it as much as possible, but it is difficult for them to follow due to their busy daily life. In this form, several interventions can be provided, such as sending a text message or electronic reminders.

Intelligent non-adherence is when patients discontinue the treatments for some reason, not necessarily a logical one. For example, a patient may feel better after a few days and decide to stop taking their prescribed medications.

The last form is ***unwitting non-adherence***, which is unintentional, when patients do not fully understand how to use the medication or the regime. Patients in this type of non-adherence do not intend to be non-adherent. In asthma, with the innovation of the inhalers industry, several devices were developed, requiring different techniques for inhaling; this resulted in poor inhaler technique becoming very common. Additionally, some asthmatic patients misunderstand or think the maintenance inhaler is the same as the reliever “as needed” inhaler.

A number of potential factors can increase the level of non-adherence. The World Health Organisation (WHO) has divided those factors into five different categories: 1) socioeconomic factors 2) healthcare team or system factors, 3) condition-related factors 4) medication-related factors and 5) patient-related factors. These categories in real life are linked, and patients could see these as either intentional or unintentional. Some of these factors are typical only for asthma patients, while the others apply to any chronic condition.

Socioeconomic and demographic factors

The patient's educational background is one of the vital factors that has a high level of correlation with medication adherence (99). The level of patients' awareness and understanding of their condition, and knowledge of which medication they need, is a good indicator of treatment effectiveness in patients with chronic asthma. The cost of medicines is another socioeconomic factor (100, 101). In asthma, the prescription of drugs is not under any exemption, so patients in England must be charged part of the cost, but in Scotland and Wales, there are no charges. Under some circumstances in England there is an exemption for certain people, e.g. those on unemployment benefits, pregnant women and those with diabetes or thyroid disease. Therefore, low income and inadequate understanding of the disease and treatments could be a determinant factor in patient adherence.

Some studies of chronic diseases have shown a significant relationship between the age and level of adherence, and they have found that the younger the patients are, the less likely they are to be adherent, when compared to older patients (102-104). Nevertheless, elderly patients are becomes non-adherence because of their risk of cognitive and physical impairment (105).

Medication-related factors

Medication-related factors include the fear of side effects, and the complexity of administration has an impact on therapy adherence. Reliever inhalers (non-selective beta-agonist containing adrenaline and isoprenaline) were administered for the first time in 1956. Despite the success of this treatment in reducing asthma symptoms, the death rate increased markedly between the ages of 5 to 34 years (106). It is suspected that the reason for the increased mortality rate was due to the misunderstanding of patients that frequent use of inhaled reliever therapy would relieve severe symptoms. The overuse of isoprenaline can lead to serious hazards, particularly cardio-vascular, and can even result in death in many patients, and that makes many asthmatic patients unwilling to take their inhaled treatments (106). A New Zealand study has found there was a correlation between fenoterol use and epidemics of asthma death (107).

Currently, the concerns regarding ICS medications are slightly different. Patients think that because short-acting β2 agonists show instant symptomatic relief they are more effective compared to ICS, which does not give immediate therapeutic effect (108, 109). Moreover, in all of the previous BTS guidelines on managing asthma, a short-acting β2 agonist was the first step, and ICS was not recommended at this step. Thus, some patients believe that the reliever medication is the first step in their treatment as it has helped them resolve the symptoms, that this could influence their beliefs about ICS. In Finland ICS has been introduced as first step, with reliever medications. However, the reason for administering is not to change their opinion (110), also in recent years the BTS guidelines have introduced the ICS as well in the first step with SABA, and it would be very interesting to see whether the patients' beliefs about ICS medication have changed.

Moreover, the route of administration is crucial for managing asthma. For instance, some inhaler devices are not suitable for patients with reduced lung function, and their effectiveness will be impaired by inadequate inspiratory flow. Inhaled medications such as Turbohalers have a higher resistance which needs high inspiratory flow (111). It has not yet been proven that there is an inhaler device that is superior or significantly better than other inhaler devices (112). On the market there are many different types and shapes of inhaler devices, and that can be confusing for both patients and healthcare providers.

Healthcare team or system factors

There is little research into the impact of health care systems or health care providers on adherence, especially in developed countries. In asthma, there was only one issue that was highlighted in the literature regarding the inhaler technique. Some studies have shown teaching sessions with a group of patients may improve the technique. However, due to the time restraints on healthcare providers, this issue will continue to grow. The primary obstacle to adherence to treatments is insufficient patient knowledge (113-115). Besides, some of the healthcare providers also have inadequate information about the proper inhaler techniques, and that will also reflect on the patient's ability to develop a correct inhaler technique (116, 117).

Condition-related factors

Adherence to any treatment can be affected by the severity of the symptoms and the disease itself. In treating asthma, severe symptoms can have advantages and drawbacks in influencing treatment adherence. Some of the most severe asthma patients think that inhaled steroids are not needed any more when they start on advanced treatments such as biological

therapies; however, other patients who turn to advanced medication become more aware of their disease (112, 114, 118). Variation has been shown in the correlation between inhaler use and symptom severity, such as patients starting to minimise the frequency of dose when symptoms are relieved or improved (119). The level of patient education is the key to patient understanding of medication and the disease.

Patient-related factors

Patient-related factors can be intentional and non-intentional. Misunderstandings of the patient or worries regarding their medication are some of the reasons behind non-adherence to treatments. In most chronic diseases, patients commonly may suffer from depression (120). Taking a regular medication is a daily reminder of illness and can be challenging for some patients. A reasonable cause of non-intentional adherence is forgetfulness (121). Clinicians should simplify the medication route for patients as this can significantly improve medication adherence (122).

1.2.3 Detecting non-adherence in asthma

As previously mentioned, it is essential to detect non-adherence to avoid poor asthma outcomes. A number of methods have been reviewed, and are categorised into two broad measures: subjective and objective methods of medication adherence. These are discussed below. All of these measures have both strengths and disadvantages.

Subjective methods

The easiest way of measuring asthma adherence is through self-reported data (123) and this can be collected while interviewing patients during patient appointments, by asking the

patient to record their medication in diaries, or simply by answering a questionnaire. Currently, questionnaires are found to be the most appropriate for the usual daily clinical practice. There are more than 58 different questionnaires that can measure adherence to medication in many conditions; however, a recent review found that only a few of the self-report surveys have published reliability data (124). In asthma, several validated scales are available for measuring adherence; the most frequently used are Morisky's 8- or 4-item (125, 126) and the Medication Adherence Report Scale (MARS) (127), which can be answered as dichotomous (yes/no) or with Likert scales, respectively (128).

In Slovenia, the Morisky scale (8 items) has been used with asthmatic patients, and it has been found to correlate with quality of life and asthma control scores (129). However, no published study has examined the validity of the Morisky scale against any other objective methods. On the other hand, the MARS scale was developed and used as a nine-item scale for several conditions including asthma (127). Then it was modified to ten items (MARS-A) and demonstrated a significant correlation with continuous electronic monitoring in adult asthmatic patients ($r = 0.42$) (130). The short version of MARS-5 was only used in child populations, where it was found to have low validity when compared to electronic monitoring devices (96).

Numerous studies have identified many limitations with self-report scales, including the fact that a patient may overestimate their adherence to avoid disappointing their health care provider. Also, it has been shown that there is poor agreement when compared to other validated objective methods such as prescription records or the number of doses remaining in the inhaler (83, 131). The main goal of self-reported scales is to identify the patient behaviour towards medications; however, it is even more important to identify the causes of

non-adherence; therefore, there is a need to develop a questionnaire that also focuses on the reasons for non-adherence.

Other self-reported tools such as patient diaries have the same limitations as questionnaires, and have not been widely used (123). Additionally, not all patients use log books or diaries, and clinicians do not prefer to check the entire logbook as this requires a considerable amount of time (132).

Objective methods

Prescription refill records

Patient prescription data describing the total of prescribed medication have been collected and can work for both ICS and OCS. The expected number of prescription records is usually calculated for a 12-month period and presented as a percentage which can be accessed from pharmacies or primary care. It is the most reliable and widely available method for checking adherence, and has been implemented in some severe asthmatic centres prior to escalation to expensive therapy (133). The limitation of this method is that it only records how many prescriptions have been collected by the patients, and this does not guarantee that the patients have used these medications. Jochman et al. (96) found no correlation between electronic devices and prescription records, and also noted that some of the patients have a 100% rate of prescription refills, but electronic monitoring as low as 27%. Another limitation of this method is that it also requires access to all patients' records or databases, and not all countries allow this access.

Pill count and weight canister

Using the dose or pill counter is the easiest and cheapest method for measuring adherence. It can be checked when patients return to the researcher or the health provider and can be calculated manually for pills only. Some of the new inhalers are made with a dose counter which indicates and reminds the patient of the remaining doses. Inhaler canisters can be weighed on a digital scale to calculate the number of doses that have been taken. However, the pill or dose counter method does not give any information about when the medication was used, or even whether the mediation was used; patients could pretend to be adherent by actuating the inhaler multiple times to dump doses before their clinic visit (92).

Direct methods

Adherence methods can also be classified into direct and indirect methods (134). All of the methods explained above are indirect methods which depend on patient concordance or honesty. The direct method could be applied by observing patients taking their medications, or more commonly by measuring medication or its metabolite concentration in any biological samples (blood, urine or saliva), which proves that the patients have used their medication (135). This method can provide adequate and valid data about patient adherence.

Direct observation

This type of measurement involves working directly with a patient, where a health provider is required to visit patients or vice versa, then observe the patient while taking the medication. It is also helpful for maintenance of inhaled therapy and for evaluating the patient's technique. Nevertheless, using this kind of measurement is not practical because it

is time-consuming in large population settings and costly. Furthermore, it may introduce bias if patients only use the medication at the time of their visit or when a health care provider visits them at their site, and this may lead to inaccurate adherence information.

At present, thanks to the widespread availability of internet services, patients can be contacted using webcam technology. Shields et al. demonstrated feasibility for using direct mobile phone observation of 12 children (136). In this study, children were asked to film and upload through an app two daily videos of themselves using their dummy inhalers, and they were assessed for inhalation technique and adherence. Using mobile technology to upload videos is a time-consuming process for the patients and their asthma nurses, and requires adequate data security.

Electronic monitoring

The new generation of electronic devices for assessing adherence is considered the most effective method at the moment. Over the last 20 years, there has been rapid development of several electronic monitoring devices which have been validated in clinical trials. Each has different capabilities and characteristics, such as providing data about the date and time of actuation or checking the inhaler technique.

The first such device to be developed was DOSER CT in 1998. It could be attached to controller medications which have documented the number of actuations only (no date and time data) with a battery memory life for 30 days only, and were mostly utilised in clinical trials of child populations (137).

After that, with the advancement of technology in the last ten years has come a new group of digital inhalers which connect the inhaler to a mobile app by using Bluetooth technology. The most commonly used electronic device to measure adherence in asthma patients is SmartInhaler (Adhrium, New Zealand), and some authors have considered it as the gold standard of objective methods of adherence in asthma (138). The main benefits of SmartInhaler are that it records the date and exact time of opening of the actuation, dose dumping cannot affect the adherence rate, and lastly it can be placed onto different types of inhalers. Moreover, clinicians can download the app to remotely asses the adherence of the patients without needing the presence of the patients in the clinic. One study assessing adherence with an audio-visual reminder in 110 adult asthmatics patients found that 18% more of the patients who were using the audio-visual reminder were adherent than the control group ($p<0.001$) (139). Despite the advantages of the SmartInhaler, there is an issue while using this device: remote monitoring is time consuming for the health care provider, and whenever poor adherence is identified a clinician needs to contact patients, which needs time, resources and patient education.

Inhaler Compliance Assessment (INCA) device

The more recent commercially available electronic device is the Inhaler Compliance Assessment (INCA) device, which has similar characteristics as SmartInhaler (date and time of the actuation data); however, INCA has a superior advantage as it contains an acoustic sensor to detect the patient inhaler technique longitudinally (140, 141). The INCA device can only be attached to the DPI Accuhaler (Diskus) inhaler, and thus is not suitable for young children. Currently, Taylor and colleagues are testing the feasibility of using INCA on MDI inhalers in

both asthma and COPD patients (142). The investigator can analyse the inhaler technique and the opening of the inhaler by listening to the acoustic files that have been saved with INCA software. The device was tested recently in an RCT by Suliman et al. on uncontrolled asthmatic patients using a high dose of ICS (step 4 and 5) for over three months (141). In this study, the intervention group randomly underwent an intensive education programme to promote adherence and inhaler technique, and the researcher provided feedback on the inhaler use during the three-month period. The control group received the same education session, but no feedback from the INCA inhaler was given. After three months, when the intervention ended, the researchers found those without the feedback had a lower adherence rate (calculated as the number of actuations and good technique) than the group with feedback (63% vs 73%; $p = 0.02$). Besides that, the INCA device identified that almost 50% still had poor asthma control and were also found to have a low adherence rate despite the monitoring. Similarly, Heaney et al. have remotely evaluated a similar population using the INCA device for one month; they found that patients who had a significant reduction in FeNO level (by 42% from baseline measurements) had a good adherence rate (≥70%) (143).

The general advantage of an electronic monitoring device is that it can possibly affect the behaviour of some non-adherent patients, because they are observed and continuously monitored (Hawthorne effect) (144), which will lead them to using their inhaler as prescribed. Therefore, these monitoring devices could also work as an intervention to encourage patients to use their inhaler. However, when interpreting such studies that examine the effectiveness of electronic monitoring devices on asthma patients one should consider the Hawthorne effect (144). Moreover, they are too expensive to be used in clinical settings, and usually have data uploading issues or battery problems, and some rely on internet connection. In Heaney

et al.'s study, after the initial evaluation (after seven days of using INCA) 35 % of the patients were not able to use the Diskus inhaler or even did not like it at all as it required additional effort by the patients, unlike the MDI inhalers (143).

Medication levels in biological samples (drug levels)

Determination of the drug levels or metabolites in any biological sample provides an objective method of assessing adherence levels; however, the concentration may vary between medications and subjects due to differences in pharmacokinetics and metabolism. Direct monitoring is the most accurate method in assessing medication adherence, but cannot discover the reasons or the behaviour behind the non-adherence, and the impact of the white coat adherence phenomenon. Despite these limitations, several studies from various perspectives corroborate that measuring drug levels or metabolites is the most suitable method to ensure the presence of the drug within the therapeutic window (145-147).

The theophylline level is commonly measured in asthma clinics by serum concentration to determine whether the patient has achieved the therapeutic index level, and also the clinician can assess the adherence level (83). Different asthma drugs such as steroids maintenance therapy were assessed similarly as theophylline by using a liquid-chromatography mass spectrometry analytical technique. Gamble et al. measured serum prednisolone and cortisol levels to identify the prevalence of non-adherence in 51 asthma patients taking prednisolone (148). They defined non-adherence as undetectable blood plasma prednisolone with normal or detectable plasma cortisol. It has been reported that cortisol level would be suppressed in patients using the prednisolone regularly (149). Twenty-three of the patients were identified as non-adherent. Of those having positive prednisolone, two patients having detectable

cortisol. However, when the researcher interviewed these two subjects, they admitted occasional use of prednisolone. Moreover, prescription refill data of ICS medications of the 23 were checked, and 15 patients filled around 50% or fewer ICS medications although they previously denied poor adherence. In a similar study in 2003, prednisolone adherence was tested after around two hours of administration for patients confirmed reversibility (n=18) and unconfirmed asthma diagnosis (nonreversible airway, n=10). Of those confirmed asthmatics, good adherence was observed in nine patients (50%), and all with unconfirmed asthma had detectable prednisolone levels. It was also shown that the mean concentration of prednisolone level on patients taking more than 15mg/day was higher than patients on <15mg/day (616 vs 456 μg. L^{-1}). The interpretation of the prednisolone levels in the blood depends on the time the last dose was taken, as the systematic bioavailability of prednisolone ranges between four- and six-hours post-dosing. It is unreliable to assess the adherence by measuring prednisolone level in serum 12 to 24 hours after administration, due to the pharmacokinetic properties of prednisolone (elimination half-life between three and five hours). Therefore, this method should be extended to non-invasive methods such as urine sampling, as this offers a larger post-dosing window for detection.

ICS medication measured directly via blood as an indicator of adherence was only reported in a single pilot study (150). In this feasibility study, blood samples were obtained from 19 severe asthma patients using only FP or BUD inhalers. Serum levels were successfully detected in all except one patient. In addition, low blood levels were reported in two patients who had poor inhaler technique. However, this method required invasive needle penetration, which can lead to discomfort or pain and possibly infections, but it warrants expansion to other non-invasive biological sampling and testing of all other inhaled steroids types to be adopted in

clinical settings. On the other hand, promising results from this pilot study tested the utility of measuring the fluticasone propionate-17 beta-carboxylic acid in urine in asthmatics after direct observation of ICS administration: urine samples were taken after 16 to 24 hours of post-ICS and all of them had detectable levels (151). More recently, a novel direct method was developed to measure the ICS residues in hair samples, and most included participants received formoterol or vilanterol (152). The authors report that 72% of the subjects have confirmed a detectable level of ICS, with a different concentration based on the colour of their hair.

Collection of biological samples

One of the major challenges for direct methods in assessing adherence to prescribed medication is the collection of suitable biological samples from patients. As mentioned in the PK section above, several factors affect the concentration levels in the biological samples such as the PK, drug dose, and factors affecting the deposition of the drug dose. A recent review by Tanna et al. on the use of hyphenated MS technique to monitor medication adherence for a diverse range of clinical conditions found blood (serum and plasma) and urine samples were the most frequently used (145). The cost of the analysis of these samples is often very high, and they need to be stored in cold storage. Moreover, drawing a blood sample needs a trained health care provider, and patients may feel some discomfort with this procedure; thus, these factors may limit the routine implementation of this method in clinical settings. On the other hand, a non-invasive method such as urine is easy to obtain and has a larger time window than other direct biological samples; however, some groups of patients may be embarrassed handling the urine container or even be reluctant to provide urine samples due to cultural or religious beliefs.

Another potentially more accessible and less invasive biological sample is saliva. This method requires a specific technique such as passive drooling to obtain the sample, to avoid any effect that modifies drug detection, such as food or drink or tooth brushing (153).

In summary, the optimal choice for selecting effective biological samples would depend on numerous factors including the volume of the sample that can ensure that the targeted analyte is detected, and the ease of sample collection from the perspective of patients and HCP.

Mass spectrometry (MS) technique in detecting medications in biological samples

Checking adherence to medications by measuring the drug concentrations in biological samples has been performed for many years ago by using immunoassays. However, immunoassay methods have been reported to suffer from non-specified interferences by the metabolite or mixed reaction issues, and there are high probabilities of receiving false-positive results (154), so, patients may be falsely considered adherent to their medication (155-157). Moreover, most of the kits of immunoassays do not apply to different specimens other than the kits produced by the manufacturers, and the traditional version of immunoassay only quantifies single target substances (158). Some studies consider the results of immunoassay analytically presumptive until confirmed with another analytical method such as gas chromatography-mass spectrometry (GC-MS) (159). Furthermore, even with the confirmation by GS-MS analysis, the accuracy of immunoassay results has dropped by 10% within a ten year period, which indicates incorrect results (false positives) from initial immunoassay measurements (160).

In the last two decades, several studies in a range of clinical conditions have directly assessed adherence by mass spectrometry (MS) (145). This method has revolutionised the analysis of different invasive and non-invasive biological samples, providing an acceptable improvement in the sensitivity and specificity for the target analyte, and it also offers the only viable measurement technique. Furthermore, it has become increasingly useful for studying mechanisms of action of drugs or therapeutic effect monitoring. The mass spectrometer is the ideal detector of the drug metabolite in complex matrices in any biological sample for assessing adherence levels, which provide data about characteristics of the substance of the interest coupled with sensitivity. In studies of adherence to medications the MS technique has been used in combination with various kinds of chromatography, the most reported of which are gas chromatography-tandem mass spectrometry (GC-MS/MS) and Liquid chromatography-tandem mass spectrometry (LC-MS/MS). These instrumental combinations are also referred to in the literature as hyphenated techniques and were described as a chemistry method of analysis that joins the mass analysis capabilities with the physical separation of either liquid or gas chromatography. In asthma, several analytical studies have assessed the feasibility of assessing ICS medications adherence by assaying it through urine, blood, hair and sputum using hyphenated techniques (151, 152, 161, 162), and each collection method has different properties of detection.

1.3 EXHALED BREATH MARKERS IN ASTHMA

Asthma is a composite of multiple phenotypes caused by numerous factors; recent GINA guidelines state it is essential to diagnose and treat asthma patients according to their phenotypes. Hence, several biomarkers have been studied to identify asthma phenotypes and improve the diagnosis process, monitor airway inflammation, and assess treatment responsiveness. A definition of a biomarker is "….any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical, or biological. The measured response may be functional and physiological, biochemical at the cellular level, or molecular interaction" (163). Monitoring and analysis of exhaled breath biomarkers in asthma has been shown to be attractive in terms of assessing and investigating inflammation in asthma. Furthermore, the ideal biomarkers should be simple, quick, repeatable and accessible and have a reasonable cost. In this section, I will review and focus on non-invasive biomarkers obtained only from multiple exhaled breath methods. The gold standard biomarkers for investigating the inflammation and airway modelling in the lungs are bronchoscopy and bronchoalveolar lavage, which is highly invasive; in contrast, some of the exhaled breaths monitors can detect biological molecules with a non-invasive approach.

1.3.1 Nitric Oxide (NO)

Nitric Oxide (NO) in the exhaled breath samples of humans was first detected in 1991 by using a chemiluminescence analyser (164). Some years later an elevation of NO in exhaled breath was noticed in asthma, compared to an average healthy person (165). It was also reported that the NO level was lower in asthmatic smokers than non-smoker controls, and that

asthmatics treated with a low dose of ICS had a similar NO level compared to healthy controls (166, 167). After that, exhaled NO became an area of interest for many researchers in the field of monitoring and diagnosis of inflammatory lung diseases.

Numerous studies have attempted to explain the specific action and the origin of NO in the body. It is now known that nitric oxide synthasess (NOS) comes in three types of isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS), and each isoform can be generated in different cell types or tissues.

The nNOS can be constitutively identified and expressed in the neurons of the brain, adrenal glands, macula densa cells in the kidney, and numbers of epithelial cells in different body organs (168). There is some evidence that the NO which formed in the central nervous system (CNS) is derived by nNOS, which implies the need for controlling blood pressure (169). The eNO is expressed in the endothelial cells and has some role in producing NO which affects vasodilation and bronchodilation (168). Both of the two isoforms (nNOs and eNOS) can generate the NO for short periods of time (seconds to minutes) in response to an increase in the calcium levels in the cells (170, 171). On the other hand, the iNOS expression is independent of the levels of intracellular calcium, and depends on binding to calmodulin, which will lead to increasing NO production. However, this binding is not only activated in iNOS alone, it also occurs in the other two isoforms (eNOS and eNOS). But iNOS has different characteristics in term of binding to calmodulin; it can bind strongly to calmodulin at very low concentration of intercellular calcium. As a result, the iNOS is not regulated by the level of calcium concentration inside the cells, but can be regulated by the gene expressions. In that case, the formation of NO by iNOS has a delayed appearance, taking several hours, and is

sustained for days; and it tends to have higher production of NOS in the cell when compared to the other isoforms (168, 170, 171).

Nitric oxide in the lung

Several studies have clarified the mechanism and the origin of the NO in the human lungs. Guo et al. in 1995 have detected iNOS mRNA abundantly in epithelial cells from healthy non-smokers (172). But this iNOS was not identified in peripheral lung tissue and even in the airway macrophages that were obtained from bronchoalveolar lavage fluid samples. In this study it was also reported for the first time that ICS could significantly down-regulate the iNOS in those particular individuals (172). In addition to this study, Guo et al. in 2000 again studied the level of NO in asthmatics, and identified that bronchiolar NO was significantly higher in asthmatics compared to healthy subjects (173). Moreover, it was also found that the lower level of iNOS was in asthmatics on ICS versus asthmatics not using ICS, and that level was close to that of healthy controls. They also assessed the level of iNOS expression after three weeks of ICS medications in three steroid naïve asthmatics, and found that the level was decreased after the three week period.

The epithelial iNOS was identified in 2004 as the primary source of nitric oxide on the exhaled breath of a human airway (174). The population of this study were 41 children (healthy controls and asthmatic subjects) sampled for epithelial cells and macrophages from the distal trachea. The iNOS was found in all of the samples, while only 14 had detectable nNOS at low levels, near the limit of detection. The eNOS was detected in 34 subjects; however, their concentration was significantly lower compared to iNOS (174). There was also a positive and significant correlation ($r=0.672$; $p=<0.001$) between exhaled nitric oxide (eNO) and the iNOS

expression, while no relation was recorded for eNO and other forms (eNOS or nNOS) (174). In spite of the previous studies above, the roles of NO and iNOS, specifically in asthmatic airways, are not fully understood.

Fractional exhaled nitric oxide

It was mentioned earlier that in some studies fractional exhaled nitric oxide (FeNO) levels were observed to be higher in asthmatics than in healthy subjects, and there was an effect from ICS medications that could reduce the FeNO in mild asthmatics (165, 167, 175). Consequently, many clinical trials were carried out to evaluate the utility of using such a method in monitoring or assessing the inflammation in a variety of respiratory conditions.

From different ranges of asthma severities, the level of FeNO has been significantly correlated to eosinophilic airway inflammation (176-179). Berry et al. have found that FeNO in atopic patients is correlated with sputum eosinophilia in steroid naïve patients (179); however, FeNO was not correlated with eosinophilia count number in patients who were using steroid medications. Other studies have investigated whether FeNO could discriminate between non-eosinophilic and eosinophilic asthma by calculating receiver-operator characteristics (ROC), and they found that FeNO with a threshold of 42 parts per billion (ppb) was a good predictor to identify high sputum eosinophilia (>3%) with sensitivity 65% and specificity 79% (180). Moreover, when FeNO levels were compared between severe refractory asthma patients with a persistent sputum eosinophilia and various asthma severities, FeNO levels were found to be higher in refractory asthmatics despite the steroid treatment ($p=0.0084$) (181). FeNO level was also studied in regard to corticosteroid responsiveness. It has been shown in mild asthma that FeNO level drops when corticosteroid treatment is administered, and rises when

steroids are discontinued (182). On the other hand, FeNO levels in severe asthma have been noticed to be elevated even with raised steroid medication (183), indicating inadequate medication or non-adherence, or the inflammatory activity is resisting the steroid (which is the less common cause) (184).

FeNO suppression tests

In 2012, McNicholl et al. assessed the utility of FeNO level to predict the non-adherence in difficult asthma after seven days of directly observing steroids treatment (185). They reviewed participants (n=146) referred to a severe asthma clinic in Belfast. Participants were classified as adherent if their prescription refills were above 80%, and non-adherent if prescription refill was below 50%. From both groups, only patients with high FeNO (>45 ppb) were included for testing the suppression level. Patients were then directly observed taking ICS for 5 to 7 days. It was found that non-adherent subjects (prescription refill <50%) had a significantly higher suppression in their FeNO level (P = 0.003), suggesting that FeNO could be used to discriminate between adherent and non-adherent asthmatics. In the validation phase of the FeNO suppression, the authors were blinded to the prescription records of 40 consecutive referrals with high FeNO receiving high dose of ICS. Non-adherence in this study was identified in 13 patients; non-adherence was defined by passing the calculated FeNO threshold. Among those, eight admitted not taking the medications as prescribed at a subsequent interview, and three denied non-adherence. The remaining two patients were considered non-intentional adherence due to poor inhaler technique (185). The measured FeNO level in conjunction with other measures of adherence such as prescription refill and adherence questionnaire can objectively identify non-adherence to ICS.

In multicentre severe asthma services in the UK, the FeNO suppression test was remotely measured in combination with electronic inhaler monitors (smart inhalers) (186). In this study, uncontrolled asthmatic patients were asked to perform a daily FeNO test at home with taking daily ICS, and the level of suppression was assessed in two periods (7 days and 1 month). Positive FeNO suppression (as illustrated in the McNicholl study above) was identified in 65% of the participants, with improvement in lung function and reduction in peripheral blood eosinophil count. The level of suppression of FeNO after using ICS for one month was almost the same as after seven days. A major limitation of this method is the complexity of performing this test. After seven days, a number of participants (around 20%) were unable to perform and complete the FeNO suppression test, while after one month, only 41 patients were able to perform the FeNO suppression test from the negative suppression group (n=71), and 89 of 130 from the positive suppression group.

1.3.2 Exhaled volatile organic compounds (VOC)

Exhaled volatile organic compounds (VOC) can be measured in humans (187). The term breathomics (breath metabolomics) is used to describe the measurements of VOCs excreted from breath specimens. VOCs originate exogenously, taken from the environment by inhalation, food and drink ingestion, or skin absorption, endogenously as products of the host, typically generated in the body, and to some extent they are derived from the microbiome (188, 189). In exhaled breath, most of the VOCs identified were derived from exogenous sources (190); however, endogenous and microbiome VOCs are of most interest clinically.

Several offline breath-sampling methodologies have been developed in the last decades. An exhaled breath sample can be collected either by the end of the tidal breath, late expiratory and mixed expiratory. The most popular collection method is the end-tidal expiratory breath, mainly because this method is closer to the conducting airway than other methods, and can give a high amount of endogenous VOCs. Mixed expiratory breath sampling covers the total exhaled breath, and that includes air not involved in the gaseous exchange (dead space air). However, all sampling methods aim to reduce contamination from the dead space.

There are many available techniques for analysing VOCs in exhaled breath, starting from methods that can only identify a small number of particular compounds to advanced technologies that can accurately detect hundreds of compounds and non-targeted methods. The selection of one of each approach depends on the research aims and the future clinical implication. Two methods were identified as the most common analysis technique, methods based on GC-MS and electronic nose (eNose). The gold standard method is GC-MS, which may be particularly useful in conditions in which VOCs have not yet been identified. In contrast, the eNOse technologies are gaining in popularity because this method is cheap and much easier than the GC-MS. VOC concentrations are usually described in trace amounts (part per million volume, or lower) (191). Because of their low level, detection of VOCs in breath samples is challenging; however, pre-concentration methods such as using thermal desorption (TD) tubes could increase the reliability of detection.

Exhaled VOC is widely used for many respiratory conditions related to inflammation, asthma, COPD, and lung cancer (192-196). This method shows some evidence that VOCs can distinguish asthmatics from healthy subjects and also can demonstrate the ability to discriminate between asthma and COPD patients (196). Ibrahim et al. studied the VOCs by

eNose in a small group of asthmatics and healthy participants (195). They found a correlation between airway inflammation phenotypes and exhaled VOCs, and found some crucial differences in VOC profiles between those groups. A prospective study to predict exacerbation reported that seven VOCs showed high accuracy (88%) in predicting asthma exacerbation 14 days after collection (197). On the other hand, Schee et al. studied the response of oral prednisone 30 mg/day for 14 days in mild to moderate asthmatics, and showed that VOCs are able to discriminate between asthma and control subjects; they also found that steroids responsiveness prediction accuracy was greater by the VOC method than by FeNO and sputum neutrophils (198). In a recent study, Brinkman et al. from U-BIOPRED cohort linked the oral corticosteroids and salbutamol detection in urine samples and exhaled breath VOCs in severe asthmatic patients (199). They were able to distinguish to a moderate degree of accuracy the VOC profiles for positive and negative steroids detection (AUROC: 82; for salbutamol; 79 for oral steroids). This study showed that VOC profiles might be useful in the future for drug therapeutics monitoring.

The major limitation of VOCs sampling and analysis is the lack of standardisation. A review article published by Lawal et al. discussed more than 100 different papers which utilised a variety of different breath VOCs collection techniques, and that included different sampling, containers and different concentration methods (200). Therefore, there is a need for establishing a standardised method which includes VOC sampling and analysis.

1.3.3 Exhaled breath temperature (EBT)

One of the features of airway inflammation is vasodilation, caused by several vasodilator mediators, e.g. histamine, bradykinin, and nitric oxide (201, 202). Such changes in airway remodelling have a positive effect on airway temperature regulation.

The first study assessing the exhaled breath temperature (EBT) was in 2002 by Paredi et al., which found that asthmatics have a higher EBT than healthy controls ($p < 0.01$), and also their exhaled breath temperature is correlated to the level of FeNO (203). They proposed a possible explanation of the elevation in EBT as the result of an increase in the vasodilation of the bronchial vessels (204). Similarly, Garcia et al. evaluated the level of EBT in three groups: controlled and uncontrolled asthmatics, and healthy controls, and found that there was a significant increase in the EBT level in uncontrolled asthmatics compared to the other two groups (205). In children, two studies reported that EBT level was noted to be significantly higher in asthmatics than the controls (206, 207) and positively correlated with sputum eosinophilia (207). In contrast to all of these studies which successfully distinguished the EBT levels among asthmatics and healthy controls, Sevensson et al. showed a trend of higher EBT in asthmatic subjects after an exercise challenge, but did not reach a statistically significant level, although this could be due to the small sample size of 20 (208).

Inhaled corticosteroids have shown some effect on bronchial circulation, specifically bronchial blood flow (Qaw) (209), and measurement of Qaw has evaluated the steroid sensitivity in asthmatics. One study assessed the effect of budesonide and salmeterol on exhaled breath temperature and bronchial blood flow (210). It found that Qaw was significantly reduced compared to the baseline 30 minutes after the inhalation of budesonide

(400 mcg), and returned to baseline levels at 60 minutes. There was a reduction, but not a significant one, in the EBT levels at a different time point post-ICS. Repeated EBT measurements were examined in asthmatics pre- and post-ICS treatments within an eight-week period, and EBT levels were significantly reduced in those who had a good response of ICS after that period (211). Further studies are required to assess the steroids medication response, and may provide valuable information.

Paredi et al. developed the first EBT device in London. It measured EBT by using fast-reacting thermal sensors connected to a computer and placed in front of the subject's mouth, which recorded the trend of the EBT in a single breath manoeuvre (203). This test required constant control of ambient temperature with minimal air movement and training on the device. Another method of assessing EBT was established by Popov et al., who designed a simple portable device which can be used in a standard indoor environment (212). The subject continuously exhales into a mouthpiece through a heated chamber until the temperature of the sink heat reaches a plateau level, to indicate the inner temperature has reached thermal equilibrium. Due to the simple use of this device and accessibility by the subject, it allowed repeated measurements over time, with the possibility of using this device at home.

1.3.4 Particles in Exhaled air (PExA)

During exhalation, there are some non-volatile compounds such as lipids and proteins which are exhaled as particles or liquid droplets that follow the air stream. In 2009 a new non-invasive technology was developed by Almstrand et al. (213) to collect these non-volatile Particles in Exhaled Air (PExA) which originated from the airways. The idea of PExA was

triggered in the early 2000s by Anna-Carin Olins's group in Sweden to find an optimised method of sampling the non-volatile particles to evaluate occupational and inflammatory lung conditions. The first version of the PExA instrument (PExA 1.0) was developed and distributed mainly to a few research centres across Europe. After that, the Swedish group updated the instrument (PExA 2.0) and it is now CE marked. The first step of developing PExA is to measure the number of different particle sizes and distribution of the particles; thus, a thermostat paper box was created and attached to an optical particle counter sensor (214). The exhaled particles in PExA are sampled by directing the aerosol particles via a nozzle to an impaction plate (215). Both the velocity and size of the particles influence the inertia and also their impaction, and regulating the velocity will affect the size of the collected particles.

Several different breathing manoeuvres were tested, and each manoeuvre generated different particle numbers and size. The tidal breathing pattern generated the lowest number of particles sampled, and their diameter was <0.3µm (216), while the higher number of particles were generated during exercise and coughing. During coughing pleural pressure is increased, which increases the flow higher than forced exhalation. In forced exhalation, the interaction between the respiratory tract lining fluid (RTLF) and the air walls creates a two-phase concurrent flow (217, 218). If the velocity of the air is more than 25 $m \cdot s^{-1}$, a mist flow can be produced from gas (218). The highest air velocity during forced exhalation is reached until the choke point which is created by the dynamic airway compression (219).

