Exhaled Biomarkers in Acute Asthma

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Thesis abstract

Objectives – The research aimed to a) determine the feasibility of conducting a study of exhaled breath biomarkers in the acute asthma setting; and b) determine whether a positive bronchial challenge test results in detectable changes in exhaled volatile organic compounds (VOC).

Methods – The *Exhaled Breath Biomarkers in Acute Asthma* feasibility study was undertaken to compare two different approaches to capturing acute asthma data. In the first, participants attending secondary care for treatment of an acute asthma attack were recruited; in the second participants who were at high risk of experiencing an attack were recruited and asked to contact the researcher should such an event occur. The *Bronchial Challenge Testing in Asthma* study was undertaken to determine the effect of mannitol dry powder inhalation on VOC in exhaled breath.

In addition to the above studies, systematic reviews of the literature on 8-isoprostane in exhaled breath condensate and exhaled breath VOC in adult asthma were conducted.

Findings – The literature reviews found insufficient evidence to confirm that EBC 8-isoprostane levels were raised in the presence of asthma or acute asthma attack; a number of exhaled VOC were found to be associated with asthma but with a high level of inter-study variation.

Breath capture studies in acute asthma proved feasible - both approaches were successful in recruiting participants and capturing breath samples, and the acceptability of breath sampling devices was similar to that of existing clinical devices. Obtaining breath samples before systemic corticosteroids were administered and identifying infectious triggers of exacerbation proved difficult. The effect of bronchial challenge on exhaled VOC was detectable but further development of methods is required to produce reliable results.

Conclusion – Designing a phase II biomarker study with the aim of validating previous studies and estimating the accuracy of predictive models appears feasible but further methodological refinement is required.

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Dedication

For Phoebe and Oscar

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Abbreviations

Table 1

AAQ	Assessment of acceptability questionnaire
ABBA	Asthma Breath Biomarker Assessment study
ABC	Asthma Bronchial Challenge study
ABG	Arterial blood gas
ACQ	Asthma control questionnaire
AE	Adverse event
A&E	Accident and emergency
ALF	Airway lining fluid
AMU	Atomic mass unit
ANOVA	Analysis of variance
AR	Adverse reaction
ARTP	Association for Respiratory Technology and Physiology
AUC	Area under the curve
AUKCAR	Asthma UK Centre for Applied Research
AUROC	Area under a receiver operating characteristic curve
BLF	British Lung Foundation
BTS	British Thoracic Society
BMI	Body mass index
COPD	Chronic obstructive pulmonary disease
COW	Correlation optimized warping
CRF	Case record file
DALY	Disability adjusted life years
DNA	Deoxyribonucleic acid
EARIP	European asthma research innovation partnership
ELISA	Enzyme-linked immunosorbent assay
ERS	European Respiratory Society
EBC	Exhaled breath condensate
FEV_1	Forced expiratory volume in one second
FDR	False discovery rate
FVC	Forced vital capacity
GC	Gas chromatography
GC x GCMS	2-dimensional gas chromatography mass spectrometry
GINA	Global Initiative for Asthma
GRC	Global Rating of Change
HDU	High Dependency Unit
HP-SPME/GC-qMS	Headspace solid-phase extraction, gas chromatography quadrupole mass spectrometry
HRA	Health research authority
ICC	Intra-class correlation coefficient
ICE	Integrated clinical environment
ICS	Inhaled corticosteroids
IPF	Idiopathic pulmonary fibrosis
ITU	Intensive treatment unit
LABA	Long acting β_2 agonist
LAMA	Long acting muscarinic antagonist
LTRA	Leukotriene receptor antagonist
LRTI	Lower respiratory tract infection
MCCV	Monte Carlo Cross Validation
MIB	Manchester Institute of Biotechnology
MS	Mass spectrometry
NHS	National health service
NICE	National Institute of Health and Care Excellence
NIH	National Institute of Health

NIHR	National Institute of Health Research
NIST	National Institute of Standards and Technology
NNUH	Norfolk and Norwich University Hospitals Foundation Trust
PAS	Patient administration service
PCA	Principal component analysis
PCR	Polymerase Chain Reaction
PC20	Provocative concentration causing 20% fall in FEV1
PEF	Peak expiratory flow
PLSDA	Partial least squares discriminant analysis
POC	Point of care
PPI	Patient and Public Involvement
pptV	Parts-per-trillion by volume Parts-per-billion by volume
ppbV	Parts-per-billion by volume
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-analysis
PROM	Patient Reported Outcome Measure
PROSPERO	International Prospective Register of Systematic Reviews
PTFE	Polytetrafluoroethylene
REC	Research Ethics Committee
ROC	Receiver operator characteristics
SABA	Short acting β_2 agonist
SAE	Serious adverse event
SD	Standard deviation
SIC	Specific inhalation challenge
SPLA	Sparse partial least square discriminant analysis
SPME	Solid phase micro-extraction
SR	Slow release
TD-SESI-ToFMS	Thermal desorption / secondary electrospray ionisation / time-of-flight mass spectrometry
Th2	Type 2 helper T cell
TIC	Total Ion Chromatogram
ToFMS	Time-of-flight mass spectrometry
VAS	Visual Analogue Scale
VOC	Volatile organic compounds
WEKA	A suite of machine learning software / algorithms hosted by the University of Waika
WHO	World Health Organisation
YLL	Years of life lost due to premature death
YLD	Years of life lived with disability

Acknowledgement of contributions

Table 2 – Acknowledgement of specific contributions

Name	Position	Contribution
Ashnish Sinha (AS)	Masters of Research student; Norwich Medical School	Literature search and data extraction for asthma breathomics systematic review (chapter 3)
Christina-Jane Crossman-Barnes (CJ-CB)	Post-graduate researcher; Norwich Medical School	Literature search, data extraction and quality assessment of papers for systematic review of 8-isoprostane in exhaled breath condensate (chapter 2)
Maxim Wilkinson (MW)	Post-graduate researcher Manchester University	Data extraction and quality assessment of papers for systematic review of asthma breathomics (chapter 3). Advice and development of sampling and shipping protocols for VOC. Development of a target list of compounds; deconvolution of GC-MS data. Advice and assistance in statistical analysis, specifically R code.
Iain White (IW)	Post-doctoral researcher Manchester University	Advice and development of sampling and shipping procedures for VOC.
Craig Ferguson (CF)	Research Technician Manchester University	Assistance with shipping. Undertaking the GC-MS processing of samples.
Kirti Vekaria (KV)	Research Technician Manchester University	Assistance with shipping. Undertaking the GC-MS processing of samples.
James Goillau (JG)	Senior Laboratory Technician, University of East Anglia	Advice and development of sampling and shipping procedures for VOC. Assistance with shipping of samples. Advice and assistance with sampling and processing of EBC samples.
Catherine Essex (CE)	Laboratory Technician, University of East Anglia	Assistance with shipping of samples.

Jonathan Tang (JT)	Research Fellow, University of East Anglia	Advice and assistance with sampling and processing of EBC samples; advice on systematic review of 8-isoprostane in EBC manuscript.
Isabelle Piec (IP)	Senior Research Associate, University of East Anglia	Assistance with processing of EBC samples and undertaking ELISA.
Melissa Goodbourn (MG)	Research Fellow; Asthma UK Centre for Applied Research, Edinburgh University	Liaison with AUKCAR PPI group.

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Statement of jointly authored publications

The research reported is my own work, carried out in collaboration with others. The design of studies was performed under the supervision of Professor Andrew Wilson (AMW), Professor Yoon Loke (YKL), Dr. Stephen Fowler (SJF) and Professor Gwyneth Davies (GAD). Studies in this thesis were the result of collaboration between the University of East Anglia; Asthma UK Centre for Applied Research; University of Manchester; and the Norfolk & Norwich University Hospital Foundation Trust.

The contribution of others to this manuscript is as follows:

Chapter 1	Introduction and background	Written by AP and reviewed by AMW and YKL.
Chapter 2	Exhaled breath condensate & 8-isoprostane	AP designed the search strategy, registered the review with PROSPERO, undertook the literature search, data extraction, quality assessment, analysis and wrote the manuscript. CJ-CB performed the literature search, data extraction and quality assessment in duplicate and reviewed the final manuscript. JT offered input on the analysis of methodologies and reviewed the final manuscript. AMW, YKL, SJF & GAD reviewed the search strategy and the final manuscript; YKL advised on the statistical analysis.
Chapter 3	Asthma breathomics	AP designed the search strategy, registered the review with PROSPERO, undertook the literature search, data extraction, quality assessment, analysis and wrote the manuscript. AS performed the literature search in duplicate. MW performed the data extraction and quality assessment in duplicate, offered input on the analysis of methodologies, classification of compounds and reviewed the final manuscript. AMW, YKL, & SJF reviewed the search strategy and the final manuscript.

Chapter 4	Developing methods	The initial study protocols were developed by AP with input from AMW, YKL, SJF, GAD, IW & MW. EBC samples were collected by AP & AS. EBC analysis conducted by AP, AS & IP. MW, IW & SJW designed the GC-MS analytical pipeline. MW conducted data pre-processing. Manuscript drafted by AP with comments and revisions from AMW, SJF, YKL & MW.
Chapter 5	Study methods	Study protocols were developed by AP with input from AMW, YKL, SJF and GAD. AP designed all study documents including participant information sheets, case report forms, non-validated questionnaires, and letters. REC and HRA approval along with subsequent amendments were obtained by AP. MW, IW & KV undertook the GC-MS analysis; MW prepared the target compound list and initial data pre-processing.
Chapter 6	ABC results	Statistical analysis conducted by AP with advice from YKL, SJF and MW. Written by AP with input from MW, YKL, AMW and SJF.
Chapter 7	ABBA results	Data analysis by AP. Manuscript drafted by AP with advice from AMW and YKL.
Chapter 8	Discussion	Written by AP; reviewed by AMW & YKL.

Chapter 1 - Background and introduction

1.1 Overview

Asthma is a chronic disorder of the airways affecting an estimated 339 million people worldwide (1). In the UK 5.4 million people are receiving treatment for asthma, generating a health service spend of approximately £1 billion *per annum* (2).

The Global Initiative for Asthma provide a disease definition, the length and descriptive nature of which reflects the complexity of the disease.

> *A heterogeneous disease, usually characterised by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness, and cough that vary over time and in intensity, together with variable airflow limitation. (3)*

Management of the condition is informed chiefly by symptoms and measures of airway calibre such as peak expiratory flow. There is currently no singe gold-standard test for the diagnosis of asthma. With no definitive cure available, the mainstay of treatment is inhaled β_2 adrenergic receptor agonists and corticosteroids, which serve to relax smooth muscle in airways and reduce inflammation respectively.

1.2 History

1.2.1 Historical descriptions and definitions

Reference to asthma-like symptoms can be found going back several thousand years; sources include the Ebers Papyrus (Egypt, 1550 BC), the Neu Ching (China, 1,000 BC), the Susruta Samahita (India, 450 BC) and Homer's Iliad (Greece, 800 BC) (4). To take just one example, the Neu Ching describes a wheezing condition which varies with the seasons (5).

The word asthma is a Greek noun derived from the verb aazeein meaning to exhale with open mouth or pant (6); while the earliest recorded use of the word is in the Iliad (5, 7) Aretaeus of Cappadocia (Greece circa 100 AD) was perhaps the first to use the term to describe what today would be called an asthma attack (4, 8).

In terms of disease understanding and therapy there was little documented progress between these early references to asthma and the 1600s when descriptions started to resemble those of our current understanding and bronchial constriction was first linked to expiratory difficulty (4).

In the 18th century descriptions included the paroxysmal nature of the condition, the excessive use of respiratory muscles, the absence of fever, and the presence of symptoms such as wheeze, cough, and phlegm (4).

The 19th century saw the identification of smooth muscle in the airways and thus a mechanism for bronchial constriction; an important step in the understanding of asthma pathophysiology. Dr Henry Salter is one of the key figures of this period; he described a "paroxysmal dyspnoea of a peculiar character with intervals of healthy respiration between attacks" (7) which is commonly cited as a key point in the refinement of the term asthma (9). He linked airway narrowing with smooth muscle contraction and also identified a characteristic cellular appearance to asthmatic sputum. In 1868 he posited the involvement of both neural and vascular elements, suggesting that inflammation of airway mucosa led to the neural stimulation of smooth muscle contraction (10). Later that century Paul Ehrlich identified the eosinophil, an inflammatory cell subsequently found to be associated with asthma. Another key figure in the history of asthma is Sir William Osler, often referred to as the Father of Modern Medicine (9); he identified familial and allergic elements to the disease as well as focussing on the role of smooth muscle in airways (7). His description of asthma issued in 1892 is largely unchanged from those today, namely spasm of the bronchial muscles; swelling on bronchial mucous membranes; inflammation of bronchioles; resemblance to hay fever; a hereditary element; commonly a childhood onset; and a variety of causes of exacerbation, including infection (9).

Allusions to allergy are prevalent throughout the history of asthma with links being drawn to occupation (Hippocrates (7)), feathers (Cardano (4)), old mattresses (Ramazinni (4)) and pollen (5, 7). However, the first clear description of seasonal asthma is reported to be that of John Bostock in 1819 (4). In the late 18th and early 19th century studies with pollen showed it was possible to provoke a local allergic reaction but the term allergy was not used until Clement von Pirquet in 1906 (4). In 1911 skin allergy testing was developed and the role of histamine in anaphylaxis was shown in 1911 (10). The identification of IgE (9) and the links between house dust mite allergy and asthma led to a reconceptualization of asthma as not only a condition of bronchoconstriction but also of inflammation and allergy.