The origin of the particles sampled from PExA is mostly driven from endogenous particles coming from the RTLF. The mechanism of the formation of these particles is not certain; however, there is a plausible theory that the formation of the particles is due to reopening of the closed airway. The peripheral airway at the early phase of expiration until the residual

volume creates a liquid menisci originating from surfactant particles. It occurs due to the instability of the liquid fluid (tendency directed to lower energy surface) and the air wall elasticity. Consequently, during the next inhalation, the liquid fluid bridge is braked and provides a shower of surfactant particles produced in the upcoming exhalation. One study has demonstrated the ideal manoeuvre for collecting PEx samples: they instructed the patient to perform tidal breathing, and the last exhalation was performed until the lung volume was empitted and then the breath was held for three to five seconds, followed by a full inhalation and exhalation, which is the optimum time of the PEx sampling (220). Similarly, another study confirmed that particle production was not altered by the changes of the exhalation flow, although in the presence of closure manoeuvre changes, it will trigger the particle numbers, particularly smaller particles on the peripheral airways with diameter size <1 μm (221). The deposition of the particles in the small airway during the breathing manoeuvre needs to be taken into account. The gravitational mechanism is the main factor responsible for particle deposition. Holmgren et al. described the size distribution by two methods: airway closure and tidal breathing manoeuvres, and found that most of the particles were located around 0.7, while smaller particles (0.2-0.5 μm) were found with airway closure (222). PExA method cannot sample particle size over 0.7 μm, and also such particles are unlikely to be derived from the small airway (223).

The utilisation of the PExA method for quantifying several exogenous components from small airways and surfactant parts has been successfully demonstrated in different research studies, and this method was also able to identify different proteins and phospholipids components in different respiratory conditions and populations (223-227). There are four types of surfactant proteins that have been identified in the lung: surfactant protein A (SPA),

surfactant protein B (SPB), surfactant protein C and surfactant protein D (SPD). In PEx sampling, SPA can be measured at a low limit of detection using immunoassays technology (227). This SPA is secreted by type 2 cells; it is the most abundant protein in the surfactant and also in PEx sampling, and can be found in humans in the alveoli and proximal airways at a very low concentration. Graaf et al. found a reduced concentration of SPA in bronco-alveolar lavage (BALF) in asthmatic patients compared to control participants (228). Moreover, it has been suggested that the surfactant dysfunction may play a role in airflow obstruction, and is potentially reversible in patients with chronic bronchitis (229).

The second most abundant protein in PEx sampling is albumin (227). Albumin is the main protein of the plasma and primarily produced in the liver. It has been described as a marker for numerous inflammatory diseases, and in some cases can be used as a clinical treatment. The level of albumin in the airways is not fully understood, although Khor et al. suggested that the albumin level in BALF may indicate increased airway vascular leakage or breakdown of the alveolar membrane (230). Interestingly, in the same study, they found that after ICS reduction, the level of albumin in BALF is significantly increased.

To date, several trials have been carried out using PExA technology by the proteomics method. In a healthy subject, albumin and SPA can be found in PEx more than in serum blood and exhaled breath condensate. Additionally, the SPA in PEx shows good repeatability in detection, as was tested from two sampling occasions within seven days. In a pilot study of COPD subjects, the level of SPA was observed to be reduced, leading to a high exacerbation rate (224). Larsson and colleagues conducted a study in an asthmatic subject with birch pollen allergy to show the impact of birch pollen exposure on the levels of albumin and SPA in PEx (231). They observed no significant difference in those proteins levels among both groups,

but the number of particles after the birch pollen exposure has decreased, which may suggest small airway inflammation that reduces the diameter and closure. Recently Soares et al. have shown high feasibility to use the PExA sampling method in different asthma severity which could allow small airway phenotypes to be identified by PEx analysis (232). Moreover, mild asthmatic patients treated with low ICS demonstrated constant albumin % compared to another group with a moderate to high ICS dose who had low albumin and SPA.

Due to the heterogeneity of severe asthma that includes various types of airway inflammation and changes of airway remodelling there is a need for further studies regarding PEx sampling to understand the mechanisms of albumin, SPA and the potential impact of steroid medications on their concentration.

Exhaled particles number and size

Moreover, counting the number of exhaled particles in every breath and the size distribution from PEx samples could be used as a potential biomarker. It has been shown that the respiratory tract lining fluids affect the particles formation process, which may change because of high inflammation and lower airway closure (233, 234). The Larsson et al. pilot study identified high variability in the exhaled number and particle mass volume between the control and the diseased subjects; however, within-subject, the variability is much lower (226). Further, the number concentration in exhaled breath may potentially be used to monitor the changes in the inflammation (226). A recent study on healthy adult subjects performing a single PExA manoeuvre found that there is no difference in particle concentration between male and female participants. However, older participants were seen

as having high exhaled particles number (235). Up to this date, no strong data have demonstrated the concentration in disease.

Particles in Exhaled Air instrument and sampling

The first generation of PExA instrument (PExA 1.0) was developed in 2012 at the Sahlgrenska Academy, the University of Gothenburg and only a few prototypes were developed and distributed in some research sites mainly in Sweden. In 2015, the company launched a new optimised and smaller PExA instrument (PExA 2.0) with the CE marked.

Almstrand et al. (236) explained the design and set-up of the PExA instrument and this briefly illustrated in the schematic representation in figure 1.4. The instrument targeted to measure particles in exhaled breath with aerodynamics range between 0.4 to 4.55 µm dimeter, these particular particles is are defined as PEx. Basically, participants breathe through a two-way non-rebreathing valve which provides air that is cleaned a HEPA filter and heated to 36 ^{0}C. There is an optical particle counter (OPC) (Grimm Aerosol Technik GmbH, Ainring, Germany) inside the box that measures the size and concentration of the particles within a one-second resolution. The exhaled particles in PExA instrument are drawn to an impactor (PM10 Impactor; Dekati Ltd., Tampere, Finland) by a vacuum pump. Inside the impactor, there is hydrophilic polytetrafluoroethylene membrane impaction substrate (PTFE, diameter 25 mm; Merck Millipore Ltd., Cork, Ireland) and exhaled particles are samples by impaction on this membrane according to the particles size. During the test manoeuvre, the exhaled flow rate and volume is measured by an ultrasonic flow meter (OEM flow sensor, Spiroson-AS, Medical Technologies, Zürich, Switzerland). hydrophilic polytetrafluoroethylene membrane impaction substrate (PTFE, diameter 25 mm; Merck Millipore Ltd., Cork, Ireland) and exhaled particles

are detected by impaction on this membrane according to the particles size. During the test manoeuvre, the exhaled flow rate and volume is measured by an ultrasonic flow meter (OEM flow sensor, Spiroson-AS, Medical Technologies, Zürich, Switzerland). Inspired air is humidified (Respiratory Humidifier Fisher & Paykel MR 700) and kept at 36 ^{o}C. The devices requires a flow of 280 mL s^{-1} and excecss gas is vented into a reservoir at the bottom of the instrument.

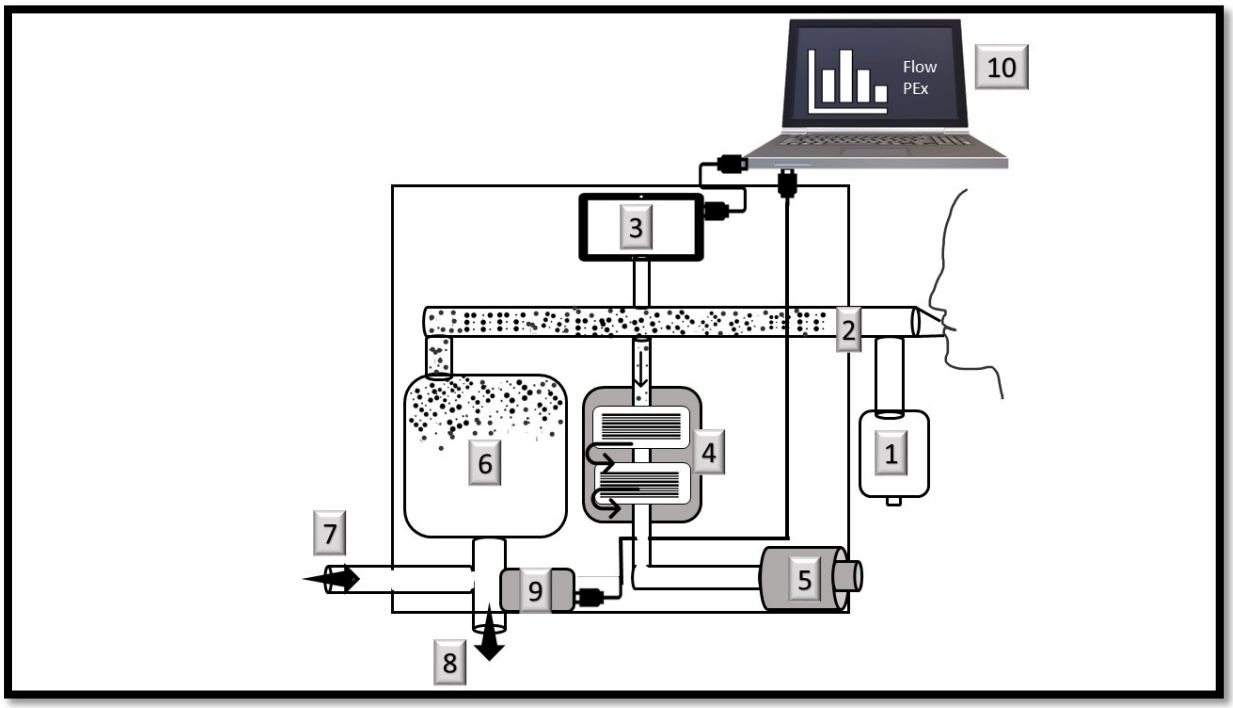


Figure 1. 4. A schematic presentation of the PExA (2.0) device; (1) tube with ambient air filter (2) 2-way non-rebreathing valve, (3) particle counter, (4) impactor with sample drawers, (5) vacuum pump, (6) exhaled breath reservoir, (7) supply of humidified air, (8) vent, (9) flow meter, (10) Laptop.

Impactor

The cascade impactor that was used is shown in figure 1.5-A. The exhaled particles were directed to the hydrophilic PTFE membrane plate by an injection nozzle. The impactors were modified to collect particles that ranged in diameter from 0.5 to 7.0 μm. This was done by placing the nozzles above the plate and also by accelerating the flow rate through the impactor. This method of sampling has showed an efficiency close to 100%. The size diameter of the PTFE membrane is 25mm and placed under the impact metal ring system and the particles is deposited mostly in the middle of the membrane. At the end of the PExA sampling, this membrane was cut using clean scalpel, the cut was around the edge of the round white filter paper (as close to the metal ring as possible) and then samples directly transferred to cryo tubes.

Particles counter

The OPC monitor and output that was used in PExA instrument is shown in figure 1.5-B. This monitor uses a laser beam that illuminates the particles resulting in light scattering which can be used to determine the concentration and the size of the particle. The OPC monitor delivered concentration values into 8 different intervals in 1 s resolution. The exhaled aerosol particles are assumed to have similar water density. Each interval was calculated by Holmgren et al. as follows 0.41-0.55; 0.55-0.70; 0.70-0.92; 0.92-1.14; 1.14-1.44; 1.44-2.36; 2.36-2.98; 2.98-4.55 μm in diameter (222).

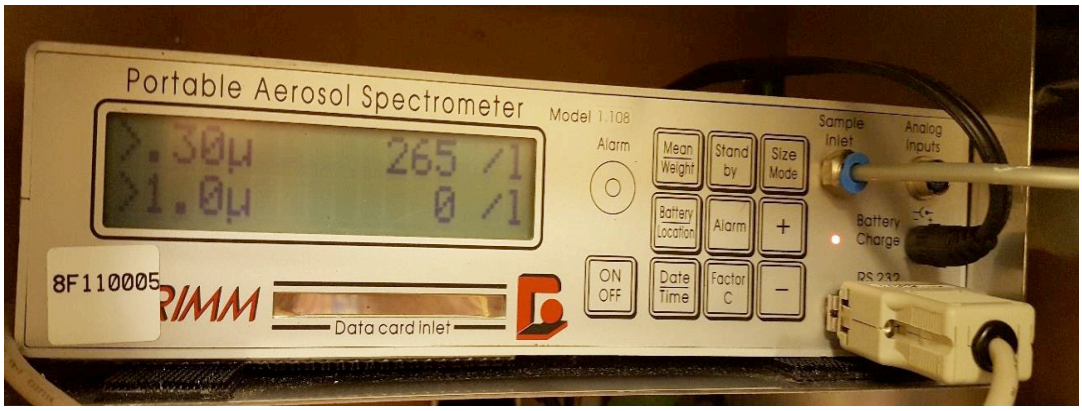


Figure 1. 5. (A) 2 stages modified cascade impactor; (B) Optical particle counter (OPC) device to measure real-time counting

1.4 AIMS OF THE PROJECT

My overarching hypothesis is that direct biological and exhaled markers may be useful in detecting adherence, and response to inhaled or oral corticosteroids in asthma. A number of aims were tested and presented here as separate chapters:

1. To identify and summarise the available literature on two objective biological methods, detection of inhaled or oral corticosteroids in body fluid and the level of exhaled nitric oxide, to assess patient adherence (Chapter 2).

2. To investigate the prevalence of poor adherence in adult participants prescribed daily oral corticosteroids by MARS questionnaire and by urinary corticosteroids detection, and compare the agreement of each method in detecting poor adherence (Chapter 3).

3. To assess the level of adherence by taking serum blood samples of patients over an eight-hour time period in high-dose ICS by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and to find any correlation between serum ICS concentration and markers of asthma severity (Chapter 4).

4. To assess the impact of the adherence rate using electronic monitoring on the serum concentration within a one-week study, and to investigate whether the blood concentration change correlated to the asthma severity markers on patients undergoing FeNO suppression test (Chapter 5).

5. To investigate whether analysis of exhaled breathing tests by using FeNO, EBT, PExA and VOC can be surrogate markers of steroid response in severe asthma patients (Chapter 6).

2 Assessment of adherence to corticosteroids in asthma by drug monitoring or fractional exhaled nitric oxide: a systematic review

2.1 ABSTRACT

Objective: Although the effectiveness of steroid treatment in controlling asthma have been well documented, high prevalence of poor adherence is identified. The optimal method in measuring adherence to asthma therapy remain unclear. This study aim is to review the literature on studies have been used detection of inhaled or oral corticosteroids in body fluid and level of exhaled nitric oxide to assess the adherence.

Design: We have searched three databases: MEDLINE (using both PubMed and Ovid), the Cumulative Index of Nursing and Allied Health Literature (CINAHL), and Web of Science. Quality of the studies were assessed using NIH checklist.

Results: A total of 2429 were screened, and 22 articles were fulfilled the inclusion criteria. Blood prednisolone with or without cortisol levels was used in seven studies as measure of adherence to oral corticosteroid suggests poor adherence is often. Whilst exhaled nitric oxide is identified in 14 studies and indicated a fall in the level after using inhaled corticosteroids and patients with good adherence.

Conclusion: Despite the prednisolone and cortisol levels commonly used in oral corticosteroids, further work is required to assess the influence of the dose and timing on the blood levels. Exhaled nitric oxide method suggest adherent patients is usually have low exhaled nitric oxide levels compared to poor adherent, however, no cut-off value was proposed to detect adherence. The accuracy in detecting adherence is a particular challenge in accuracy of the method. The promising findings of FeNO suppression testing should be explored in further studies in regard to adherence and sensitivity.

2.2 INTRODUCTION

Asthma is the most common chronic inflammatory airway disease, affecting more than 300 million people worldwide (237). Inhaled corticosteroids (ICS) are the foundation of daily asthma control medication, and in severe asthma are needed at high doses, often in combination with daily oral corticosteroids (OCS) (238). A high prevalence of poor adherence has been identified in all asthma severities, which impacts exacerbation and hospitalisation rates, quality of life, pulmonary function, and healthcare costs (237, 239-242). Unrecognised poor adherence likely leads to inappropriate medication escalation with associated side-effects and costs.

Measuring adherence to asthma medications is recommended in national and international guidelines (238, 243), but represents a significant challenge. Methods of monitoring adherence should be accurate, easy-to-use and cost-effective. They can be classified into objective and subjective methods, but all have drawbacks, and none would be considered as a 'gold standard'. The most common subjective methods of adherence include self-report questionnaires, patient diaries, and assessment by healthcare providers. This is known to be unreliable, as patients particularly tend to overestimate their adherence (3). Objective assessments, which include pill counting, prescription pick-ups, and electronic device monitoring (smart inhalers) may also overestimate adherence where patients pick up or actuate their inhalers without inhaling the drug as prescribed. The effect of corticosteroids medications (pharmacodynamics) on blood biomarkers in airway diseases such as blood eosinophils (148, 244, 245) or cortisol (246) may be useful in ascertaining efficacy and toxicity, but this may not directly reflect adherence. The fractional exhaled nitric oxide test (FeNO) is

an alternative biological non-invasive method used as a surrogate marker of ICS adherence (184, 247, 248), due to the effect of ICS on nitric oxide synthase (249).

Direct drug monitoring in biological fluids is probably considered the most reliable and accurate method, as it at least provides evidence that the patient has taken the medication sometime in the recent past, according to the specific assay and pharmacokinetics. Numerous studies have used blood assays to identify nonadherence in asthma patients regarding prescribed daily OCS by measuring the prednisolone and cortisol levels (148, 250, 251), and more recently, a similar approach has been reported for ICS (252).

We have conducted a systematic review aiming to identify and summarise the available literature on two objective biological methods that have been used to assess the adherence to either ICS or OCS in adults and children with asthma: 1. The detection of inhaled or oral corticosteroids in body fluid, and 2. The level of exhaled nitric oxide. Due to the heterogeneity of the studies within each adherence method included, a meta-analysis was not conducted.

2.3 METHODS

Search strategy

This systematic review was performed according to PRISMA (the preferred reporting items for systematic reviews and meta-analyses) guidelines (253). The search was completed by 9 February 2019. The databases searched were MEDLINE (using both PubMed and Ovid), the Cumulative Index of Nursing and Allied Health Literature (CINAHL), and Web of Science. The terms used were (asthma OR asthmatic) AND (adherence OR compliance OR concordance) AND oral corticosteroids OR inhaled corticosteroids) AND (exhaled nitric oxide OR FeNO). We screened the entire reference list from each eligible study, and Google Scholar was used to find any relevant citing articles. The study selection was performed based on the following inclusion and exclusion criteria.

Inclusion criteria

- A clear diagnosis of asthma based on a physician's diagnosis or international guidelines.
- Studies published in full.
- Primary research involving direct corticosteroids monitoring and the level of exhaled nitric oxide as a marker of adherence.

Exclusion criteria

- Other indirect methods of adherence (e.g., blood eosinophils or measures of adrenal suppression).
- Non-human studies.

- Review articles.
- Research in abstract form only.
- Non-English language publications.

Data collection

The first author screened all the titles and abstracts to exclude non-relevant articles. All the selected studies were screened in full-text to assess the eligibility. The last author (SF) confirmed the eligibility. Any disagreement in study selection between the authors was resolved by discussion.

Quality assessment

We used the National Heart, Lung, and Blood Institute of the National Institutes of Health (NIH) quality assessment tools for observational, cohort and cross-sectional studies, and controlled intervention studies (254). The first (FA) and the second (AP) reviewers evaluated the included studies individually.

Synthesis of results

Due to the different methodologies between the included studies, a meta-analysis was not conducted.

2.4 RESULTS

We identified 3899 studies through all databases. After removing duplicates, the titles and abstracts of 2429 articles were screened. The full texts of 39 potential articles were retrieved for further evaluation; 17 fulfilled the inclusion criteria. Reference list checking and Google Scholar screening added five studies, resulting in 22 studies included in total (Figure 2.1).

The relevant characteristics and findings of the selected articles are reported in Tables 2.1-2.5. The studies were conducted in seven countries: 14 in the United Kingdom and Ireland, three in the Netherlands, two in the USA, and one each in Germany, Denmark, and Spain. No relevant studies were found before 1998. The sample size of the included studies ranged from 17 to 529 participants. 12 studies included only children, nine adults, and only one both.

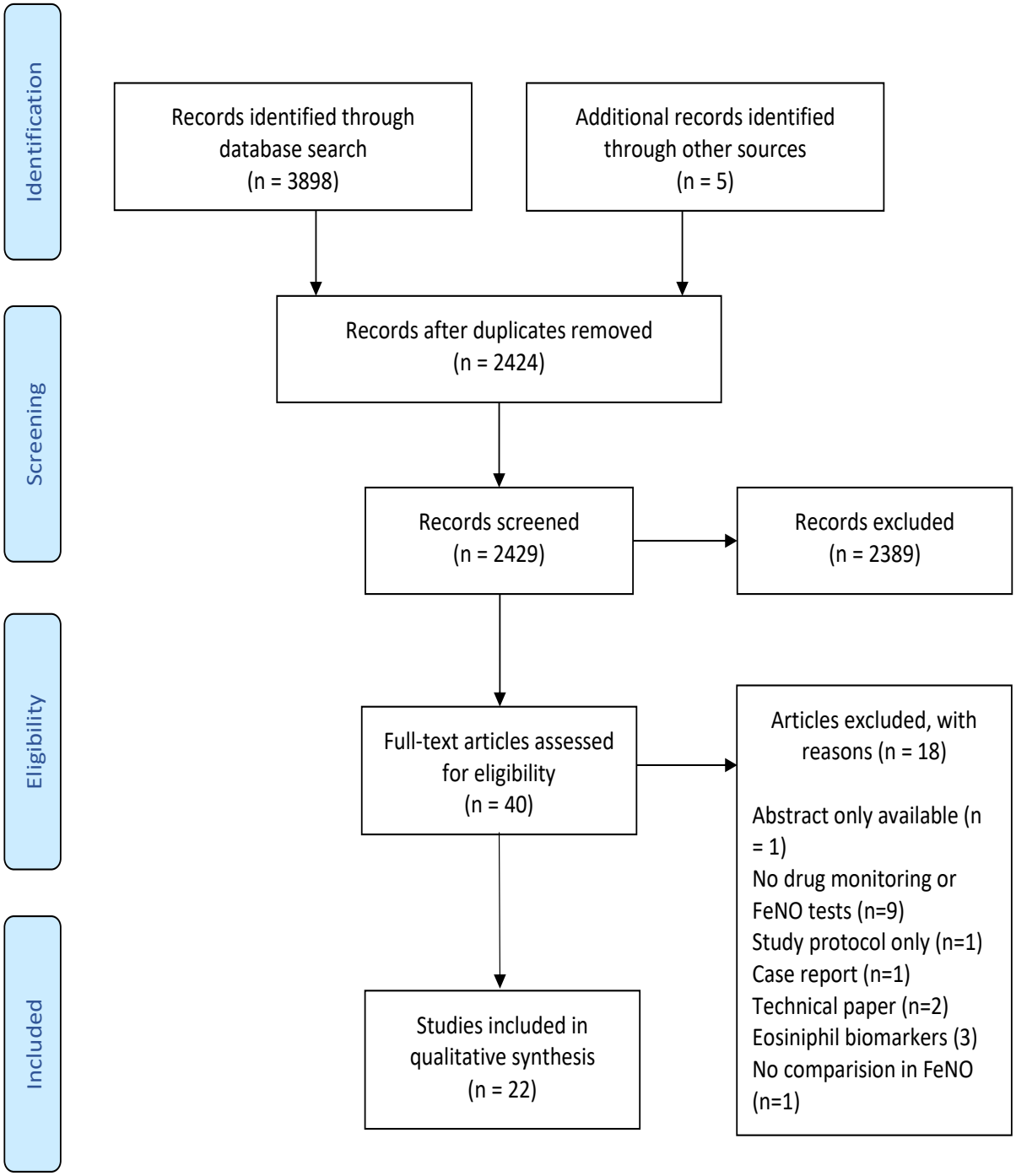


Figure 2. 1. PRISMA flow diagram summary of study selection process.

Quality assessment

Cohort and cross-sectional designs were most common (n=19); the remaining three studies were randomised controlled trials (RCTs). Generally, most of the studies (n= 14) were ranked as poor-quality (see appendix tables A2.1and A2.2). Of the observational cohort and cross-sectional studies, five ranked as fair (148, 184, 255-257), two were good (258, 259), and 12 studies ranked as poor-quality (250-252, 260-268). Among the randomised controlled studies, one ranked as good (269), while two rated as poor (247, 270).

Adherence methods

Oral corticosteroid adherence was assessed by direct measurement of blood levels in seven studies (148, 250, 251, 260-262, 264), and ICS levels in one (252) (tables 2.1 and 2.2). Thirteen studies (184, 247, 255-257, 259, 263, 265-270) used the FeNO test to indirectly assess adherence (tables 2.3-2.5).

Corticosteroid adherence: assessment by direct monitoring of drug levels

Methods used for corticosteroid detection

Oral prednisolone was the only OCS used among the included studies, which typically reported blood prednisolone level in addition to cortisol (148, 250, 251, 261, 262, 264), applying various cut-offs to each as shown in tables 2.1 and 2.2. One study reported ICS levels over eight hours after witnessed inhalation (150). The analyses of prednisolone and cortisol concentrations were done by either high-performance liquid chromatography methodology (HPLC) or LC/MS, although important analytical parameters such as the limits of detection (LoD) or quantification (LoQ) were not mentioned. George et al. reported serum ICS levels

measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a LoQ of 25ng/L (252).

Adherence rates by direct corticosteroid detection

Four studies investigated a daily dose of 40mg prednisolone given as an 'interventional' drug (table 2.1). Overall poor adherence rates were between 8% and 25% (250, 251, 261, 264). Three further studies reported 'real-world adherence' in patients who were prescribed regular daily prednisolone at enrolment (148, 260, 262). Stirling et al. reported three of 15 (20%) participants had undetectable prednisolone in the blood two hours after dosing (260). Robinson et al. assessed adherence in patients referred with "difficult" asthma and compared adherence rates between those with and without a subsequently confirmed diagnosis. They found that half of the participants (9/18) with confirmed asthma were non-adherent, while none of the 10 patients in the non-asthma group were considered as having poor adherence (262). In a cross-sectional study in Northern Ireland, the prevalence of poor adherence to OCS was 23/49 (47%). Of those with undetected prednisolone, 35% had a good level of adherence based on ICS prescription refill >80% in the last six months (148).

In the ICS study (252), all participants were witnessed using their inhaler and had inhaler technique rated. One patient observed to have a poor inhaler technique did not have a detectable level of ICS. Eighteen of 19 (95%) participants had detectable ICS (budesonide or fluticasone propionate) in the serum eight hours after witnessed inhalation.

Table 2. 1. Interventional studies reporting adherence to corticosteroids by direct serum measurement.

First author, year	Participants* and asthma severity (ICS dose)	Study description	Corticosteroid route, dose, duration and timing (where given)	Adherence measure and cut-off	Adherence results
Payne et al. 2001 (250)	n=27, children Budesonide ≥ 1000mcg/day or equivalent	Assessing adherence to prednisolone in patients treated with two weeks of OCS by measuring serum prednisolone and cortisol levels.	Oral prednisolone (40mg daily) The time between the last dose and OCS measurement was variable.	Serum prednisolone and cortisol level by HPLC. Good adherence: Last dose < 24 hours: detectable prednisolone + cortisol level <100nM. Last dose > 24 hours: cortisol level <100nM (no LOD given)	Two patients (2/27) had undetectable prednisolone or high cortisol levels. Prednisolone and cortisol levels not reported.
Payne et al. 2001 (261)	n=31, children Budesonide ≥ 1600 mc/day or equivalent	Assessing prednisolone adherence in patients treated with two weeks OCS by measuring serum prednisolone and cortisol levels.	Oral prednisolone (40mg daily) The time between the last dose and OCS measurement was variable.	Serum prednisolone and cortisol level by HPLC. Good adherence: Detectable prednisolone + cortisol level <100nM. (no LOD given)	Non-adherence to prednisolone was identified in five patients. Prednisolone and cortisol levels not reported.
Lex et al. 2007 (264)	n=12, children and adolescents	Assessing prednisolone adherence in patients treated with two weeks OCS by measuring serum	Oral prednisolone (40mg daily)	Serum prednisolone and cortisol level by HPLC. Good adherence:	Three patients were considered as non-adherent.

	Budesonide ≥ 1600 mcg/day or equivalent	prednisolone and cortisol levels.	The time between the last dose and measurement was variable.	Detectable prednisolone + cortisol level <100nM. (no LOD given)	Prednisolone and cortisol levels not reported.
Bossley et al. 2009 (251)	n=65, children Budesonide ≥ 800 mcg/day or equivalent µcg	Asthmatic patients referred to difficult asthma clinic; response (lung function, inflammation, symptoms) to corticosteroids (oral or IV) measured after two weeks. Adherence to OCS was assessed by the detection of prednisolone and cortisol in serum blood.	Oral prednisolone (40mg daily) The time between the last dose and measurement was variable.	Serum prednisolone and cortisol level; analytical method analysis not given Good adherence: Last dose < 24 hours: detectable prednisolone + cortisol level <100nM. Last dose > 24 hours: cortisol level <100nM (no LOD given)	12 patients (12/65) were non-adherent. Prednisolone and cortisol levels not reported. Adherence was confirmed in all children who a clinical corticosteroid response.

*We only considered those who have participated in the adherence measurements (not all the participants of the study). BDP: beclomethasone dipropionate; HPLC: High Performance Liquid Chromatography; LoD: Level of Detection; OCS: Oral corticosteroids; ICS: Inhaled corticosteroids.

Table 2. 2. Studies reporting adherence to corticosteroids by direct serum drug measurements in patients on long term treatment.

First author, year	Participants * and asthma severity (ICS dose)	Study description	Corticosteroid route, dose, duration and timing (where given)	Adherence measure and cut-off	Adherence results
Stirling, 1998 (260)	n=17, adult. BDP ≥ 2000 mcg/day or equivalent	Assessing the level of adherence to OCS by serum prednisolone level.	Levels measured 2 hours after prednisolone dosing (dose not reported).	Serum prednisolone by HPLC. Good Adherence: detectable prednisolone (no LoD given).	Prednisolone level in three patients (3/15) was undetectable. Mean (SD) levels 344 (285) nmol/l.
Robinson, 2003 (262)	n=18 "Confirmed asthma" n=10 "unconfirmed asthma" BDP ≥ 1000 mcg/day or equivalent.	An observational study in uncontrolled patients on prednisolone attending the 'difficult asthma' clinic.	Mean (range) dose of prednisolone in 'confirmed asthma' 15 (12.5-125) mg/day. Mean (range) daily dose of prednisolone in 'unconfirmed asthma' 30 (2-60) mg. Levels measured 2 hours after prednisolone dosing (unsupervised).	Serum prednisolone and cortisol by HPLC. Good adherence: detectable prednisolone AND 'normal' cortisol in patients on at least 15 mg/day. (Reference range and LoD not given).	Confirmed Asthma: nine patients "poorly adherent" (six with undetectable OCS and normal cortisol; three with detectable OCS and 'normal' cortisol); mean (SD) prednisolone concentration (≥ 15mg/d) 616 (143) ng/mL, and (<15mg/d) 465 (90) ng/ml. Unconfirmed asthma: none were classed as non-adherent; mean (SD) prednisolone concentration (≥ 15mcg/d) 624 (125) ng/L, and (<15mg/d) 397 (136) ng/mL.

Gamble, 2009 (148)	n=51, adult Mean (SD) BDP equivalent 1388 (550) mcg/day.	Measuring the prevalence of non-adherence to OCS among severe asthma and its relationship with asthma outcomes	Mean (SD) dose prednisolone 15.6 (11.1) mg/day. Levels measured 2-4 hours after prednisolone dosing (unsupervised).	Serum prednisolone and cortisol (methods of analysis not given). Good OCS adherence: detectable prednisolone and undetectable cortisol (LODs not given; no reference given for cortisol level). Good ICS adherence: prescription refill ≥ 50%	Poor OCS adherence in 45% (23/51). Mean (SD) prednisolone level of the adherent group was 194 (160) ng/ml with undetected cortisol. Poor ICS adherence in 35% (8/23) Eight patients (8/26) who were adherent to OCS were filling <50% or fewer ICS prescriptions. Note: QoL score and hospital anxiety mean scores are higher in patients filling ≤ 50% in the preceding 12 months
George, 2017 (252)	n=19, adult. BDP ≥ 1000 mcg/day or equivalent.	Determining the feasibility of liquid chromatography-mass spectrometry in detecting ICS over eight-hours post-dosing.	Inhaled fluticasone propionate dose range: 1000 to 1600mcg; budesonide dose range: 400-800 mcg Directly observed and inhaler technique noted	Prescription refill for ICS in the last 6 months. ICS level measured by LC-MS/MS. Good ICS adherence: prescription refill >80%; detectable ICS (LoD=10.0 ng/ml)	18 subjects (18/19) had detectable serum ICS 8-hours post-dosing. ICS levels not reported. ICS adherence rate 'undetectable baseline drug' in 52% (10/19).

*We only considered those who participated in the adherence measurements (not all the participants of the study). BDP: beclomethasone dipropionate; HPLC: High Performance Liquid Chromatography; LoD: Level of Detection; OCS: Oral corticosteroids; ICS: Inhaled corticosteroids.

Adherence to inhaled corticosteroids and exhaled nitric oxide

There was marked heterogeneity in the 14 studies that measured FeNO levels. Three different designs were used: 1. studies investigating the association between a single FeNO measurement and ICS adherence (n=5); 2. studies examining the association between repeated FeNO measurements ICS adherence (n=7); and 3. studies using the "FeNO suppression test" as a surrogate marker of adherence in patients using ICS (n=2). All studies used the FeNO test accompanied by different objective adherence methods, for example prescription refill, pill count for a specific period, or self-reported questionnaires.

Single FeNO test and adherence

Five studies measured FeNO level at a single time point and reported FeNO in adherent and non-adherent patients (256, 257, 265, 266, 268). Methods such as self-reported questionnaires, electronic devices, and patients' diaries were used in assessing the adherence rate. ICS dose and route were not controlled (i.e. participants used their usual medication). Cano et al. considered adherence satisfactory if parents reported that at least 75% of prescribed doses were taken (265), and found that adherence was the only independent predictor of FeNO in patients treated with ICS after adjusting for age, gender, BMI, and ICS dose. Scott et al. defined poor adherence where the patient missed at least two doses of ICS per week (266); they found no difference in FeNO by adherence levels. A validated medication adherence self-report scale (MARS), reported by the parents of participants, was used to identify poor adherence to ICS in two studies (256, 257). Both of the studies reported an association between adherence and FeNO, Koster et al. (256) noted that FeNO was lower in adherent compared to nonadherent patients. Vijverberg et al. investigated several confounding factors for FeNO, and found that adherence was an independent factor in

patients with high FeNO (>25 ppb) (257). One study evaluated adherence over six to 12 months by smart inhalers and reported an inverse correlation between adherence and FeNO ($r = -0.41$; $p = 0.001$) (268).

Table 2. 3. Changes in FeNO (cross-sectional) in observational studies monitoring adherence by other methods

First author, year	Participants * and asthma severity (ICS dose)	Brief study description	Adherence measure and cut-off	Adherence results
Cano, 2010 (265)	n=67, children. Budesonide 200-800mcg/day or equivalent.	Identifying the main determinants of airway inflammation.	Self-reported by patient or parents. Good adherence: if over 75% of the prescribed doses taken.	Good adherence rate: 83%. Lower FeNO with ICS *versus* no ICS, [median (IQR) 27.5 (15.0-51.5) *versus* 41.8 (19.9-62.5) ppb. FENO independently predicted adherence to ICS.
Scott, 2010 (266)	n=65, adult. ICS dose not reported.	Assessing the effect of atopy on FeNO.	Self-reported. Good adherence: if participants reported forgetting ICS once a week or less.	Good adherence rate: 50% (30/59). No difference between atopic and non-atopic asthma adherence vs good adherence.
Koster, 2011 (256)	n=527, children. ICS dose not reported.	Investigating factors affecting adherence.	MARS questionnaire completed by parent. Good adherence: cut off ≥21.	Good adherence rate: 57%. Factors associated with good adherence: low FeNO and young age.
Vijverber, 2012 (257)	n=529 children. ICS dose not reported.	Assessing FeNO as a marker of uncontrolled asthma and adherence.	MARS questionnaire completed by parent. Good adherence: cut off ≥21.	No overall adherence rate reported. Children with FeNO >25 ppb had lower adherence.

				FeNO measurements not reported for both groups (adherent and non-adherent).
Klok, 2016 (268)	n=60, children. Low-moderate fluticasone propionate.	FeNO level assessed as a marker for ICS adherence.	Smartinhaler-recorded dosages Good adherence: If over 80% of the prescribed doses taken.	Overall median (IQR) adherence: 82 (56-90) %. Low FeNO associated with good adherence. High FeNO (>25ppb) predicted poor adherence with a sensitivity of 41% and specificity of 94% when ICS adherence < 80%.

*We only considered those who have participated in the adherence measurements (not the whole participants of the study). OP: Oral prednisolone; LoD: Level of Detection; OCS: Oral corticosteroids; ICS: Inhaled corticosteroids; pMDI: pressurised metered dose inhale

Repeated FeNO measurements and adherence

Longitudinal FeNO measurements and adherence were reported in seven studies conducted over three to 12 months (247, 255, 258, 263, 267, 269, 270). Beck-Ripp et al. measured FeNO three times over 16 weeks and compared with estimated adherence by dose-counting (247). Any patients with a history of suboptimal adherence or poor inhaler technique were excluded. In the first four weeks, children were prescribed high dose inhaled budesonide, which was then withheld between the fifth and the eighth week (the washout period). In the last eight weeks, patients were randomised to 200mcg daily inhaled budesonide versus no ICS and a correlation was identified (r^2 = 0.59; p=0.0003) between ICS dose counts and the reduction percentage of FeNO. Another study measured FeNO in 30 children and evaluated adherence by checking the diaries of the participants (number of daily doses missed) in each visit for six months (263). FeNO levels were significantly lower in those regularly using ICS versus the poorly adherent group (34 vs 130 ppb, p=0.001).

Similarly, in 2006, children used a data logger attached to their pMDI for one month and attended four visits to test the level of FeNO in each visit (255), but no link was demonstrated. Strandbygaard et al. (270), reported data from pharmacy records alongside FeNO for 12 weeks and randomised patients to short message services (SMS) daily intervention reminder, or control (no intervention). However, despite the non-SMS group having poorer adherence over 12 weeks, both groups of patients (SMS and non-SMS) had significant improvements in FeNO. A large cohort study investigated the relationship of adherence by measuring the medication possession ratio (271) and the level of FeNO in 12 months and found adult and children with high FeNO levels tend to have low adherence rate at the baseline. After one year, those with high FeNO had a significant increase in adherence (267). One good-quality

RCT compared the FeNO level with adherence. Adherence was evaluated at each visit by counting the missed doses in a Diskus device and adherence was considered poor if <50% was taken during the treatment trial (269). A recent longitudinal observational study assessed the level of adherence in children by attaching an electronic monitoring device (Smart inhaler) to the participants' inhalers and by MARS questionnaire (258). There was an improvement in FeNO at the end of the trial in all participants, but no between the good and poorly adherent groups.

Table 2. 4. Repeated FeNO measurements in studies monitoring adherence by other methods

First author, year	Participants* and asthma severity (ICS dose)	Brief study description	Adherence measures and cut-off	Adherence results
Beck-Ripp, 2002 (247)	n=15, children. Budesonide 200-400mcg/d	Measuring FeNO to determine if it could be used as a potential marker of inflammation	Dose counting, calculated as doses are taken/doses prescribed×100 (%). Good adherence: If over >65% of the prescribed doses taken.	Mean (SD) FeNO pre-ICS 14.0 (1.2) ppb; post-ICS 7.7 (2.5) ppb. Positive correlation between ICS adherence and % reduction in FeNO (r2 = 0.59; p=0.0003). Adherence rate after eight weeks of ICS not reported.
Claudia, 2004 (263)	n=30, Children ICS dose estimated range (84-2000)mcg BDP equivalent	To determine the utility of FeNO in assessing asthma control and ICS adherence.	Diaries (number of doses per day and number of days used as prescribed) then recorded as a percentage. Good adherence: if self-reported adherence was >75% of their prescribed regime.	Poorly adherent patients had high FeNO compared to adherent patients (130 vs 34 ppb, p=0.001) Negative correlation between FeNO and adherence (r=-0.76, p=0.001).
Katsara, 2006 (255)	n=20, children ICS (dose not reported)	Assessing the relationship between FeNO levels and ICS compliance in asthmatic children.	A data logger was attached to the inhaler (recorded time and date of all actuations) Good Adherence: Day compliance >60%.	No correlation between adherence and mean FeNO (r = 0.05, p = 0.67). No overall adherence rate reported. No repeated FeNO measurements reported

Szefler, 2008 (269)	n=276, adolescent and adult. Fluticasone dose range: 100-500mcg/day	Determine if FeNO could increase the effectiveness of asthma treatment	Dose counter. Good adherence: if 50% of the prescribed dose taken.	Mean (SD) adherence rate in the study was 89 (28) %. FENO lower in adherent group (geometric mean 23.9 vs 30.8).
Strandbygaard, 2010 (270)	n=26, adult. Fluticasone ICS (dose not reported) GINA 2 (n=8) GINA 3 (n=16) GINA 4 (n=2)	The impact of daily SMS text message reminder on asthma treatment and change of FeNO over 12 week period	Dose counter. Good adherence: Dose count on Seretide Diskus within week 4 until week 12. Pharmacy record (cut-off not reported)	In the intervention group adherence rate did not change; in the control group adherence decreased from 84 to 70%. FeNO fell in both groups. With no between-group difference Data on pharmacy records not given.
Price, 2013 (267)	n=226, adult and children. BDP 100-400 mcg/day or equivalent	Evaluating FeNO in predicting steroid-responsiveness.	Medication possession ratio. (Defined as a number of days' supply of therapy/number of days in the total prescribing period × 100%) Good adherence: Unknown	Low baseline adherence to ICS associated with high FeNO (adult >50 ppb, children >35 ppb) In the following year, participants with high FeNo had improved adherence.
Jochmann, 2017 (258)	n=93, children and adolescents ICS dose range: 100-3200 mcg	Electronic ICS monitoring devices in identifying non-adherence.	Smart inhaler attached to ICS Good adherence: if ⩾80% of the prescribed dose taken. MARS-5 (parent-reported). Good adherence (MARS): Unknown	39/93 (42%) participants adherent. Baseline visit: no difference between adherent and non-adherent groups in FeNO.

	BDP/day or equivalent			Improvement in FeNO in all participants at the end of monitoring. (34 vs 21ppb; p= 0.001). Patients with good adherence and asthma control test >20 showed a significant reduction in FeNO in the follow-up visit (median: 18 vs 11ppb). No correlation identified between MARS questionnaire and adherence.

*We only considered those who have participated in the adherence measurements (not the whole participants of the study). ICS: Inhaled corticosteroids.

FeNO suppression test as a marker of adherence

Two studies reported an application for FeNO testing in assessing adherence to ICS in patients with severe or uncontrolled asthma (184, 259). This method measures the daily level of FeNO along with direct observation or monitoring of participants using a high daily dose of ICS over five to seven days, or one month. McNicholl et al. first reported the feasibility of using the FeNO suppression test to predict non-adherence in patients with "difficult asthma" and high FeNO levels (≥45ppb) (184). They determined a positive FeNO suppression test where the FeNO level dropped beyond a threshold (log10 ΔFeNO ≥ 0.24), calculated by change from mean log10 FeNO (day 0 – 1) to mean log10 FeNO (day 6 – 7). This threshold demonstrated 78% sensitivity and 92% specificity for non-adherence, determined by ICS prescription refill-rates over the previous six months (adherent if >80%). They noted a high reduction of FeNO levels among the poorly adherent group (<70% of prescription refill), and the mean level of FeNO suppressed from 79 to 47 ppb (p= 0.003) after seven days of observed ICS. Likewise, Heaney et al. used the FeNO suppression test and the same threshold for FeNO suppression testing over seven days and four weeks and monitored adherence by a smart-inhaler device attached to Diskus inhaler, which recorded the sound of the inhaler opening alongside the date, time, and technique (259). FeNO in 65% (130/201) of the participants was suppressed (>42% reduction from baseline) after one week of ICS. However, almost 40% of participants were not able to perform FeNO suppression test and were excluded.

Table 2. 5. FeNO as marker of adherence (cross-sectional and suppression studies)

First author, year	Participants* and asthma severity (ICS dose)	Brief study description	Adherence measure and cut-off	Adherence results
McNicholl 2012, (184)	n=146, adult. BDP 800-2000mcg/day equivalent.	Assessing daily level of high FeNO suppression after using high dose of ICS for seven days as marker in identifying non-adherence.	Good ICS adherence: prescription refill ≤ 80% ICS. Good OCS adherence: detectable prednisolone and undetectable cortisol (LODs not given; no reference given for cortisol level).	At baseline: no correlation between baseline FeNO level and adherence; poor adherence rate: 76%. Five days of observed ICS: 13/40 of the participants were non-adherent. 42% fall in FENO over five days of treatment with high dose ICS indicated a previous non-adherence. Seven days of observed ICS: the non-adherent group (n=13) had a reduction in FeNO compared to the adherent group (p =0.003).
Heaney, 2018 (259)	n=290, adult BDP 800-2000mcg/day equivalent. OCS (n=129)	The utility of FeNO suppression testing in clinical care using remote monitoring technologies.	Diskus inhaler connected with INCA device. Good adherence: defined as ≥70% FeNO levels: reduction by 42% from baseline FeNO level day 7 (positive FeNO suppression test).	FeNO after seven days: Positive FeNO suppression identified in 130 patients [median FeNO= 88 ppb (64-127) vs 32ppb (21-46)] 45 (63%) patients from the negative FeNO suppression group (n=70) on daily prednisolone. FeNO after four weeks: Positive FeNO suppression group (n=85); good adherence rate: 63% Negative FeNo suppression group (n=40); good adherence rate: 67% FeNO level at 7 days associated with FeNO after four weeks in good adherence group

*We only considered those who have participated in the adherence measurements (not the whole participants of the study). ¥ Reduction by 42% from baseline FeNO level day 7. OCS: Oral corticosteroids; ICS: Inhaled corticosteroids.

2.5 DISCUSSION

We have reviewed studies using objective biological methods for adherence monitoring in asthma, specifically through drug concentration assays in blood or measurement of exhaled nitric oxide. Our key findings are: 1. measurement of blood prednisolone levels, with or without serum cortisol, is commonly used as an adherence measure in studies, and if accurate suggests adherence to oral prednisolone is often poor, even within clinical trials; 2. whilst exhaled nitric oxide typically falls with inhaled corticosteroid use, and is generally found to be lower in adherent *versus* non-adherent patients, there appears to be no reliable cut-off that can be used to classify adherence; 3. it may be that a fall in FeNO following monitored ICS use can identify previous non-adherence, although this needs to be prospectively validated.

Although prednisolone detection was commonly used as marker of adherence, sometimes with simultaneous measurement of cortisol, none of the included papers cite data that support this assumption over the range of prednisolone doses used, and the timing of the sample post-dose. Indeed many studies did not report the dose or timing. Although prednisolone can likely be reliably detected for at least eight hours following a 40mg dose (272), this cannot be assumed to hold for the lower doses that are commonly used for maintenance in severe asthma (273). If this measure is to be used in clinical practice, further dose ranging pharmacokinetic studies are required in this patient group. One study reporting the level of ICS within an eight-hour window in serum as a marker of adherence (252) found that they could be detected in all but one of 19 patients. Whilst promising, this study was performed in patients taking only budesonide or fluticasone, and similar studies are needed

for other commonly used ICS. Corticosteroids in biological samples were typically detected by either LC-MS or HPLC. The mass analytical detector in LC-MS gives a much higher sensitivity (~ about 1000-fold) with a LoD in the low ng/mL range (274, 275), although it is relatively high-cost and requires a skilled operator. In many of the included studies we found that key methodological details and performance parameters, such as LoD or LoQ, were not given, thus making the application and generalisability of the findings difficult.

Exogenous corticosteroids have an adrenal suppressive effect (250, 276), and cortisol level was reported in several studies as a surrogate for adherence to oral corticosteroids (148, 250, 251, 261, 262, 264), although using varied and non-validated cut-offs. Many factors can influence cortisol levels, such as circadian rhythm, ICS use, and the variability of cortisol values between individuals (277, 278), and so (as for prednisolone levels above) studies are required to validate timings and cut-offs to support their clinical use in patients with severe asthma.

Most of these studies reporting direct serum monitoring were of poor or fair quality, with small sample sizes and cross-sectional designs. Prednisolone administration was almost always unsupervised, and so it is unknown whether lack of detection represents non-adherence or failure of the test. As expected, low adherence rates were more common in real-world study designs (20% to 50%) when compared to interventional studies (8% to 25%).

Most studies reporting a single measurement of FeNO (256, 257, 265, 268), or repeated measures (247, 263, 267, 269), found higher levels in poorly adherent subjects. There are not enough data available to be able to propose a single cut-off, or a clinically meaningful change (fall) over time, for clinical use. This may be in part because important confounding factors such as age, height, gender and atopy were not taken into consideration (279). In

addition the “gold standard” adherence in these studies often had significant limitations. A number of studies measured the level of adherence by diaries or self-report questionnaires (256, 257, 263, 265, 266). The accuracy and reliability of such methods are low (280), with patients typically over-estimating their adherence (92, 241, 281). The FeNO suppression test may be able to identify patients with previous poor adherence (184), and may be useful in a clinical setting at least in patients with elevated FeNO levels (>45ppb) (184, 259), where adequate resources are available. Further, non-suppressors may be identified with relative corticosteroid insensitivity who may require alternative therapeutic strategies.

Several limitations of this systematic review need to be considered. The included studies were selected based on the method of adherence from either direct drug measurement or measuring the FeNO level, and these methods cannot be compared to each other. Most studies were low-quality with high heterogeneity across the studies, including patient characteristics (in particular asthma severity and age), and cut-off values of both adherence methods. Methods not included in the review, but of potential use, include the detection of corticosteroid metabolites in the urine, as reported in (282). In this study fluticasone propionate-17beta-carboxylic, was found in the urine of all 30 included subjects 16- to a 24-hours after witnessed inhalation of fluticasone propionate. A further single case report also found that fluticasone propionate and beclomethasone dipropionate were detectable in and induced sputum sample (161). Further improvements to methods that assess the hypothalamic-pituitary-adrenal (HPA) axis may also prove useful; Smy et al. found that hair cortisol during ICS treatment was reduced, and proposed that this as a useful surrogate marker of adherence (283).

In conclusion we have reviewed the use of serum exogenous corticosteroid and cortisol detection, and of FeNO, as biological measures of adherence in asthma. Further work is required to adequately define the influence of drug dose- and timing-dependent factors on prednisolone and cortisol levels, in order to support their use in clinical and research practice. Although FeNO is usually lower in adherent patients, there are no data available to allow a single cut-off to be proposed. FeNO suppression testing merits further investigation both as a marker of adherence and steroid insensitivity.

3 Medication adherence in patients with severe asthma prescribed oral corticosteroids in the U-BIOPRED cohort

3.1 ABSTRACT

Introduction: Whilst estimates of sub-optimal adherence in asthma range from 30 to 50%, no ideal method for measurement exists; the impact of poor adherence in severe asthma is likely to be particularly high.

Objectives: We aimed to determine the prevalence of suboptimal adherence detected using self-reporting and direct measures, and the association of suboptimal adherence with disease activity.

Methods: Data were included from individuals with severe asthma taking part in the U-BIOPRED study prescribed daily oral corticosteroids. Participants completed the MARS, a five-item questionnaire used to grade adherence on a scale from 1 to 5, and provided a urine sample for analysis of prednisolone and metabolites by liquid-chromatography mass spectrometry.

Results: Data from 166 participants were included in this study, mean (SD) age 54.2 (11.9) years, FEV_1 65.1 (20.5) % predicted, 58% female. 37% completing the MARS reported sub-optimal adherence, and 43% with urinary corticosteroid data did not have detectable prednisolone or metabolites in their urine. Good adherence by both methods was detected in 35% participants who had both performed; adherence detection did not match between methods in 53%. Self-reported high-adherers had better asthma control and quality of life, whereas directly-measured high-adherers had lower blood eosinophils.

Conclusion: Low adherence is a common problem in severe asthma, whether measured directly or self-reported. We report poor agreement between the two methods suggesting some disassociation between self-assessment of adherence and regular oral corticosteroid use, which suggests that both approaches would be useful in clinical practice.

3.2 INTRODUCTION

Severe asthma is defined where the disease is not controlled despite treatment with high dose inhaled corticosteroids (ICS) plus second line therapies, or where treatment with systemic corticosteroids is required to bring about control (5). It comprises up to 10% of the asthma population, but contributes disproportionately to the burden of disease in terms of morbidity, exacerbation rate, quality of life and healthcare costs (284, 285). The diagnosis of severe asthma assumes that the prescribed medication is taken, and decisions leading to treatment escalation are often made on the basis of presumed inadequate benefit. This is despite the evidence that suboptimal adherence is known to be common, although the estimated prevalence varies widely (4). Low levels of adherence are associated with poor symptom control, lung function, and increased exacerbation frequency, as well as high costs (286-288).

Adherence is defined by the World Health Organization as "'the degree to which the person's behaviour corresponds with the agreed recommendations from a health care provider" (85). Measuring adherence to medication in asthma is challenging. Prescription refill-rates can be used to determine whether an appropriate number of inhalers have been collected, but do not indicate whether the medication has been taken, and are not available to treating clinicians in many healthcare systems (289). Self-reported adherence, through questionnaires such as the Medication Adherence Report Scale (MARS), rely on accurate patient recall and reporting (290). Electronic inhaler monitoring devices are being developed and used in research (291), (and becoming available for clinical use in some healthcare systems), but few record inhalation as well as actuation (185). Direct measures of adherence

such as detection of drug in biological samples are not widely available or validated (148, 252).

The Unbiased BIOmarkers for the Prediction of Respiratory Disease Outcomes (U-BIOPRED) project is a collaboration between public and private sectors, which aims to identify new phenotypes and targets in patients with severe asthma who are often prescribed systemic corticosteroids (292). During the baseline visit, we collected urine samples for measurement of corticosteroids and metabolites, and also asked participants to fill the MARS adherence questionnaire. In the present study we aimed to investigate: 1. the prevalence of poor adherence in adult participants prescribed daily oral corticosteroids by each of these methods; 2. the performance of the MARS questionnaire in predicting adherence by urinary corticosteroids detection; and 3. the clinical characteristics of adherent and non-adherent participants identified by each method.

3.3 METHODS

Design and participants

This study used the cross-sectional data from the U-BIOPRED cohort (292). We included adults with severe asthma participating in the baseline visit of the study, who were currently prescribed daily oral corticosteroids. Severe asthma was defined where patients had uncontrolled symptoms and/or frequent exacerbations despite high intensity asthma treatment (at least 1000 mcg/day fluticasone or equivalent) (16). The inclusion criteria stated that adherence should be assessed prior to inclusion in the study, but there was no explicit requirement to exclude patients who were poorly adherent. Patients were not asked to withhold prednisolone and were not told that it specifically would be measured. As it is usual practice to prescribe prednisolone to be taken in the morning, then we would expect samples to have been taken within 8-10 hours of dosing.

Asthma Control Questionnaire (ACQ), Asthma Quality of Life Questionnaire (AQLQ) and Hospital Anxiety and Depression Scale (HADS) were administered, and participants underwent measurement of spirometry and fractional exhaled nitric oxide (FeNO) test at 50ml/sec. Sputum was induced using hypertonic saline inhaled via ultrasonic nebulizer and analysed by a standard protocol to measure the differential cell count (293). Venous blood samples were analysed for differential white cell count.

Adherence Measurements

In the MARS questionnaire, five items assess how participants use their medicines, which includes unintentional and intentional behaviours: 1, “I forget to take them”; 2, “I alter the

dose”; 3, “I stop taking them for a while”; 4, “I decide to miss out a dose”; and 5, “I take less than instructed”. Each item was answered using a five-graded response scale, ranging from very often (1) to never (5). The sum was calculated for each participant ranging from 5 to 25. If the total score of MARS was below 23, the participant was considered non-adherent (294). It is important to note that MARS is non-specific to particular medications.

Urine samples were collected on the same day as the MARS questionnaire, and analysed for prednisolone, prednisone and their metabolites, and for cortisol, by liquid-chromatography mass spectrometry.

Chromatographic analysis:

The sample preparation for determination of corticosteroids was performed on a robotic liquid-handling platform (Microlab STAR, Hamilton Robotics, Bonaduz, Switzerland). The corticosteroids were analysed from a simple preparation using a 1mL aliquot of urine fortified with internal standards and subsequently hydrolyzed using β-glucuronidase (*E. coli*). Purification was performed using mixed mode solid phase extraction (SPE) in 96 well plate format. The analysis of the extract was performed with reversed phase liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS, Thermo Q-Exactive, Thermo Fisher Scientific Inc, Waltham, USA). Acquisition of raw LC-HRMS data was performed in full scan mode at a resolution of 35000 with polarity switching (295). The limit of detection (LoD) for all these compounds (prednisolone, prednisone, methylprednisolone, 16α-OH-prednisolone, 20β-dihydro-prednisolone, and cortisol) was 1 ng/mL. At this LoD prednisolone and its major metabolites would be detectable for over 24h after a 10mg oral dose (296).

Statistical analysis:

The datasets for this analysis were downloaded from tranSMART, an open-source knowledge management platform (297) on November 2018. Differences in clinical variables between adherent and non-adherent groups [including ACQ, forced expiratory volume in 1 second (FEV1), HADS, FeNO, and blood biomarkers] were investigated using parametric t-tests if normally distributed or Mann-Whitney U tests if nonparametric, or Chi-square tests if categorical. For assessing the agreement between MARS questionnaire and urinary corticosteroids detection, Cohen Kappa test was used. All statistical analysis was performed using SPSS for MAC version 22 (SPSS, Chicago, IL). A *p*-value of less than 0.05 was considered significant. The performance characteristics of the MARS (cut-off less than 23 out of 25 indicating non-adherence) predicting undetected urinary corticosteroids were calculated.

3.4 RESULTS

Participant characteristics

A total of 166 participants currently prescribed daily oral corticosteroids were included in this cohort study (figure 3.1). The median (IQR) daily dose of oral corticosteroids was 10.0 (7.5-20.0) mg. Demographic details are shown in table 3.1. In summary this cohort contained a majority of females, with clinically significant airflow obstruction (mean forced expiratory ratio 61%), a high BMI and a heterogeneous smoking history.

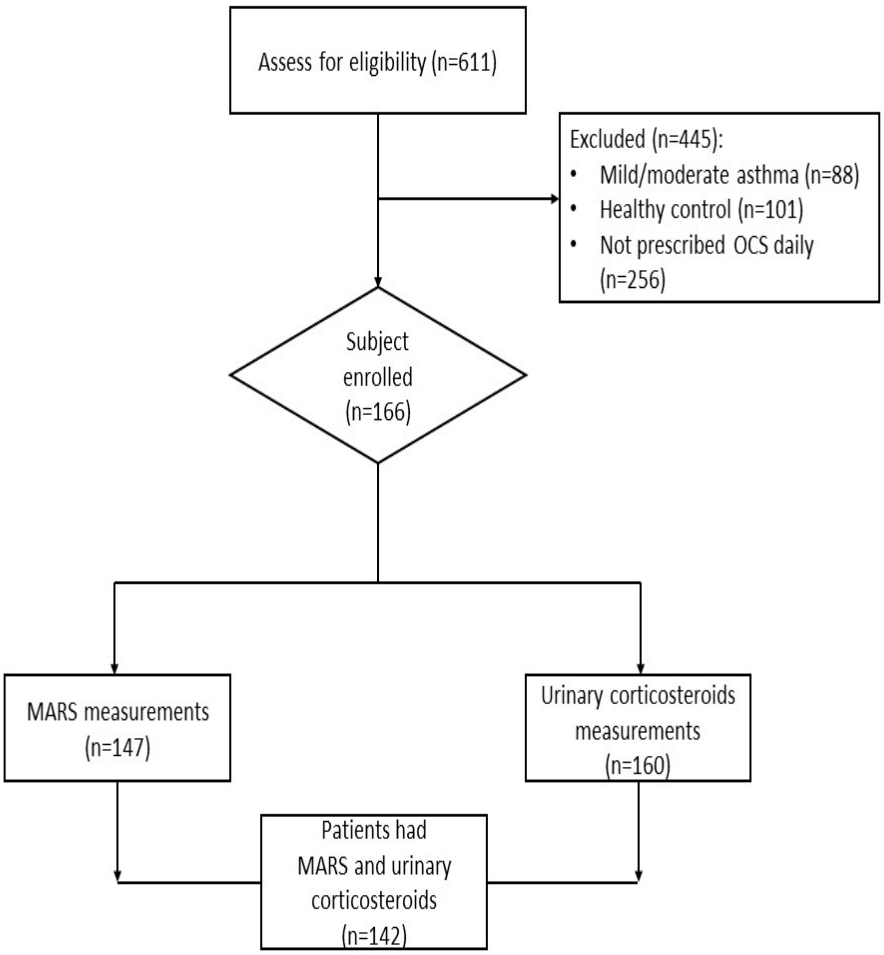


Figure 3. 1. Study CONSORT diagram

Table 3. 1. Participant characteristics

	Participants using oral corticosteroids
Subjects, n	166
Daily prednisolone dose, mg	10.0 (7.5-20.0)
Females, n (%)	96 (58)
Age, years	54.2 ± 11.9
BMI (kg/m^2)	30.1 ± 6.5
FEV_1 % pred. (pre-BD)	65.1 ± 20.5
FVC % pred. (pre-BD)	86.5 ± 18.9
FEV_1/FVC % (pre-BD)	61.3 ± 13.1
Exacerbations over the previous year, n	3 (2-5)
Smoking status, n (%)	105 (63) non-smoker
	54 (32) ex-smoker
	7 (4) current smoker
Smoking history, pack years	12.7 (4.8-22.5)
Intubation ever, n (%)	15 (9)
ICU admission over the previous year, n (%)	8 (5)

Data are expressed as mean ± SD, median (interquartile range) or n/N (%). BMI: body mass index; FEV_1: forced expiratory volume in 1 second; BD: bronchodilator; FVC: forced vital capacity; ICU: intensive care unit.

Self-reported adherence measured by MARS questionnaire

Complete MARS data were available from 147 participants, of which 54 (37%) were classed as having poor self-reported adherence (median score = 20, IQR = 19-22). The prescribed dose of prednisolone was not different between individuals who were classed as having good- or poor-adherence (Table 3.2). Likewise, no differences were observed in the urinary prednisolone level between groups, nor in the frequency of absence of detectable urinary cortisol. The poorly-adherent group had statistically- and clinically-significant worse asthma control and quality of life than the group with good adherence. Whilst there were no differences in lung function or inflammatory biomarkers between groups, there were high levels of airflow obstruction and inflammatory biomarkers across both adherence categories.

Objective adherence measured by urinary corticosteroid detection

Urinary corticosteroids and metabolite data were available for 160 participants, of which 69 (43%) did not have detectable levels in their urine, despite the prescribed daily dose of prednisolone or prednisone being similar to those with detectable levels (Table 3.2). Other prednisolone metabolites (methylprednisolone, 16α-OH-prednisolone, and 20β-dihydro-prednisolone) were detected in 11 of the 91 who have corticosteroids detected. Almost all (89%) of patients with detectable urinary corticosteroid metabolites had undetectable urinary cortisol, compared to around half (51%) of those with undetectable metabolites (chi squared $p \leq 0.05$). There were no differences in asthma control, quality of life, exacerbation frequency, or in any of the HADS domains, between individuals with detectable urinary corticosteroids levels and the individuals with undetectable levels. Lung function parameters were similar between groups. There were differences in inflammatory biomarkers between groups, with sputum (percentages) and blood (counts) neutrophils significantly higher, and

blood eosinophils (counts) significantly lower in patients with detectable urinary corticosteroids metabolites. Of note, even in those with detectable urinary corticosteroid metabolites, the median (IQR) sputum eosinophils were still well above the normal range at 5.2 (0.8-15.9) %.

A daily prednisolone dose of at least 10 mg was prescribed in 100 participants, of whom 41 % (n=40) had undetectable corticosteroids in urine, compared to 44% (n=19) of the 44 patients prescribed less than 10mg (chi squared p = 0.744). Moreover, no correlation was observed between daily dose of prednisolone and the quantity of prednisolone in urine (Spearman's r= 0.095, p = 0.264).

Table 3. 2. Characteristics of adherent and non-adherent participants assessed using the Medication Adherence Rating Scale (MARS) or objective urinary corticosteroids metabolites.

		MARS (n=147)			Urinary metabolites (n=160)		
		Adherent	Non-adherent	Significance	Adherent	Non-adherent	Significance
Demographics	Subjects, n	93 (63%)	54 (37%)		91 (57%)	69 (43%)	
	Daily prednisolone dose, mg	10.0 (7.5-15) (n=82)	10.0 (8.7-20) (n=45)	P=0.846	10.0 (7.5-18.7) (n=81)	10.0 (7.5-20) (n=59)	P=0.940
	Females, n (%)	53 (57%)	31 (57%)	NA	49 (54%)	44 (63%)	NA
	Age, years	55.1 ±11.9	51.8±11.9	P=0.198	54.0± 12.7	54.8± 11.0	P=0.667
	BMI (kg/m2)	30.5 ±7.1	29.4 ± 5.7	P=0.336	30.0±6.5	29.9± 6.7	P=0.965
Asthma control, quality of life and exacerbations	ACQ-average	2.6±1.4 (n=89)	3.1±1.2 (n=51)	P=0.015	2.7±1.3 (n=81)	2.9±1.4 (n=59)	P=0.291
	AQLQ	4.7±1.2 (n=89)	4.2±1.3 (n=53)	P=0.020	4.7±1.2 (n=82)	4.4±1.2 (n=60)	P=0.193
	Exacerbations over the previous year, n	3.0 (2.0-4.0) (n=80)	3.0 (2.0-6.0) (n=42)	P=0.085	3.0 (2.0-5.0) (n=74)	3.0 (1.7-4.2) (n=62)	P=0.449
Hospital Anxiety and Depression Score	Total	12.4±8.8 (n=93)	14.0±8.3 (n=52)	P=0.306	12.9±9.2 (n=89)	12.0±7.5 (n=67)	P=0.529
	Anxiety	6.9±4.9 (n=93)	7.8± 4.7 (n=52)	P=0.302	7.2±5.1 (n=89)	6.6±4.2 (n=67)	P=0.454
	Depression	5.5±4.4 (n=93)	6.2±4.4 (n=52)	P=0.383	5.7±4.6 (n=89)	5.4±4.1 (n=67)	P=0.702
Lung function	FEV_1 %pred.	66.0±21.4 (n=92)	62.0±20.1 (n=53)	P=0.264	66.6±21.4 (n=89)	62.7±19 (n=68)	P=0.239
	FVC %pred.	87.9±20 (n=92)	83.5±18.7 (n=53)	P=0.195	87.7±18.8 (n=89)	85.3±19.8 (n=68)	P=0.454
	FEV_1/FVC	60.6±12.9	61.1±13.9	P=0.819	62.0±13.7	59.8±11.9	P=0.328
Biomarkers	FeNO	33 (22.0-53.0)	28 (15.7-72.5)	P=0.924	33 (18.6-53.0)	29 (19.5-77.0)	P=0.177

	(n=83)	(n=51)		(n=80)	(n=65)	
Sputum eosinophils, %	3.5 (1.0-18.9) (n=40)	5.0 (0.2-19.7) (n=24)	P=0.720	5.2 (0.8-15.9) (n=42)	5.0 (1.9-31.5) (n=32)	P=0.261
Sputum neutrophils, %	66.5 (44.1-86.7) (n=40)	63.9 (30.3-93.6) (n=24)	P=0.650	69.5 (47.9-86.3) (n=44)	44.6 (27.2-71.8) (n=33)	P=0.011
Blood eosinophils (X 10^3/ul)	0.19 (0.1.0-0.4) (n=93)	0.17 (0.1.0-0.4) (n=51)	P=0.649	0.1 (0.04-0.3) (n=90)	0.30 (0.1-0.5) (n=66)	P=0.001
Blood neutrophils (X 10^3/ul)	7.1 (4.9-8.7) (n=93)	6.60 (4.0-8.4) (n=51)	P=0.539	7.4 (5.6-9.2) (n=90)	5.30 (3.8-7.4) (n=66)	P=0.001
Urinary prednisolone (ng/mL)	1579.7 (866.6-4458.9) (n=43)	1561.1 (587.6-2834.9) (n=30)	P=0.466	1577.1 (690.7-3064.7) (n=79)	NA	NA
Detectable urinary cortisol n (%)	26 (28%)	13 (24%)	p>0.05	10 (11%)	34 (49%)	p≤0.05

Data are expressed as mean ±SD, median (interquartile range) or n (%); BMI: body mass index; FEV_1: ACQ: Asthma Control Questionnaire; AQLQ: Asthma Quality of Life Questionnaire; FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity

Agreement between methods for classifying adherence

One-hundred and forty-two participants had urinary corticosteroid metabolites analysed and completed the MARS questionnaire (Table 3.3). The sensitivity and specificity of MARS to predict urinary corticosteroids detection were 59% and 31% respectively. The associated positive and negative predictive values were 69% and 41% respectively. There was poor agreement between the methods for determining medication adherence (kappa test = -0.106, p = 0.268).

Table 3. 3. Agreement between MARS and urinary corticosteroids detection for classifying adherence

		Urinary prednisolone metabolites		Total
		Detectable	Undetectable	
MARS	Good-adherence (≥23)	49 (35%)	41 (28%)	90 (63%)
	Poor-adherence (<23)	34 (23%)	18 (13%)	58 (37%)
Total		83 (58%)	59 (42%)	142

MARS, medication adherence rating score

3.5 DISCUSSION

Poor adherence to oral corticosteroids is a major contributory factor to poor symptom control and hospitalisations (288, 298); poor adherence to ICS has been linked to death from asthma (3). Despite recommendations that medication adherence should be routinely checked in primary care (299), the optimal method to assess adherence is not clear. This is the first study to compare self-reported adherence using the MARS questionnaire to adherence determined by urinary corticosteroid metabolite detection, in individuals with severe asthma prescribed daily oral corticosteroids. Our data show that MARS overestimates adherence to oral corticosteroids considering urine corticosteroid metabolites as the gold standard comparator. We identified poor-adherence in approximately 40% of individuals using each method. Interestingly however, the methods showed poor agreement, and the low-adherers, identified via each method, were different in around half of all cases. Patient self-assessed as having poor-adherence had worse asthma control and quality of life compared with self-reported good-adherers, whilst objectively-determined poor-adherers do not appear to have more severe/uncontrolled disease. Importantly, patients with good-adherence, assessed via either method, still displayed significant disease burden and raised inflammatory biomarkers, consistent with severe refractory asthma. Whilst the optimal method to assess medication adherence remains open to debate, we identified that medication adherence remains sub-optimal in a large number of severe asthma patients, which should be considered by prescribers and discussed with patients during asthma reviews, particularly prior to the initiation of novel and expensive therapies such as biological therapies or bronchial thermoplasty (148, 300).

The identification of sub-optimal medication adherence occurred despite the application of the U-BIOPRED definition of severe asthma, recommending the exclusion of other, recognisable reasons for having 'difficult' asthma such as clinical evidence of poor adherence (301). Using the self-reported MARS questionnaire to determine adherence, 37% of the population had poor medication adherence. Previously, poor self-reported medication adherence using the MARS questionnaire has been observed in 69% of inner city asthmatic adults (302) and 27% of children with persistent asthma (303). Given the plethora of factors that may affect medication adherence (patient characteristics such as age, gender, socio-economic level and ethnicity, social support, patient knowledge, psychological state and patient's willingness to participate in self-management (304), the divergence in adherence in our cohort of severe asthma patients is of no great surprise.