Although bronchoconstriction and the role of smooth muscle had already been identified, bronchial hyper-responsiveness in asthma was first described by Curry in 1946 (10); work which led to the subsequent development of the histamine challenge as a diagnostic test in asthma.

In addition to inflammation and bronchoconstriction, structural changes may occur in the airways, particularly in those with more severe disease (9). In the early 20th century morbid anatomical examinations revealed mucous impaction, airway wall thickening, smooth muscle hypertrophy, submucosal oedema, and infiltration by eosinophil and neutrophils; mucosal denudation and basement membrane thickening were later identified (10). It is likely that these changes in the epithelium affect the barrier function of the airways; coupled with changes in the cellular immunity this is posited to make those with asthma more vulnerable to respiratory virus infection (9). More recent pathophysiological developments have been biochemical in nature, examining the immunology and biochemical mediators of inflammation in increasing depth; the clinical application of which can be seen in the latest drug therapies (see section 1.5).

The emphasis placed upon the different elements of asthma has varied over time likely influenced by a combination of both physiological understanding and available treatments. Asthma definitions have been described as swinging from a focus on bronchoconstriction in the 1960s, to inflammation in the 1990s, and back to a more balanced appreciation of both in the 2000s (11). In the 2010's the discussion has centred on disease heterogeneity and the extent to which a single umbrella term such as asthma is useful at all (11-13).

1.2.2 Historical prevalence and incidence

The UK has had a charity dedicated to researching the cause and cure of asthma since 1927 when Asthma UK (originally the Asthma Research Council) was founded (14). It is noted by McFadden (10) that although associated with a high morbidity, historically asthma had a relatively low mortality. Indeed, Siegel (15) reports that in the 19th century asthma deaths were considered rare and mortality rates in the young remained stable throughout the early 20th century up until the beginning of an asthma 'epidemic' in the 1960s. While arguments have been posited for an increase in the incidence of asthma – including westernisation, urbanisation, pollution, the hygiene theory (13) and improved diagnosis / reporting – it is frequently argued that the 1960s epidemic of asthma deaths may have, at least in part, been due to the effectiveness of bronchodilator treatment in improving symptomology leading to an over-reliance on bronchodilators. Not only might these have masked worsening underlying disease and delayed help-seeking (16) but their cardiotoxic effects were underappreciated and may have contributed to mortality (9).

More respiratory-selective β_2-agonists were subsequently developed while at the same time trials were showing the positive effect of corticosteroids on asthma management; results which led to increasing awareness and uptake of preventative inhalers in the 80s.

1.2.3 The development of respiratory function tests and asthma monitoring

The spirometer was invented by John Hutchison in 1846 and entered more widespread clinical use in the 1950s in the form of the vitalograph (4). Performing and interpreting spirometry is a specialised skill; in the UK accreditation with the Association for Respiratory Technology and Physiology (ARTP) is recommended. Spirometry therefore tends not to be conducted on a regular basis in primary care but rather at annual asthma reviews or specialist secondary care centres.

A cheaper, more portable means of measuring lung function was developed after world war two in the form of the peak expiratory flow (PEF) meter (10); this entered common usage in the 1960s and 70s. PEF meters allowed regular home monitoring, making variable airflow obstruction easier to detect.

Personalised asthma action plans designed to empower patients in the management of their own asthma were first introduced in 1990 (17); these are now a key part of asthma management guidelines and can be used with either symptoms or peak expiratory flow readings to guide patient action.

Assessment of inflammation by sputum differential cell count (specifically the percentage of eosinophils) has been used widely in research (18) and is well established as a marker of airway inflammation (19) and disease activity (20). Directing clinical management (for example titration of steroid treatment) according to the percentage of sputum eosinophils has been found to improve outcomes (21-24) and BTS/SIGN guidelines recommend considering this in the management of patients with difficult asthma. The technique of inducing sputum is however time consuming, semi-invasive and requires laboratory facilities; perhaps because of this its uptake in clinical practice has been limited (25).

Exhaled breath gas analysis is another means of non-invasive airway assessment containing, as it does, a number of molecules indicative of inflammation. Exhaled breath gas markers including nitrite, nitrate and volatile organic compounds (VOC) have been the target of much research, but only one compound - Nitric oxide (NO) – has made the leap into clinical use. The fraction of exhaled nitric oxide (FeNO) is - alongside sputum eosinophils - one of the best established markers for disease (20).

It is recommended by the National Institute for Clinical Health Excellence (NICE) for the diagnosis and monitoring of patients with asthma whose symptoms are unresponsive to corticosteroids. Although widely used in research its clinical use has been inconsistently recommended by asthma guidelines (26).

1.2.4 The history of drug therapies

McFadden describes the modern pharmacologic era for asthma as starting in the 1920s (10). Prior to this a variety of treatments were employed; as with early treatments for many diseases some of the ingredients are likely to have been deleterious to health while others may have been of benefit. Some of the inhalants used for asthma included smooth muscle relaxants, mucolytics, vasoconstrictors and cough suppressants.

Anticholinergics were present in some of the herbs of the 19th century (4); these inhibit the parasympathetic nervous system by blocking the cholinergic nerve activity which contributes to smooth muscle contraction and airway narrowing (27). Ipratropium is a modern anticholinergic which may be prescribed as a pressurised metered dose inhaler (pMDI) or used in a nebuliser.

Adrenergics were first used in asthma in 1900 and adrenalin was first used via an inhaler in 1929 (4). Modification of the drugs occurred with analogues being developed (rather than extracted directly from the adrenal gland). The pMDI was subsequently invented and in 1956 was used to deliver an adrenalin analogue isoproterenol (a β-adrenoreceptor agonist)(10). These had unfortunate cardiotoxic effects which – as previously stated - may have contributed to the 1960s asthma death epidemic. Less cardiotoxic, respiratory selective, and longer acting β_2 agonists were developed, including the still widely used salmeterol and formoterol. Trials of dry powder inhalers were first carried out in 1967 (28).

Methyl xanthines are present in coffee, recommended as a treatment for asthma by William Withering in 1786 (4). In 1921 the bronchodilator effect of methyl xanthines was demonstrated, with theophylline being used for asthma the following year and aminophylline in 1944. Cromones are another group or medications derived from a plant which had been used traditionally as an antispasmodic. Cromoglycate and nedocromil sodium are both available today an inhalers.

The treatments mentioned so far focus on tackling bronchoconstriction and were initially used to treated acute episodes; as longer acting formulations were developed and asthma better understood, regular use began in an attempt to prevent asthma attacks from starting (10); there was, however, no treatment to tackle the inflammatory pathophysiology which characterised many asthmas until the advent of glucocorticoids.

Corticosone was first extracted from the adrenal gland in 1936 with successful trials in asthma in the 1950s and its first use as an aerosol the following year (4). Oral corticosteroids were used for asthma in 1956. Aerosolised steroids (beclomethasone and betamethasone) followed in the 1970s (10) transforming the management of asthma (29).

More recent pharmacologic developments include leukotriene receptor antagonists. These gained a licence for use in asthma in the 1990s and work by blocking the action of leukotrienes - an inflammatory mediator in the immune pathway involved in allergy.

The newest class of drugs are the monoclonal antibodies or 'biologics' which include omalizumab, mepolizumab, benralizumab reslizumab, dupilumab and tezepelumab. Omalizumab binds to IgE preventing its action on immune cells thereby retarding allergic reactions; the others all target interleukins or cytokines - chemical mediators involved in the inflammatory cascade (commonly in the production of eosinophils). Omalizumab (Xolair) was approved for use in the UK in 2005, and mepolizumab (Nucala) in 2015.

1.3 Epidemiology and disease burden

1.3.1 Prevalence and incidence

Asthma is one of the most common non-communicable diseases; it affects an estimated 339 million people worldwide (8); and morbidity and mortality are high despite treatment that is effective in the majority of patients (13). Prevalence in the UK is thought to be between 7% and 9% (30) placing it amongst the highest in Europe. Asthma UK estimate that in excess of 5 million people in the UK currently receive treatment for asthma (30); of these, 200,000 are thought to have severe asthma, responding poorly to a combination of bronchodilators and steroids. The British Lung Foundation (BLF) report that in 2012 incidence of asthma was 36% higher and prevalence 11% higher in the most deprived communities when compared to the least deprived. They suggest damp housing, fungal spores, pollution and second-hand smoke may be contributing factors (31).

1.3.1 Disease burden

The NHS spend on asthma is estimated at £1 billion *per annum* (32). A large proportion of costs are likely to come from acute secondary care – Rodrigo et al (33) report that only 20% of asthma patients in the U.S. have ever been admitted to hospital with an acute exacerbation, yet these patients account for over 80% of the asthma healthcare costs. In the UK for the year 2016/17 there were a reported 77,124 hospital admissions for asthma (30). While the mortality rate from asthma exacerbations has declined since the 1960's, there has been relatively little progress in the last 10 years (34).

With the advent of social media, blogging, and vlogging - and with the activities of asthma charities - it is very easy to find personal accounts of the lived experience of asthma. There is a body of work on long term conditions and their effect on the individual, conducted from a number of perspectives – physiological, psychosocial, and socioeconomic. Similarly, a great deal of research has been conducted into disease management and the design of medical care systems for long term conditions. People are living longer but are increasingly likely to have multiple long-term conditions; acknowledgement of this is one of the key drivers of the NHS Long term Plan (35) which includes personalised and digitally enabled care. It is worth noting that relapsing-and-remitting diseases may pose their own distinct difficulties, for example in terms of medication adherence and psychology. Moreover, unlike some other long term conditions such as diabetes, asthma does not attract free prescriptions in the UK. The Global Asthma Network reported 23.7 million disability adjusted life years (DALYs) globally as a result of asthma in 2016 and state that this has not changed since 1990 (8). They ranked asthma 16th in the leading causes of years lived with disability (YLD) globally.

1.4 Asthma definitions and diagnosis

1.4.1 Defining asthma

"*What is this thing called love? – or, defining asthma"* Gross (36)

Defining a disease with multiple endophenotypes creates difficulty. The above editorial title by Gross (cited by Sakula (4)) suggests that – as with love – clinicians and patients know what asthma is, but find it hard to provide a definition that reflects the multitude of possible forms in which it exists. In slightly less prosaic terms The Lancet describes it as "one of the most elusive of all common chronic disorders"(13). It is increasingly recognised that the term asthma represents a heterogenous set of clinical conditions (37); definitions of athma are thus frequently wide ranging and descriptive in nature.

The National Institute of Health use the following definition - "a chronic inflammatory disorder… that is complex and characterised by variable and recurring symptoms, airflow obstruction, bronchial hyperresponsiveness and an underlying inflammation" (38). Many of the earliest definitions centred on bronchoconstriction; however as the importance of inflammation and eosinophils were established, the focus came to rest more on inflammation as the defining characteristic, as exemplified by the NIH definition. As discussed in section 1.2.1 the most recent asthma definitions have swung back to a more balanced view of both bronchoconstriction and inflammation (11) such as this from the Global Initiative for Asthma (GINA):

> *A heterogeneous disease, usually characterised by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness, and cough that vary over time and in intensity, together with variable airflow limitation. (3)*

The fact that it is *usually* characterised by inflammation reflects the existence of variable airflow obstruction in the absence of active inflammatory processes. The fact that what was once thought to be a defining disease characteristic is not ubiquitous has added impetus to the argument that the label 'asthma' might better conceptualised as an umbrella term or perhaps abandoned entirely having outlived its utility (12).

1.4.2 Clinical features of asthma and diagnosis

BTS/SIGN guidelines (39) identify the following clinical features, the presence of which increase the probability of an asthma diagnosis:

- History of recurrent episodes
- Variable peak flow when symptomatic and asymptomatic
- Symptoms of wheeze, cough, breathlessness and/or chest tightness
- Personal or family history of atopic conditions

The guidelines recommend undertaking a structured clinical assessment of asthma probability based on the above clinical features; spirometry; and the comparison of diagnostic tests undertaken when symptomatic and asymptomatic (in order to assess variability).

A number of tests may be conducted including reversibility testing (where lung function is assessed before and after a nebulised SABA), bronchial challenge testing, FeNO, blood eosinophil count, allergy testing and IgE level. Positive results increase the likelihood of an asthma diagnosis being appropriate but none are diagnostic or definitive when taken in isolation.

1.4.3 Acute asthma

Such is the level of variability that the notion of a 'mild' exacerbation has been deemed unworkable; most definitions of such fall within normal levels of symptom variation (40). The BTS/SIGN guidelines use the categories moderate, severe and life threatening based on a number of physiological signs including PEF, respiratory rate, ability to complete sentences and blood gases. A strong argument has been made for the abandonment of the term asthma exacerbation in favour of lung attack (12); with authors arguing that this would reverse the trivialisation of the condition and more accurately convey the potentially fatal consequence of the event.

Acute asthma exacerbations or asthma attacks have three main types of trigger - viruses, bacteria or allergens (41), although exercise, irritant exposure or a change in weather conditions can also trigger variability in symptoms (3). Lower respiratory tract infections are more commonly viral but these are clinically indistinguishable from those of a bacterial origin (42) creating the potential for overuse of antibiotics in the management of acute asthma. Each trigger may act through a different mechanisms but with a final common pathway – that of inflammation and/or bronchoconstriction (43).