Adherence rates were similar when assessed using the self-reported MARS questionnaire and using urinary prednisolone detection. Importantly, however, the 'poor-adherers' were different in around half of cases. Our results highlight the sensitivity and specificity for good-adherence on the MARS questionnaire to identify individuals with detectable urinary prednisolone metabolites were 58% and 32%, respectively. These results indicate that relying solely on self-reported adherence would not be a useful assessment method in clinical practice. Whilst this is the first study to utilise the detection of urinary prednisolone metabolites to objectively assess medication adherence, our results are in line with adherence levels determined by blood plasma prednisolone detection in severe asthma (148). It has been shown that challenging patients who claim to be adherent to medication, with objective evidence of poor-adherence, in the form of blood prednisolone results or prescription refill rates, can facilitate frank and honest discussions on medication adherence

(148). We envisage a similar utility of urinary corticosteroid detection, which has the additional advantage of being less invasive than blood-sampling and potentially offer a larger post-dosing window for detection (296). Prednisolone metabolites are mostly excreted in the urine, and the peak concentration usually occurs after 4-8 hours (305), whilst the peak concentration for plasma prednisolone occurs much earlier (1.5-2 hours) and becomes undetectable after 8-10 hours (306). It seems likely that self-reported adherence contributes complimentary information; possible explanations for those reporting poor adherence but with detectable corticosteroid levels includes sporadic poor adherence to systemic corticosteroids therapy, or good adherence to these drugs but poor adherence to others, such as inhaled medication.

Blood cortisol levels have also been used as surrogates for prednisolone adherence. It has been studied that prednisolone has an effect in the cortisol suppression level in asthma patients (149, 307). Therefore, adherence level was considered satisfactory if patients have detectable prednisolone and undetected cortisol level. However, if no prednisolone detected with normal or detectable cortisol level, it is hard to confirm the level of adherence as there are no published data (to our knowledge) that investigated the pharmacokinetics of prednisolone in different biological samples (blood or urine) in this particular group; hence this method cannot reliably used in this way. Further, cortisol suppression is an indirect measurement of adherence and cannot discriminate between the source of exogenous steroids (e.g. high dose ICS *versus* low dose OCS), or indeed from primary hypoadrenalism.

Comparing the clinical characteristics between good-adherers and poor-adherers provides some interesting insights. Firstly, self-reported poor-adherers had worse asthma control and quality of life compared to self-reported good-adherers. Although it is perhaps unsurprising

that poor-adherence would be associated with reduced asthma control and quality of life, these differences were observed despite no difference in urinary corticosteroid levels, nor any difference in lung function or inflammatory biomarkers. Possible explanations could be that patients with poor disease control and quality of life may be more self-analytical, or that they would be more likely to notice (and therefore report) when they had missed a dose of medication.

Somewhat surprisingly, there were no differences in markers of asthma control, quality of life or severity between those with and without detectable urinary corticosteroids. It may be that patients "self-regulate" their daily dose of corticosteroids in order to maintain relative disease stability. However, the patients with poor adherence measured in this way still had frequent exacerbations and poor control, and may represent a group in whom targeting of adherence as a "treatable trait" could potentially have an impact on these important outcomes. The relatively high blood eosinophil counts in these patients do suggest that regular corticosteroid therapy might be clinically effective (308, 309). On the other hand, the finding of persistently raised median sputum eosinophils even in those with detectable corticosteroids levels suggests that some of these patients may represent a truly corticosteroid-insensitive phenotype (310), and we propose that the concomitant measurement of corticosteroids in biofluids should be advocated in studies investigating this phenotype in future.

Many techniques are available to assess adherence to asthma medication, however there is currently no gold standard (311). This study benefits from using two such methods, but each technique has its own limitations. The MARS questionnaire is a validated tool to assess medication adherence with good test-retest reliability in asthma (302), however its concordance with alternative objective measures has had mixed results (303). In the current

study, we administered the MARS questionnaire to determine adherence to asthma medication in general, rather than to oral corticosteroids specifically. It has been shown that adherence may vary between types of asthma treatment, therefore a patient's response to the MARS questionnaire may not reflect their oral corticosteroid adherence *per se*. Mass spectrometry is highly sensitive for urinary prednisolone metabolites, with detection possible up to 24 hours after a 10 mg dose, and 72 hours after 40mg (296). We did not record the specific formulation of oral corticosteroids taken; it is known that enteric coating slows the absorption of prednisolone (310), and could therefore adversely have affected the sensitivity of the assay in this regard. A patient with occasional or sporadic medication use may therefore be categorised as having good-adherence if they only took their medication on the days preceding the urine sample. Objective measures could have been further enhanced by the inclusion of direct measurement of inhaled corticosteroids metabolites in both blood and urine (252, 282).

Conclusion

Using self-reported and objective adherence methods we identified poor adherence in a significant number of patients with severe asthma. We identified poor concordance between the methods, which questions the validity of relying solely on self-reported adherence in clinical practice. Importantly, inflammatory biomarkers remained significantly raised in patients with good adherence to medication, which may represent a population of truly refractory asthmatics. We propose a combination of self-reported and objective measures of adherence are utilized in clinical practice, to initiate discussions on medication adherence and identify 'steroid-unresponsive' patients for research and for novel biologic treatments.

4 Serum Inhaled Corticosteroid Detection For Monitoring Adherence In Severe Asthma

4.1 ABSTRACT

Background: Daily inhaled corticosteroids (ICS) are fundamental to asthma management, but adherence is low.

Objectives: 1. to determine whether liquid chromatography tandem mass spectrometry (LC-MS/MS) could be used to detect ICS in serum; 2. to investigate whether serum levels related to markers of disease severity.

Methods: We collected blood samples over an 8 hours period from patients with severe asthma prescribed at least 1000mcg daily of beclomethasone dipropionate (BDP) equivalent. Following baseline sampling, patients were observed taking their usual morning dose. Subsequent blood samples were obtained 1, 2, 4 and 8 hours post-inhalation and analysed by LC-MS/MS. Correlations between serum ICS levels and severity markers were investigated.

Results: 60 patients were recruited, 41 female, 39 prescribed maintenance prednisolone, mean (SD) age 49 (12) years, FEV_1 63 (20) %predicted. 8hours post-inhalation, all patients using budesonide (BUD, n=10) and BDP (15), and all but one using fluticasone propionate (FP, 28) had detectable serum drug levels. Fluticasone furorate was detected in two patients (of four), ciclesonide in none (of seven). Low adherence by prescription refill (less than 80%) was identified in 43%. Blood ICS levels correlated negatively with exacerbation rate, and (for FP only) positively with FEV_1 %predicted.

Conclusion: Commonly used ICS can be reliably detected in the blood at least 8hours after dosing. Higher exacerbation rates and poorer lung function (for FP) were associated with lower blood levels.

4.2 INTRODUCTION

Inhaled corticosteroids (ICS) are the cornerstone of treatment in asthma (5). Poor adherence to ICS is recognised in 30% to 70% of people with asthma (83, 312), and associated with exacerbations, hospital admissions, morbidity and declining lung function (313, 314). It is also considered one of the major causes of treatment failure (5), and adherence checks are therefore recommended before stepping up, and especially before considering treatment with monoclonal antibodies (4).

Although a significant concern in the management of asthma, measuring the level of adherence is challenging (25), with no method considered as a gold standard. Subjective measurements such as self-reporting show that patients tend to overestimate their adherence (315). Prescription refill rate represents an objective method whereby the number of collected doses in a specific period are calculated as a percentage of total amount prescribed (5, 316), with the patient usually considered adherent if the number of collected doses are between 80 and 100% (317, 318). The most obvious drawback of this method is that even with 100% pick-up, there is no way to confirm whether the patient has taken the treatment. Recently, electronic monitoring devices have been developed, including where the patient is provided with feedback (e.g. *via* a mobile application) (258). Such systems have shown to improve adherence and reduce the exacerbation rate in people with poorly controlled asthma (319).

Direct biological measurements of adherence (i.e. drug assay) could give complimentary information though measurement of drug exposure (320), although disease-specific data on pharmacokinetics are required to guide interpretation of results. High-performance

has been widely used as a method for sensitive and specific drug measurement in clinical laboratories, and has significant advantages over immunoassays (321). LC-MS has shown high accuracy in detecting the level of urinary fluticasone propionate (FP) in mild-moderate asthma (151).

In this study, we assessed the level of adherence by taking serum blood samples over an 8 hourstime period in patients with severe asthma treated with high dose ICS by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The secondary aim to identify any correlation between serum ICS concentration and markers of asthma severity. Preliminary results of this study have been reported in abstract form (322).

4.3 METHODS

Participants

Patients under the care of the regional severe asthma service at Wythenshawe Hospital, Manchester University NHS Foundation Trust were considered for recruitment. Inclusion criteria were: asthma diagnosis confirmed by severe asthma multidisciplinary team; treated with high dose ICS [1000mcg beclomethasone dipropionate (BDP)/day equivalent minimum) at a stable dose for at least two months. Exclusion criteria were: patients unwilling or unable to participate in the study. Ethical and R&D approvals were obtained (refs: M2014-23; IRAS107832; R&D study ID: 2005TM013) and patients provided written informed consent. Demographic and clinical data including smoking history, medications, exacerbation rate were collected from the patient and their clinical notes.

Study design

The study was conducted during a single visit over approximately 8.5 hours. At baseline (typically between 8 and 9am) spirometry and fractional exhaled nitric oxide (FeNO) were recorded, and a venous blood sample collected. Patients were then observed using their usual morning dose of ICS, and inhaler technique assessed by the researcher using a specific inhaler checklist (323). Repeat venous blood samples were collected at 1, 2, 4 and 8 hours post-inhalation.

Measurements

Exhaled nitric oxide

A Niox device (Aerocrine, Sweden) was used to measure FeNO at a constant flow rate of 50ml/s. Patients were asked to inhale deeply via mouthpiece connected to a screen and exhale for 10 seconds, with visual feedback provided to guide exhalation flow, in compliance with the ATS/ERS recommendations (248).

Spirometry

Spirometry was performed using a handheld digital spirometer (In2itive, Vitalograph, UK) which allows measurement of FEV_1 and FVC, stores data on the digital device and is synchronised to Vitalograph Spirotrac software. Digital spirometers were calibrated on every study day using a 3 L syringe to within ± 3%.

Adherence

Prescription refill records for all participants were collected from their General Practitioner (GP). It was calculated as the number of taken prescribed doses of medication divided by the actual number of doses prescribed and multiplied by 100. The optimal adherence rate was defined as the refill of more than 80% for the previous 6 months minimum (317, 318).

Venous blood sampling and storage

Venous blood was drawn *via* a size 22G cannula, into a 5 ml EDTA tube, then after 30-60 minutes centrifuged (centrifuge 5702; Eppendorf, Darmstadt, Germany) for 10 minutes at a

speed of 3500rpm at 20^{o}C. Extracted serum was transferred into 1.5ml cryotube vials and stored in -80°C freezer prior to analysis.

Inhaled corticosteroid analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Sample preparation

Steroid stock solutions (Alsachim, Illkirch-Grafenstaden, France) of 1 mg/mL were prepared by dissolving BDP, budesonide (BUD) and ciclesonide (CIC) in ultra-pure methanol, and fluticasone propionate (FP) and fluticasone furoate (FF) in dimethylsulfoxide (Fluka, Poole, UK) and stored at -20^{o}C for up to 6 months. Separate stock solutions were prepared for standards and quality controls (QCs). Working standards and weighed-in QCs were prepared by diluting the stock solution with phosphate buffered saline (PBS) pH 7.4 (Sigma, Poole, UK) containing 0.1% (w/v) bovine serum albumin (BSA, Sigma, Poole, UK) to give concentrations of 0-10,000 ng/L. Aliquots (300 μL) of these were stored at -30^{o}C for up to 6 months. In addition, serum QCs (12.5, 50, 200, 800 ng/L) were used to assess imprecision in matrixed samples. Deuterated steroids for use as internal standards (Alsachim, Illkirch-Grafenstaden, France) were prepared at a working concentration of 10 μg/L each in ultra-pure methanol.

Standard, QC or sample (250 μL), 20 μL of working internal standard and 100 μL de-ionised water were added to a supported liquid extraction plate (SLE+ Biotage, Uppsala, Sweden). After elution with dichloromethane into a deep well plate, the solvent was evaporated to dryness and the extract was reconstituted with 75 μL of 70% ultra-pure methanol/water. The plate was heat-sealed and transferred directly to the Acquity autosampler (Waters, Manchester, UK) for analysis, 30 μL of sample was injected.

Chromatography & solid phase extraction

The mobile phases utilised were: A. distilled water with 2 mmol/L ammonium acetate containing 01.% formic acid (Sigma, Poole, UK); and B. methanol (ultra pure grade, Fluka, Poole, UK). The analytical column used was a 2.1 x 50 mm 1.8 μm Waters Acquity UPLC HSS C18 SB column coupled to a Waters Vanguard filter. Steroids were eluted from the analytical column with a linear gradient starting from 50% mobile phase B at a flow rate of 0.4 mL/min, rising to 90%b over 2.5 minutes. After 2.5 minutes, the composition was stepped up to 100% B for 0.6 minutes before returning to starting conditions. Both guard and analytical columns were maintained at 50^{o}C. Run time injection to injection was 4.3 minutes.

Mass spectrometry

The eluate was injected directly into a XEVO^{TM} TQ-XS tandem mass spectrometer (Waters, Manchester, UK), using MassLynx NT 4.1 software for system control. The mass spectrometer was operated in electrospray positive mode, the capillary was maintained at 1.0 kV and the source temperature was 150^{o}C. The desolvation temperature and gas flow were 600^{o}C and 1000 L/hr respectively. The source offset was maintained at 50 V. The quantifier transitions identified were *m/z* 431.2> 323.3, *m/z* 465.2>279.2, *m/z* 501.2>313.2, *m/z* 539.2>313.2 and *m/z* 541.3>147.1 for BUD, BDP, FP, FF and CIC respectively. The internal standards had transitions of *m/z* 439.2>323.1, *m/z* 470.3>279.2, *m/z* 506.2>313.2, *m/z* 543.2>293.2 and *m/z* 548.2>323.2 for D8 BUD, D5 BDP, D5 FP, D5 FF and D7 CIC respectively. Transitions were monitored in multiple reaction monitoring (MRM) mode, with a dwell time of 0.025 seconds.

Elution times were as follows, BUD 2.2 minutes, BDP and FP 2.4 minutes, FF 2.6 minutes and CIC 3.05 minutes. Matrix effects were minimal and recoveries were 93%, 113%, 97%, 102%

and 103% for BUD, BDP, FF, FP and CIC respectively. Lower limit of quantitation was 10 ng/L for all steroids except for CIC which was 50 ng/L. All steroids were linear to at least 10,000 ng/L. Between-batch imprecision showed coefficient of variation (CV) less than 8% for all steroids except CIC at a concentration of 50 ng/L, CIC gave a CV of 6.6% at 200 ng/L.

Sample Size Calculation

The primary outcome of the study was to estimate the proportion of patients where ICS could be detected at 8 hours. Based on our previous study (20), expecting detectable ICS in 90% [95% confidence intervals (CI) ± 10%] at 8 hours, we would need a sample size of 62.

Statistical Methods

Data analyses were performed using SPSS software version 22 (IBM corporation, Chicago, IL, USA) and Prism version 7 for graphical figures (Graph Pad Software INC., La Jolla, CA, USA). Non-parametric data were expressed as median (Q1; Q3) and parametric as mean [standard deviation (SD)]. Serum concentration for FP, BUD, BDP, CIC, and FF were plotted against time. Pharmacokinetic (PK) properties (AUC_{0-8}), maximum concentration (C_{max}) and time to maximum concentration (T_{max}) were calculated. The trapezoid test determined the AUC. Non-normally distributed data were log-transformed to normalise where possible. Correlation (r) between continuous normally distributed data was performed using Pearson's correlation coefficient and if non-normally distributed Spearman's test. Partial coefficient were calculated for the relationship between PK (total AUC_{0-8}, incremental AUC_{1-8}, C_{max}, T_{max}) and clinical parameters (age, gender, BMI, exacerbation rate, and FEV_1 %predicted) after adjusting several potential confounding factors. The significant level (p) was set to <0.05.

Using repeated measurements for each patient, mixed linear models were applied to examine the actual changes from baseline serum concentration over time adjusted by the three main ICS inhaler-types (FP, BUD and BDP). All the data models were calculated using STATA V.13 and data are presented as means (95% CI).

4.4 RESULTS

Demographics

We recruited 60 patients with severe asthma, mean (SD) age 49.0 (12.3) years, 69% females and 65% patients prescribed maintenance (daily) oral corticosteroids. Poor adherence based on GP prescription refill rates (less than 80% within minimum six months) was identified in 47% (n=28) of the participants. Inhaled FP was used most commonly used (43%), while 23%, 15%, 11% and 8% were using BDP, BUD, CIC and FF respectively. Full demographics are presented in table 4.1.

Table 4. 1. Clinical characteristics of study participants

	All participants	ICS type					p-value (all)	p-value¥ (FP-BUD-BDP)
		FP	BUD	BDP	CIC	FF		
Subjects, n* (%)	60	28 (43%)	10 (15%)	15 (23%)	7 (11%)	5 (8%)	NA	NA
Female, n (%)	41 (69%)	19 (68%)	8 (80%)	9 (60%)	7 (100%)	3 (60%)	0.338	0.576
Age, years	49.1 ± 12.1	47.3 ± 13.7	52.7 ± 6.5	48.5± 12.4	52.0 ± 9.8	50.4 ± 14.3	0.741	0.495
BMI, kg/m²	30 (27-36)	33 (28-39)	28 (27-31)	27 (25-29)	29 (27-34)	34 (26-39)	0.027	0.006
Exacerbations, n in last year	2 (1-6)	3 (1-10)	3 (2-6)	1 (0-2)	1 (0-2)	1 (1-4)	0.010	0.011
Hospital admissions, n in last year	1 (1-4)	2 (1-4)	1 (1-4)	1 (1-3)	1 (1-4)	1 (1-4)	0.456	0.347
Daily prednisolone use, dose (Q1-Q3) mg/day, (n)	13 (8-20); (n=39)	7.5 (0-15) (n=18)	10 (5-20) (n=8)	5 (0-15) (n=9)	10 (5-15) (n=6)	20 (0-55) (n=2)	0.740	0.437
BDP equivalent daily ICS dose, median (IQR) mcg	2000 (1700-2000)	2000 (2000-4000)	2000	1600 (800-1600)	4000	2000	<0.001	<0.001
Spacer use, n	12	5	0	6	1	0	NA	NA
Poor inhaler technique, n	5	3	2	0	1	0	NA	NA

GP prescription refill %	80 (58-100)	77 (58-89)	80 (54-80)	75 (66-100)	92 (58-100)	92 (66-100)	0.686	0.750
FEV_1, mean ± SD % pred	63 ± 19.4	65 ± 18.3	61 ± 20.3	59 ± 25.1	53 ± 9.9	68 ± 11.1	0.529	0.587
FVC mean ± SD % pred	75 ± 20.0	73 ± 19.8	86 ± 20.4	71 ± 22.9	75 ± 22.3	72 ± 11.2	0.780	0.450
FeNO, median (IQR) ppb, (n)	24 (11-50), (n=43)	28 (8-41) (n=18)	20 (6-45), (n=4)	45 (14-76), (n=14)	33 (13-43), (n=7)	15 (8-28), (n=5)	0.515	0.392

*Five patients out of 60 were taking two types of ICS**.** ¥ p-value for comparison of FP,BUD and BDP inhalers only. Definition of abbreviation: BMI: body mass index; $FEV_{1:}$ forced expiratory volume in one second; FVC: forced vital capacity; FeNO: fractional exhaled nitric oxide. Data presented as mean (±SD) or median (Q1-Q3).

Inhaled corticosteroid detection in blood

At 8 hours blood ICS were detected in 96% of participants taking FP, 100% BUD, 100% BDP, 75% FF and 0% CIC. As the individual pharmacokinetics are ICS-specific, we present each separately.

Fluticasone propionate

Blood samples were collected from 27 of 28 individuals prescribed FP (one provided urine only; data not shown here); 74% (n=20) 1000mcg and 26% (n=7) 2000 mcg daily as either Seretide or Flixotide. Eighteen patients were on metered-dose inhaler (36), five with a spacer, and nine on dry powder inhaler (DPI). At baseline, typically 10-12 hours after previous dose and before witnessed ICS administration, we were able to detect serum FP in 21 participants (78%). Eight hours after witnessed ICS, FP was detectable in all but one (96%). Most of the participants (23/27) had peak serum concentration 1-2 h after FP administration (supplementary figure A4.1.a). Inhaled FP detection in serum of individuals over 8 hours is shown in supplementary table A4.1. We examined the level of serum concentration changes from baseline by linear mixed model (figure 4.1). The mean change at each time point after FP was above the LoD; however, the lower 95% CI at 4 and 8 post-ICS were below the LoD.

Poor inhaler technique was identified in three participants, two of whom had low serum concentration of FP compared to the other participants (supplementary figure A4.2). Four patients had <50% inhaler pick up, and a further 10 had 50-79% pick up; three of these 14 (21%) had undetectable baseline FP, *versus* three of the 13 (23%) with good (≥80% pick up) adherence.

Budesonide

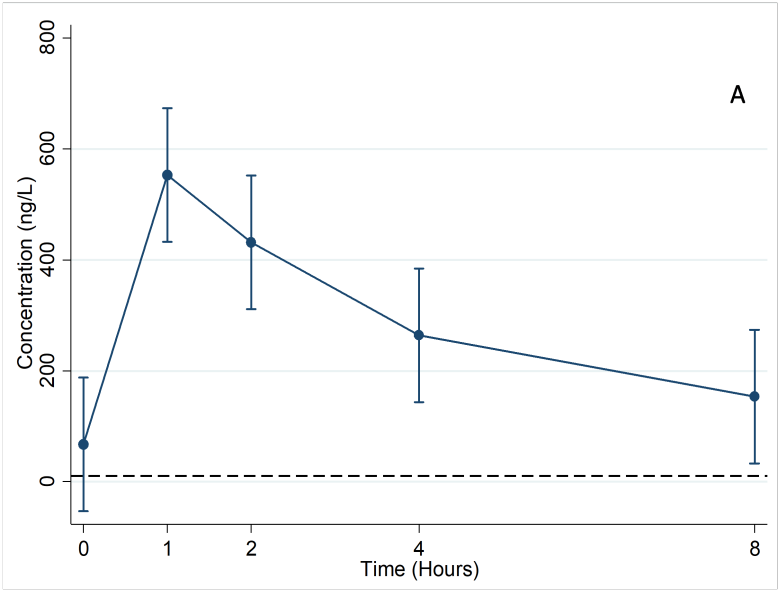
Nine individuals used DPI Symbicort (400/12) and one DPI DuoResp Spiromax (320/9). Seven (70%) had detectable BUD at baseline (pre-morning ICS dosing). Serum BUD was detected in all patients after 8 hours post-ICS dosing. The maximum concentration of serum BUD occurred one hour after inhalation (supplementary figure A4.1.b). From the mixed linear model, the lower 95% confidence limit was above the LoD at all time points (figure 4.1). Detection rates at each time point are shown in supplementary table A4.2.

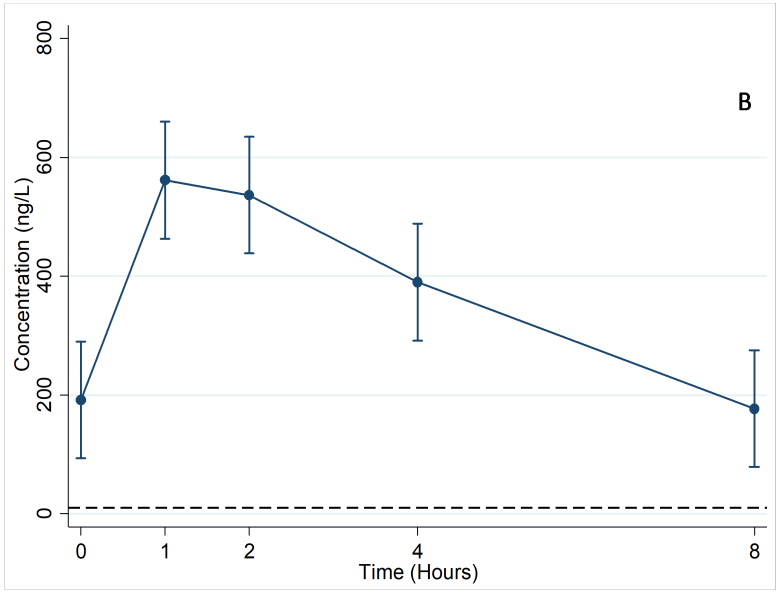
Two participants had poor inhaler technique with BUD (supplemental figure A4.2). A good (≥ 80%) prescription refill rate was observed in six participants, one of whom had undetectable baseline level. Two of the four subjects with low adherence (≤ 80%), had undetectable level at baseline.

Beclomethasone dipropionate

The majority of participants in the BDP group (11/15) used MDI Fostair®, three DPI Fostair NEXThaler®, and one Clenil ®. Serum ICS was not detected in one patient at baseline. Serum BDP was detectable in all participants 8 hours after using the inhaler. The highest concentration among most of the participants took place after 1 hour of using the medication (supplementary figure A4.1.c); serum detection of individuals over the eight-hour period is shown in supplementary table A4.3. Results of mixed linear modelling indicated the lower 95% CI was above the LoD (figure 4.1).

All participants taking BDP had acceptable inhaler technique. Satisfactory prescription refill rate (≥80%) was recorded in 46% (n=7). Undetectable baseline levels were noted in one patient (considered as adherent based on a prescription refill).


A
Concentration (ng/L)
0
200
400
600
800
0
1
2
4
8
Time (Hours)


B
Concentration (ng/L)
0
200
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Time (Hours)

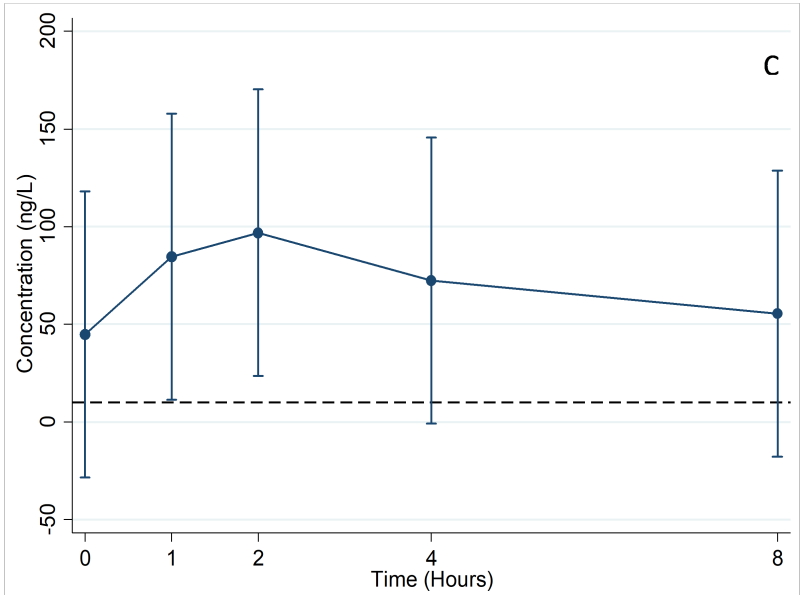
C
Concentration (ng/L)
-50
0
50
100
150
200
0
1
2
4
8
Time (Hours)

Figure 4. 1. Linear mixed model predictions for mean (95% Cls) serum concentration of: A. fluticasone propionate (FP); B. budesonide (BUD); and C. beclomethasone dipropionate (BDP). Dashed line indicates limit of detection.

Ciclesonide

Seven patients used MDI Alvesco at a dose of 80 or 160 mcg. Three patients used it along with another ICS (FP, BUD or BDP). We were not able to detect serum CIC at 8 hours post-ICS in any participant; one patient had a detectable level at baseline (supplementary table A4.4). Good inhaler technique was observed in all of patients using CIC. Four out of the seven patients were collecting 50-79% of their ICS prescriptions, and the rest collecting more than 80%.

Fluticasone furorate

All four patients were prescribed one daily puff of DPI Relvar (184/22). Fluticasone furorate was not detected at baseline in any samples, and in two patients after 8 hours (supplementary table A4.5).

All four patients had good inhaler technique; two collected <80% of prescriptions.
Due to low numbers and low levels of detection, CIC and FF were not included in further analyses.

Pharmacokinetics and adherence

We compared the pharmacokinetics [C_{max}, AUC, baseline concentration (C_{base})] between participants with a suboptimal rate of prescription refill with participants filling above 80% of prescription refill in the past 6 or 12 months. There were no differences between these two groups in any pharmacokinetic variables for each individual inhaler (supplementary table A4.6).

Serum pharmacokinetics and markers of asthma severity

Pharmacokinetic variables were log transformed to normalise prior to analysis. We investigated relationships between serum pharmacokinetics [C_{base}, AUC for FP, BUD and BDP both together (n = 52) and individually], and markers of asthma severity.

Baseline concentration

Higher log-C_{base} ICS concentration correlated with lower exacerbation frequency (figure 4.2), and with lower BMI and ICS dose (table 4.2). After adjustment for other covariates, none of these correlations remained significant. For FP there was a positive correlation (r = 0.384; p = 0.048) between serum concentration at baseline and FEV_1 % predicted (figure 4.3).

Area under the curve

The AUC for BDP and BUD was considerably higher than for FP (supplementary figure A4.3). Because of the strong association between the AUC and baseline levels for each inhaler we used incremental AUC (iAUC). This was calculated by subtracting the area under the baseline level. Higher iAUC correlated with lower BMI (supplementary figure A4.4), exacerbation rate (figure 4.2), and ICS dose (table 4.3). After adjustment, only correlation with morning ICS dose remained significant. The FEV_1 % predicted was associated with low iAUC concentration for FP individuals (r = 0.401; p=0.038) (illustrated in figure 4.3). This correlation remained significant after adjustment for covariates (r = 0.433; p= 0.044). No significant correlations of iAUC for BUD and BDP against asthma markers were identified as shown in table 4.3.

Table 4. 2. Correlation coefficients (p value) of $logC_{base}$ of participants using FP, BUD and BDP inhalers and markers of asthma

	All			FP	BUD	BDP
	Unadjusted	Adjusted¥				
Age	0.188 (p = 0.181)			**0.436 (p=0.023)**	0.405 (p=0.246)	-0.400 (p=0.139)
BMI	**-0.379 (p=0.006)**	Exacerbation	-0.156 (p=0280)	-0.378 (p=0.052)	-0.635 (p=0.066)	0.261 (p=0.348)
		ICS dose	-0.233 (p=0.103)			
Exacerbation	**-0.298 (p =0.032)**	BMI	-0.275 (p=0.053)	-0.249 (p=0.210)	-0.006 (p=0.986)	0.220 (p=0.431)
		ICS dose	-0.302 (p=0.031)			
FEV1	0.000 (p = 0.999)			**0.384 (p=0.048)**	-0.305 (p=0.391)	0.123 (p=0.704)
Prednisolone dose	-0.069 (p = 0.625)			0.006 (p=0.974)	-0.408 (p=0.242)	0.411 (p=0.128
Total daily ICS dose*	**-0.397 (p = 0.004)**	BMI	-0.184 (p=0.202)	-0.093 (p=0.645)	NA	0.164 (p=0.560)
		Exacerbation	-0.135 (p=0.345)			

*Dose converted to BDP equivalent. ¥ Adjusted correlation controlled by BMI, Exacerbation, and Total daily ICS dose. NA: all patient having the same dose

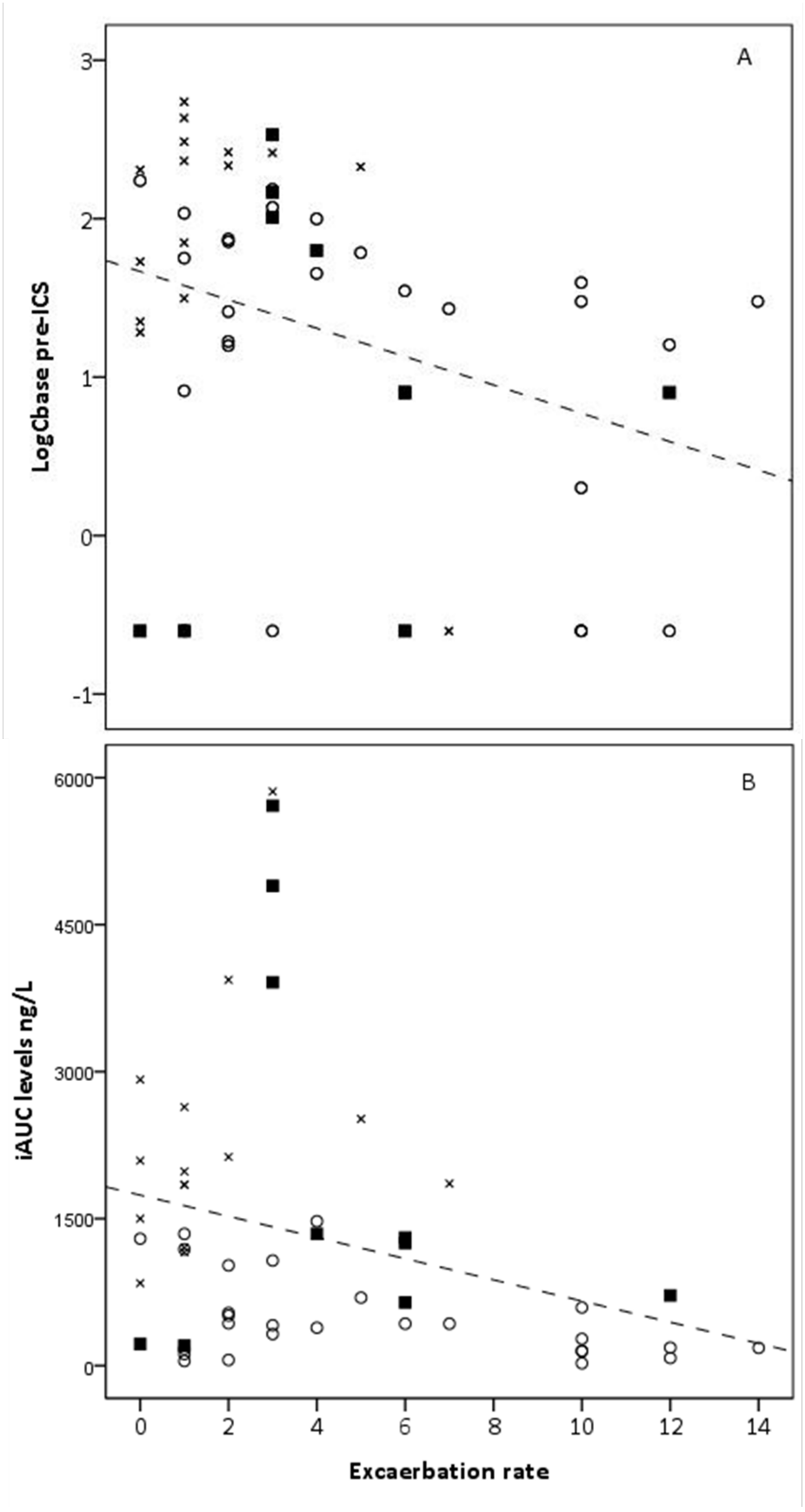

Figure 4. 2. Scatter plots showing the log-serum baseline concentration (Cbase) (A) and incremental area under the curve (iAUC, B) for FP (open circle), BUD (solid square) or BDP (X) against exacerbation rate ($logC_{base}$ r = -0.298; p = 0.032; iAUC r = -0.297 p = 0.032).