1.4.4 Pathophysiology of asthma and aetiology

Pathophysiology

Much of the pathophysiology of asthma has been discussed in section 1.2.1 (the history of the disease). It is characterised by three inter-related elements – those of inflammation, bronchial hyperreactivity or hyperresponsiveness (BHR), and airway remodelling.

BHR is defined by Joos et al as an abnormal increase in airflow limitation due to smooth muscle contraction following exposure to non-allergic stimulus (44).

Bronchial inflammation may be characterised by a variety of inflammatory cells including, eosinophils, mast cells, lymphocytes and neutrophils, which may infiltrate any or all layers of the bronchial wall (45); the predominant inflammatory cell may vary with neutrophilic, eosinophilic and paucigranulocytic as possible endotypes.

Eosinophilic inflammation is a result of type 2 helper T-cells (Th2) producing inflammatory cytokines; Th2 cytokines include those interleukins responsible for the production of IgE and eosinophils in both atopic reactions and inflamation. Mucous hypersecretion may also be a feature. However, non-eosinophilic asthma has also been identified; Th2-low, it has instead been linked to activation of Th1 and Th17 cells (46).

Bronchial remodelling is characterised by stuctural changes in elements of the airway wall such as basement membrane thickening and smooth muscle hypertrophy or fibrosis. It is associated with longer standing disease but can be present early on and sometimes in the absence of inflammation (47). Mutiple pathways may be involved in each of these processes but it is the chemical mediators of inflammation and allergy which have received the greatest attention with drugs being developed to target different parts of the allergic pathway and/or inflammatory cascade.

The aetiology of asthma has not been entirely elucidated but increasingly it is considered - like many other conditions – to be a result of gene-environment interactions (47). A large hereditabe component has been evidenced by twin studies but the rapid increase in asthma prevalence suggests environmental factors (48). Amongst the environmental factors posited are diet, allergen exposure and the hygiene hypothesis. The latter is supported by evidence of a negative association between childhood infections and allergic disease later in life; exposure to microbial products such as endotoxins having a positive effect on the development of the immune system. Similarly allergen exposure in early life may have a protective effect (49). However, microbial agents have been posited to have negative effects also, with infection of the airways being implicated in the subsequent development of asthma (48). In recent years there has been a growth in research into the microbiome and the role this may play in both the development and maintenance of a healthy immune system. Genetic studies have reported a large number of genes to be associated with asthma (multiple interacting genes, some with protective associations, some causative (50)) but relatively few have been replicated (51); genome-wide association studies have however pointed to potentially new aspects of disease pathogenesis such as epithelial barrier function. It has been suggested that therapeutics should focus on increasing airway resistance to the inhaled environment / environmental insults rather than continuing to focus on inflammation (52). At present there is no unifying theory to explain the aetiology (49).

Phenotypes

A phenotype is an observable and measurable disease characteristic – for example airway eosinophilia; whereas endotype referes to a sub-specification of disease based on underlying pathophysiological mechanism (53).

Deliu points out that the same observable features of a disease can be produced by differing underlying mechanisms, thus differing endotypes may have the same phenotype; alternatively different observable features can be the result of a single underlying pathophysiological process after it has been mediated via different individuals' unique biological systems.

The endo-phenotype schema perhaps most commonly used is based upon Th2 status (see figure 1) (46).

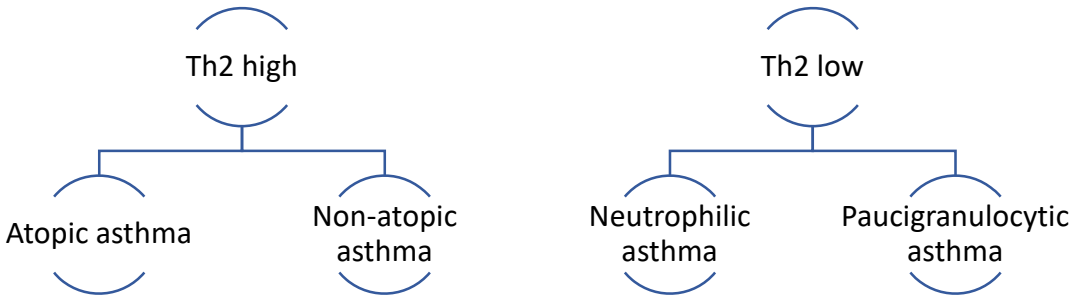


Figure 1 – A commonly used asthma phenotyping schema based on the presence or absence of helper T cells.

The Th2-high classification is a broad umbrella category capturing several different conditions including (but not limited to) atopic asthma, non-atopic asthma, exercise induced asthma, aspirin exacerbated respiratory disease, and late onset eosinophilic asthma. There is no definite consensus on conditions that clearly fall within the Th2-low group, but it is thought to potentially include neutrophilic, paucigranulocytic, mixed granulocytic, smoking associated, and obesity associated asthma.

This system of categorisation has yet to be integrated into national clinical guidelines. GINA state that although recognisable clusters based on demographic features or clinical characteristics exist, with the exception of severe asthma and the new biologic drugs there has been little evidence to establish a strong relationship between these clinical patterns and underlying pathophysiological processes (endotypes). They currently recognise the following phenotypes:

- Allergic asthma – typically of childhood onset with a family history of allergic disease / atopy; typically eosinophilic and responsive to steroids.
- Non-allergic asthma – No associated allergies; airway inflammatory profile may be eosinophilic, neutrophilic or paucigranulocytic; may be less responsive to steroids.
- Adult onset asthma – More common in women, tending to be non-allergic and responding less well to steroids.
- Asthma with persistent airflow limitation – non-reversible airflow limitation likely due to airway wall remodelling in long-standing asthma
- Asthma with obesity – highly symptomatic but with low levels of airway inflammation.

- Occupational asthma – response to an inhaled irritant at work to which a patient is sensitive or has become sensitised.

1.5 Treatment

There are numerous guidelines for the pharmacological management of asthma including both the Global Initiative for Asthma (GINA) and the American Thoracic Society (ATS). In the UK there are the BTS/SIGN guidelines and the guidelines published by the National Institute for Health and Care Excellence (NICE); occasionally these guidelines deviate from one another (54).

BTS/SIGN guidelines recommend a stepped approach to asthma management (as shown in figure 2); increasing or reducing treatment according to control. BTS/SIGN define control as an absence of symptoms, normal lung function and no limitation to normal activities.

It is worth noting Haldar et al (21) identified a group of patients with high risk of asthma attack but low symptom expression.

Amongst such at-risk patients, BTS/SIGN recommendations may not be sufficient to identify disease control; the use of a biomarker such as FeNO may be of benefit.

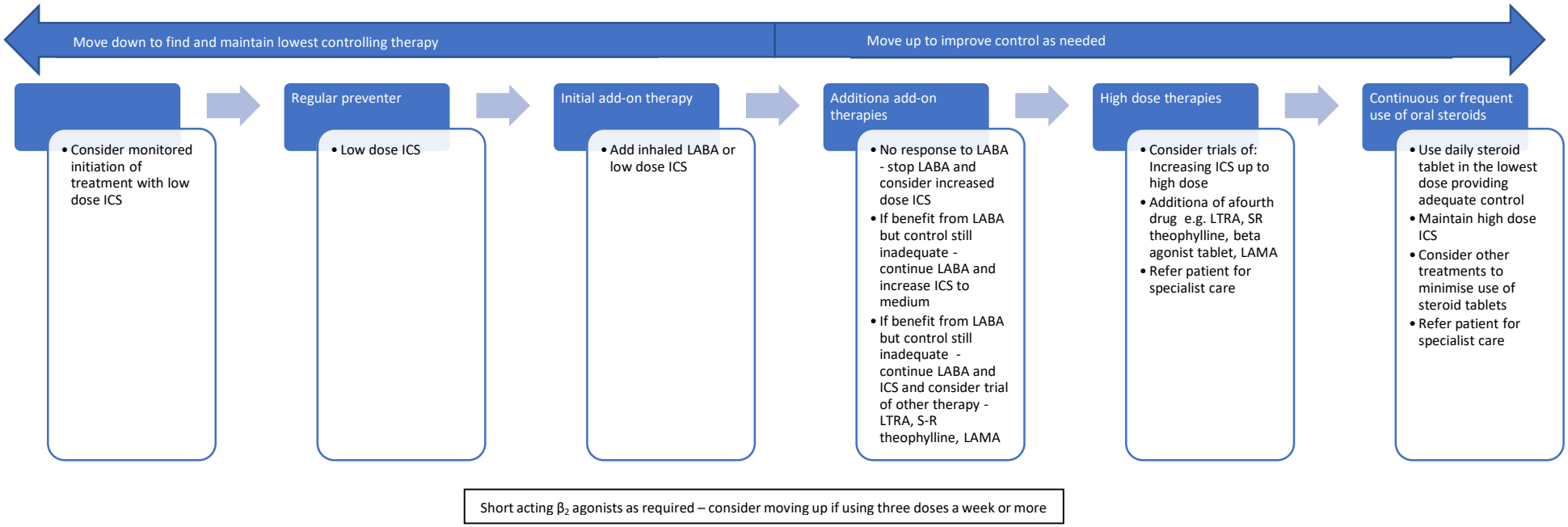


Figure 2 – BTS/SIGN treatment guidelines. Adapted from the 2019 guidelines.

At the top level of treatment, BTS/SIGN guidelines suggest consideration of other steroid-sparing drugs; the new wave of 'biologic' medicines would fall under this category.

There are other treatments not covered in the algorithm:

- Bronchial thermoplasty, used to treat bronchial wall thickening. Radiofrequency pulses are used to reduce smooth muscle mass in the bronchial wall and also to target nerve endings and neuroendocrine epithelial cells (55); although this may reduce severe exacerbations and hospital admissions for up to five years (39) there is an increased risk of exacerbation immediately after treatment (55) and it is recommended for the treatment of non-responsive asthma only (39).
- Azithromycin – a macrolide antibiotic thought to have possible anti-inflammatory effects. Previously limited in use to those with a non-type 2 inflammation (for example paucigranulocytic or neutrophilic), there has been some suggestion that it may have wider applications (16).
- Magnesium sulphate – used in the acute setting where exacerbation has been refractory to treatment. Magnesium acts as a smooth muscle relaxant / bronchodilator. Ramsahai et al report little benefit from nebulised magnesium (16) but cite a Cochrane systematic review reporting a small improvement in lung function and reduced hospital admissions where IV magnesium sulphate is used.

Other aspects of disease management include assessment of therapy adherence, trigger avoidance advice, and personalised asthma action plans to empower self-management.

Patients are concerned about the long term effects of treatments, particularly inhaled bronchodilators and corticosteroids. This is evident from the results of a Priority Setting Partnership on Asthma (56) which ranked information on the adverse effects of these medications as the number one priority.

1.6 Biomarkers

1.6.1 An Overview

A biomarker may be defined as a "characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (57). Applications include diagnosis; indicating clinical prognosis; and/or predicting or monitoring response to therapy.

The SIGN/BTS clinical guidelines (25) recommend that diagnosis of asthma be based on symptoms and spirometry while peak expiratory flow readings should be used for ongoing disease monitoring. These are measures of airway calibre however, not measures of underlying pathophysiological change or activity. The ability to directly assess disease activity may improve diagnostic certainty, facilitate phenotyping and inform treatment algorithms used in the management and prevention of acute asthma exacerbations thereby reducing disease burden.

Inflammation can be assessed directly by analysis of lung biopsy or broncho-alveolar lavage. Both of these are undertaken via bronchoscopy which is expensive and invasive, and therefore unsuited to the purpose of regular clinical monitoring. As discussed in section 1.2.3, assessment of inflammation by induced sputum differential cell count is well established as a marker of airway inflammation (19) and disease activity (20) and directing clinical management according to percentage of sputum eosinophils has been found to improve outcomes (21-24). The technique is however time consuming and semi-invasive, and its uptake in clinical practice has been limited (25). The need to quantify inflammation by less invasive means has been pursued through a number of avenues including the analysis of sputum, exhaled breath condensate (EBC), exhaled breath gases, blood, plasma and urine.

Blood tests offer a more standardised approach than some of the emerging technologies currently do - for example serum proteins such as eosinophilic cationic protein or leptin use existing, reproducible laboratory technologies - however it is argued that they are less sensitive and slower to respond to changes within the airways (47) than an exhaled breath sample; moreover they are more vulnerable to being affected by systemic conditions. In contrast, circulating levels of epithelial proteinases may act as an early warning for exacerbation; however the application would need to be less invasive if it were to be used for routine self-management.

Urinary leukotrienes have been used in their ratio with FeNO to identify a subset of patients more responsive to LTRA (58), while urinary markers of lipid peroxidation and eosinophil-oxidised oxidation were found by another study to be associated with asthma (59). These studies used nuclear magnetic resonance (NMR) analysis which is not widely available and - as with serum – urinary metabolites may be less responsive to airway change and vulnerable to systemic confounders.

Other non-invasive airways assessments using EBC or exhaled breath gases as the sample medium are not yet part of standard clinical practice. There are a number of reasons for this including practical issues (the time and facilities required for some tests), a lack of studies (in the case of emerging technologies) and conflicting evidence of benefit.

1.6.2 Exhaled Breath Gas

Exhaled breath is a mixture of air from the alveoli – the site of gaseous exchange – and air from the anatomical "dead-space" where there is no such exchange; the ratio being approximately 350ml/150ml respectively (60). Amongst the breath tests which have made the leap into clinical use are C-urea for helicobacter pylori infection and nitric oxide for airways inflammation in asthma (60). The fraction of exhaled nitric oxide (FeNO) is measurable using commercially developed equipment and has become widely used; alongside sputum eosinophils it is one of the best established markers for disease (20). However, FeNO monitoring equipment requires controlled breathing which may prove an obstacle for some patients; in one study (61) only 70% of participants experiencing an acute exacerbation of COPD or asthma were able to successfully complete the FeNO test (compared with 100% using an EBC collection device). FeNO is a single biomarker reflecting a single pathological process (47); it is argued by some that its use as an inflammatory marker in non-atopic asthma is limited because it is associated primarily with allergic and eosinophilic inflammation (62); it is not yet recommended in all guidelines; it is not recommended for use in the care of acute asthma; nor is it of use in non-eosinophilic phenotypes; and systematic reviews conducted on studies of FeNO guided asthma treatment have had conflicting results (63-68). Other exhaled breath gas markers including nitrite, nitrate and volatile organic compounds (VOC) have been the target of research. Due to the complex aetiology of asthma and multiple possible phenotypes an argument can be made that a single biomarker or exhaled gas may be limited in its utility.

Recent developments in monitoring have led to the development of equipment which is able to produce a profile of multiple VOC in exhaled breath and their relative concentrations (69); a 'breath-print' which has been used in the identification of disease phenotypes.

Studies suggest that VOC may be able to differentiate asthma from other airways diseases (70-72) and guide asthma treatment; in one study (73) it outperformed both FeNO and sputum eosinophil count in predicting steroid responsiveness. This field is still in its infancy and no study has yet considered this measure during an acute exacerbation of asthma.

1.6.2 Exhaled Breath Condensate (EBC)

In addition to gases, exhaled breath contains aerolised airway lining fluid which in turn contains a number of molecules indicative of inflammation (74). Exhaled breath may be collected and condensed, and – importantly - this can be undertaken during normal tidal breathing; it is therefore not dependent on patient effort and can be undertaken in the acute setting. EBC has been analysed for its acidity (pH) and the presence of leukotrienes, prostaglandins, hydrogen peroxide and other markers of inflammation and oxidative stress. A recent review of EBC pH (75) suggests acidity is a good but non-specific marker of numerous diseases, not limited to respiratory.

Oxidative stress is known to play an important role in the pathogenesis of asthma (76) and markers of oxidative stress can be found in both exhaled breath gas and EBC. Oxidative stress is defined as "a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential damage" (77)(pxc-xvi). The damage caused by oxidants may result in apoptosis or cellular necrosis; oxidants can induce mucous secretion or alter remodelling of extracellular matrix and they can cause smooth muscle contraction and bronchoconstriction (78, 79). All of the above may contribute to asthma exacerbations or death.

Amongst the many markers of oxidative stress the compound 8-isoprostane has the advantage of being relatively stable and suitable for batch analysis. It is a prostaglandin-F_2-like compound formed by the peroxidation of arachidonic acid (80) in cell membrane phospholipids. This non-enzymatic reaction is catalysed by free radicals and is thus a potential marker of oxidative status. It has been shown to be higher in asthmatics than non-asthmatics (80-82); higher in severe asthma than in moderate asthma (83); higher in moderate asthma than in mild asthma (84, 85); able to predict asthma severity (84); and responsive to treatment with corticosteroid therapy (86, 87). Others have, however, reported contradictory findings (88, 89) and the majority of these studies are cross-sectional in design making it difficult to draw inferences about causation. The results do however suggest that 8-isoprostane has potential as a biomarker in that it may be capable of both indicating an abnormal process and response to treatment.

1.6.3 Markers of Infection

The results of bacterial cultures take too long to guide initial therapy so there is a clinical need to develop methods of phenotyping attacks in order to facilitate appropriate tailoring of treatment (including safely withholding antibiotic therapy).

Point of care tests for the identification of viral or bacterial pathogens are in development with the most rapid using nucleic acid amplification techniques, however, these have not yet entered clinical use due to issues relating to interpretation, sensitivity and specificity (90, 91).

Examination of VOC within exhaled breath gas has the potential to inform this subject. VOC profiles can classify the dominant sputum inflammatory profile in asthma (70) and were found to be associated with the presence of lower respiratory tract bacterial infection (LRTI) in ventilated patients (69). Inflammatory mediators dissolved within EBC may also inform this subject; soluble triggering receptor expressed on myeloid cells 1 (sTREM-1) - a neutrophil cell signalling receptor (92) - can be used to identify the presence of bacterial infection (93). It has been found to be elevated in serum and to correlate with blood neutrophil numbers during asthma attacks (94). It has not yet been measured in EBC but is significantly higher in the exhaled ventilator condensate of patients with ventilator acquired pneumonia, when compared to those without (95). Interleukins have previously been measured in EBC; Interleukin-33 is a non-specific pro-inflammatory cytokine that has recently been implicated in rhinovirus induced asthma attacks (96). This has been identified in BALF but not yet studied in EBC. These are both promising biomarkers for use in acute asthma management. Furthermore the engagement of toll-like receptor cells by lipopolysaccharides produced by gram-negative bacteria have been shown to alter the production of VOC offering potential markers of infection (97).

1.6.4 Beyond single markers

Coumou & Bel (98) note that the varying strength of correlation between surrogate airway-eosinophilia markers and sputum eosinophil count may be due to the populations studied and the fact that there are multiple pathways resulting in eosinophilia. It is argued that a panel of biomarkers is likely needed to capture the variance in underlying disease pathophysiology and enable categorisation into endophenotypes (47). Wadsworth et al argue that these might include a wider array of markers; moving away from those purely focussed on inflammation to encompass other disease components such as structural epithelial changes, mucous hyperplasia and myofibroblast proliferation (47).

Ramsahai et al (16) suggest that heterogeneity exists not only in the underlying disease but also in acute exacerbation and argues for the expansion of precision medicine in asthma to enter the treatment of exacerbations; for example using macrolide antibiotics in those exacerbations associated with neutrophilic asthma and evidence of bacterial infection.

It is hoped that exhaled breath analysis capable of identifying and quantifying a range of markers reflective of multiple pathophysiological pathways might address this as yet unmet clinical need.

1.7 Patient priorities for research

In a mixed-methods asthma study Caron-Flinterman et al (99) reported medication side-effects to be the number one patient concern, while more knowledge on the origins of asthma was their number one research priority. Similarly, a James Lind Alliance Priority Setting Partnership (100) reported the adverse effects of medication to be the number one priority, while number two and three were questions about the most effective ways to manage asthma.

Biomarker research is directly relevant to disease management and – through accurate monitoring and medication titration - relevant to patient's medication concerns, precision medicine being one potential route by which non-beneficial medications can be eliminated from a patient's treatment regimen.

The European asthma research innovation partnership (EARIP) (101) gathered stakeholders (including patients, health professionals, researchers and industry representatives) to discuss research priorities. They reported that exploring the value of VOC to phenotype asthma and predict clinical outcomes (such as asthma attack or response to treatment) would be of great interest. Masefield et al (102) published 15 key research priorities identified by EARIP in a roadmap for asthma research and development. Number one was to identify, understand and better classify the different forms of asthma, and their effect on airway inflammation. Number six was to develop tools for quick, accurate and low-cost diagnosis to distinguish asthma from other causes of breathlessness, cough and wheeze. Numbers eight and nine were to evaluate new ways of measuring airway inflammation in monitoring asthma; to identify biomarkers for exacerbations, and to understand the interactions between biomarkers, risk and comorbidities.

1.8 Overview of thesis and rationale

Asthma is a chronic disease with potentially life threatening exacerbations or attacks; the majority of patients have disease that responds well to conventional therapy but a significant minority have severe or refractory disease. It is a heterogeneous disease with multiple endophenotypes which have not been fully elucidated and no gold standard for diagnosis.

Moreover, during acute attack standard assessment methods such as spirometry and biomarker assessment such as FeNO are challenging for patients to perform (103). After very little change in the mainstay of treatment a number of new 'biologic' drugs have been developed targeting specific elements within immuno-inflammatory pathways. Set against this background, the need for biomarkers to diagnose, phenotype and direct treatment is apparent. Input from PPI groups suggests that patient priorities for research are well aligned with these clinical needs. To be adopted in a clinical context, simple methods of assessment which are relatively non-time consuming at the point of care are required, with non-invasive methods being preferable. Exhaled breath collection is non-invasive and – in the case of FeNO – has already entered into clinical use in a way that the more invasive, time-consuming sputum eosinophil count has not achieved. Metabolomic analysis holds great promise in heterogeneous disease due to the ability to apply an inductive, non-targeted approach to biomarker identification.

Acute exacerbations of asthma may be triggered through a number of mechanisms including infection. It is thought that bacteria are responsible for relatively few exacerbations nonetheless many patients receive antibiotics as bedside tests to determine the presence and type of infective agents (or other trigger) are lacking. Exhaled VOC and compounds within EBC hold promise for use in phenotyping exacerbations, with potential application in both the acute and non-acute settings.

Ransohoff (104) in work based on Sullivan et al (2001) suggests five phases to biomarker research (in a parallel to the three phase approach used for drug studies). Phase one is a pre-clinical exploratory study designed to identify promising directions, the primary aims of which are 1) to identify leads for potentially useful biomarkers, and 2) to prioritise the identified leads. Phase two is clinical assay and validation – the primary aim of this stage is to estimate the true and false positive rates, or ROC curves for subjects with and without a condition.

This thesis aims to:

1) Summarise the literature on promising methods of exhaled breath analysis in asthma.
2) Assess the feasibility of conducting phase one or two exhaled breath biomarker studies of asthma in the acute setting. A secondary objective was to collect data on exploratory outcomes including the ability of biomarkers to distinguish between controlled and exacerbated states.

3) Determine the ability of exhaled breath VOC sampling and analysis to detect those changes induced by bronchial challenge testing (namely the release of inflammatory mediators triggered by bronchial challenge using mannitol dry powder).

Summary

The ability to directly assess inflammatory state may improve diagnostic certainty, facilitate phenotyping and inform treatment algorithms used in the management and prevention of acute asthma exacerbations thereby reducing disease burden. The development of a non-invasive, easy-to-use test measuring airway inflammation and/or infection, and its validation in clinical practice would provide potential opportunities for more effective management of exacerbations, reduced medication usage, and reduced hospital admissions. This could lower healthcare costs and enhance the lives of patients living with asthma. The two studies comprising this thesis fall within the phase one stage of biomarker research - establishing the feasibility of an acute study and identifying leads for potentially useful biomarkers. Service users have confirmed that they believe the research topic of the PhD is worthwhile. They aided the design to make it more acceptable to patients and reviewed the lay summary / patient information sheet in order to improve its accessibility.

1.9 Conclusion

This chapter has provided a disease description, outlined clinical questions which need addressing and provided an overview of the current state of research into exhaled breath biomarkers. **Chapter two** examines EBC as a sample medium and 8-isoprostane as a potential asthma biomarker within this. It presents a systematic review of the EBC 8-isoprostane literature in adults with asthma. **Chapter three** is comprised of a systematic review of the literature on exhaled breath gases – specifically VOC – in adult asthma.

The review reports on both the results of such studies and the methods used (including a summary of both sample processing and statistical methods). The later chapters of this thesis present two studies of exhaled breath analysis in acute asthma which were conducted by the author at the Norfolk & Norwich University Hospital Trust. **Chapter four** describes the development of the breath sampling and analytical methods used within these studies. In **Chapter five** the protocol and methods used for the two studies are presented. First the feasibility study *Exhaled Breath Biomarkers in Acute Asthma (ABBA);* this study aimed to determine the feasibility of conducting exhaled breath analysis in the acute setting and compares two different recruitment and assessment methods. Second *Bronchial Challenge Testing in Asthma: The Effect of Mannitol Dry Powder Inhalation on Volatile Organic Compounds in Exhaled Breath (ABC).* This study aimed to determine whether inflammatory changes induced by bronchial challenge testing can be detected using exhaled breath samples and the analysis of volatile organic compounds. **Chapter six** presents the results from the ABC study while **chapter seven** present the results of the ABBA study. **Chapter eight** concludes the thesis with a discussion of both studies, the experimental results and study limitations.

Chapter 2 – Exhaled breath condensate and 8-isoprostane

Accepted for publication as:

Peel, AM. Crossman-Barnes, CJ. Tang, J. Fowler, SJ. Davies, GA. Wilson, AM. Loke, YK (2017) Biomarkers in Adult Asthma: a Systematic Review of 8-Isoprostane in Exhaled Breath Condensate *J. Breath Res.* https://doi.org/10.1088/1752-7163/aa5a8a

Peel, AM. Crossman-Barnes, CJ. Tang, J. Fowler, SJ. Davies, GA. Wilson, AM. Loke, YK (2016) Biomarkers in Adult Asthma: A Systematic Review of 8-Isoprostane in Exhaled Breath Condensate. British Thoracic Society Winter Meeting, Oral Presentation. 7/12/2016

Peel, AM. Crossman-Barnes, CJ. Tang, J. Fowler, SJ. Davies, GA. Wilson, AM. Loke, YK (2016) 8-isoprostane in Exhaled Breath Condensate: A Systematic Review in Adult Asthma. Asthma UK Annual Scientific Meeting, Oral Presentation. 9/11/2016

Protocol registered as:

Peel, AM. Crossman-Barnes, CJ. Loke, YK. Wilson, AM. Exhaled Breath condensate 8-isoprostane in adult asthma: a systematic review protocol. PROSPERO: International prospective register of systematic reviews. 2016. CRD42016027312. Available from https://www.crd.york.ac.uk/prospero/display_record.php?ID=CRD42016027312.