Table 4. 3. Correlation coefficients (p value) of iAUC of participants using FP, BUD and BDP inhalers and markers of asthma

	All			FP	BUD	BDP
	Unadjusted	Adjusted¥				
Age	0.191 (p= 0.174)			0.352 (p=0.072)	0.456 (p=0.185)	-0.307 (p=0.266)
BMI	**-0.488 (p=<0.001)**	Exacerbation	-0.217 (p=0.345)	-0.320 (p=0.353)	-0.613 (p=0.079)	-0.270 (p=0.331)
		ICS dose	**-0.308 (p=0.029)**			
Exacerbation	**-0.297 (p=0.032)**	BMI	-0.232 (p=0.105)	-0.320 (p=0.104)	0.043 (p=0.905)	0.401 (p=0.139)
		ICS dose	-0.257 (p=0.069)			
FEV1	0.078 (p=0.593)			**0.401 (p=0.038)**	-0.359 (p=0.309)	-0.385 (p=0.216)
Prednisolone dose	-0.103 (p=0.469)			0.018 (p=0.930)	-0.502 (p=0.140)	0.006 (p=0.984)
Total daily ICS dose*	**-0.644 (p=0.001)**	BMI	**-0.422 (p=0.002)**	-0.206 (p=0.302)	NA	0.279 (p=0.314)
		Exacerbation	**-0.377 (p=0.006)**			

*Dose converted to BDP equivalent. ¥ Adjusted correlation controlled by BMI, Exacerbation, and Total daily ICS dose. NA: all patient having the same dose

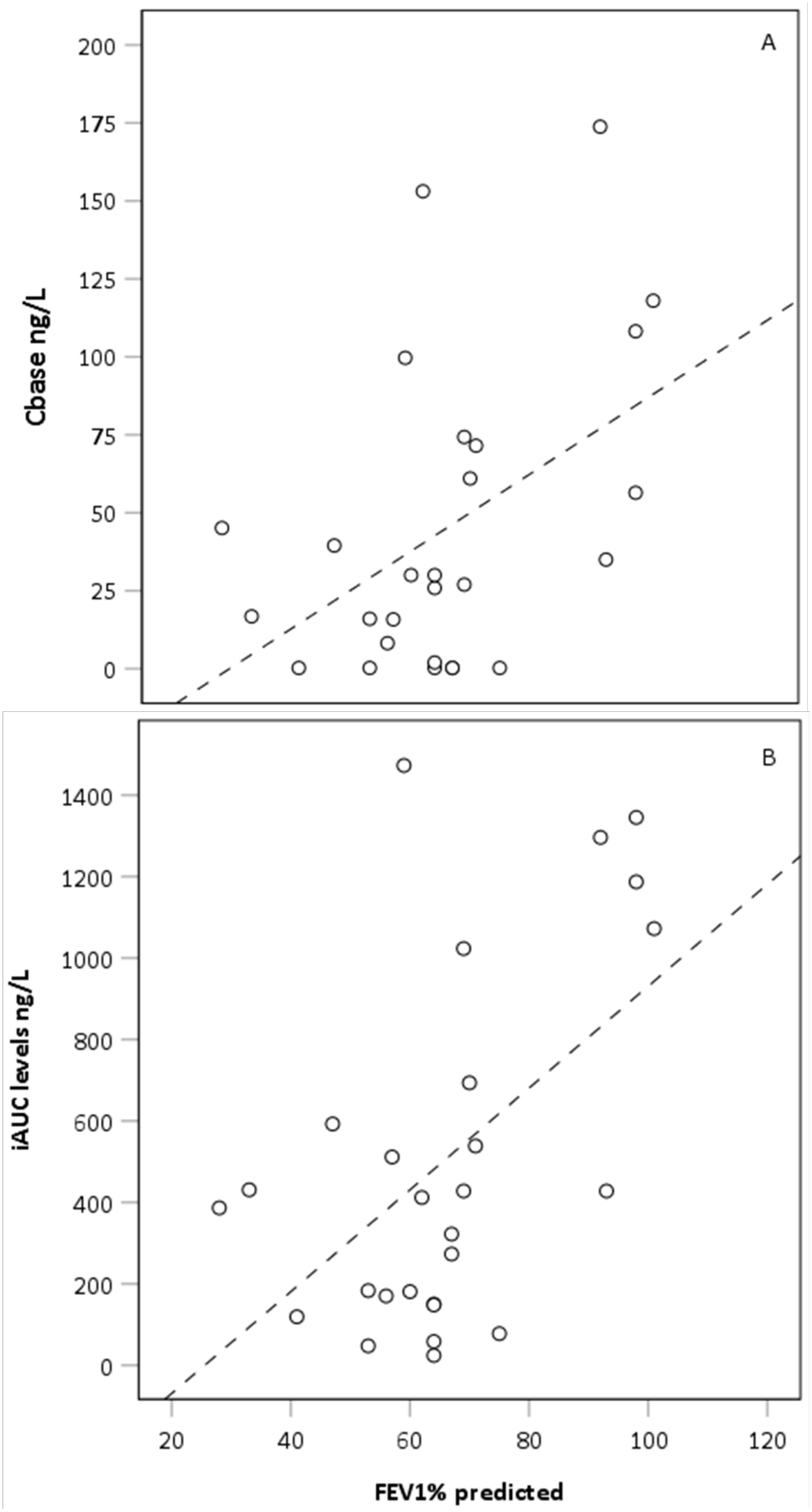

Figure 4. 3. Scatter plot showing baseline serum blood concentration (A) and iAUC (B) against % predicted FEV_1 % for FP (C_{base} r = 0.384; p = 0.048; iAUC r = 0.401; p = 0.038).

4.5 DISCUSSION

Whilst indirect methods, such as prescription refill or self-report diaries, have commonly been used to identify non-adherence to ICS in asthma, the only direct and therefore reliable way of determining ICS exposure is through detection in biological samples. In this observational study, we have examined the feasibility of using LC-MS/MS to detect ICS in serum after direct observation of drug administration. Up to 8 hours after inhalation, we were able to detect ICS in the serum of all patients using BUD and BDP, and all but one using FP. In contrast, we could not reliably detect CIC or FF in samples, although the number of participants taking these ICS was low. The lower 95% CI for predicated levels of BUD and BDP were above the LoD at 8 hours. The implication is that the test will have clinical utility in a typical daytime outpatient clinic; if the patient has undetectable serum ICS (BUD or BDP) there is a lower than 1 in 20 chance that they have taken their ICS that morning.

Reliable detection of ICS in the serum may have significant advantages over other methods of detecting adherence. Electronic monitoring devices such as the Inhaler Compliance Assessment Device (INCA) are gaining popularity in assessing adherence and currently considered the most accurate and reliable method (324). The INCA, unlike most other devices, is designed to detect inhalation as well as actuation, allowing an estimation to be made of whether each dose has been taken. However, the INCA relies upon an acoustic sensor and automated detection software, and could therefore be prone to false positives or negatives, where either an environmental sound has been picked up, or an inhalation sound missed. Further, the INCA device is only available for the DPI Diskus (325), and there are many different inhaler devices on the market for ICS that cannot currently be monitored (326, 327).

The most commonly used indirect assessments of adherence are prescription-refill counting and self-assessment. The former is only useful if the source of all prescriptions is traceable, and even then will be prone to overestimate adherence as no measure is made of actual dosing (320). Self-assessment is also known to overestimate adherence, although the responses to some questionnaires are able to give insights into possible reasons behind non-adherence in an individual and could therefore potentially guide intervention (303, 328, 329).

Unsurprisingly there was marked variability in serum pharmacokinetics between ICS-types and between patients, although the mean serum concentrations of FP, BUD and BDP were similar to previously reported (330-333). The main sources of variation in PK relevant to this study are likely to be deposition (lung *versus* oropharyngeal), oral bioavailability (the fraction of a drug that is not completely metabolised on first pass through the hepatic circulation), lipophilicity (and hence lung residency time) and volume of distribution(40). The higher detection rates for BUD and BDP that we have reported are likely a consequence of the interaction between a number of these factors. First, the oral bioavailability of FP, CIC and FF are similarly low (less than <1%), while in BUD and BDP are much higher (11% and ~15% respectively) (40, 52),meaning more drug can enter the circulation unchanged via this route. Second, FF and FP have significantly longer lipophilicity and lung residence time than BUD and BDP (52, 334), and will therefore have lower systemic bioavailability, at least during acute dosing. We did not measure any surrogate for corticosteroid toxicity, such as serum or urinary cortisol, as over half of our patients were prescribed maintenance systemic corticosteroids, which would likely have masked any suppression attributable to ICS.

We did not find any significant difference in pharmacokinetics between patients based on inhaler type (DPI or MDI) or spacer use. It may be that these make little absolute contribution to total systemic absorption in the context of the other pharmacokinetic factors, such that no difference was found in our relatively small sample size. Although we assessed inhaler technique, a poor technique was seen in so few patients that it is difficult to draw any conclusions. It is likely that a poor technique that results in more mouth deposition would result in particularly low blood levels for FF or FP, which have almost 100% first pass hepatic metabolism.

We found an inverse relationship between AUC concentration and annual exacerbation rate, both in the group overall (i.e. including BDP, BUD and FP) and in those taking FP alone. It is likely that the relationship with FP was dominant in the overall analysis, because in fact there was a positive relationship (albeit non-significant) for BUD and BDP when analysed individually. We also noted a positive correlation between FEV_1 and serum FP, and again the opposite (non-significant) relationship for BUD and BDP. This may be because, as noted above, serum FP is more reflective of lung deposition and absorption than for BUD and BDP. Two possible explanations could contribute to this relationship between higher blood FP, fewer exacerbations, and better FEV_1: 1. the higher systemic ICS exposure leads to better outcomes; or 2. patients with less airflow obstruction and less damaged airway epithelium absorb more drug. The first explanation may be less likely, as the relationship did not hold for BUD and BDP, which had much higher serum levels. The second is supported by previous studies that have also demonstrated a correlation between serum FP (but not BUD) levels and FEV_1 (335), and shown lower levels in asthma *versus* healthy controls (333).

Therapeutic drug monitoring is already in clinical use in many areas including epilepsy, cardiovascular disease, and infection, usually performed as in the present study by methods based on LC-MS. Although detecting drugs in this way requires time and expertise, and is potentially costly, it is still likely valuable in patients with severe disease, especially those being considered for expensive and/or potentially toxic treatments. Indeed, monitoring of oral corticosteroids (prednisolone) is already used [and recommended by GINA (4) as an adherence measure in severe asthma, even without any supporting data available such that we here provide for ICS (148, 260).

There were a number of limitations to our study. We were unable to recruit many patients taking CIC and FF, due to their infrequent use in the UK. In addition we did not measure the active metabolite of CIC, desisobutyryl-ciclesonide (des-CIC) (336, 337). Ciclesonide has a relatively short plasma half-life (40) and hence we were unable to detect it in any samples beyond 2 hours post-dosing, in line with previous findings (338). We were able to detect FF at later time-points but not reliably, probably due to its PK properties as previously mentioned, and the relatively low total daily dose of 184mcg.

We anticipated recruiting more than 60 patients within 12 months from the beginning of the recruitment. Nevertheless, the progression was slower than expected due to some reasons. Firstly, all of the blood samples were taken from specific venous cannula was inserted for the study purpose only. However, venous access for severe asthma patients was challenging. No more than one attempt was made and if unsuccessful then the patient was excluded from the study to minimise discomfort to the patients. Secondly, a few patients were suddenly requested to withdraw from the study after a few attempts of FeNO and lung function tests.

In conclusion we have shown that commonly used ICS (BUD, BDP, and potentially FP) can be reliably detected in the blood at least 8 hours after dosing and could therefore have a potential clinical application as a direct measure of adherence in severe asthma. Higher exacerbation. rates and poor lung function were found in those with lower blood levels of FP, likely due to lower lung absorption in such patients. Future work should look to improve the sensitivity of assay for drugs prescribed at lower doses, and include measurement of active metabolites such as des-CIC.

5 Serum inhaled corticosteroids detection and electronic monitoring of adherence

5.1 INTRODUCTION:

The adherence rate to inhaled corticosteroids (ICS) in severe asthma is low and associated with symptoms, poor lung function, high exacerbation rate and costs to health care systems (339-341). However, detection of non-adherence is challenging. Subjective methods such as patient self-report or healthcare practitioner estimation usually overestimate adherence (342), as does prescription refill-counting (343). Direct drug monitoring through biological sampling can confirm adherence, but consideration of individual drug pharmacokinetics is essential for interpretation of results.

High FeNO may indicate poor adherence to ICS and in this circumstance FeNO suppression testing may be considered. It involves daily home FeNO measurements with directly observed or recorded ICS administration for seven days. This test shows a high prediction in identifying nonadherence in patients with difficult asthma who are not responding to ICS (184, 186). In some centres it has been implemented as part of routine care for difficult asthma patients with poor asthma control and a high FeNO (≥45 ppb). We have previously reported serum detection of ICS as a potential marker of adherence over 8 hours post-dosing, and identified that patients prescribed long-term inhaled fluticasone propionate (FP) have considerably more variation in the baseline concentration (pre-ICS) than patients using either beclomethasone or budesonide, likely due to variable systemic accumulation of the drug, reflecting long-term adherence and inhaler technique. We also noted a correlation between higher ICS levels and better lung function / lower exacerbation rate. We hypothesised that this could either be due to higher levels of ICS in the blood reflecting higher drug deposition

in the lungs, and leading to better outcomes; or those with better lung function and less airway wall damage absorbing more drug.

We therefore sought to determine blood ICS levels in patients undergoing routine FeNO suppression testing to investigate if: 1. blood ICS levels increase over a week of monitored dosing; 2. markers of asthma severity are associated with blood ICS levels, as a surrogate for lung absorption; 3. baseline ICS levels, and change in ICS levels over one week, are associated with ICS efficacy over that week. During FeNO suppression FP is used, which has particular pharmacokinetic characteristics that make it a suitable drug for this study. First, it has high first-pass hepatic metabolism, thus serum levels largely reflect lung absorption (52). Second, there is less variability in levels over the four-hours window post-dosing compared to budesonide and beclomethasone dipropionate (322), which means the timing of sampling may not be so critical.

5.2 METHODS:

Study design and participants

This was a prospective observational study, involving participants with severe asthma attending the FeNO suppression clinic at Wythenshawe Hospital. Patients were referred with uncontrolled asthma symptoms despite high prescribed doses of ICS and elevated FeNO. If FeNO was at least 45 ppb and the other study criteria were fulfilled (BTS step 4 or above, 18 years or older, non-smoker), the researcher gave a written and verbal description of the study, and participants gave written informed consent. The study was approved by the South-East Exeter research ethics committee (REC reference: 18/SW/0058).

Comprehensive demographic and clinical data were gathered from each patient and their notes. Then, the asthma nurse instructed the patients how to use the Flixotide 500 Accuhaler and the patient was observed taking the first two puffs. Blood was then collected (within 30 minutes), followed by study tests repeated over 2 hours. At the baseline (30-60 minutes following ICS administration), several measurements were taken, including: FeNO, exhaled breath temperature (EBT), particles in exhaled air (PExA), exhaled volatile organic compounds (VOC), blood sampling and spirometry. Further selected measurements were repeated at 30, 60 and 120-minutes' post-baseline, illustrated in figure 5.1. The exhaled breath tests (EBT, VOC and PExA) were performed in order to investigate pharmacodynamic effects of inhaled FP. Further details are given in chapter 6. At the end of the first visit, patients were sent home with Flixotide Accuhaler to be taken two puffs in the morning along with a daily FeNO recording. At the second visit (day 7), patients returned to the hospital and further tests took place.

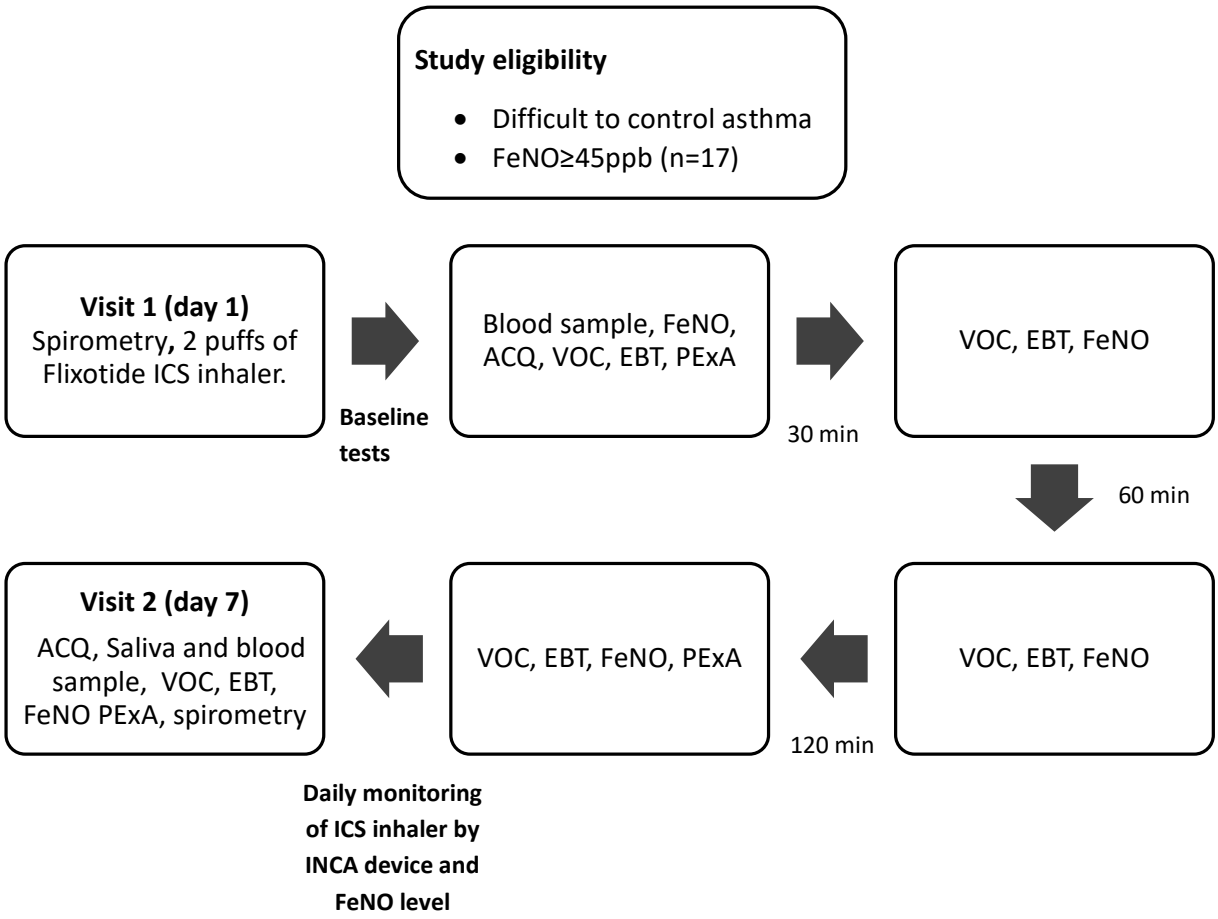

Figure 5. 1. Time and order of study procedures.

Assessing inhaler use by acoustic monitoring

We assessed the daily use of ICS by INCA technology developed by Costello et.al (Royal College of Surgeons in Ireland, Dublin), attached to an Accuhaler DPI (GlaxoSmithKline, Brentford, London, UK). The INCA is removable and contains a battery and microphone. At the time of opening the Accuhaler, a magnetic switch operates the INCA device, and date and time of opening is recorded. Once the activation begins, the device starts recording the sound by a microphone from the opening until the inhaler is closed or 90 seconds has elapsed, whichever happen first. The recorded audio files (.wav format) are stored in the INCA device. Participants were given the Accuhaler to be used as two puffs once a day for a week.

Once the participants returned the Accuhaler on day 7, the INCA device was removed from the inhaler by the asthma nurse or the researcher and connected to a computer to upload adherence data. In the device software, a bar chart is created to depict dose counts for each study day (see example appendix figure A5-1) as well as a scatter plot for inhaler technique (see example appendix figure A5-2). The software considers a good inhalation if all of the following occur: the inhaler being opened and primed, sufficient flow and time of inhalation followed by approximately 10 seconds breath-hold.

In addition to the audio files, several graphs were created to show the dose counts for each participant and the time and day of the inhaler and if good or poor technique was taken. In case of unsatisfactory technique registered by INCA software, the researcher listened to the audio files to cross-check the software registration of sounds.

Asthma control questionnaire (ACQ)

We used the ACQ-6, which consists of five items about asthma symptoms and one for reliever inhaler use in the preceding week (344). Patients describe the severity of symptoms based on a scale of 0-6 (the higher score is worse), with the sum score divided by 6. The most common cut-off for poor control is 1.5 (345, 346).

Fractional exhaled Nitric Oxide (FeNO)

FeNO was measured by using the portable nitric oxide analyser Niox Mino (Aerocrine AB, Solana, Sweden) at an exhalation flow rate of 0.05 L/s. FeNO measurements were performed according to the American Thoracic Society and European Respiratory recommendations (248).

Home FeNO monitoring

The INCA device was connected to a Flixotide 500 Accuhaler administered as two puffs daily. At the day of the enrolment (Day 1), we first instructed and then observed the patients in using the Niox Mino device. Each patient enrolled in this study was issued a FeNO machine with mouthpieces and carrying bag to perform a daily test. Patients were asked to use the daily Flixotide inhaler (two puffs) after each FeNO test until Day 6. Longitudinal measurements of FeNO were recorded along with the date and time of the test. At day 7, participants returned to the severe asthma clinic to attend the second visit and performed the last FeNO test without taking the Flixotide inhaler.

The FeNO suppression testing was considered as positive suppression if $Lg_{10}\Delta FeNO \geq 0.24$. This threshold reference number was established by McNichol et al in 2012 (184).

Formula for calculation of FeNO suppression:

Log10ΔFeNO = [mean (Log10 FeNO Day0, Log10FeNODay 1)] - [mean (Log10 FeNO Day 4, Log10 FeNO Day 5)].

if $Lg_{10}\Delta FeNO \geq 0.24$ (nonadherent); if $Lg_{10}\Delta FeNO < 0.24$ (adherent).

In other words, a positive test in identifying nonadherence where there was at least a 42% drop in FeNO level between the mean value at Day0/Day 1 *versus* Day4/Day5.

We ran the FeNO suppression test over 7 days, and a positive FeNO suppression level was definied in the same way, but usingdata from Day 6/Day 7 as the end data-point.

Blood Sampling

A venous blood sample was collected from patients to measure the level of ICS in serum. One blood sample was taken on each visit. The timing of blood sample in the first visit was after 30 minutes to 1 hour post ICS. For second visit, we asked the participants about the last time of inhaler was used, and cross-checked with the last actuation recorded by the INCA. Blood samples were processed and analysed as detailed in chapter 4.

Spirometry

Spirometry was measured by Vitalograph spirometer (Vitalograph, Maids Moreton, and Buckingham, UK). The test was performed when the participants were seated, and nose clips were placed. Patients were instructed to perform a full inspiration to total lung capacity and then a forced fast exhalation until residual volume as long as possible through the mouthpiece. Several repeated measurements were taken to record the FEV1, FVC, and FEV1/FVC ratio and the best one was recorded. Then predicated scores of FEV1 and FVC were calculated based on age, sex, race, and height.

Statistical analysis:

We performed statistical analyses using SPSS version 23 and Graphpad Prism version 8. Data were non-normally distributed and presented as median with interquartile range (Q1-Q3). Comparison between clinical measurements at day 1 and day 7 were performed by using Wilcoxon signed-rank test. Correlation between adherence data or levels of ICS and clinical severity markers were calculated using Spearman's correlation coefficient (r). Comparisons between positive and negative FeNO suppression groups were made using Mann-Whitney test.

5.3 RESULTS:

Demographics:

Twenty patients were recruited from the severe asthma clinic; two were withdrawn due to difficulties in performing the FeNO measurement and one due to anxiety. Table 5.1 shows the demographics of the remaining 17 patients. Of these 17 two subjects did not attend the second visit. In the second visit. Daily FeNO measurements are shown in appendix figure A5-3. There was an improvement in ACQ and FeNO, but not FEV1 or blood eosinophils, over seven days (table 5.2).

Adherence by INCA device

The median (IQR) adherence rate based on the INCA software analysis was 50 (14-86) % (table 5.3). Unsatisfactory adherence from the INCA software analysis (<70%) was identified in 10 (67%) of 15 participants. In light of an apparent discrepancy between FeNO suppression data and recorded doses we manually checked the sound files. For example in Appendix table A5-2 it can be seen that participant A5 experienced a 58% fall in FeNO and quadrupling of serum ICS concentration over a week, despite only having one correct dose recorded by the software. On manual checking of the audio files it was clear that the sound of an adequate inhalation had been missed by the software on 11 of the 12 dosing occasions. Manual checking of each file revealed missed sensing of at least one dose in all but three patients. Among the remaining 12 patients, five had taken all 12 correct doses by the aural evaluation, whereas they were counted below the half of prescribed doses by INCA software analysis. The INCA software analysis considered some correct doses as unsatisfactory due to: noise in the background, holding breath for more than 10 seconds, early closure of the inhaler before

the exhalation, and very quiet inhalation. For the rest of the analyses only data from the checked audio files were used.

Table 5. 1. demographic information of the study participants

Subjects, n	**17**
Male, n (%)	12 (70%)
Age, years	49 (37-57)
BMI, kg/m^2	27 (23-29)
FEV$_1$, % pred.	68 (53-84)
FVC, % pred.	79 (67-97)
Number of comorbidities	0 (0-1)
Exacerbations over the previous year, n	1 (0-1)
Daily ICS type, dose, (n)	FP, 1000mcg, (3) BDP, 800mcg (10) FF, 2000mcg (4)
Daily prednisolone dose, mg, n	10 (5-30) , 7
Smoking status, n	Ex-smoker 4 Non-smoker 13
Prescription refill, %	100 (58-100)

Data are expressed as median (interquartile range) or n (%). BMI: body mass index; FEV$_1$: forced expiratory volume in 1 second; FVC: forced vital capacity; FP: fluticasone propionate; BDP: beclomethasone dipropionate; FF: fluticasone furorate.

Table 5. 2. Clinical study measurements before and after FeNO suppression

	Day 1	**Day 7**	**p**
ACQ	2.8 (1.3-4.0)	1.2 (0.4-3.4)	**0.023**
FEV_1 % predicted	68 (53-84)	70 (49-86)	p=0.683
FeNO ppb	90 (58-117)	40 (18-61)	**<0.001**
EOS x10^9cells/L	0.39 (0.23-0.80)	0.44 (0.18-0.72)	p=0.859

All data are shown as median (IQR). ACQ: asthma control questionnaire; FEV1%: forced expiratory volume in 1 second; FeNO: fractional exhaled nitric oxide; EOS: blood eosinophils

Table 5. 3. Summary of adherence of each study participant by INCA software and manual (aural) checking

		Median (IQR)
INCA software data	**Missed doses**	0 (0-5)
	Technique error	5 (2-6)
	Device error	0 (0-1)
	Total number correct doses	6 (2-10)
	Adherence rate (%)	50 (14-86)
Audio file inspection	**Total number correct doses**	12 (6-12)
	Adherence rate (%)	100 (50-100)

Association between adherence (by INCA) and markers of severity

We investigated any correlation between the number of correct doses by aural inspection with patient characteristics and severity markers (table 5.4). There was an association between number of comorbidities and INCA-adherence. We also noted a correlation between adherence and better asthma control at day 7, but with no other markers of severity at day 7, or indeed with change in these markers over the week of treatment (table 5.5)

Table 5. 4. Spearman's correlation coefficient (r; p) between asthma characteristics, severity markers and adherence measures by INCA device

	INCA-confirmed doses taken
Age	r=0.509; p=0.052
BMI	r=-0.285; p=0.303
Exacerbation rate	r=-0.294; p=0.287
Number of comorbidities	**r=-0.593; p=0.020**
ACQ	r=-0.113; p=0.637
Prednisolone dose (n = 7)	r=-0.320; p=0.537
Prednisolone dose (n = 15)*	r=-0.017; p=0951
Prescription refill	r=0.299; p=0.279
FEV_1 %	r=-0.274; p=0.343

ACQ: asthma control questionnaire; BMI; body mass index; FEV_1 %: forced expiratory volume in 1 second. * including data from patients on 0 mg.

Table 5. 5. Correlation coefficient (r; p) between severity markers at day 7, change in severity markers over one week, and adherence

		Aural inspection correct dose
ACQ	**Day 7**	**-0.536; p=0.039**
	% change	-0.204; p=0.466
	Absolute change	-0.102; p=0.717
EOS	**Day 7**	0.097; p=0.790
	% change	-0.118; p=0.746
	Absolute change	-0.003; p=0.992
FEV1%	**Day 7**	-0.295; p=0.285
	% change	-0.360; p=0.206
	Absolute change	-0.362; p=0.204
FeNO	**Day 7**	-0.077; p=0.786
	% change	-0.102; p-0.718
	Absolute change	-0.122; p=0.644

ACQ: Asthma control questionnaire; EOS: blood eosinophils; FEV_1%: forced expiratory volume in 1 second; FeNO: fractional of exhaled nitric oxide.

Monitoring FeNO suppression testing

We dichotomised into "positive" and "negative" suppression (reduction in FeNO by <42%) according to (184) (figure 5.2). Median (IQR) baseline FeNO was 113 (64-139) ppb in the "positive" and 69 (59-96) in the "negative" groups (Mann-Whitney p=0.232). Baseline ACQ was higher in those with positive FeNO suppression (p=0.041), although ACQ improved in both groups by 50% over the week (table 5.6). Other notable, although not statistically significant, findings in the "positive suppression" group also suggest that these might have more severe disease: they had worse lung function and higher daily prednisolone dose; they also took more correct doses of ICS than the negative group.

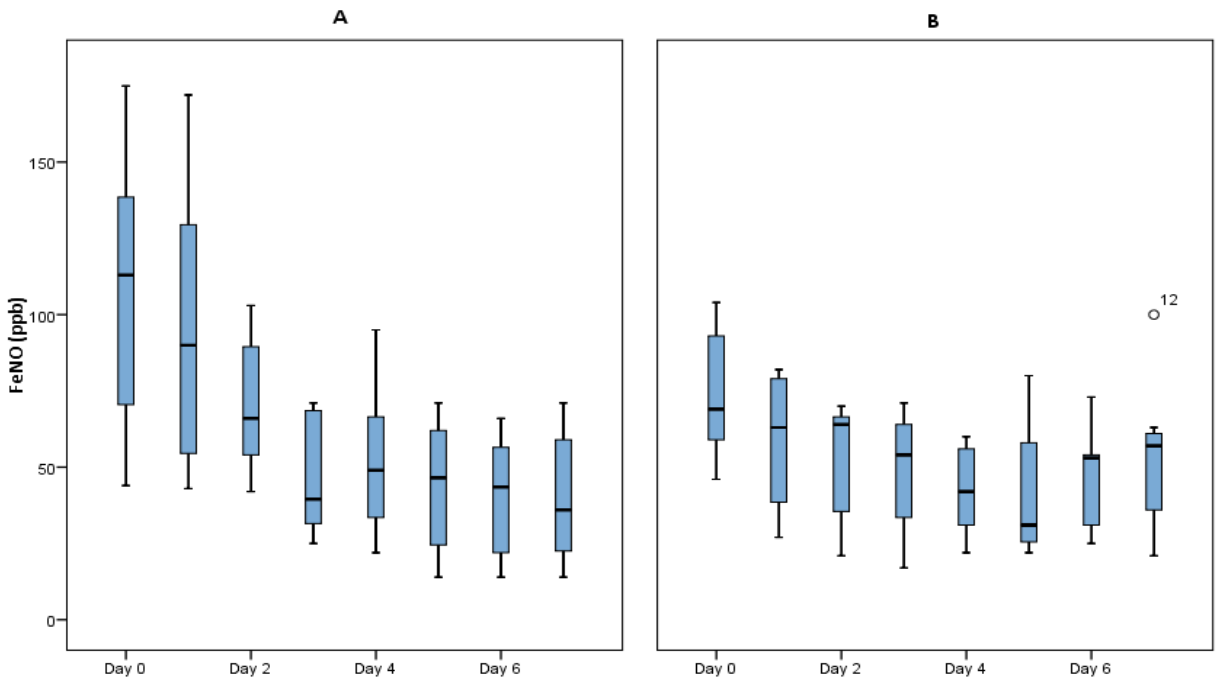

Figure 5. 2. Box-plot diagram showing the Fractional exhaled nitric oxide (FeNO) levels for 7 days between patients with a positive suppression test (A, n=8) and negative suppression test (B, n=7).

Table 5. 6. Severity markers comparisons between subjects performing FeNO suppression test.

	Positive FeNO suppression test (n=8)	Negative FeNO suppression test (n=7)	p Value
BMI	26 (21-32)	26 (25-28)	0.536
Exacerbation	1 (0-2)	1 (0-1)	0.779
ACQ baseline	**4.0 (1.9-4.6)**	**2.4 (0.2-3.2)**	**0.040**
ACQ day 7	2.0 (0.4-4.2)	1.2 (0.4-2.6)	0.536
Prednisolone, n	4	3	0.398
Prednisolone dose (n = 7)	20 (7-30)	6 (5-6)	0.267
Prednisolone dose (n = 15)*	3 (0-25)	0 (0-5)	0.536
Prescription refill	100 (45-100)	80 (66-100)	0.867
FEV_1 %	63 (46-68)	75 (58-96)	0.165
FVC%	74 (67-79)	83 (56-108)	0.318
Correct doses by INCA	6 (4-10)	6 (2-12)	0.867
Correct doses by inspection audio files	12 (8-12)	6 (0-12)	0.152

All data are shown as median (IQR); ACQ: asthma control questionnaire; FEV_1: forced expiratory volume in 1 second; FVC: forced vital capacity. * including data from patients on 0 mg.

Inhaled corticosteroid detection

Serum FP was detected in all participants at both visits (Appendix table A5-2). There was a negative correlation between the timing of the last dose and the concentration of ICS in serum at day 7 (r= -0.640, p=0.010, figure 5.3).

The median (IQR) of serum concentration of FP at the second visit was 1082 (586-1878) ng/L *versus* 561 (342-674) ng/L at the first visit (p= 0.020, figure 5.4). All patients except three had a greater than 50% increase in serum FP concentration by the second visit. The median (IQR) of patients having received ≥80% of ICS doses was 1651 (862-2257) ng/L and if those receiving <80% was 653 (451-992) ng/L (p= 0.026). The detected serum levels at day 7 was positively associated with number of ICS doses taken within one week (r=0.611, p=0.015, see figure A5-5)

Correlations between asthma severity markers and serum FP concentration are shown in tables 5.7 and 5.8. There was a significant correlation between the change in serum levels (%) and the predicted FEV_1 (r=-0.574; p=0.032). Further, a significant association was found between the ICS concentration at day 1 and the level of change (%) of ACQ (r=-0.554; p=0.032, see Figure 5.6)

Table 5. 7. Relationship between baseline asthma severity markers and serum concentration of FP

	Baseline serum concentration	**Day 7 serum concentration**	**Change of serum concentration (%)**	**Change of serum concentration (absolute)**
Age	r=-0.509; p=0.316	r=-0.357; p=0.191	r=0.468; p=0.079	r=0.439; p=0.101
BMI	r=0.007; p=0.303	r=-0.118; p=0.676	r=-0.168; p=0.550	r=-0.043; p=0.879
Exacerbation rate	r=0.003; p=0.992	r=-0.029; p=0.919	r=-0.179; p=0.522	r=0.092; p=0.745
Number of comorbidities	r=-0.117; p=0.654	r=-0.077; p=0.784	r=-0.187; p=0.503	r=0.014; p=0.960
ACQ	r=0.351; p=0.182	r=0.106; p=0.708	r=0.013; p=0.965	r=0.061; p=0.829
Prednisolone dose (n =7)	r=0.606; p=0.149	r=-0.147 p=0.781	r=-0.294; p=0.571	r=-0.294; p=0.571
Prednisolone dose (n = 15)*	r=-0.169; p=0.516	r=0.394; p=0.146	r=0.267; p=0.337	r=0.313; p=0.256
Prescription refill	r=-0.272 p=0.327	r=-0.149 p=0.595	r=0.272; p=0.327	r=0.144; p=0.610
FEV_1 %	r=0.193; p=0.491	r=-0.468; p=0.091	**r=-0.574; p=0.032**	r=-0.371; p=0.191

ACQ: asthma control questionnaire; BMI; body mass index; FEV_1 %: forced expiratory volume in 1 second. * including data from patients on 0 mg.