2.1 Introduction

The previous chapter provided an overview of exhaled breath assessments, covering the variety of methods currently employed for this task, and highlighting the multitude of biomarkers and sample mediums which have been investigated.

This chapter focusses more narrowly on exhaled breath condensate as the medium and 8-isoprostane as the biomarker. A systematic review, this chapter has been published as Peel, AM, Crossman-Barnes, CJ, Tang, J. Fowler, SJ, Wilson, AM, Loke, YK. *Biomarkers in Adult Asthma: A Systematic Review of 8-isoprostane in Exhaled Breath Condensate* (2017) Journal of Breath Research, Vol 11, Number 1. It has also been presented under the same title at the British Thoracic Society Winter Conference 2016 and under the title *8-Isoprostane in Exhaled Breath Condensate: A Systematic Review in Adult Asthma* at the Asthma UK Centre for Applied Research Annual Scientific Meeting 2016.

Collecting and analysing the condensate from exhaled breath (EBC) as a non-invasive measure of disease activity, has been studied since the early 1980's (105). Several different commercial devices are available and this methodology has been adopted in a number of studies looking at an ever growing number of potential biomarkers.

Oxidative stress is thought to play an important role in asthma as both a causative factor and a result of inflammation (76, 106). It results from the action of reactive oxygen species (ROS), formed through the addition of an electron/s to oxygen. These ROS - also known as free radicals - are so named because they react easily with other molecules, such as the phospholipids in cell membranes, with damaging effects. The presence of ROS is not abnormal; their production is a part of common metabolic activity and there are physiological levels at which ROS may play a role in cell signalling (79). Oxidative stress occurs where there is a failure of homeostasis due either to an excess of ROS (such as may occur during inflammation) or to a lack of antioxidants; this can cause cellular damage, proinflammatory mediator release, mucous secretion, remodelling of extracellular matrix, smooth muscle contraction and bronchoconstriction (78, 79, 106).

The reaction of ROS with other molecules is so rapid that their direct measurement is difficult; however, end products of ROS 'attack' are more stable and may be useful as surrogate markers for oxidative stress. Arachidonic acid within the phospholipids of cell membranes is converted, through the action of oxidants, into isoprostanes – prostaglandin-like compounds. 8-isoprostane was first identified in 1990 (107) and is a prostaglandin-F_2-like compound specific to oxidative stress, stable, and measurable in EBC (108-110). Paediatric studies of EBC 8-isoprostane have been the subject of a systematic review (111) which found the majority of studies reported a significant association between 8-isoprostane and asthma, however, as biomarker thresholds vary with age (112), there is a need to review the adult literature. A systematic approach was selected in order to view the evidence as whole, and to identify common themes as well as inconsistencies that may only become apparent through evaluation of the entire dataset. The review aimed to assess the evidence regarding the efficacy of EBC 8-isoprostane as a biomarker – its ability to identify disease, disease severity and response to treatment.

2.2 Methods

Study design

The study protocol was registered with the International Prospective Register of Systematic Reviews (PROSPERO) (registration number CRD42016027312). The primary objective of the review was to assess the ability of 8-isoprostane to identify and distinguish between a) those with asthma and healthy controls b) levels of asthma severity, and c) response to treatment. A secondary objective was to determine possible thresholds appropriate to a diagnosis of asthma or classification of severity.

Search Strategy

A search strategy was developed using terms relating to asthma, exhaled breath condensate and 8-isoprostane (see table 3).

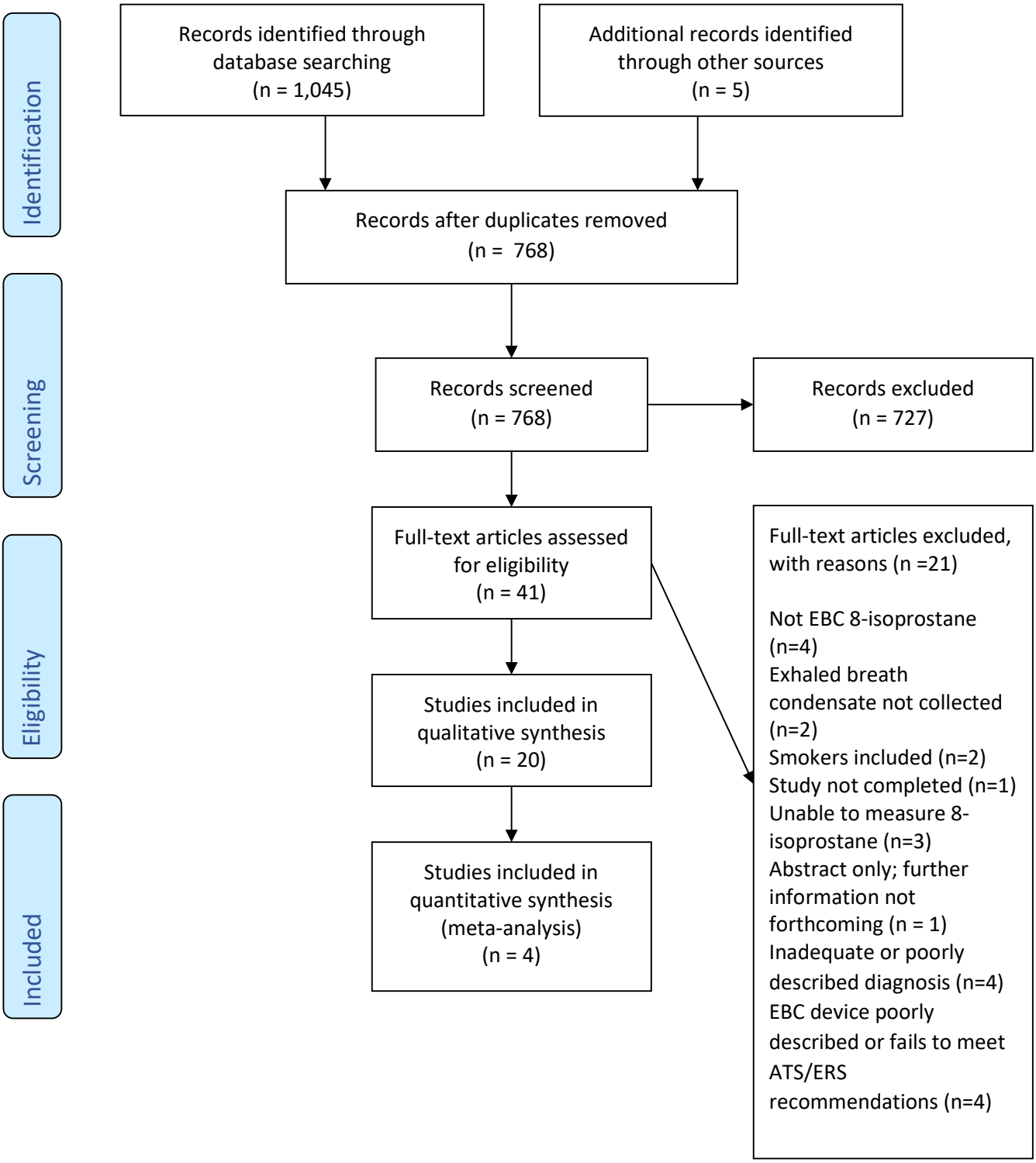
Table 3 – Search terms for 8-isoprostane in exhaled breath condensate

Terms relating to the condition of interest - asthma	**Asthma* OR "Bronch* hyperreactivity"**
Terms relating to the collection method - exhaled breath condensate.	"Exhaled breath condensate" OR "Breath test*" OR "Lung function test*" OR "Expired air"
Terms relating to the biomarker of interest - 8-isoprostane	*isoprostane* OR Dinoprost* OR *prost* OR "Lipid peroxid*" OR *prostaglandin*
Master search string (adapted for use in individual databases required)	(Asthma* OR "Bronch* hyperreactivity") AND ("Exhaled breath condensate" OR "Breath test*" OR "Lung function test*" OR "Expired air") AND (*isoprostane* OR Dinoprost* OR *prost* OR "Lipid peroxid*" OR *prostaglandin*)

The strategy was modified as required for individual databases and implemented in the following online databases: Cochrane, Embase, PubMed, Lilacs, Scopus, ClinicalTrials.gov, Open Grey and ProQuest.

Two reviewers (AMP & CJCB) screened titles and abstracts for inclusion, resolving discrepancies through discussion with a third reviewer (YKL). The screening and selection process is described in a PRISMA flow chart (see figure 3).

Figure 3 – PRISMA flow diagram: 8-isoprostane in exhaled breath condensate. Steps taken in the identification of studies for inclusion in review.



Eligibility criteria

Inclusion / exclusion criteria are described in table 4 below.

Table 4 – 8-isoprostane in EBC: inclusion & exclusion criteria

Inclusion criteria	Exclusion criteria
Abstract in English	Review articles
Primary data	Studies including paediatric patients
Quantitative data	Studies of occupational asthma
Diagnosis of asthma according to recognised guidelines	Studies of current smokers
EBC 8-isoprostane measured	In vitro studies
Human subjects	Use of a custom EBC device with insufficient description or which fails to meet ATS/ERS guideline recommendations (113).
Adult participants (aged 18+)	Studies published as comment / letters will have a request for further information made; they will be excluded if further detail is not forthcoming.

Studies were excluded if the EBC collection device failed to meet ATS/ERS construction guidelines (114) (or was described insufficiently to determine this), or if the method of asthma diagnosis failed to meet recognised guidelines or was incompletely described. An exception to this was the use of nose-clips; although this was recommended, the guidelines state that there were no data underpinning this recommendation. A study by Vass et al (115) published since the guidelines found no significant difference between samples collected with or without nose-clips (although 8-isoprostane was not one of the mediators studied).

During the initial screening process several conference abstracts were found. On contacting the authors it was confirmed that the results had not been published more fully elsewhere but insufficient information was forthcoming to determine suitability for inclusion. In order to avoid selective dissemination bias an analysis of these papers was included.

Data extraction and quality assessment was conducted by two reviewers independently (AMP and CJCB). Data were extracted directly into SPSS (116); papers were assessed for quality and risk of bias (117); and the overall strength of evidence was assessed (118, 119). Discrepancies between the two reviewers were resolved through discussion with a third (YKL).

Statistical methods

The objective was to produce a quantitative synthesis using methods appropriate to the data extracted and to assess statistical heterogeneity using the I^2 statistic. Open-Meta Analyst was used to conduct a random effects meta-analysis of mean difference (between asthma and control groups) for those studies reporting continuous data with a mean and standard deviation (SD). Where the SD was not reported it was calculated from confidence intervals or standard error (except where data had been transformed). In studies with multiple arms data was combined. Papers which presented their results as a median and range could not be included.

2.3 Results

The number of papers identified through the database search was 1,045; a further five were identified through reference searches (see PRISMA diagram, figure 3). This was reduced to 768 on removal of duplicates. Title and abstract screening resulted in 41 papers which was reduced to 20 after screening full texts. Study characteristics are summarised in table 5.

Table 5 – 8-isoprostane in EBC: study characteristics and results

Author	Publication type	Country	N =	EBC device	Method of analysis	Study focus	Severity of asthma population	Significant difference between asthma & controls?	Significant difference between asthma severities?
Battaglia et al (2005)	Journal	Netherlands	31	EcoScreen	ELISA	Small airway function	Mild	-	-
Brussino et al (2010)	Journal	Italy	32	RTube	ELISA	Allergen challenge	Mild	$p<0.001$	-
Carpagnano et al (2006)	Journal	Italy	26	EcoScreen	ELISA + GC-MS	GORD	Mild persistent	-	-
Fritscher et al (2012)	Journal	Canada	67	RTube	LC-MSMS	COPD & asthma	Mild persistent	NS	-
Gratziou et al (2008)	Journal	Greece	28	EcoScreen	ELISA	Seasonal allergic rhinitis & asthma	Mild (previously untreated)	$p<0.05$	-
Head & Mickleborough (2013)	Journal	USA	7	EcoScreen	LC-MS	Supplements	Mild-to-moderate	-	-
Komakula et al (2007)	Journal	USA	114	RTube	ELISA	BMI	Moderate-to-severe	NS	-
Kostikas et al (2002)	Journal	Greece	50	Custom device	ELISA	pH	Mild + moderate	-	-
Mastalerz et al (2011)	Journal	Poland	21	EcoScreen	GC-MS	Aspirin sensitivity	Mild-to-moderate	-	-
Mastalerz et al (2015)	Journal	Poland	53	EcoScreen	GC-MS	Aspirin sensitivity	Moderate	-	-
Mickleborough et al (2013)	Journal	USA	20	EcoScreen	ELISA	Supplements	Mild-to-moderate	-	-
Piotrowski et al (2011)	Journal	Poland	52	EcoScreen	ELISA	Asthma severity	Severe + never treated	NS	NS

Samitas et al (2009)	Journal	Greece	62	EcoScreen	ELISA	Asthma severity	Mild + moderate + severe	*p*<0.001	*p*< 0.01
Shimizu et al (2007)	Journal	Japan	62	EcoScreen	ELISA	GORD	Moderate	*p*<0.05	-
Sood et al (2013)	Journal	USA	14	RTube	ELISA	Allergen	Mild atopic	NS	-
Zhao et al (2008)	Journal	Japan	64	EcoScreen	ELISA	GORD	Mild	*p*= 0.034	-

Potentially eligible studies (conference abstracts)

Gemicioglu et al (2014)	Conference abstract	Turkey	19	No info	No info given	Smokers & non-smokers	Newly diagnosed	-	-
Holguin & Fitzpatrick (2009)	Excerpt in review article	USA	125	RTube	No info given	BMI	Moderate-to-severe	NS	-
Sedlak et al (2013)	Conference abstract	Czech Republic	61	EcoScreen	LC-MS	Inflammatory phenotype	Severe refractory	*p*<0.001	-
Sedlak et al (2012)	Conference abstract	Czech Republic	20	No info	LC-MS	Oral steroids	Difficult-to-control	*p*<0.001	-

p< = a significant relationship reported
NS = a non-significant relationship reported
\- = not analysed or not reported
N = number of participants in asthma and healthy control groups eligible for inclusion
ELISA = Enzyme-linked immunosorbent assay
GC-MS = Gas chromatography mass spectrometry
LC-MS = Liquid chromatography mass spectrometry
GORD = Gastro-oesophageal reflux disease

2.3.1 Quality assessment

Results of the Quadas-2 quality assessment can be found in table 6. It was not possible to assess the risk of bias arising from patient selection methods or from the conduction of the index test (EBC collection); in all but one paper description of patient sampling and/or recruitment methods was absent, and in only one paper was it clear whether the laboratory analysis of EBC was conducted by someone blinded to the participants' asthma status. The time between reference and index standards was not clearly stated in five of the papers. The larger the interval the greater the risk of a change in condition between the two assessments and potential misclassification of asthma severity; asthma assessment within 1 week of EBC collection was deemed to be acceptable. Participant drop-out occurred in very few studies.