Table 5. 8. Correlation coefficient (r; p) between severity markers at day 7, change in severity markers over one week, and serum FP levels

		Baseline serum concentration	Day 7 serum concentration	Change of serum concentration (%)	Change of serum concentration (absolute)
ACQ	**Day 7**	-0.007; p=0.980	-0.395; p=0.145	-0.386; p=0.155	-0.392; p=0.149
	% change	**-0.554; p=0.032**	-0.421; p=0.118	-0.296; p=0.283	-0.450; p=0.092
	Absolute change	-0.422; p=0.117	-0.299; p=0.279	-0.147; p=0.602	-0.336; p=0.220
EOS	**Day 7**	-0.055; p=0.881	-0.370; p=0.293	-0.309; p=0.385	-0.297; p=0.405
	% change	-0.091; p=0.803	-0.236; p=0.413	-0.248; p=0.489	-0.370; p=0.293
	Absolute change	-0.055; p=0.881	-0.292; p=0.413	-0.249; p=0.487	-0.407; p=0.243
FEV1%	**Day 7**	0.394; p=0.131	-0.420; p=0.111	-0.446; p=0.446	-0.400; p=0.140
	% change	0.024; p=0.935	-0.182; p=0.551	-0.156; p=0.594	-0.200; p=0.493
	Absolute change	-0.051; p=0.863	-0.174; p=0.551	-0.152; p=0.603	-0.223; p=0.444
FeNO	**Day 7**	0.194; p=0.456	0.367; p=0.179	0.268; p=0.334	0.274; p=0.324
	% change	0.216; p=0.439	0.086; p=0.761	0.029; p=0.919	0.055; p=0.845
	Absolute change	0.186; p=0.508	-0.307; p=0.265	-0.307; p=0.265	-0.214;p= 0.443

ACQ: Asthma control questionnaire; EOS: blood eosinophils; FEV_1%: forced expiratory volume in 1 second; FeNO: fractional of exhaled nitric oxide.

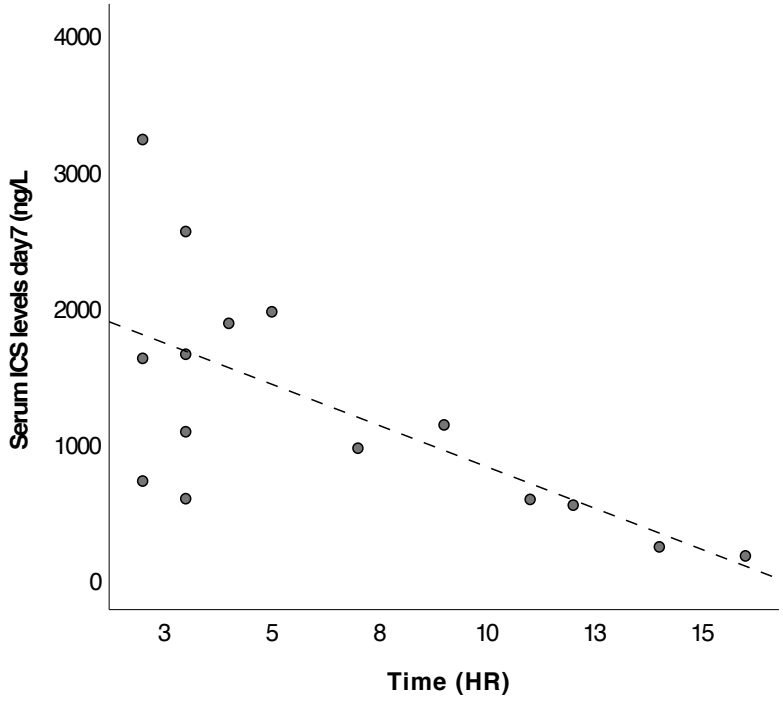

Figure 5. 3. Scatter plot showing the correlation between timing of the last FP dose taken *versus* serum concentration at day 7 (r= -0.640, p=0.010).

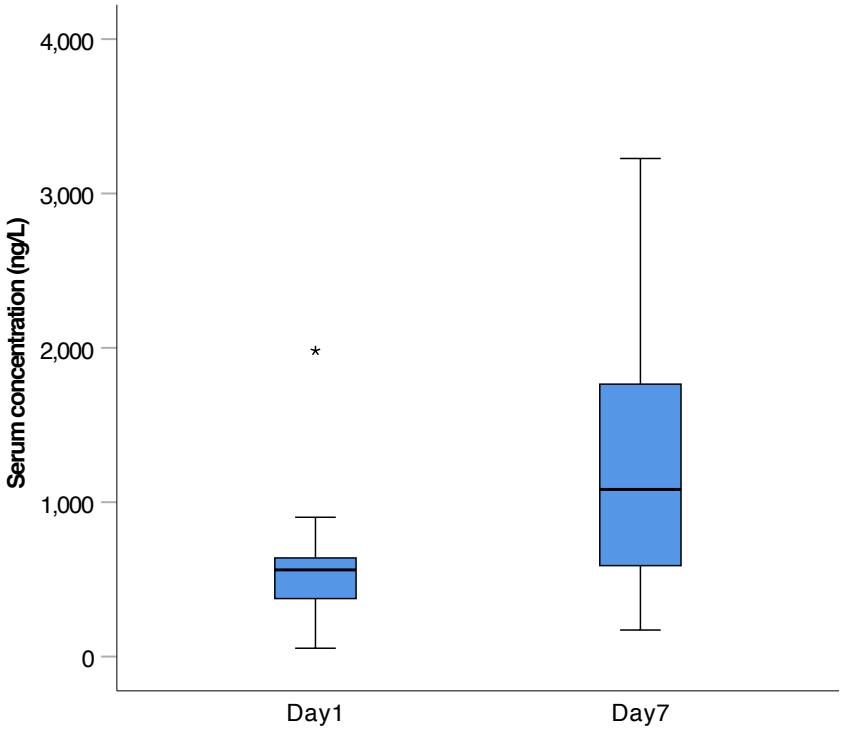

Figure 5. 4. Box plot of serum concentration at the baseline visit (day 1) and second visit (day 7) (p=0.020).

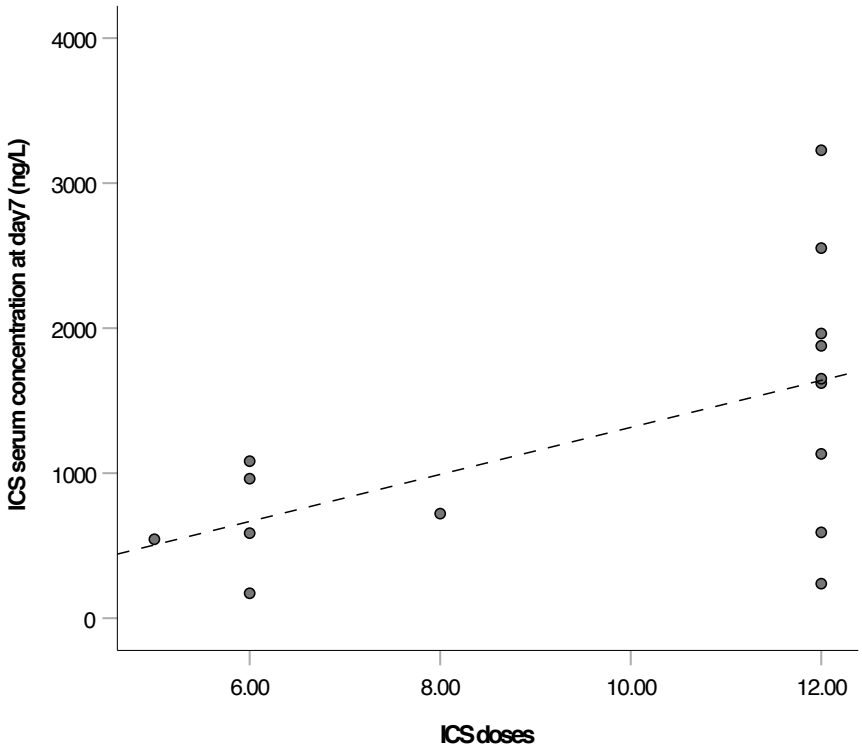

Figure 5. 5. Scatter plot showing the correlation between the number of doses were taken over one week *versus* serum concentration at day 7 (r=0.611; p=0.015).

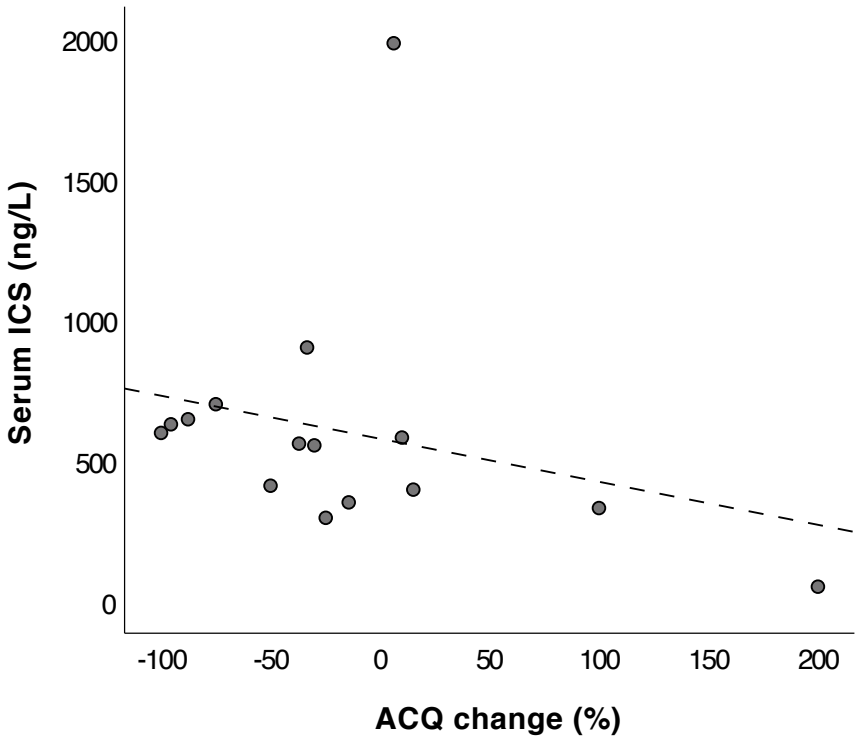

Figure 5. 6. Scatter plot showing the correlation between ACQ change (%) *versus* serum concentration at day 1 (r=-0.554; p=0.032).

5.4 DISCUSSION:

In the current study, for the first time, we have reported blood concentration of FP over one week of treatment in parallel to an electronic monitoring method for confirming adherence. Serum FP was detectable at both visits in all patients and we found that there was a significant increase in ICS concentration after one week of ICS use. The ICS serum concentration at the second visit was correlated to number of inhalations taken over the week, and the time since the last dose taken, but not to the level of FeNO suppression. We also demonstrated that higher ICS concentrations in the blood after the first dose were associated with higher improvement of asthma control over a week. Direct drug monitoring in the blood in patients using inhaled FP could be clinically useful in assessing the level of adherence.

We found approximately 2.5-fold higher median serum FP levels after one week in those taking at least 80% of ICS doses than in those taking less than 80%. The average timing of post-dosing blood sampling for the first visit and 2nd visit was 1 hour and 6.5hours respectively. Form our previous study (322), serum FP concentration peaked at approximately 2 hours and cleared within 4-8 hours. However, in the current study we found that ICS concentrations of median sampling time 6.5 hours (at day 7) were much higher from 1 hour sampling time (at day 1). These discrepancies suggest that repeated FP doses may accumulate in the blood over days; similar findings have been reported with FP over four weeks (347). Whelan et al. have found that serum FP levels almost doubled between one and six weeks after initiation (348).

It has been indicated from previous studies that worse lung function is associated with lower blood ICS levels (150, 331). We did not find the same in the current study, but noted that people with worse lung function at the baseline visit had a subsequent greater change in serum concentration over seven days. One possible explanation is that there is a greater improvement in lung absorption as the ICS leads to improvements in small airway lung function and inflammation over a week, which is likely to happen to a greater extent in those with more impaired airways than in those who have relatively normal airways at baseline. Whilst we did not see improvement in lung function over one week, we did see an improvement in inflammation (FeNO).

Another important association was found between higher serum levels at baseline with greater improvement of ACQ. This finding suggests that better ICS absorption (as a surrogate for lung deposition) may predict subsequent changes in asthma control. Furthermore, better asthma control at the end of the study was also associated with more frequent use of ICS. This association was expected as several studies have addressed the effect of daily ICS use on controlling asthma symptoms (349, 350).

Although, the FeNO suppression test with remote dose monitoring may be a useful test to identify patients with poor adherence (184), Heaney et al. found the daily use of INCA inhaler and FeNO test was not possible in almost 40% of the study participants, due to forgetting to do daily FeNO, or critical missing of daily ICS doses (186). In our study, two (12%) of the participants could not complete the test, because the forgot to measure daily FeNO. We also identified those with positive FeNO suppression had taken double the number of correct doses compared to negative suppression group. Symptoms in both groups improved by around 50% over one week (not statistically significant), but there was no suggestion of

improvement in lung function and blood eosinophils. The “FeNO suppressors” had markers suggestive of worse asthma severity at baseline, including higher FeNO, prednisolone dose, ACQ and lower lung function, compared to unsuppressed subjects. Suppression in this case could therefore be because of previous poor adherence, or relative corticosteroid insensitivity requiring very high doses of ICS. Further work is needed to understand better why those with a “negative” suppression test still had an apparent improvement in asthma control with little FeNO reduction. it might that the proposed threshold for FeNO suppression (42%) cannot accurately distinguish between negative and positive individuals, as still there was moderate drop in FeNO levels in most of those with a “negative” test.

In this study younger age appeared to associated with poorer ICS adherence, as has been previously reported (351, 352). However, it was also noted asthmatics above 50 years age poor adherence is common (353). Therefore, there is no specific or typical age associated with patient poor adherence. We also identified patients having multiple diseases were not using daily ICS medications. In asthma the presence of multiple comorbidities have been previously shown to influence treatment use and disease control (354).

The INCA software analysis program generates an audio file to evaluate if the patients use their inhaler correctly. This process identifies specific events from the acoustic files including blistering and breath sounds (exhalation and inhalation); it then calculates the number of doses taken correctly (355). Failure in performance in any of these stages will be detected as technique error. Thus, in this study and Heaney et al., adherence was assessed by audio file inspection rather than the INCA software analysis. This was done due to several limitations of the INCA software. The software registered many good inhalations (assessed manually) as a technique error and therefore unsatisfactory. Disagreements between the human rater and

the software were identified previously at the time of INCA development (355), where it was reported that the INCA algorithm detected around 83% of the correct doses checked manually. In our study, the INCA software detected correct doses was lower (65%), possibly because in the previous study a large number of participants already had experience in using the Accuhaler. Checking each audio file is time consuming and not feasible particularly in clinical settings or large-scale clinical trials.

Our study has some limitations; this includes the relatively small sample size. The exact timing of the blood sampling was variable among participants in both visits. We had assumed before starting the study that the timing of final ICS dose pre-sampling would be relatively similar in all participants at the second visit, when in fact we found significant variability, which will affect the interpretation of the day 7 serum level. Surprisingly, the median concentration of FP in serum was much higher when compared to the previous studies (150, 322, 331, 347), and this might be due some limitations of blood assays method that been developed. If we had measured the "background" ICS concentration before ICS use at the baseline visit, it may add some explanation to the elevated ICS levels. Background measurements might rule out any potential limitation of blood assays that we developed. For example, if the levels were below or equal to the LoD, then, we can rule out that there was any limitation. Moreover, the pre-ICS level can estimate the absorption rate of ICS, and later we could compare to severity markers. Since we have multiple comparisons, false discovery rate (FDR) calculations should be used to rule out type I error. Finally, the electronic monitoring devices may have a positive impact on adherence, as participants are aware that they are being monitored ("white coat" adherence) (356).

In summary, we have demonstrated that FP concentration in serum increases with adequate daily use of ICS over one week. Direct blood monitoring method could be implemented with the current routine FeNO suppression clinic among different asthma services to predict adherence level accurately. Further future studies with adequate patient numbers and other types and doses of ICS are required.

6 The effect of inhaled corticosteroids on exhaled breath biomarkers in severe asthma

6.1 INTRODUCTION

Asthma is the most common chronic inflammatory lung disease, and characterised by paroxysmal airway inflammation and smooth muscle hyper-responsiveness resulting in variable airway obstruction. Regular inhaled corticosteroids (ICS) and as required β_2-agonists are the primary therapy in managing and controlling asthma; however, responsiveness to treatment varies due to the heterogeneity of the disease.

Since several different inflammatory phenotypes have been identified in asthma, pathological and clinical features have been investigated in regards to ICS response. For example, it has been shown that after the ICS administration, patients have reduced levels of sputum eosinophils (357, 358), which then increase once treatment is withdrawn (359) or stepped down (360). Recently, Fractional exhaled nitric oxide (FeNO) has been used to monitor ICS response in asthma at all levels of severity (248, 361, 362). The level of FeNO in mild asthma drops when corticosteroid medication is administered and rises when steroids are discontinued (182). On the other hand, FeNO levels in severe asthma may remain elevated even in patients prescribed high dose ICS (183), indicating non-adherence or, less commonly, relative steroid insensitivity. After the promising results of FeNO as a biomarker of ICS response, there is a great interest in other non-invasive biomarkers that can easily predict steroid treatment responsiveness and aid in phenotyping asthma.

One of the features of airway inflammation is increased bronchial blood flow, caused by mediators such as histamine, bradykinin, and nitric oxide. Asthmatics have a higher exhaled breath temperature (EBT) when compared to healthy controls (363). EBT has been evaluated

to assess steroid response in asthma, although the results are inconclusive, with a non-significant reduction in breath temperature 30 minutes post-ICS administration (203).

More recently, the measurement of Particles in Exhaled Air (PExA) has been developed as a non-invasive tool by Almstrand et al*.,* (213), sampling particles which arise from the peripheral airways. An exhaled aerosol containing liquid particles of respiratory tract lining fluid (RTLF) is formed in the small airways when performing a breathing manoeuvre that induces airway closure and re-opening (364-366). In a recent study, asthmatics exposed to birch pollen to induce inflammation were found to have a reduction in exhaled particle numbers after the exposure (367), postulated to be due to the adverse effect of inflammation on the repeated small airway opening and closure that is thought to occur during the manoeuvre.

Exhaled volatile organic compounds (VOC) that originate from both endogenous and exogenous sources may provide an alternative method for monitoring airway inflammation (368) and assessing asthma control (369). Schee et al. studied the response to oral prednisone in patients with mild/moderate asthma and found that VOC patterns differed between steroid-responsive and steroid-unresponsive patients (370). Analysis of exhaled VOCs could therefore identify inhaled steroid response and be used to assess ICS adherence.

In this study, our objective was to investigate whether exhaled breath tests can be used as surrogate markers of ICS response in severe asthma patients by using a range of methodologies FeNO, EBT, PExA and VOCs.

6.2 METHODS:

Study design and setting

The design of the study was prospective. We recruited severe asthma patients with elevated FeNO (≥45ppb) attending the FeNO suppression clinic (see chapter 5) at Wythenshawe Hospital, Manchester. All participants gave written informed consent. Th study received ethical approval from the South-East Exeter research ethics committee (REC reference: 18/SW/0058).

The study was conducted during two visits over one week. At the baseline visit, full medical history was obtained. Then, each patient inhaled two puffs of Flixotide 500 Accuhaler, followed by measurement of FeNO, EBT, VOC, and PExA. These exhaled measurements were repeated after 30, 60 and 120 minutes post-baseline. All FeNO, VOC, and EBT measurements were done prior to spirometry and PExA tests to avoid any effect of the forced exhalation manoeuvres on the results (371). The full study design and procedures were explained previously in chapter 5.

Clinical tests performed in this project

The following tests were performed at each study visit:

Breathing tests

Fractional exhaled Nitric Oxide (FeNO)

Described in chapter 5

Exhaled breath temperature (EBT)

Exhaled breath temperature was measured by the X-Halo device (Delmedica) which has been validated by Poppa et al. (372). Before EBT measurements were performed, body temperature and room temperature were recorded. During sampling the device was connected through a cable to a mobile phone loaded with the relevant application and connected to the internet. The manufacturer of X-Halo recommended that the device should not be used immediately after consuming any food, so patients were asked to avoid eating for 1 hour before the baseline test (373). Sampling was conducted according to the following procedure: patients were requested to inhale freely through their nose and exhale through the mouthpiece of the device whilst maintaining a normal tidal breathing rhythm. During sampling they were instructed to keep their mouth on the sampling nozzle until the device indicated the test was complete. The period of the manoeuvre was approximately 1 min.

Exhaled Volatile Organic Compounds (VOCs)

Exhaled VOC samples were collected from each subject by using the Respiration Collector for In Vitro Analysis (ReCIVA ®) (Owlstone, Cambridge, UK). The device is connected with a Clean Air Supply Pump for ReCIVA (CASPER) which provides a continuous flow of 40 L min^{-1} of VOC free air to the ReCIVA. During sampling, the ReCIVA directed the airflow from the exhaled breath onto four sorbent tubes packed with Carbograph 1TD/Carbograph 5TD (Markes International, Llantrisant, UK). The ReCIVA allows the collection of a specific breath fraction by constant monitoring of the pressure inside the mask. This allows only the end tidal portion of breath to be collected, minimising contamination from the mouth and airway deadspace. Prior to use thermal desorption (TD) tubes were conditioned using a TC20 (Markes

International, Llantrisant, UK) for 60 min at 320 °C. The TD tubes were sealed before and after sampling to prevent contamination with exogenous compounds.

Before sampling, background air samples were collected to assess any VOC contamination from the breath sampling setup (e.g. CASPER air supply or the face mask). This reference sample was collected by strapping the ReCIVA to a glass head and setting the pumps to "always on" allowing the collection of 500 ml of gas at a flow rate 200 ml min^{-1}.

During sampling patients were advised to perform regular tidal breathing to allow the collection of 500 ml of gas at a flow rate 200 ml min^{-1} which took between 6 and 10 minutes depending on breathing rate. Once the sampling was completed the sorbent tubes were removed from the ReCIVA to be recorded on the CRF and stored immediately in the refrigerator at 4 °C. The analysis and storage of the tubes were done at the University of Manchester by Thermal Desorption – Gas Chromatography – Mass-Spectrometry (TD-GC-MS).

Particles in Exhaled Air (PExA)

The PEx mass was collected using PExA 2.0 device (PExA, Gothenburg, Sweden). All of the participants were trained to do the PExA breath manoeuvre. Firstly, participants wore nose clips and breathed normally in and out via a mouthpiece connected to a two-way valve in the PExA instrument. After that, patients emptied their lungs through a deep exhalation until the residual volume (RV) and held their breath for five seconds. Patients then inspired rapidly until their total lung capacity and then breathed out steadily (maximum exhalation flow of 2000 L/s). Patients were asked to breathe normally for 2 minutes until the next breath sample.

During this time they were provided filtered air to prevent any contamination from the ambient air. Patients were asked to repeat this manoeuvre until a target mass of PEx had been collected. Normally 10 to 15 manoeuvres were performed by each participant to collect at least 50-100ng. However, there were a few patients who were unable to provide sufficient mass after 15 repeated manoeuvres; these individuals were not excluded from the PExA data. An example of expiratory flow and estimated exhaled particles that were collected in each breath manoeuvre can be seen in appendix figure A6-1.

SPA and albumin detection in PEx

All samples were kept in a cryotubes and stored at -80 ^{0}C until the analysis. After the last patient recruited, the samples were sent from North West Lung Research lab at Wythenshawe hospital to Sahlgrenska Academy, University of Gothenburg, Sweden for lipids and proteins analysis.

Chemical analysis and SPA and albumin assay

An extraction buffer of 10 mM of Phosphate-Buffered Saline (PBS) was prepared, it contains 1% Bovine serum albumin (BSA) w/v and 0.05% TWEEN® 20 (Thermo Scientific, Rockford, IL, USA). Next, a 140 µl of extraction buffer was pipetted into the half PEx filter and 160 µl to the whole PEx filter, this was followed by 400 rpm shaking for one hour at 37 °C (Eppendorf Thermomixer comfort, Eppendorf AG, Hamburg, Germany).

Further, we split the sample volume as follows; 40 µl for SP-A and albumin respectively, the rest was retained as a backup sample. Extracted PEx samples were stored at -20°C for one day

in the SP-A sample and two days for albumin prior their analysis. The analysis was conducted by the Enzyme-Linked Immunosorbent Assay (ELISA). Minor modifications were applied to the manufacturer's instructions for SP-A ELISA (BioVendor, Brno, Czech Republic) and a human albumin ELISA kit from Immunology Consultants Laboratory, Inc. (Portland, OR, USA) (374). Before analyzing the samples, an 80 µl assay dilution buffer was added to them. In order to unify the sample matrix, a 1:2 ratio was prepared between Extraction buffer and assay dilution buffer in all PEx samples, controls and standard samples.

The incubation period for SP-A lasted for two hours at 37 °C with 300 rpm of shaking, and for one hour for albumin samples at room temperature and 300 rpm of shaking. Finally, a nine minutes reaction time was given to each assay. The coefficient of variation (CV) for the SP-A was 0.5 ng/ml and was 0.9 for the albumin. The limit of quantification (LoQ) for the SP-A and albumin were 0.1 and 0.8 (ng/L) respectively.

Statistical analysis

We performed the statistical analysis using SPSS version 23 and Graphpad Prism version 8. Data were not normally distributed and presented as median with interquartile range (Q1-Q3). Comparison between repeated measurements at day one was done by Friedman test. Comparison between the mean of 2 hours measurements at day 1 vs day 7 were performed by using paired Wilcoxon test. Correlation were calculated using Spearman' correlation coefficient (r). In VOCs analysis, data were imported and normalised to the internal standard and log transformed; we used the false discovery rate (FDR) test of (0.1) using the Benjamini–Hochberg method.

6.3 RESULTS:

Demographics results

Seventeen patients with severe asthma and treated with at least 1000 mcg per day of BDP equivalent were recruited; five patients could not perform PExA and/or FeNO tests. Demographics of the remaining 12 are summarised in Table 6.1.

Table 6. 1. Participant characteristics

ID	Age	Sex	FEV_1%	FVC%	BMI	Exacerbation rate	Comorbidities	Smoking
A-1	49	M	84	89	29.3	2	1	Non-smoker
A-2	63	M	43	56	25.8	1	0	Ex-smoker
A-3	72	M	38	58	27.1	1	0	Ex-smoker
A-4	18	M	46	69	21.0	1	2	Non-smoker
A-5	54	M	88	97	21.9	0	1	Non-smoker
A-6	45	M	NA	NA	23.6	0	1	Ex-smoker
A-7	42	M	58	83	26.8	0	0	Non-smoker
A-8	40	F	68	76	52.3	6	4	Non-smoker
A-11	34	F	70	108	36.6	1	1	Non-smoker
A-12	46	M	108	120	22.9	1	0	Non-smoker
A-15	26	M	79	98	28.7	0	0	Non-smoker
A-16	60	F	96	11	28.9	3	1	Ex-smoker
Total or Median (IQR)	45 (36-59)	M=9 F=3	70 (46-88)	83 (58-98	27 (23-29)	1 (0-1)	1 (0-1)	Ex=4 Non=8

FEV_1%: forced expiratory rate in 1 second; FVC: forced vital capacity; BMI: body mass index.

Fractional exhaled nitric oxide measurements

FeNO tests were performed at four occasions during the first visit. These measurements fell within two hours of post-baseline measurement (which was approximately 0.5-1 hour post-ICS inhalation). The median (IQR) of FeNO at baseline, 30 min, 60 min, and 120 min was 87 (59-124), 85 (68-137), 102 (66-143), and 97 (68-145) ppb respectively, with no significant variability in FeNO values between these time points (Friedman test, p=0.378). FeNO levels on the second visit fell compared to the mean FeNO on day1 [median (IQR) day 7: 50 (31-71) ppb versus day 1: 87 (58-136) ppb, p=0.009] (Figure 6.1).

The mean FeNO levels at day 1 and day 7 were negatively correlated to the FEV_1 at day 1 (r= -0.793 p=0.004; r= -0.675 p=0.032 respectively, see figure 6.2).

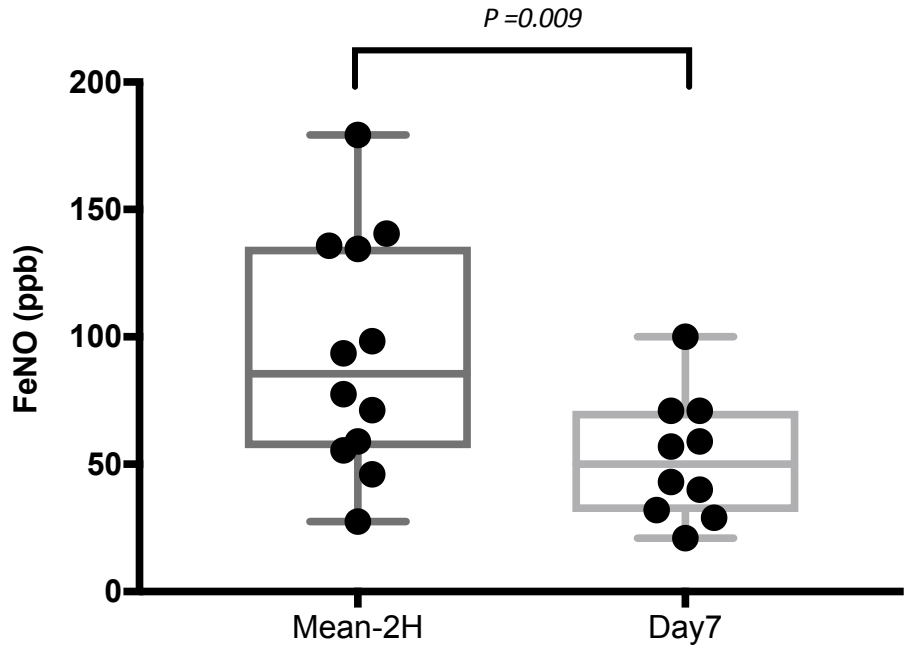

Figure 6. 1. Box whisker showing fractional exhaled nitric oxide (FeNO) levels at day 1 (mean of four readings over 2 hours) and at day 7 for all patients.

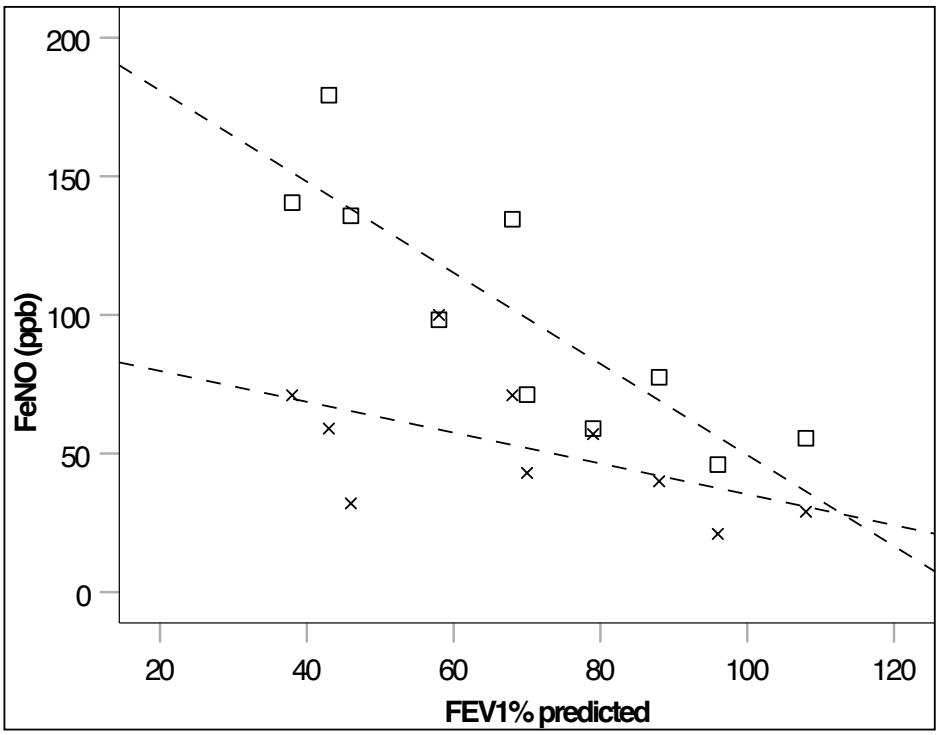

Figure 6. 2. Relationship between the level of FeNO at each visit and FEV_1 % predicted at day 1. Mean 2 hours of day 1 represented as open square and day 7 is x symbol.

Exhaled breath temperature

One patient had exhaled breath temperature consistently below 31°C at day 1 – this was clearly an outlier and therefore data were removed from subsequent analysis. For the remainder, there was significant change in EBT over 2 hours post ICS administration (p= 0.017) (Figure 6.3). All participants except two had reduced EBT at day 7; the median (IQR) was 34.03 (33.02-34.42) °C and 33.55 (32.33-33.88) °C for day 1 and day 7 respectively, and this was statistically significant (p=0.017).

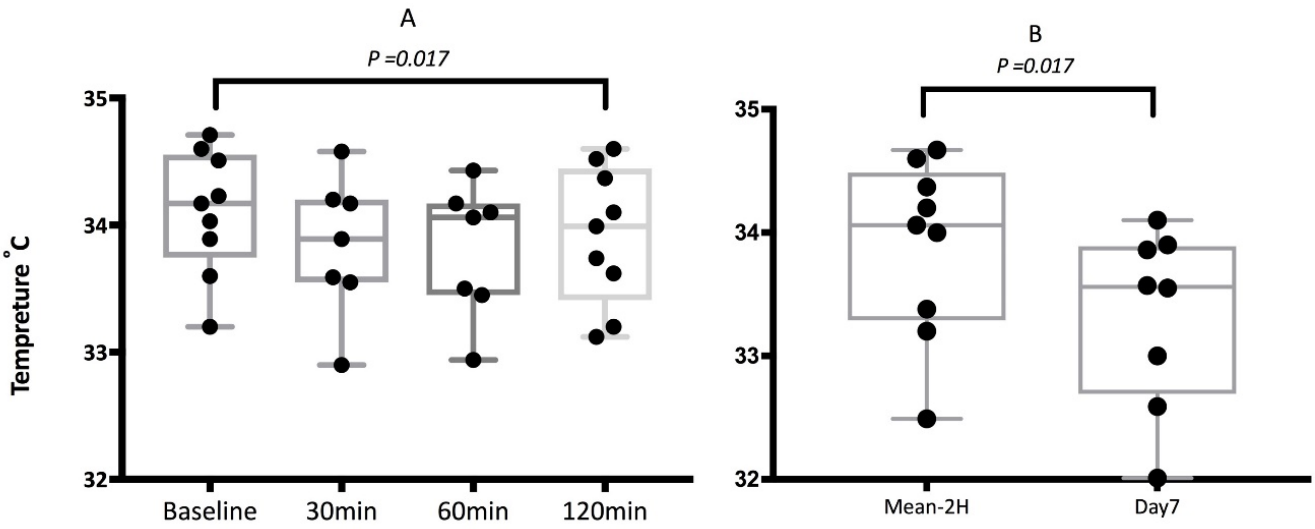

Figure 6. 3. Box blot of exhaled breath temperature (EBT) levels at different time point within 2 hours (A) and mean 2 hours) and at the day 7 (B). Note: We excluded one patient with EBT level below <31°C.

Particles in exhaled air

Ten participants each provided three PExA samples; two at visit 1 and one at visit 2 (day 7). The median (IQR) of the PEx mass at baseline and after 2 hours from baseline were 19.6 (7.2-64.2) ng and 25.4 (18.2-61.9) ng respectively (p=0.575). We measured the average (mean) of day 1 levels to compare it to the day 7, and this was also not statistically significant (see table 2).

No significant difference in the concentration of SPA and albumin between the mean of visit one and visit 2 (summary measurements illustrated in table 6.2, figure 6.4). Further, there were no significant difference in the calculated SPA (weight %) and albumin (weight %) between day 1 and day 7 (figure 6.5).

The mean of generated PEx and the mean particles per breath manoeuvre within 2 hours at day 1 correlated with the % predicted FEV_1 (r=0.717, p=0.030; r=0.750, p=0.020) (Figure 6.6). However, this association was not observed at day 7. We also noted a possible (non-significant) negative relationship between the levels of FeNO in both visits and the collected PEx (ng) at baseline only (*r*=-0.612 p=0.060, *r*=-0.635 *p*=0.091 respectively) (Figure 6.7). Because of the association identified previously between FeNO and FEV_1, we assessed the correlation between PEx and FeNO or FEV_1 using partial correlation. The FEV_1 and PEx were still highly correlated after adjusting for FeNO (*r*=0.746 p=0.089), while PEx and FeNO was non-significant after adjusting for FEV1 (*r*=-0.393 p=0.384).

The mean measurements of PEx mass at the visit one was positively correlated to the levels of SPA and albumin (r=0.806, p=0.005; r=0.709, p=0.002; see appendix figure A6-2).