Table 6 – 8-isoprostane in EBC: QUADAS-2 quality assessment

Study	RISK OF BIAS				APPLICABILITY CONCERNS		
	PATIENT SELECTION	**INDEX TEST**	**REFERENCE STANDARD**	**FLOW AND TIMING**	**PATIENT SELECTION**	**INDEX TEST**	**REFERENCE STANDARD**
1. Battaglia 2005	?	?	☺	☹	☺	☺	☺
2. Brussino 2010	?	?	☺	☺	☺	☺	☺
3. Carpagnano 2006	☺	?	☺	☺	☺	☺	☺
4. Fritscher 2012	?	?	☺	?	☺	☺	☺
5. Gratziou 2008	?	?	☺	☺	☺	☺	☺
6. Head 2013	?	?	☺	☹	?	☺	☺
7. Komakula 2007	?	?	☺	☺	☺	☺	☺
8. Kostikas 2002	?	?	☺	☺	☺	☺	☺
9. Mastalerz 2011	?	?	☺	☺	☺	☺	☺
10. Mastalerz 2015	?	?	☺	☺	?	☺	☺
11. Mickelborough 2013	?	?	☺	?	?	☺	☺
12. Piotrowski 2011	?	?	☺	☺	☺	☺	☺
13. Samitas 2009	?	?	☺	☺	?	☺	☺
14. Shimizu 2007	?	?	☺	?	☺	☺	☺
15. Sood 2013	?	☺	☺	?	☺	☺	☺

16. Zhao 2008	?	?	☺	?	☺	☺	☺
Potentially eligible studies							
(conference abstract/review excerpt)							
a. Gemicioglu 2014	?	?	☺	☺	☺	?	☺
b. Holguin 2009	?	?	?	?	☺	?	?
c. Sedlak 2013	?	?	?	?	?	☺	?
d. Sedlak 2012	?	?	?	?	?	?	?

Key

☺ = high risk / high level of concern regarding applicability

? = unclear risk / unclear level of concern regarding applicability

☹ = high risk / high level of concern regarding applicability

2.3.1.1 Variability: Pre-analytical

One study (Samitas et al (83)) coated the condenser surface of their EBC collection device in Tween-20 (a non-ionic surfactant) to reduce eicosanoid adherence. They report 8-isoprostane concentrations which are towards the higher end of results within this review. The extent to which this was due to the use of Tween-20 is unclear; Sood et al (120) examined this method and found no significant difference in 8-isoprostane between samples collected with or without Tween.

Three studies (Battaglia et al (121), Fritscher et al (122) and Sood et al (120)) undertook or cited 8-isoprostane recovery rates obtained from spiking tests; all were over 90%. Sood et al found that concentrating their samples by lyophilisation had no effect on recovery rates, whereas Battaglia et al found lower rates when they used an immunoaffinity sorbent and lyophilisation.

Kostikas et al (123) cooled their condensing surface to minus 10°C whereas other studies used minus 20°C. This study was included as it does not contravene ATS/ERS recommendations and evidence on the effect of temperature on EBC 8-isoprostane collection is conflicting (124-126).

Not mentioned in the ATS/ERS guidelines but specified by Cayman in their enzyme-linked immunosorbent assay (ELISA) information (127) is the use of an anti-oxidant - butylated hydroxytoluene (BHT) - for EBC samples which are being frozen and stored for later analysis.

This is to prevent further (in vitro) oxidative formation of 8-isoprostane. The majority of studies using ELISA kits stored their samples for later analysis but none reported the use of BHT.

Relatively few studies reported the length of time samples were stored for but Samitas et al evaluated the stability of 8-isoprostane at -80°C and found no significant difference in samples tested at one, four and eight weeks (although an upward trend could be noted).

2.3.1.2 Variability: Analytical

For their ELISA, Cayman cited a sensitivity of 3pg/ml and inter-assay variation rates of 10-24% however this validation was not undertaken in EBC. Sood et al (120) found the intra-assay CV in EBC to be 37.7% compared to 6% in buffer diluent. They concluded that interference from the EBC matrix was possible; the extent to which this might be a confounder in other studies is unclear as Sood et al's analysis was conducted on a lyophilised, concentrated EBC sample. The majority of studies in this review cite intra-assay and inter-assay CV <10%.

Several studies utilised mass spectrometry techniques as their method of analysis – GCMS and LC-MS/MS methods offer improved sensitivity and selectivity over immunoassays, hence they are often regarded as the superior method for measurement of isoprostanes (128-130). Fritscher et al (122) report the limit of detection with LC-MS to be 0.05-0.1pg; while Mastalerz et al (131) report that of GC-MS to be between 0.17 and 0.89pg/ml. The results found by studies using mass-spectrometry frequently fell below the lower detection limit of immunoassays. Two papers compared the results produced by ELISA methods with a) GC-MS (Carpagnano et al) and b) radioimmunoassay (Sood et al). Sood et al report discordance between methods while Carpagnano do not.

The absence of prime certified standard reference materials (SRM) produced by accredited bodies (such as NIST) for the production of calibration curves is a further source of potential inaccuracy and inter-laboratory variation.

2.3.1.3 Grade Assessment

A GRADE assessment was completed (using GradePro GDT (132)) for the 12 studies reporting on both asthma and control groups (see table 7). The strength of the evidence pertaining to the differentiation of disease status was judged to be very low due to the inconsistency and imprecision of results.

Table 7 – 8-isoprostane in EBC: GRADE evidence profile

Setting: Adult non-smokers in any clinical setting.

Bibliography: Battaglia, Hertog, Timmers et al (2005); Brussino, Badiu, Sciascia et al (2010); Carpagnano, Resta, Ventura et al (2006); Fritscher, Post, Rodrigues et al (2012); Gratziou, Rovina, Makris et al (2008); Komakula, Khatri, Mermis et al (2007); Kostikas, Papatheodorou, Ganas (2002); Piotrowski, Majewski, Marczak et al (2011); Samitas, Chorianopoulos, et al (2009); Shimizu, Dobashi, Zhao et al (2007); Sood, Qualls, Seagrave et al (2013); Zhao, Shimizu, Dobashi et al (2008).

Quality assessment							Impact	Quality	Importance
№ of studies	**Study design**	**Risk of bias**	**Inconsistency**	**Indirectness**	**Imprecision**	**Other considerations**			
Is exhaled breath condensate 8-isoprostane capable of differentiating between healthy controls and those with asthma?									
12	Mixture of observational and experimental studies	Not serious	Very serious [1]	Not serious	Very serious [2]	Publication bias strongly suspected; all plausible residual confounding would reduce the demonstrated effect [3]	Cases (asthma) 353; controls 229.	⊕◯◯◯ VERY LOW	CRITICAL

1. Significant unexplained variability in results; I-squared test for statistical heterogeneity = 94
2. Large variance in study data
3. Probable publication bias

Very low quality – Little confidence in the effect estimate; the true effect is likely to be substantially difference from the estimate of the effect.

2.3.1.4 Quality assessment summary

For the majority of included papers there are no concerns over applicability to the review question but the risk of bias in the studies is largely unclear and there are unresolved methodological questions. Overall assessment of the evidence grade is very low.

2.3.2 Quantitative synthesis

2.3.2.1 Prediction of asthma attack or treatment response

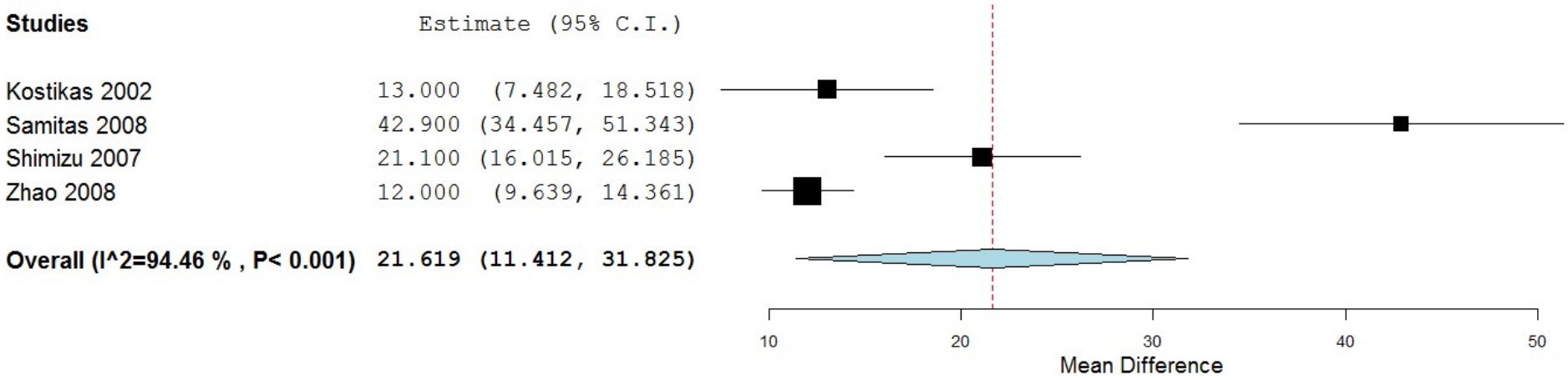
There were no studies examining the strength of association between 8-isoprostane concentration and frequency of asthma attack, nor studies examining the ability of 8-isoprostane to predict the risk of attack or response to treatment.

2.3.2.2 Differentiation of disease status

There was a large degree of clinical heterogeneity; studies examined different asthma phenotypes and severities, and utilised different interventions (including provocation tests and treatments). Given the broad study question being addressed, studies were considered sufficiently homogenous for meta-analysis despite these differences.

Using Open Meta Analyst (133) a random effects meta-analysis of mean difference between groups (see figure 4) was performed. The estimated mean difference was +21.62 pg/ml in those with asthma (standard error 5.21). The *p*-value of <0.001 suggests statistical significance, and the lower bound of the meta-analytical point estimate - 11.4pg/ml – is above the detection limit for the ELISA (2.8 to 7pg/ml). However, the I^2 test result - 94 - suggests a considerable degree of statistical heterogeneity, and the estimated mean difference (21.62pg/ml) should be viewed in light of the overall range of averages for EBC 8-isoprostane which varied from 0.25pg/ml to 78.10pg/ml.

Figure 4 – Random effects meta-analysis showing a significant mean between-group difference in 8-isoprostane levels (asthma versus controls). Weighted mean difference of 8-isoprostance levels between asthma and control groups showing significantly higher levels (+21.62 pg/ml (SE 5.21, p<0.001)) in asthma patients, but with considerable heterogeneity I2 = 94.46%.



2.3.3 Qualitative synthesis

Ten papers (n = 419) reported average 8-isoprostane levels to be higher in asthma than in healthy controls, while five papers (n = 389) reported averages to be the same or higher in controls.

Of the ten studies reporting higher concentrations in asthma, only seven (n=329) reported the difference to be statistically significant. However, of the three which were excluded, two (123, 134) simply omitted to report the significance level, while the third study – Sood et al (120) - was not powered to detect a between-group difference in 8-isoprostane concentration.

With the exclusion of conference abstracts, five papers (n=248) report a significant difference, and five papers (n=278) report either no significant difference or higher concentrations amongst controls. All papers scored similarly in their quality assessment.

Table 8 presents the results of the included papers.

Table 8 – 8-isoprostane in EBC study results: averages & variance

			Average EBC 8-isoprostane level	
Author	**Units (measure of central tendency)**	**Measure of variance**	**Asthma (variance)**	**Control (variance)**
Battaglia, Hertog, Timmers et al	pg/ml (median)	Range	2.10 (1.6 - 2.7)	3.6 (2.9 - 7.6)
Brussino, Badiu, Sciascia et al*	pg/ml (geometrical mean)	95% confidence interval	21.56 (19.92 - 23.35)	16.43 (15.50 - 17.41)
Carpagnano, Resta, Ventura et al	pg/ml (median)	Range	17.90 (8.9 - 23.8)	6.9 (5.6 – 9.7)
Fritscher, Post, Rodrigues et al	pg/ml (median)	Range	0.60 (0.4 - 2.0)	0.9 (0.2 - 1.7)
Gratziou, Rovina, Makris et al	pg/ml (median)	Interquartile range	39.0 (4.0 - 125)	18.5 (4 - 37)
Head & Mickleborough	pg/µl (mean)	Standard error	3.08 (+/- 1.5)	_
Komakula, Khatri, Mermis et al*	pg/ml (mean)	95% CI	11.0 (9.6 - 12.4)	11.0 (8.0 – 13.8)
Kostikas, Papatheodorou, Ganas	pg/ml (mean)	Standard deviation	33.0 (11)	20.0 (7)
Mastalerz, Sanak, Kumik et al	pg/ml (mean)	Standard deviation	0.25 (+/- 0.12)	_
Mastalerz, Januszek, Kaszuba et al AERD & ATA (two asthma groups within study)	pg/ml (median)	Interquartile range	0.28 (0.19 - 0.49) 0.54 (0.35 – 1.65)	_ _
Mickleborough, Vaughn, Shei et al	pg/ml (mean)	Standard deviation	46.40 (+/- 15.1)	_
Piotrowski, Majewski, Marczak et al Severe asthma & Never treated asthma (two groups within study)	pg/ml (median)	Interquartile range	3.8 (2.5 – 10.73) 4.67 (2.5 – 27.92)	6.93 (2.5 - 12.98)
Samitas, Chorianopoulos, et al	pg/ml (mean)	Standard error	59.30 (+/- 4)	16.4 (+/- 1.6)
Shimizu, Dobashi, Zhao et al	pg/ml (mean)	Standard error	27.70 (+/- 2.3)	6.6 (+/- 1.2)
Sood, Qualls, Seagrave et al*	pg/ml (mean)	Standard deviation	2.50 (+/- 0.99)	1.54 (+/- 1.39)
Zhao, Shimizu, Dobashi et al	pg/ml (median)	Interquartile range	16.20 (11.7 – 19.1)	3.5 (2.6 – 7.9)

* Log transformed data

Potentially eligible studies (conference abstracts)				
Gemicioglu, Duman, Akdeniz et al	No units given (mean)	Standard deviation	135.72 (+/- 38.85)	
Holguin & Fitzpatrick	pg/ml (mean)	95% confidence interval	Unable to extract data	_
Sedlak, Cap, Kacer et al	No units given (?)	No measure of variance given	Result not directly cited	_
Sedlak, Cap, Kacer et al	pg/ml (?)	No measure of variance given	78.10	_

Results from those papers reporting a median and those reporting a mean are displayed in figures 5 and 6 respectively. Even when looking only at those studies reporting a significant between-groups difference, there is a considerable overlap of results between studies - the range of values for controls in one study being similar to those for asthma in another. This degree of statistical heterogeneity precludes the determination of threshold values.

Figure 5 – Median levels of 8-isoprostane in asthma and control groups; between study overlap in the two groups is apparent. The values for controls in one study falls within those for asthma in another; statistical heterogeneity which prevents the determination of a diagnostic threshold.

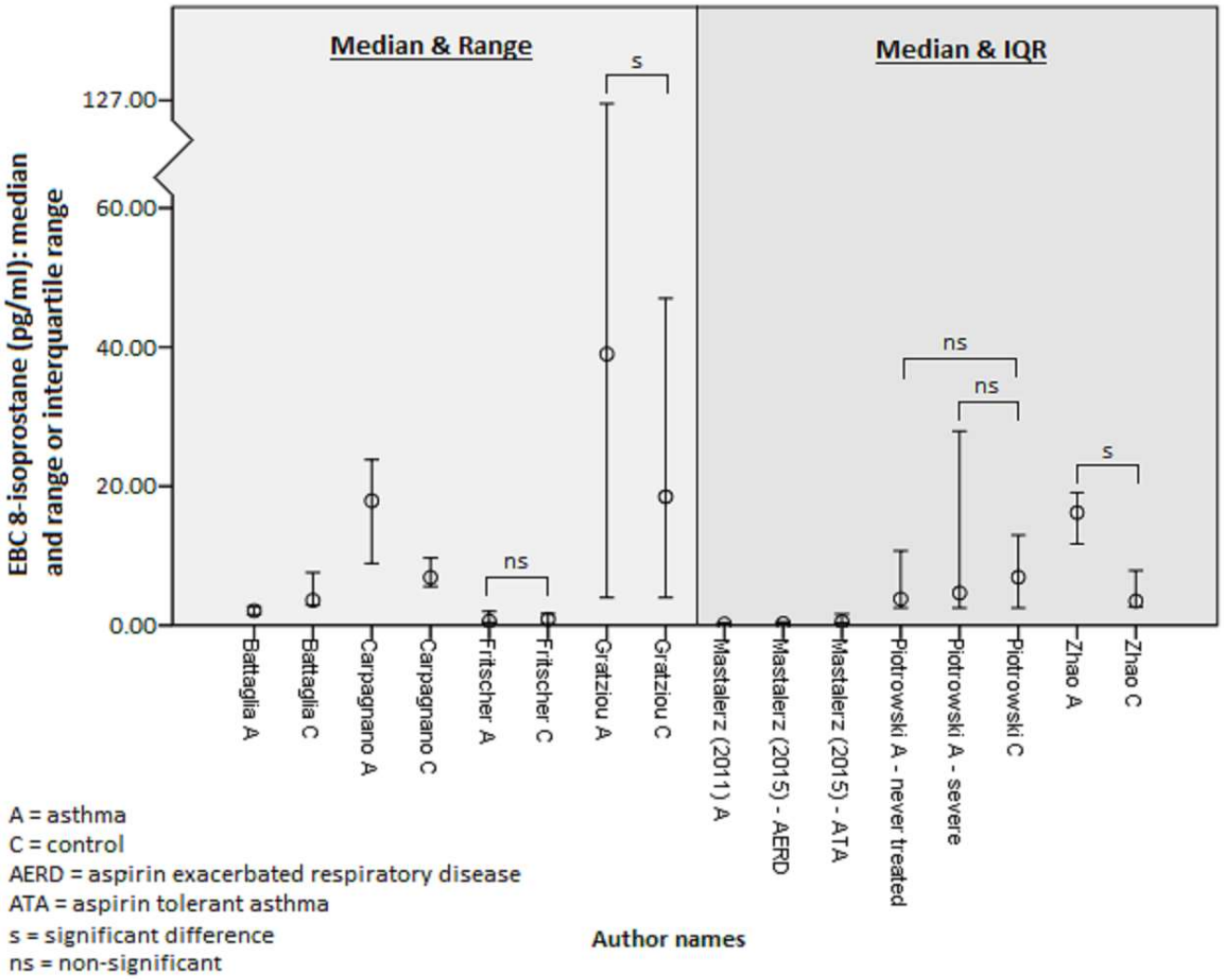
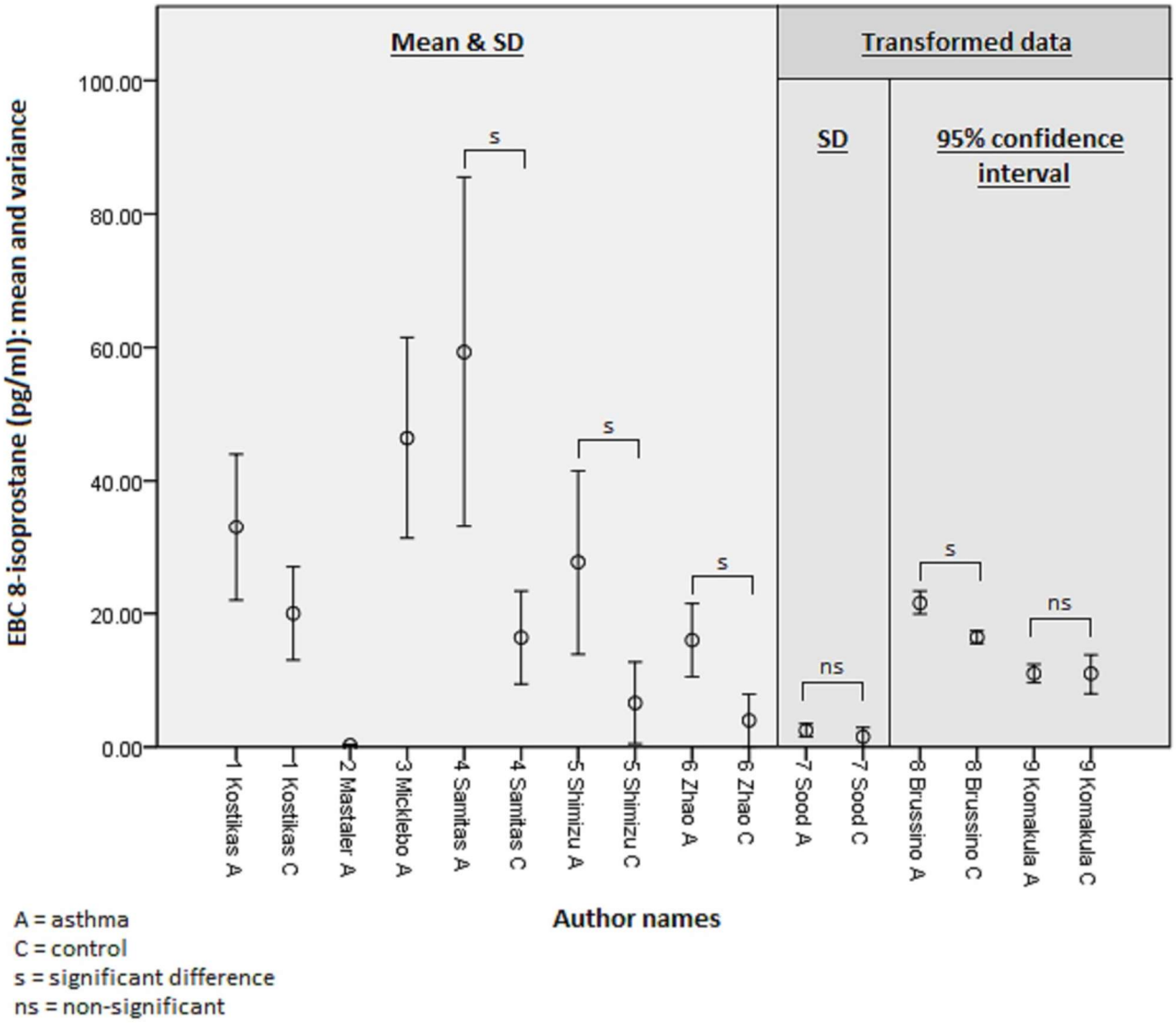


Figure 6 – Mean levels of 8-isoprostane in asthma and control groups; between study overlap in the two groups is apparent. The values for controls in one study falls within those for asthma in another; statistical heterogeneity which prevents the determination of a diagnostic threshold.



There was a large degree of overlap in 8-isoprostane concentration between severities of asthma. This may be attributable to between-study methodological differences, however, three studies (83, 88, 122) made within-study comparisons of severity. Samitas et al (83) report a significant difference between the severe and milder asthma groups, whereas Piotrowski et al (88) report a small, non-significant difference (0.87 pg/ml). Kostikas et al (135) report a difference of 15 pg/ml but do not comment on its statistical significance.

Both Brussino et al (136) and Sood et al (120) investigated the effect of allergen challenge on EBC 8-isoprostane concentration. Brussino et al reported a statistically significant increase while Sood et al reported no such change.

Gratziou (137) studied patients with seasonal allergic rhinitis and concurrent asthma, reporting significantly higher levels of 8-isoprostane during pollen season, and a significant decrease after treatment with nasal corticosteroids. Mastalerz et al (131, 138) conducted a pair of studies in which patients with aspirin intolerant asthma (AIA) or aspirin exacerbated respiratory disease (AERD) were subjected to an aspirin challenge; they found no significant difference in 8-isoprostane after challenge.

Baseline measures of pulmonary function (spirometry) were commonly reported in order to characterise study populations; their relation to EBC 8-isoprostane was less commonly examined. Eight studies conducted such an analysis, of which only two reported a significant (negative) correlation. Similarly, baseline blood eosinophil count was reported by five papers but analysed in relation to 8-isoprostane by only one (reporting no correlation). FeNO was measured by six studies; four assessed the degree of correlation with EBC 8-isoprostane only one of which yielded a statistically significant (positive) association. Two papers undertook sputum analysis; one reported on the relationship with EBC 8-isoprostane - no significant association was found.

2.3.4 Subgroup analysis

Methodological heterogeneity has been suggested as one of the factors inhibiting clinical use of EBC (114). Those papers included in the meta-analysis all used ELISA as their method of analysis but represent a mixture of asthma severities and EBC collection devices. A sub-group analysis of EBC collection and analytical methods was used as a means of exploring this heterogeneity.

Five of the seven studies using the EcoScreen reported a positive difference between asthma and control groups; four were statistically significant, the fifth was not reported upon. Of the four studies using the RTube, two reported a positive difference between groups of which one was statistically significant.