Table 6. 2. PExA sampling parameters and protein concentration in all study visits

	Baseline	120 minutes	Day 7	p value	Mean 2H vs day 7 p value
Time (min)	10.5 (2.3)	10.7 (2.6)	11(3.7)	0.882	0.819
Mass PEx (ng)	19.6 (7.2-64.2)	25.4 (18.2-61.9)	47.5 (10.5-109.5)	0.607	0.575
Particles per exhalation	102507 (560-164007)	126590 (53811-243112)	189327 (30727-305091)	0.417	0.779
SPA (ng/L)	0.8 (0.3-2.0)	1.5 (0.7-1.9)	1.4 (0.5-2.5)	0.882	0.624
Albumin (ng/L)	0.6 (0.4-1.5)	1.4 (0.7-1.8)	1.5 (0.9-2.3)	0.417	0.263

Data expressed as median (interquartile range). PEx: mass collected from exhaled particles; SPA: Surfactant protein A concentration.

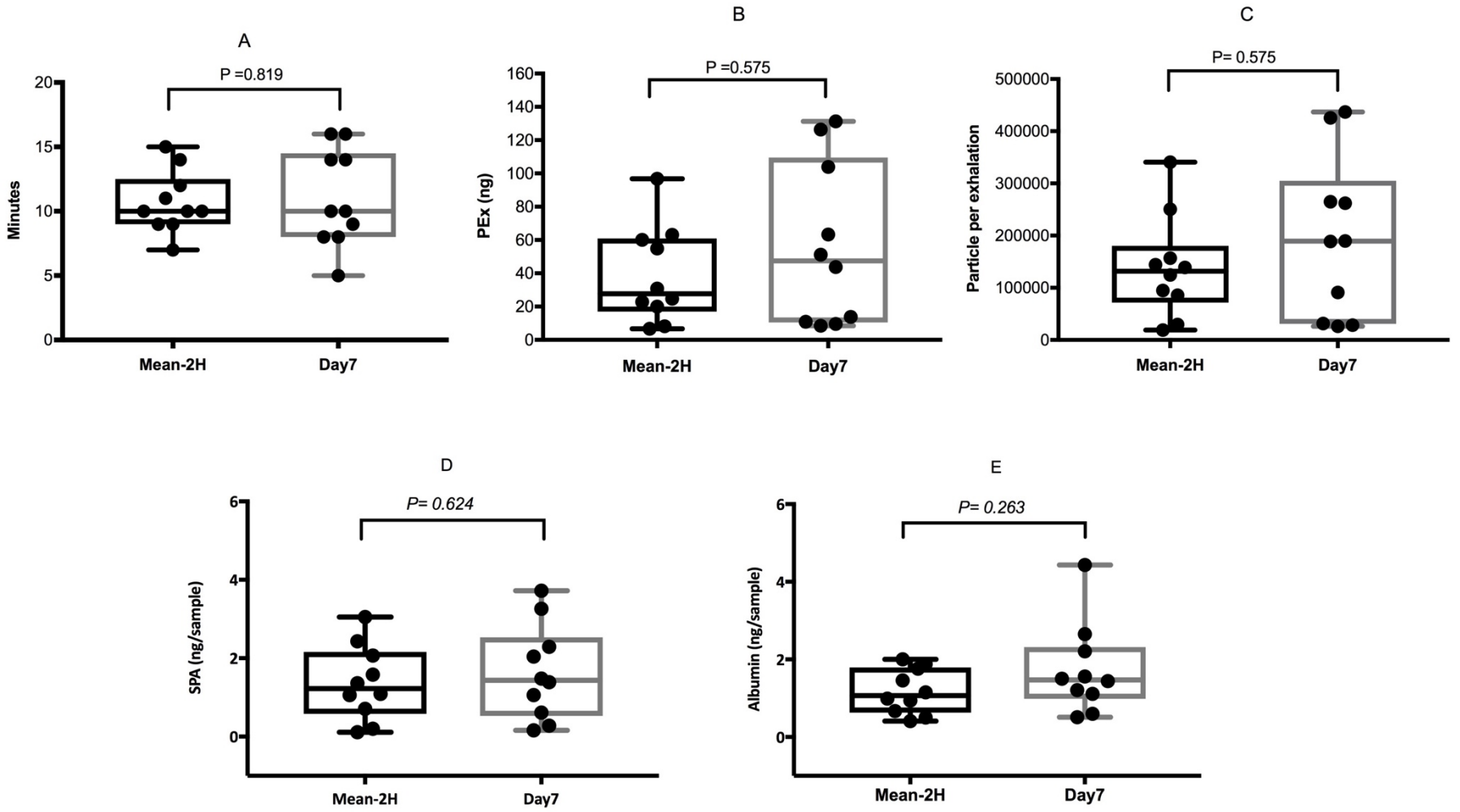

Figure 6. 4. Box whisker showing individual data at visit 1 and visit 2 for (A) the time required to sample PEx (B) number of PEx (ng) (C) particles per exhalation (D) Total number of Surfactant protein A (ng/sample) (E) Total number of Albumin (ng/sample).

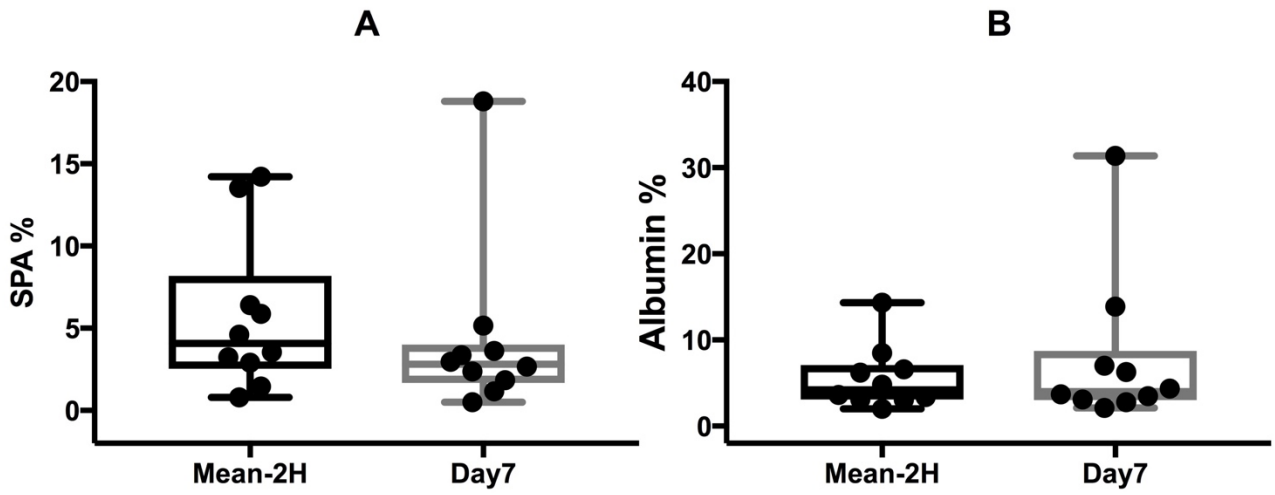

Figure 6. 5. box plot showing the difference between visit 1 and visit 2 in (A) SPA % (p=0.989) and (B) Albumin % (p=0.674).

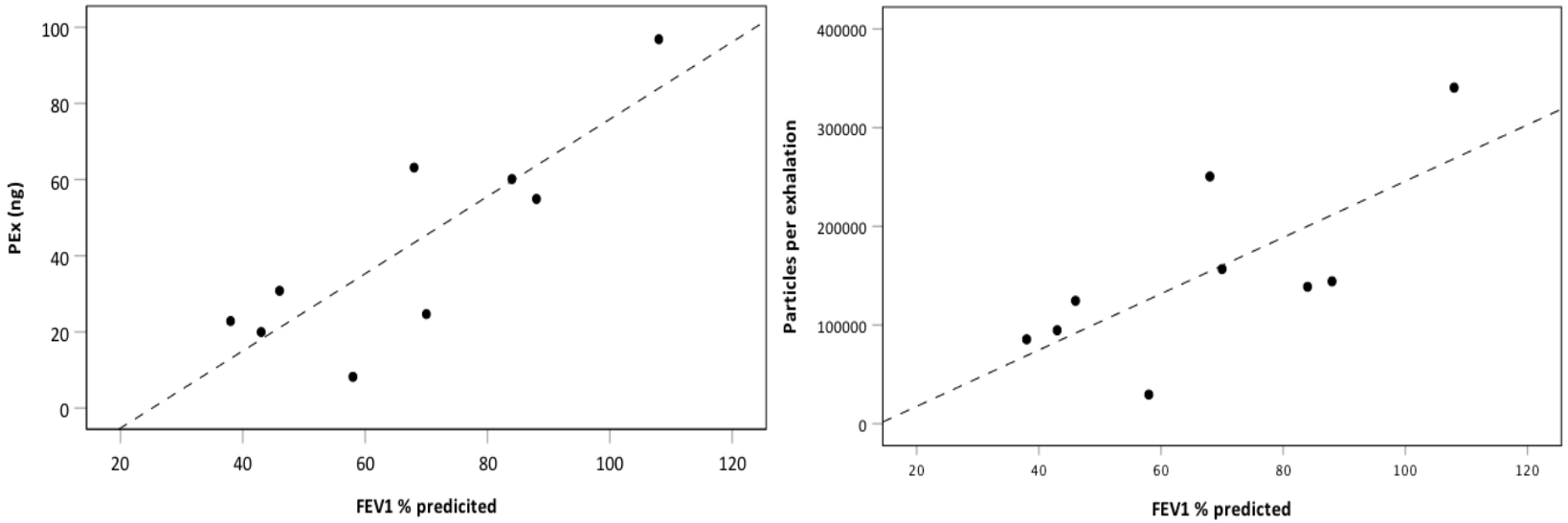

Figure 6. 6. A) Relationship between the mean level of collected PEx at visit 1 and FEV_1 predicted (r=0.717 p=0.030). (B) Relationship between the mean number of particles per exhalation at visit 1 and FEV_1 predicted (r=0.750 p=0.020).

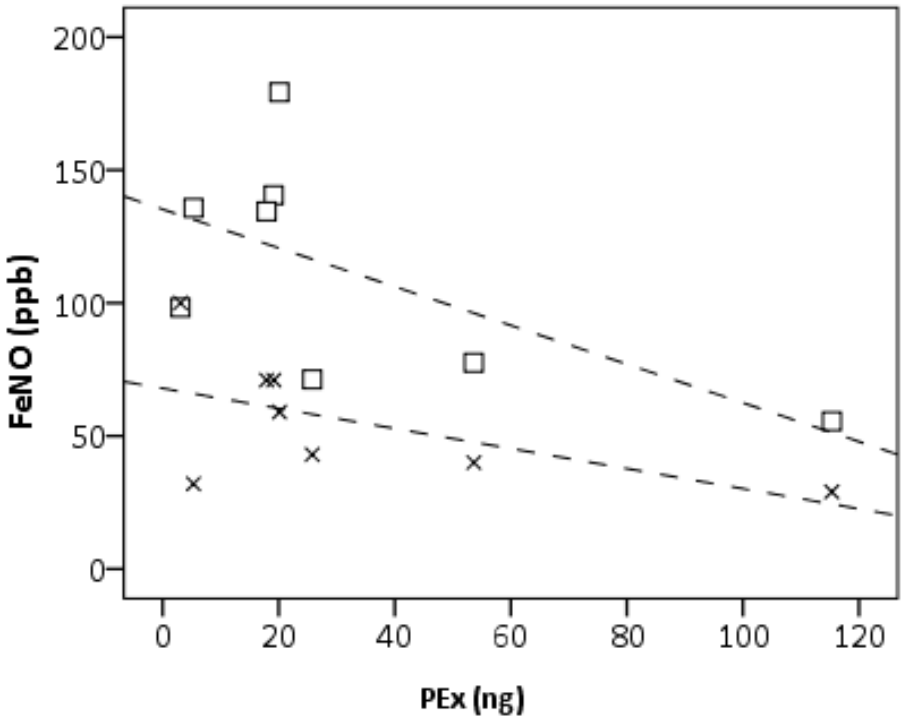

Figure 6. 7. Relationship between the level of FeNO at both visits and collected PEx (ng) at baseline (*r*=-0.612 p=0.060, *r*=-0.635 *p*=0.091 respectively). Mean 2 hours represented as open square and day 7 is x symbol).

Volatile organic compounds

Data from one participant (A15) were removed as VOC samples were collected at day 7 only. We compared the four VOC samples collected at day 1 (within 2 hours), and identified 14 significantly different VOCs. However, once an FDR was applied only six compounds met q<0.1. Using the National Institute of Standards and Technology (NIST) library three compounds were identified: ethyl benzene, o xylene, and tetradecane (Figure 6.8).

Then we compared the mean results of the VOCs from day 1 with the levels observed in day 7. After the NIST library identifications, two significant VOCs were identified: furfural and xylene (Figure 6.9) but neither of these had FDR q<0.1.

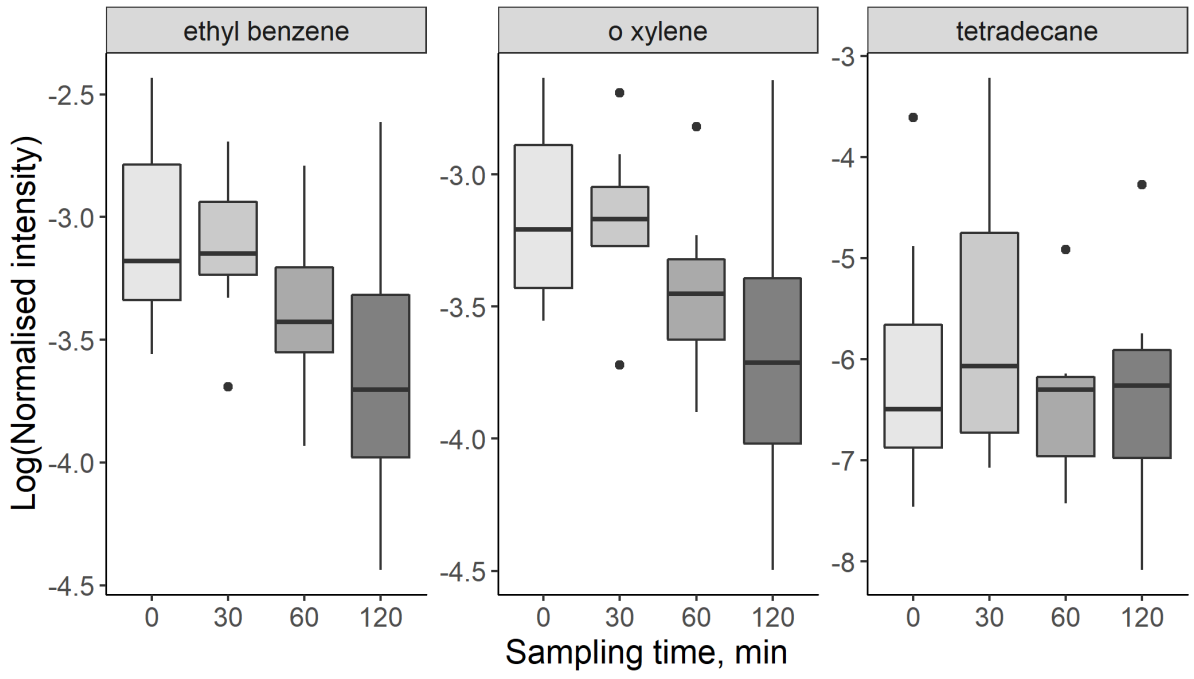

Figure 6. 8. Box and whisker plots comparing measurements in day 1 for the VOCs that had a false discovery rate q<0.1

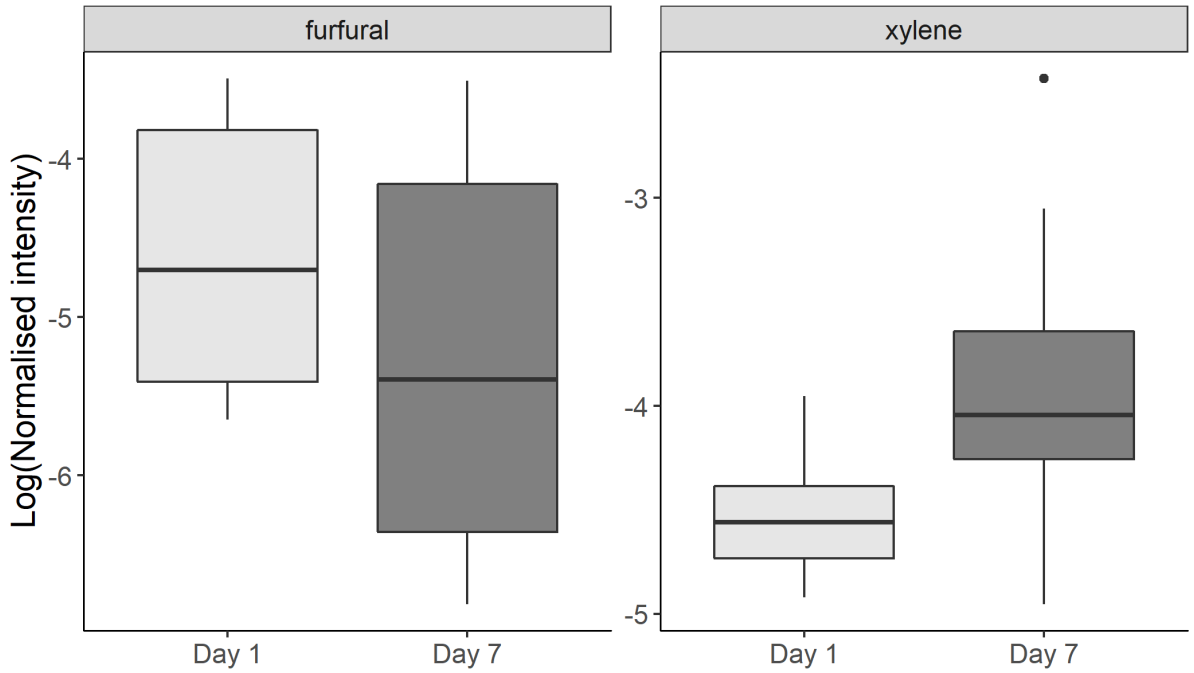

Figure 6. 9. Box and whisker plots comparing mean measurements of day 1 with day 7. Note these compounds have false discovery rate above >0.1.

6.4 DISCUSSION

This study examined various non-invasive exhaled breath profiles that have been proposed for assessing ICS responsiveness. Four methods of analysis were applied, FeNO, EBT, PExA and exhaled VOCs. Using FeNO, as expected significant reduction was found in the levels following seven days of ICS treatment. This study also demonstrated for the first time a rapid impact of ICS on EBT. For VOCs, we have shown that there was a clear variability in the pattern of some compounds following ICS use. In contrast, we do not observe any change any difference in all of the PExA parameters. Our study indicates that different exhaled biomarkers profiles in at least FeNO, EBT and VOCs might be useful for evaluating ICS response.

Inhaled corticosteroids are used to treat airway inflammation in asthmatics. It has previously been shown that FeNO is correlated with the degree of the inflammation and is an accurate predictor of ICS response. Our results are in line with the previous literature that showed daily use of ICS suppresses FeNO (186, 317). We have not shown any difference in FeNO levels over 2 hours after ICS administration and measurements were almost identical indicating high reproducibility. Hence, FeNO measurements over short time nature unlikely to reflect ICS responsiveness. To our knowledge, no studies have reported FeNO within 2 hours interval. The study has also found high FeNO values were inversely correlated with FEV1 % predicted. This correlation is consistent with those of other studies and further research should be done to investigate this association (375-377).

We have shown for the first time that there is a significant short term reduction of EBT after ICS administration and also after seven days of ICS use. It has been suggested the airway

inflammation and remodelling may increase bronchial vascularity and that may lead to elevated heat exchange in the airway (363, 378). Inhaled corticosteroids can cause acute reduction (within 30 to 90 min) in the bronchial blood flow (47), which may resulting in reduced airway heat. Thus, EBT may be clinically useful to assess acute responsiveness to ICS medication over a very short time period. Moreover, since we have also observed a significant reduction in EBT levels after seven days of ICS administration and most of the patients had used more than 50% of their ICS, it could merit further investigation as a possible marker of ICS adherence .

We demonstrated that ICS medications do not show any significant effect on PEx protein biomarkers (SPA and albumin) over a short and long time-period. Our results were similar to Larsson et al. who reported no difference between asthmatics using ICS and those not on ICS (367). Further, the acquired mass of proteins were lower than found in earlier studies (367, 379, 380), probably was due to high correlation between the collected PEx samples to SPA and albumin demonstrated in this study and this association was previously reported (380). Exhaled proteins are formed and transported via exhaled aerosol particles which are highly dependent on the subjecting breathing (236, 381).

Despite the limitation of the low collected PEx mass, we have shown a % predicted FEV_1 was strongly and positively correlated with PEx and particles per exhalation collected within the 2 hours; however, this was not observed in at the second visit. Although the FEV1 parameters related to the level of obstruction in the large airway, they may also reflect the obstruction in small airways. The reduction in the lung function is associated with air trapping and may reduce the closure and opening of the airways required to produce PEx (382). Further, the inflammatory processes that occur in asthmatic small airways and alveoli may change their

mechanical characteristics and lead to airway closure and opening occurring more proximally, leading to fewer particles produced. On the other hand, a potential relationship was found between FeNO at both visits and PEx mass, although non-significant after adjusting for lung function, indicating that lung function is the main driver for PEx mass.

We have demonstrated changes in a few exhaled VOCs over 2 hours and one week of ICS administration. From the two hours comparison, ethyl benzene, xylene, and tetradecane compounds were the only highlighted compounds identified, and O-xylene and furfural from the difference between the two visits. Xylene has previously been shown to discriminate between asthma and healthy controls (383), o-xylene compounds were observed in Ibrahim et al. related to the level of asthma control (369), and tetradecane in allergic asthmatics (384). To our knowledge, this is the first assessment of these compounds in relation to ICS responsiveness. Therefore, we suggest further work to be done to confirm the change in these compounds after ICS use. If successful, it might be worthy to be investigated on a different group of asthma patients with a range of ICS dose.

The main strength of this study was that the use of ICS medication was recorded for each participant during the two visits. Secondly, most of our patients included were proven to be steroid responsive, as FeNO levels dropped at the second visit in 10 out of 12 cases.

The limited number of participants enrolled in this study was the main limitation. Moreover, we intended to perform the first tests of all exhaled measurements before the ICS use at the baseline visit; however, this was not achievable due conflicts with patient's clinic schedule. Another potential limitation was noted from the repeated measurements design at narrow interval (2 hours). PExA test was particularly hard to be done twice in some subjects at this

interval as it required high effort and time. Although the study has demonstrated a pattern change in some of VOCs profiles, however, the test was not evaluated in placebo group to guarantee the change was due to ICS use or by other factors.

In summary, this study demonstrates that FeNO, exhaled VOC and breath temperature levels are affected after ICSs use. Furthermore, collected PEx mass is associated with lung function and suggest the PExA test is potentially useful in clinical settings. Future larger studies are required to validate and reproduce our findings.

7 General discussion

In the last chapter, I will summarise and discuss the findings from each chapter, particularly the results related to the hypothesis and aims stated at the end of Chapter 1. I will also make some suggestions for future research studies based on my findings.

Nonadherence to steroid medications is prevalent across asthma severities. However, it is challenging to detect nonadherence. If we can identify the nonadherence to steroids medications, then we may be able to address the patients concerns and reasons for not taking the drugs. Subsequently, better adherence can hopefully be achieved. Although several subjective and objective assessment tools of adherence are available, we do not yet have any good tools that accurately identify nonadherence. In the UK, the most common method that has been used in many clinical asthma services is prescription refill records, calculated according to how many doses the patient has taken (commonly within 6 or 12 months) divided by the number of doses prescribed. The main drawback of this method is measuring the average adherence over time, as this disregards the time gaps between doses and checking patient technique. Thus, the prescription refill method provides a poor reflection of patient adherence. The thesis investigated further novel biomarkers of corticosteroid medications adherence and responsiveness. This was conducted by using direct blood levels to measure adherence rates and several exhaled breath biomarker profiles to assess ICS responsiveness.

Summary and conclusions of thesis studies

Chapter 2: Systematic review

Two objective biological methods for assessing adherence among asthma patients have been demonstrated over the last decade: detection of ICS or OCS in body fluids, and the level of exhaled nitric oxide. These methods have shown interesting data in estimating the level of adherence. Therefore, we have conducted a systematic literature review aiming to identify previous studies which focused on the use of direct drug monitoring or FeNO in assessing adherence, and we were able to derive important results. Firstly, the prevalence of poor adherence was common in most of the studies. Secondly, despite the successful detection of oral prednisolone in blood serum, some limitations were identified. The analytical method of blood detection was not adequately described; however, if mentioned, the detection level and cut-off values were variable among the studies. The PK and PD parameters of the prednisolone and timing of the last dose were not reported in most of the studies. These findings indicate the need for standardisation across all analytical methods and further studies on PK and PD parameters in particular in patients with severe asthma. Thirdly, the FENO levels tend to be higher in the non-adherent patients compared to the adherent; however, no referenced values can be suggested to distinguish between them. In FeNO suppression studies (in severe asthma) it has been shown that FeNO levels dropped after daily ICS use, which suggests previous poor adherence, or less likely, inadequate treatment.

Chapter 3: Oral corticosteroid detection and the MARS self-assessment questionnaire

Next, we compared the prevalence of nonadherence using two methods: a self-reported questionnaire and urinary corticosteroid (and metabolites) detection. In this study, we demonstrated that both methods showed a similarity in the prevalence of poor adherence. However, after we considered the urinary corticosteroids as a gold standard method, the MARS questionnaire was found to overestimate patient medication use. This was observed in around half of the individuals self-reporting good adherence on MARS while they had undetected prednisolone metabolites.

Using a self-reported questionnaire, we found that patients with suboptimal adherence had worse asthma control and quality of life compared to adherent patients. In contrast, based on the urinary corticosteroids detection, we found no difference between good and poorly adherent patients regarding asthma control; however, adherent patients had lower blood eosinophils.

The results of this chapter confirmed previous findings that the self-reporting questionnaire does not necessarily reflect adherence, and would overestimate adherence rates in clinical settings. Nevertheless, the subjective report of adherence remains important, as it affords a better understanding of patients' perception about the frequency of medication use. Oral corticosteroid adherence has been previously examined by Gamble et al. in the form of blood detection with prescription refill rates (148). They have found that patients denied poor adherence, and then they admitted not using their medication after prednisolone detection. We believe these issues could be overcome with urinary corticosteroid detection as it also

has some advantages over blood sampling such as being less invasive and providing a longer post-dosing window.

Chapter 4: Serum inhaled corticosteroids detection

In the next study, we examined the feasibility of using direct drug detection of the most commonly used ICS inhalers by using LC-MS/MS after observing inhaler use and checking technique. We were able to detect steroids in serum after eight hours of use in patients BDP and BUD inhalers in the blood while FP was detected in all except one. In contrast, only two of the four patients using FF had detectable levels. The fifth ICS inhaler was CIC; none of the individuals using this inhaler had detectable levels and this was not due to the behaviour of the patients adherence. We measured only the CIC prodrug, which can usually be only detected within the two hours after ICS use (338). We would likely have been able to detect des-CIC (the active metabolite of CIC) more easily, as this can be detected for 12 hrs post dosing (337).

While the main aim of this work was to examine the feasibility of using blood detection as a direct method of measuring adherence, we found a significant association between a higher serum concentration within eight hours and the annual exacerbation rate and high FEV1 for patients on FP. This association was in line with a previous study (335) and suggested higher ICS systemic ICS exposure was associated with improved severity measurements.

Chapter 5: Serum inhaled corticosteroid detection and electronic monitoring of adherence

Since we have demonstrated promising results using drug detection for monitoring adherence, it would be useful to investigate this method in a real-world study and compared it to other validated adherence tools. We carried out a longitudinal study over one week, to investigate the adherence rate by using acoustic electronic monitoring inhalers and compared it with blood detection of FP in patients attending the FeNO suppression clinic. We were able to detect FP at both visits in all patients and a significant increase in ICS concentration was observed after a week. Furthermore, after the inspection of the audio files from the INCA device, patients with a higher number of correct doses had a higher and significant increase in the ICS levels in the blood. Furthermore, the time of the last ICS dose also correlated with the blood concentration after one week. From these findings, we have confirmed the huge variability of the baseline FP concentration that we reported in Chapter 4. We have also shown that improvement of asthma control was associated with higher ICS concentration over a week.

The FeNO suppression test has been identified previously as sensitive and specific to predict poor adherence (317); we found the median number of correct doses was almost double in the positive FeNO suppression groups (>42%) compared to the unsuppressed group. However, no difference was identified in serum ICS concentration, lung function and blood eosinophil over one week of ICS treatment. Moreover, we noted the negative FeNO suppressors still had some degree of FeNO reduction with an apparent improvement in asthma control, which may suggest that we cannot rely on the FeNO threshold value alone (42%) to predict the level of adherence.

We envisage that blood detection could provide unbiased and more reliable adherence data compared to the current adherence method (i.e. self-reported questionnaire or prescription refill data) in patients on the three most common ICS inhalers (BUD, BDP and FP). However, it is essential to be aware there is a possibility of bias in detecting steroids and metabolites in the last 24 hours if patients take the medication only before the upcoming tests (white-coat adherence).

Chapter 6: The effect of inhaled corticosteroids on exhaled breath biomarkers

In this study, we examined the effect of ICS treatment over two hours and one week on FeNO, EBT, PExA and VOCs levels. Exhaled breath temperature levels were significantly reduced after one hr of the first dose and subsequently after one week of ICS use. We found an apparent variability in the pattern of some VOCs compounds. The level of FeNO, as expected from previous studies, dropped after seven days of ICS use. There was no observed effect on the PEx mass number and SPA and albumin concentration in either visits.

Direction of future studies

1. We have shown in Chapter 3, 4, and 5 the ability to detect oral or inhaled steroids in blood or urine to assess adherence. However, these studies involved only severe adult asthma patients. Thus, we do believe that to generalise our findings; future studies should explore in mild or moderate asthma severity.
2. After using biological samples to detect inhaled or oral steroid medications, good adherence was considered when patients had a detectable level. But from our study in Chapter 5 study, we found that FP concentrations can be accumulated (over one week) with the daily use of medication. Therefore, further research is needed to study the pharmacokinetics of all steroid treatments in all levels of asthma severity to understand the ICS absorption and accurately distinguish patients not using medication or even those with occasional use.
3. We only investigated the inhaled or oral steroids metabolites in blood and urine; other possible biological sampling such as capillary blood or saliva could be explored in further work to monitor adherence.
4. Further studies should investigate the cost-effectiveness of using biological samples in monitoring adherence.
5. We have demonstrated that exhaled breath markers represent a promising new approach in evaluating ICS responsiveness, and some of our results supported this theory. The VOCs and EBT methods disclosed further validation of its reproducibility and the results could be compared to a control group.
6. Exhaled breath particles have recently been proposed as an alternative method for testing for drug abuse. In an exploratory study of testing drugs (including

amphetamines, methamphetamines, cannabis, cocaine and heroin), exhaled breath testing was found to provide a high detection rate, compared to self-reporting, and blood and urine analysis (385). Therefore, future studies can utilise breath analysis to examine the feasibility of using exhaled particles to work as a potential marker of direct therapeutic effect and subsequently measure the level of adherence to ICS.

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Appendix:

Table A2.1: results of quality assessment of observational included studies

Year	Author	Q1	Q2	Q3	Q4	Q5	Q 6	Q 7	Q 8	Q 9	Q 10	Q 11	Q 12	Q 13	Q 14	QUALITY
1998	Stirling (260)	Y	Y	Y	Y	N	CD	Y	N	N	N	Y	CD	Y	N	POOR
2001	Payne (261)	Y	Y	CD	Y	N	NA	Y	N	Y	N	Y	Y	Y	N	POOR
2001	Payne (250)	Y	Y	CD	CD	N	CD	Y	N	Y	N	Y	CD	Y	N	POOR
2003	Robinson (262)	N	Y	CD	Y	N	NA	Y	N	N	N	N	CD	Y	N	POOR
2004	Delgado (263)	Y	N	CD	Y	N	Y	Y	Y	N	Y	Y	CD	CD	Y	POOR
2006	Katsara (255)	Y	N	CD	Y	N	Y	Y	Y	Y	Y	Y	CD	Y	N	FAIR
2007	Lex (264)	Y	Y	CD	Y	N	NA	Y	N	Y	N	Y	NA	Y	N	POOR
2009	Bossley (251)	Y	Y	Y	Y	N	CD	Y	N	Y	N	Y	CD	Y	N	POOR
2009	Gamble (148)	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	CD	Y	Y	FAIR
2010	Cano (265)	Y	Y	Y	Y	N	N	Y	N	N	N	Y	Y	Y	Y	POOR
2010	Scott (266)	Y	N	Y	Y	N	CD	Y	N	N	N	Y	CD	NA	Y	POOR
2011	Koster (256)	Y	Y	Y	Y	N	CD	Y	N	Y	Y	Y	CD	Y	Y	FAIR
2012	Vijverberg (257)	Y	Y	CD	Y	N	NA	Y	NA	Y	NA	Y	NA	Y	Y	FAIR
2012	McNicholl (184)	Y	Y	N	NA	N	N	Y	N	CD	Y	Y	CD	Y	N	FAIR
2013	Price (267)	Y	N	CD	CD	N	Y	CD	Y	Y	Y	Y	NA	NA	N	POOR
2017	Klok (268)	Y	N	CD	Y	N	Y	Y	Y	Y	Y	Y	CD	CD	Y	POOR
2017	George (252)	Y	N	CD	Y	N	NA	Y	N	N	N	N	CD	Y	N	POOR
2017	Jochmann (258)	Y	Y	CD	Y	N	Y	Y	Y	Y	Y	Y	CD	Y	Y	GOOD
2018	Heaney (259)	Y	Y	Y	Y	N	N	Y	N	Y	Y	Y	CD	Y	N	GOOD

Abbreviation: Y: Yes; N: No; : CD: cannot determine; NA: Not applicable. Q1. Was the research question or objective in this paper clearly stated? Q2. Was the study population clearly specified and defined? Q3. Was the participation rate of eligible persons at least 50%? Q4. Were all the subjects selected or recruited from the same or similar populations (including the same time period)? Were inclusion and exclusion criteria for being in the study prespecified and applied uniformly to all participants? Q5. Was a sample size justification, power description, or variance and effect estimates provided? Q6. For the analyses in this paper, were the exposure(s) of interest measured prior to the outcome(s) being measured? Q7. Was the timeframe sufficient so that one could reasonably expect to see an association between exposure and outcome if it existed? Q8. For exposures that can vary in amount or level, did the study examine different levels of the exposure as related to the outcome (e.g., categories of exposure, or exposure measured as continuous variable)? Q9. Were the exposure measures (independent variables) clearly defined, valid, reliable, and implemented consistently across all study participants? Q10. Was the exposure(s) assessed more than once over time? Q11. Were the outcome measures (dependent variables) clearly defined, valid, reliable, and implemented consistently across all study participants? Q12. Were the outcome assessors blinded to the exposure status of participants? Q13. Was loss to follow-up after baseline 20% or less? Q14. Were key potential confounding variables measured and adjusted statistically for their impact on the relationship between exposure(s) and outcome(s)?

Table A2.2: results of quality assessment of included randomised control studies

Year	Author	Q 1	Q 2	Q 3	Q 4	Q 5	Q 6	Q 7	Q 8	Q 9	Q 10	Q 11	Q 12	Q 13	Q 14	QUALITY
2002	Beck-Ripp (247)	Y	CD	CD	N	CD	Y	Y	CD	CD	Y	Y	Y	Y	N	POOR
2008	Szefler (269)	Y	Y	Y	N	CD	CD	N	N	CD	Y	Y	Y	Y	Y	GOOD
2010	Strandbyg-aard (270)	N	Y	Y	N	N	Y	N	Y	Y	Y	Y	N	Y	N	POOR

Abbreviations: Y: Yes; N: No; : CD: cannot determine; NA: Not applicable. Q1. Was the study described as randomized, a randomized trial, a randomized clinical trial, or an RCT? Q2. Was the method of randomization adequate (i.e., use of randomly generated assignment)? Q3. Was the treatment allocation concealed (so that assignments could not be predicted)? Q4. Were study participants and providers blinded to treatment group assignment? Q5. Were the people assessing the outcomes blinded to the participants' group assignments? Q6. Were the groups similar at baseline on important characteristics that could affect outcomes (e.g., demographics, risk factors, co-morbid conditions)? Q7. Was the overall drop-out rate from the study at endpoint 20% or lower of the number allocated to treatment? Q8. Was the differential drop-out rate (between treatment groups) at endpoint 15 percentage points or lower? Q9. Was there high adherence to the intervention protocols for each treatment group? Q10. Were other interventions avoided or similar in the groups (e.g., similar background treatments)? Q11. Were outcomes assessed using valid and reliable measures, implemented consistently across all study participants? Q12. Did the authors report that the sample size was sufficiently large to be able to detect a difference in the main outcome between groups with at least 80% power? Q13. Were outcomes reported or subgroups analyzed prespecified (i.e., identified before analyses were conducted)? Q14. Were all randomized participants analyzed in the group to which they were originally assigned, i.e., did they use an intention-to-treat analysis?

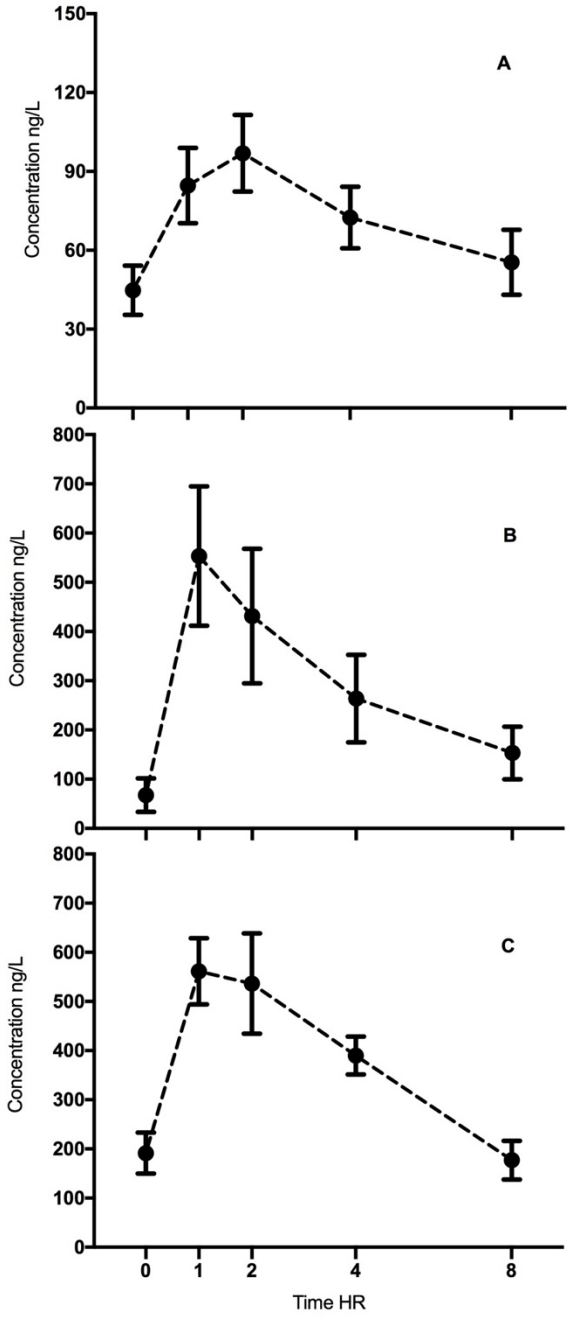

Figure A4.1: mean (SE) serum concentration-time graph of (a) fluticasone propionate, (b) budesonide, and (c) beclomethasone dipropionate in subjects with severe asthma.

Table A4.1: Fluticasone propionate group: detection over time, adherence level and inhaler technique (n=27)

FP dose	Inhaler device	GP PR%	Technique	Times (hours.)				
				0	1	2	4	8
250mcg	MDI	+	+	+	+	+	+	+
	MDI	+	+	+	+	+	+	+
	DPI	+/-	+	+	+	+	+	NA
	MDI	+/-	+	-	+	+	+	+
	DPI	+	+	+	+	+	+	+
	MDI	+/-	+	+	+	+	+	+
	MDI	+/-	+	-	+	+	+	+
	MDI	+/-	+	+	+	+	+	+
	DPI	+	+	+	-	+	+	NA
	MDI	-	+	+	+	+	+	+
	DPI	+/-	+	+	+	+	+	+
	MDI	+	+	+	+	+	+	+
	MDI	+	+	-	+	+	+	+
	MDI	-	+	+	+	+	+	+
500mcg	MDI	+	+	+	+	+	+	+
	MDI	+/-	+	+	+	+	+	+
	MDI	+/-	-	+	+	+	+	+
	DPI	+/-	+	+	+	+	+	+
	DPI	-	+	+	+	+	+	NA
	MDI	+	+	-	+	+	-	-
	MDI	+/-	+	-	+	-	-	+
	MDI	-	+	+	+	+	+	+
	MDI	+	+	+	+	+	+	+
	MDI	+	-	+	+	+	+	+
	DPI	+	-	-	+	+	-	+
	DPI	+	+	+	+	+	+	+
	DPI	+	+	+	+	+	+	+
% patients with detectable ICS				77%	96%	96%	89%	96%

FP: fluticasone propionate; (+): detected ICS; (-): Undetected ICS; NA: missing; inhaler technique: good technique: (+) poor Inhaler technique (+); prescription refill (PR) %: (-):<50%; (+/-):50-79%; (+):>80%.

Table A4.2: Budesonide group: detection over time, adherence level and inhaler technique (n=10)

BUD dose	GP PR %	Technique	Times (hours.)				
			0	1	2	4	8
400mcg	-	+	-	+	+	+	+
	+	-	-	+	+	+	+
	+/-	+	-	+	+	+	+
	+	+	+	+	+	+	+
	+	+	+	+	+	+	+
	-	+	+	+	+	+	+
	+	+	+	+	+	+	+
	+	+	+	+	+	+	+
	+	+	+	+	+	+	+
320mcg	+/-	+	+	+	+	+	+
% patients with detectable ICS			70%	100%	100%	100%	100%

BUD: budesonide; (+): detected ICS; (-): Undetected ICS; NA: missing; inhaler technique: good technique: (+) poor Inhaler technique (+); prescription refill (PR) %: (-):<50%; (+/-):50-79%; (+):>80%.

Table A4.3: Beclomethasone dipropionate group: detection over time, adherence level and inhaler technique (n=15)

BDP dose	GP PR %	Technique	Times (hours.)				
			0	1	2	4	8
100mcg	+	+	+	+	+	+	+
	+	+	-	+	+	+	+
	+/-	+	+	+	+	+	+
	+/-	+	+	+	+	+	NA
	-	+	+	+	+	+	NA
200mcg	+/-	+	+	+	+	+	+
	-	+	+	+	+	+	+
	+	+	+	+	+	+	+
	+/-	+	+	+	+	+	NA
	+	+	+	+	+	+	NA
	+/-	+	+	+	+	+	+
	+	+	+	+	+	+	+
	-	+	+	+	+	+	+
	+	+	+	+	+	+	NA
400mcg	+	+	+	+	+	+	+
% patients with detectable ICS			93%	100%	100%	100%	100%

BDP; beclomethasone dipropionate; (+): detected ICS; (-): Undetected ICS; NA: missing; inhaler technique: good technique: (+) poor Inhaler technique (-); prescription refill (PR) %: (-):<50%; (+/-):50-79%; (+):>80%.

Table A4.4: Ciclesonide group: detection over time, adherence level and inhaler technique (n=7)

CIC dose	GP PR %	Technique	Times (hours.)				
			0	**1**	**2**	**4**	**8**
160mcg	+	+	-	-	-	-	-
	+	+	-	+	+	-	-
	+/-	+	+	-	-	-	-
	+/-	+	-	-	-	-	-
	+	+	-	+	-	-	-
	+/-	+	-	+	-	-	-
80mcg	+/-	+	-	-	+	-	-
% patients with detectable ICS			14%	43%	28%	0%	0%

CIC; Ciclesonide; (+): detected ICS; (-): Undetected ICS; NA: missing; inhaler technique: good technique: (+) poor Inhaler technique (-); prescription refill (PR) %: (-):<50%; (+/-):50-79%; (+):>80%.

Table A4.5: Fluticasone furorate group: detection over time, adherence level and inhaler technique (n=4)

FF dose	GP PR %	Technique	Times (hours.)				
			0	1	2	4	8
184mcg	+	+	-	+	-	+	-
	+	+	-	-	+	+	+
	+/-	+	-	+	+	+	+
	+/-	+	-	+	+	-	-
% patients with detectable ICS			0%	75%	75%	75%	50%

FF: fluticasone furorate; (+): detected ICS; (-): Undetected ICS; NA: missing; inhaler technique: good technique: (+) poor Inhaler technique (-); prescription refill (PR) %: (-):<50%; (+/-):50-79%; (+):>80%.

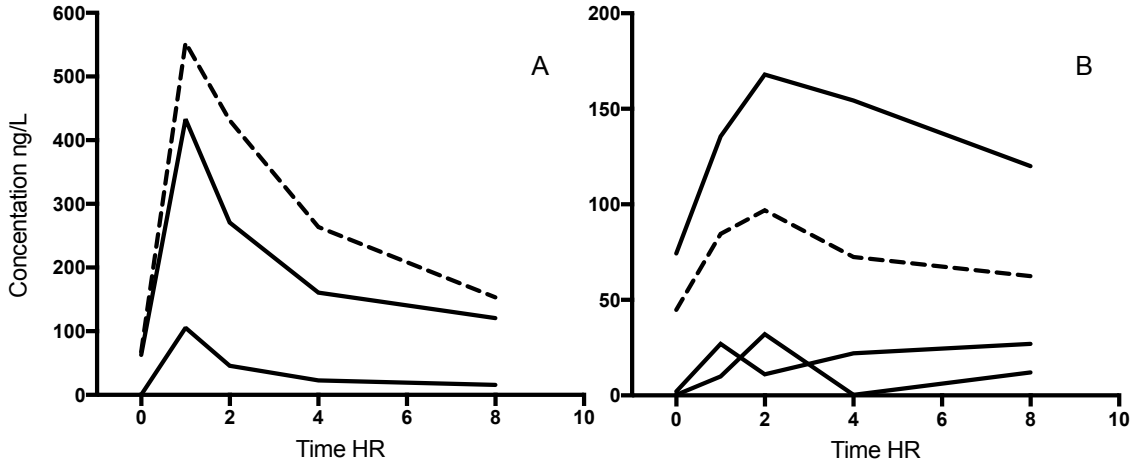

Figure A4.2: Serum concentration time graph for patients with poor inhaler technique, (A) is fluticasone propionate patients and (B) is budesonide patients, solid lines represent patients' ICS concentration and dash lines is the mean concentration for all patients.

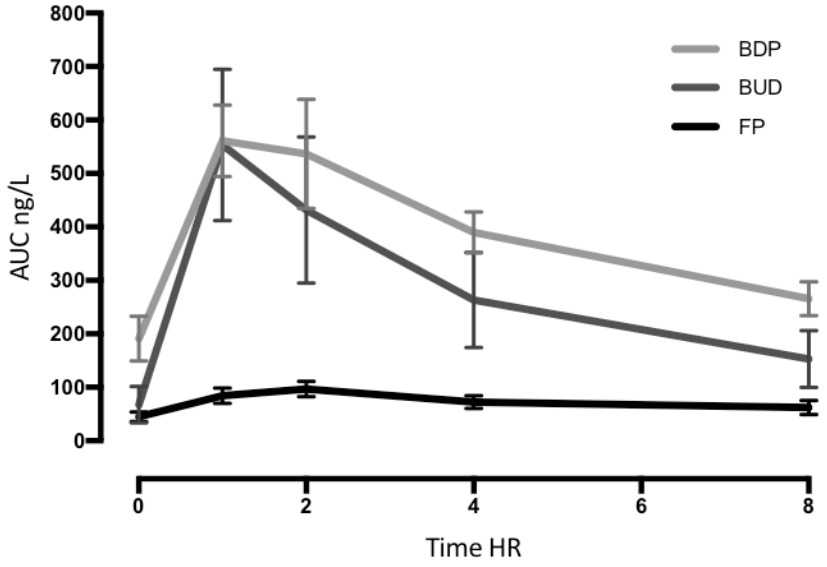

Figure A4.3: The mean (SEM) of Area under the curve (AUC) for BDP, BUD, FP inhalers

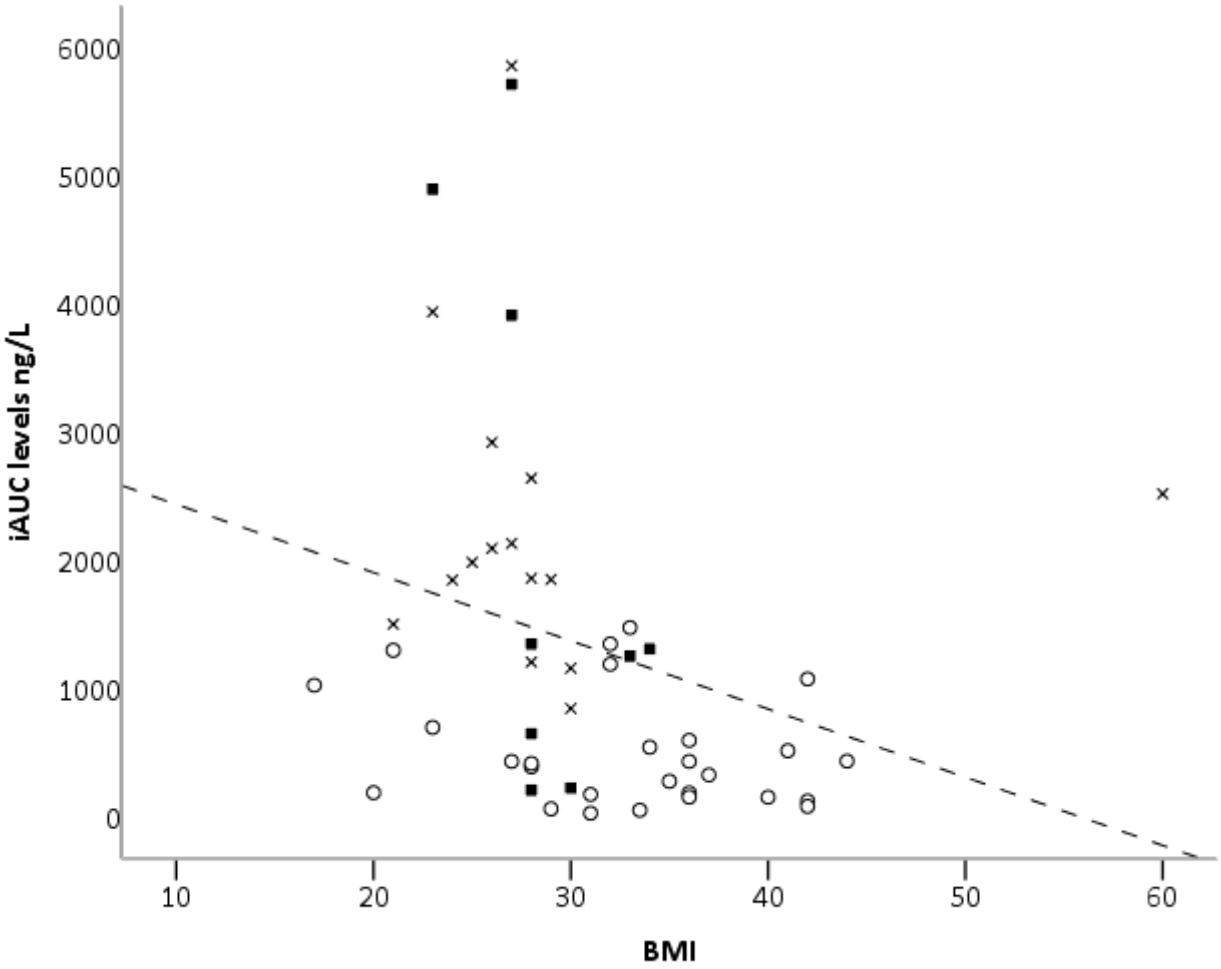

Figure A4.4: Scatter plots showing the incremental area under the curve (iAUC) against body mass index (BMI) for FP (open circle), BUD (closed black square) or BDP (X) (r = -0.488 (p=<0.001).

Table A4.6: Pharmacokinetics comparison between participants with a high and low rate of prescription refill (FP, BDP, BUD inhalers):

	All (n=52)			**FP (n=27)**			**BUD (n=10)**			**BDP (n=15)**		
	PR ≥80% (n=26)	PR <80% (n=26)	p value	PR ≥80% (n=13)	PR <80% (n=14)	p value	PR ≥80% (n=6)	PR <80% (n=4)	p value	PR <80% (n=7)	PR <80% (n=8)	p value
Prescription refill, %	92 (80-100)	58 (33-75)	< 0.001	88 (80-100)	58 (38-66)	< 0.001	80 (80-83)	51 (36-70)	0.010	100 (92-100)	70 (36-75)	< 0.001
C_{base}, ng/L	37 (7-125)	42 (13-158)	0.713	30 (1-53)	28 (12-101)	0.488	55 (6-194)	5 (0-49)	0.257	203 (22-260)	214 (37-401)	0.536
AUC, ng/L	833 (256-2474)	1202 (427-1668)	0.784	285 (151-642)	517 (200-1326)	0.280	2955 (723-5802)	1130 (384-1569)	0.357	2462 (2210-3365)	2025 (1538-2989)	0.281
C_{max}, ng/L	202 (65-684)	273 (85-420)	0.621	66 (35-144)	122 (47-196)	0.350	693 (256-1211)	305 (138-419)	0.257	655 (497-881)	482 (404-724)	0.281

PR: prescription refill; C_{base}: baseline concentration; AUC: Area under the Curve; C_{max}: maximum concentration. Data presented as median (IQR).

Table A5-1: demographic information of the study participants

ID	Age	Gender	FEV1%	FVC%	BMI	Exacerbation rate	Number of comorbidities	Smoking
A-1	49	M	84	89	29.3	2	1	Non-smoker
A-2	63	M	43	56	25.8	1	0	Ex-smoker
A-3	72	M	38	58	27.1	1	0	Ex-smoker
A-4	18	M	46	69	21.0	1	2	Non-smoker
A-5	54	M	88	97	21.9	0	1	Non-smoker
A-6	45	M			23.6	0	1	Ex-smoker
A-7	42	M	58	83	26.8	0	0	Non-smoker
A-8	40	F	68	76	52.3	6	4	Non-smoker
A-9	49	F	65	67	34.0	0	1	Non-smoker
A-10	50	M	74	79	25.8	0	0	Non-smoker
A-11	34	F	70	108	36.6	1	1	Non-smoker
A-12	46	M	108	120	22.9	1	0	Non-smoker
A-13	55	M	67	88	25.6	0	0	Non-smoker
A-14	22	F	53	79	21.3	2	0	Non-smoker
A-15	26	M	79	98	28.7	0	0	Non-smoker
A-16	60	F	96	11	28.9	3	1	Ex-smoker

A-17	68	M	63	74	27.4	0	0	Non-smoker
Median (IQR)	49 (37-57)	M=12 F=5	68 (53-84)	79 (67-97)	27 (23-29)	1 (0-1)	0 (0-1)	Ex-smoker=4 Non-smoker=13

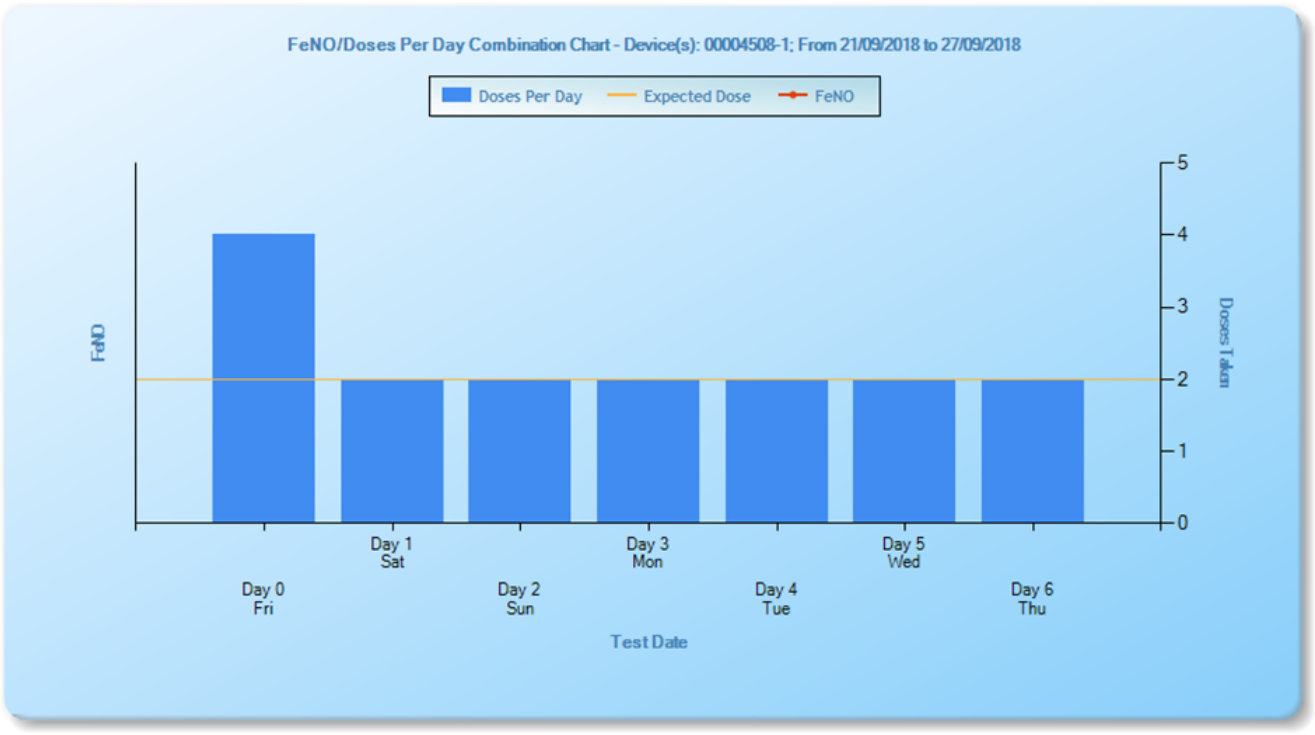

Figure A5-1: Bar chart created by INCA software describing the number of doses taken per day-amber line is the number of doses prescribed by clinician. Note FeNO measurements not illustrated in figure.

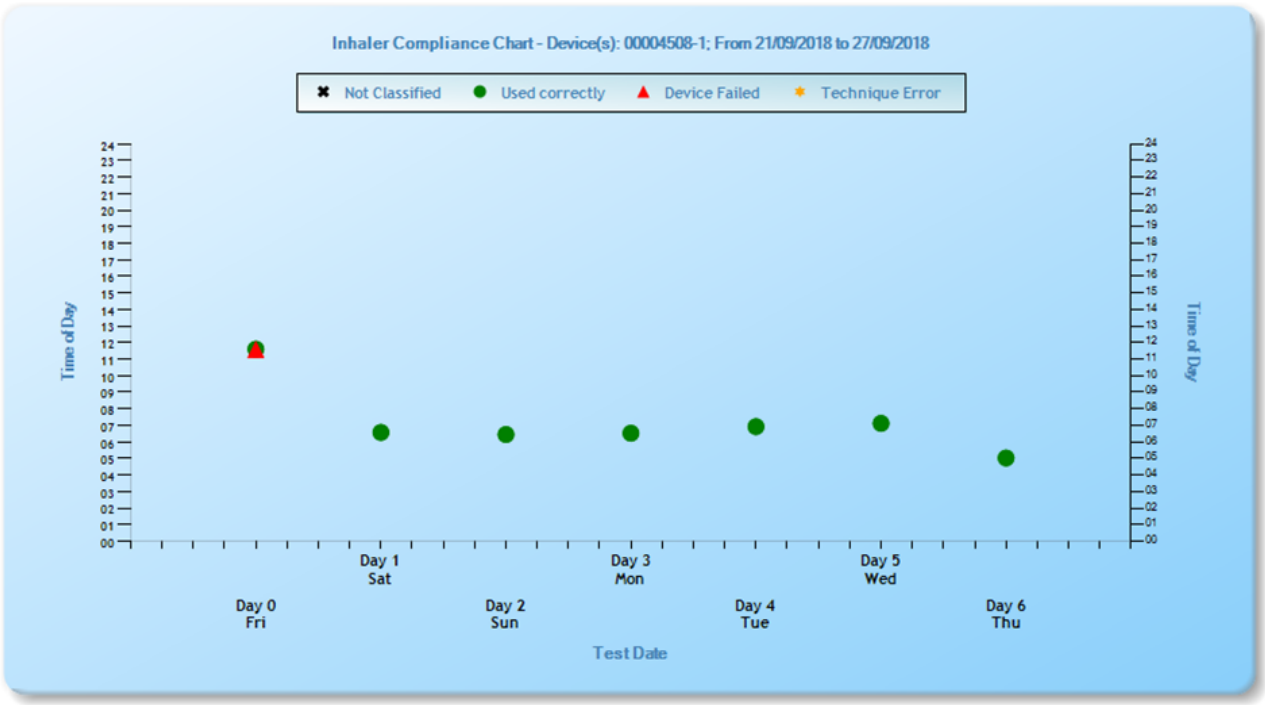

Figure A5-2: the INCA device created scatter blot showing the date and time of inhaler use and analysis of technique.

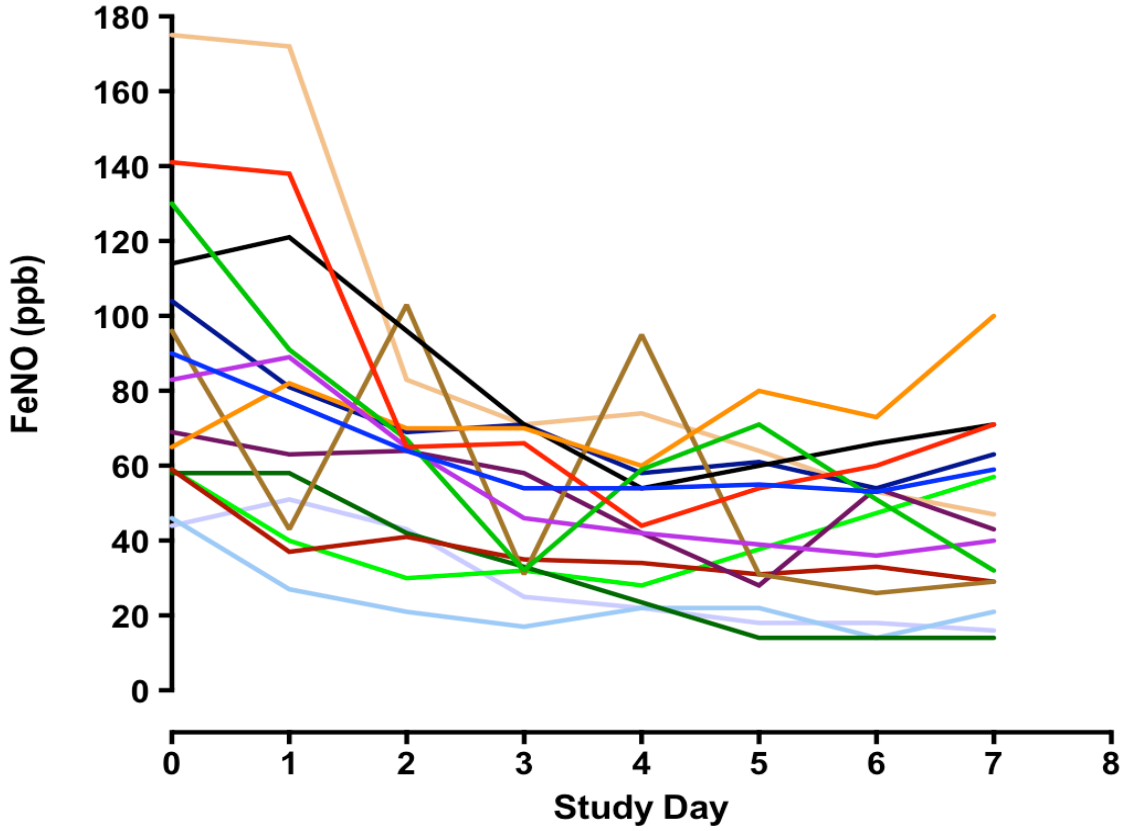

Figure A5-3: Individual patients' FeNO measurements over seven days.

Table A5-2: Summary of adherence of each study participants

ID	Prescription refill rate (%)	INCA software				Total correct doses by audio file inspection	FeNO suppression level (%)	Day 1 serum concentration (ng/L)	Day 7 serum concentration (ng/L)	Day 7 time between last dose and blood sample, hours
		Missed doses	Technique error	Device error	Total correct doses					
A-2*	80	0	0	0	12/12	12/12	32	54	2552	3
A-3	100	0	6	0	6/12	12/12	53	599	1963	5
A-4*	100	5	3	1	4/12	5/12	53	352	544	12
A-5	100	0	12	0	1/12	12/12	58	701	3227	2
A-7	100	0	0	1	12/12	12/12	-20	647	1878	4
A-8	41	2	6	0	4/12	6/12	43	582	1083	3
A-9	100	0	2	0	10/12	12/12	44	561	1621	2
A-10	100	0	5	0	7/12	8/12	33	412	721	2
A-11	80	0	5	1	2/12	6/12	25	555	962	7
A-12	100	5	7	1	0/12	12/12	29	332	238	13
A-13	16	0	7	0	7/12	12/12	76	398	1133	9
A-14	58	0	1	0	11/12	12/12	71	629	1651	3

A-15	16	2	4	0	6/12	6/12	11	1981	172	16
A-16	66	5	5	0	2/12	6/12	29	902	586	11
A-17	100	0	4	0	8/12	12/12	69	298	592	3

*patients already prescribed FP-containing inhalers

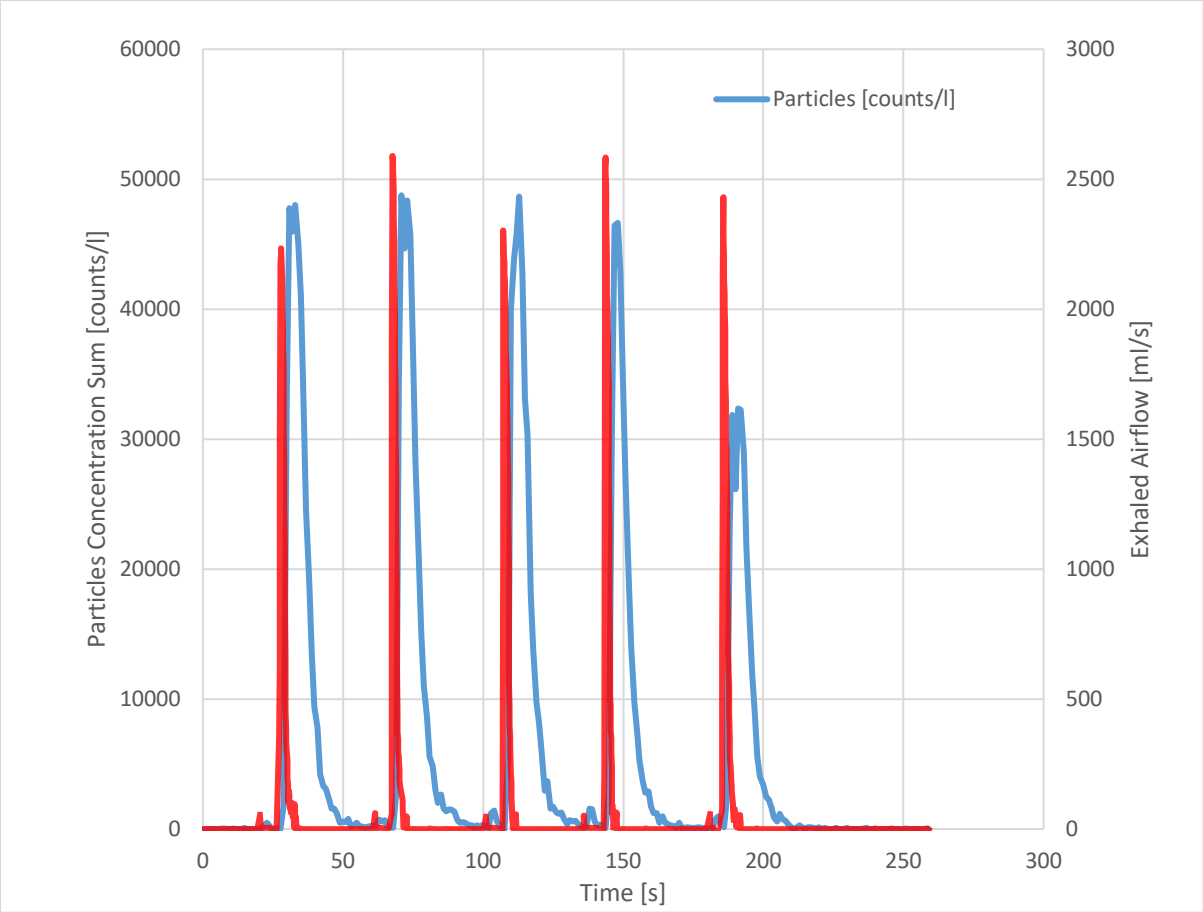

Figure A6-1: Particles concentration and exhaled airflow output graph shown in the laptop at the end of breath manoeuvre

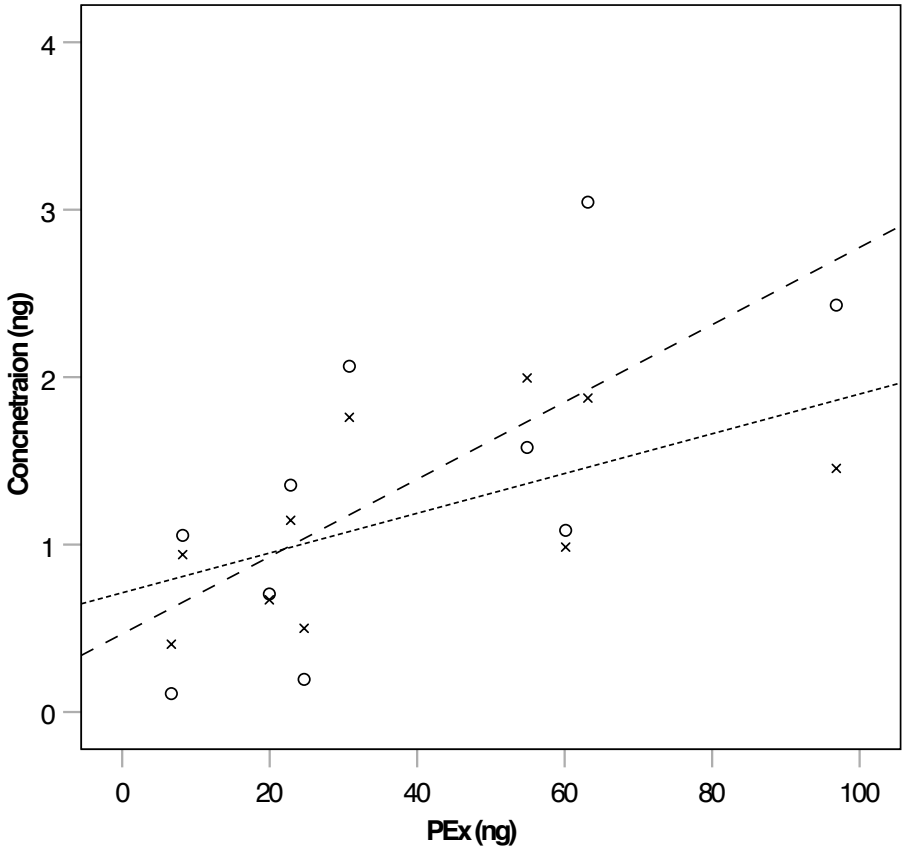

Figure A6-2: Relationship between mass PEx collected at visit 1 and Surfactant protein A (represented as open circle) and albumin protein (represented as X symbol).