The condensing surface of the RTube is polypropylene while on the EcoScreen it is teflon. Several papers have looked at the possible impact of device and condensing surface upon EBC results (124-126, 139-143). Czebe et al (125) compared the RTube and EcoScreen and concluded that both temperature and condenser surface had an impact on biomarker levels. Soyer et al (124) found similar results although neither study examined 8-isoprostane. Rosias et al (126) did study 8-isoprostane and concluded that condenser surface did have an effect but that there was no difference between polypropylene and teflon. Moreover they concluded that temperature difference between the two did not appear to have a significant effect on 8-isoprostane collection. Based on current studies of methodology it is not possible to be certain that choice of device explains any of the heterogeneity in the results.

Regarding analytic method, if the outlier generated by the inclusion of conference abstracts (Sedlak et al (144)) is excluded, the results from mass spectrometry exhibit a smaller range and are considerably lower than the majority of results from ELISA. However, Carpagnano et al (134) – the only study to confirm their ELISA results using gas chromatograph-mass spectrometry - report no discrepancy between the two measures; this is in line with previous studies (80). That analytical method is responsible for a degree of heterogeneity in the results is plausible but cannot be stated with certainty.

The inclusion of papers studying mild or intermittent asthma - in which there may be little or no oxidative stress – might explain the lack of consistently observed difference between asthma and control groups.

A sub-group analysis comparing moderate-to-severe asthma with controls was conducted to explore this possibility (see table 9). Results were inconclusive; of the eight included papers (83, 88, 123, 144-148) five reported a between-groups difference (four of which were statistically significant) while three reported no difference.

Table 9 – 8-isoprostane in EBC sub-group analysis: moderate or severe asthma

	8-isoprostane concentration levels	
	Asthma > controls	Controls $\geq$ asthma
All papers	5 studies (n = 253)	3 studies (n = 273)
Conference abstracts removed	3 studies (n=174)	2 studies (n =166)

2.4 Discussion

This review highlights a lack of comparability between studies, as well as evidence gaps which create difficulties in determining 8-isoprostane thresholds for diagnosis or severity classification of asthma. The clinical value of EBC 8-isoprostane as a quantitative assessment of oxidative stress in asthma remains unclear due to variability in results and inadequate standardization.

The previously published paediatric review (111) reported more consistent findings - five of the six identified studies found a significant difference between asthma and healthy control groups. However, the studies exhibited a similarly large degree of variance in their results (ranging between 4.2 – 56.4pg/ml for asthma and 2.6 – 34.2pg/ml for control groups).

The ATS / ERS taskforce of 2005 (113) was set-up to address variability in EBC results and lack of standardisation in methods. They suggested two likely contributors to variability - varying EBC dilution levels and biomarkers being at the lower end of assay sensitivity. That there exists a large degree of variance in 8-isoprostane concentration levels even where studies have used the same EBC collection method would support these assertions.

Ahmadzai et al in 2013 (149) discuss three possible methods of calculating a dilution factor, none of which has established itself as a gold standard and none of which were used in the studies comprising this review. Only one study (131) used a dilution factor, giving their results in both pg/ml and parts per million of palmitic acid. It remains to be seen whether this improves reproducibility.

It has been suggested that lyophilisation may be useful for reducing variability by concentrating samples thereby raising biomarkers away from the lower end of assay sensitivity. There are a lack of studies examining the reliability and reproducibility of this method (149). Unfortunately the only studies in this review to have used this approach (Battaglia et al (121) and Sood et al (120)) concentrated their samples to differing levels (threefold and fourteenfold respectively). Furthermore, Sood et al reported an intra-assay CV of 37.7% and an inter-day CV of 71.6% when using this method.

The validity of any assessment of diagnostic test accuracy rests upon the accuracy of the reference standard to which it is compared; studies where diagnosis was conducted according to recognised guidelines were included.

A large number of exclusions were due to lack of diagnostic clarity; many undertook spirometry as a study measure rather than a diagnostic assessment and - unless reviewed by a physician and judged against a clearly described standard - can't be accepted as diagnostic confirmation. Furthermore, guidelines stress the importance of variable airflow obstruction to diagnosis; this cannot be assessed by a single spirometry measurement thereby complicating the process for any study wishing to have a rigorous diagnosis as the basis for inclusion.

Of concern were studies where it was neither explicitly stated that smokers were excluded, nor was smoking status featured in the participant description. There were six studies in which this occurred and over which there must be some concern that data might include that from smoking participants. This would be a potential confounder; there is evidence that EBC 8-isoprostane is significantly higher in smokers compared to healthy controls (150) and may increase in an acute smoking response (151).

Another potential confounder is the effect of food and drink; 16 of the studies did not mention fasting prior to tests. The ATS/ERS guidelines (114) state that eating and drinking do not affect the non-volatile components of EBC as far as is known, but Ahmadzai (149) point out that food & drink may elevate levels of oxidants in body fluids and has the potential to influence oxidant concentrations in EBC (although they identify no studies describing any such effect on 8-isoprostane). The extent to which this might constitute a confounder is unknown.

Several authors confirmed they were unable to measure 8-isoprostane in a majority of their samples (152-154). Of those studies in this review which reported undetectable samples the percentage ranged from 16% (Komakula et al) to 50% (Piotrowski et al). Not all papers made clear the cause of missing data (whether an inability to obtain EBC samples or an inability to detect 8-isoprostane) nor how this was handled in the analysis. Gratziou et al (137) gave non-detectable levels of 8-isoprostane a value of 3.9pg/ml (the lower limit of assay detection) while Sood et al ascribed undetectable levels a value half the lower detection limit; neither state how many cases this applied to. If these samples came predominantly from healthy controls, raising them might obfuscate any difference between asthma and controls.

In chapter four of this thesis the researchers own experiences with detecting 8-isoprostane in EBC are detailed; in a study of patients with idiopathic pulmonary fibrosis, 8-isoprostane was detectable in only four out of 49 samples, with none yielding results that were above the lower limit of quantification for the enzyme-linked immunosorbent assay (ELISA) used.

The absence of oxidative stress is a potential explanation of inability to detect 8-isoprostane. This might be the case for studies of mild or intermittent asthma. The use of provocation tests or the study of moderate-to-severe asthma is one potential approach to this problem but the results of such studies were no less conflicted.

Although not one of the primary study objectives, factors for which an association with 8-isoprostane was reported were examined. The majority of studies which assessed GORD and BMI reported a significant association with 8-isoprostane. It is possible that these are important confounders which may need to be controlled for in future studies.

2.4.1 Limitations

By employing rigorous inclusion criteria for asthma diagnosis and EBC methodology several 'key' papers were excluded, including that of Montuschi et al (80) frequently cited by others as justification for their methodology. These exclusions are justified as the use of rigorous inclusion criteria are crucial for a review of diagnostic test accuracy.

Inability to assess the risk of bias in key domains of the QUADAS-2 quality assessment tool makes any conclusions from this review necessarily tentative. Furthermore, it was possible to conduct meta-analysis of only four studies due to the frequent use of median, range, and log-transformed data.

The increasing ability to examine several biomarkers - for example Sedlak et al (144, 145) – creates a risk that non-significant findings may go unreported unless high reporting standards are adhered to. Hussain et al mention EBC 8-isoprostane in the methods section of a conference abstract (155) but not in the results, nor anywhere in the full published paper (156); suggesting that 8-isoprostane was either undetectable or the results were non-significant. Although these may constitute a publication bias, the under-representation of negative findings makes the lack of positive findings in this review more robust.

2.4.2 Occupational asthma

Writing, registering and following a protocol is an important tool for limiting the risk of bias in systematic reviews. In the protocol for this review occupational asthma (OA) formed part of the exclusion criteria, being deemed sufficiently atypical to warrant separate consideration. However, an argument can be made that excluding an asthma phenotype as 'atypical' makes little sense given the heterogeneity of the condition and absence of a 'typical' asthma.

Furthermore, studies of occupational asthma using a specific inhalation challenge (SIC) - where the putative causative agent is used in a broncho-provocation test - might offer greater assurance of oxidative stress in the airways of the participant at the time of assessment. In light of these considerations the results of the initial literature search were subsequently revisited and studies of occupational asthma identified. A single reviewer (AMP) conducted a literature search (6th September 2016) using the same databases and search terms as the original study with the addition of 'OR occupation*' in the asthma part of the search string. Twenty six papers were identified. Sixteen were excluded on the basis that they studied healthy participants (with occupational exposure to potentially sensitising agents, as opposed to formally diagnosed occupational asthma); four were reviews; three did not study EBC 8-isoprostane, and one was not available in an English translation. Two papers were suitable for inclusion (157, 158). In the study by Klusackova et al (2008) only six participants had a positive SIC, thus the study was underpowered to draw any significant conclusions. In Pelclova et al (2014) 8-isoprostane levels were raised in those with occupational asthma as compared to controls.

2.5 Conclusion

There is a trend towards higher EBC 8-isoprostane concentrations in subjects with asthma compared to controls. Twice as many studies reported higher levels amongst those with asthma than did not. However the strength of this evidence is weak and the number of studies reporting a significant difference was the same as that reporting none. A random effects meta-analysis found a significant difference between groups however its rigour is compromised by the small number of studies and substantial statistical heterogeneity.

Concentrating EBC samples may address some of the variability and difficulty arising from the use of ELISA. However, the central issue of calculating EBC dilution cuts across analytical methods and a gold standard is still to be determined. It will be essential to develop accurate, reliable and standardised methods of both EBC collection and 8-isoprostane analysis if its use as a biomarker in asthma is to be properly evaluated.

While research into EBC continues, interest in exhaled breath gases (volatile organic compounds) is growing. A search conducted on the 14th September 2019 using Google Scholar and limited to publications in 2019 returned 807 hits for "exhaled breath condensate" compared to 1,530 for "exhaled volatile organic compounds". In the next chapter **(chapter 3)** the literature on exhaled volatile organic compounds is examined in relation to adult asthma.

Chapter 3 – Asthma Breathomics

Accepted for publication as:

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Peel, A. Sinha, A. Fowler, S. Loke, Y. Wilkinson, M. Wilson, A. (2019) Asthma breathomics – a systematic review of exhaled volatile organic compounds associated with diagnosis and disease characteristics. British Thoracic Society Winter Meeting, Poster Presentation. 04/12/2019

Peel, AM. Loke, YK. Wilson, AM. (2018) Asthma Breathomics – Promising Biomarkers in Need of Validation (letter to Editor). Pediatric Pulmonology, 2018; 1-3. DOI: 10.1002/ppul.23941

Protocol registered as:

Peel, AM. Loke, YK. Wilson, AM. Asthma Breathomics: A Systematic Review Protocol. PROSPERO: International prospective register of systematic reviews. 2017. CRD42017082727. Available from http://www.crd.york.ac.uk/PROSPERO/display_record.php?ID=CRD42017082727

3.1 Introduction

The previous chapter reviewed current evidence for the use of 8-isoprostane in exhaled breath condensate (EBC) in adult asthma. While results suggested there may be a difference in EBC 8-isoprostane levels between those with asthma and healthy controls, the evidence is currently too weak to reach a firm conclusion and true tests of diagnostic test accuracy in an undiagnosed population have not yet been conducted.

This chapter reviews those studies which have applied an analysis of exhaled breath VOC to adults with asthma. A systematic review, it has been published in Respiratory Medicine as Peel, A. Sinha, A. Fowler, S. Loke, Y. Wilkinson, M. Wilson, A. (2020) *Volatile organic compounds associated with diagnosis and disease characteristics in asthma*. It has also been presented as a poster at the British Thoracic Society Winter Meeting (2019) under the title *Asthma breathomics – a systematic review of exhaled volatile organic compounds associated with diagnosis and disease characteristics*.

A review of paediatric asthma breathomics was published in 2017 (159) however this was not based on a systematic literature search and it limited itself to publications from the preceding ten years. A systematic search was conducted in order to determine whether the published review had identified all relevant studies. This is presented within this chapter and was published as Peel, AM. Loke, YK. Wilson, AM. (2018) Asthma Breathomics – Promising Biomarkers in Need of Validation (letter to Editor). Pediatric Pulmonology, 2018; 1-3. DOI: 10.1002/ppul.23941

The identification and or quantification of metabolites offers an alternative route to diagnosis and disease management. Metabolites are low molecular weight (typically defined as <1500 amu[1]) organic and inorganic chemicals produced by cellular processes (including pathophysiological processes). The term 'metabolome' refers to the entire set of metabolites associated with a biological system (160). Change in the metabolome reflects change in underlying cellular activity (161) - disease pathophysiology can alter the relative concentrations of metabolites produced, or produce metabolites which are absent in health (162) - metabolomics is thus gaining traction as a means of biomarker discovery in disease (163).

Volatile organic compounds (VOC) are carbon-based, low molecular weight compounds, volatile at room temperature. The study of endogenous VOC generated by metabolic processes within the body and exhaled on the breath is commonly referred to as breathomics (164). Such studies produce data on a large number of compounds permitting inductive, hypothesis-generating approaches in which data are interrogated in order to identify disease-induced metabolomic permutations (165) without the prior identification of a candidate marker. This approach has been applied to many diseases including asthma.

Rufo et al (166) conducted a systematic review of the asthma breathomic literature in 2014, identifying 18 studies which reported on diagnostic accuracy. In a meta-analysis of six studies they calculated a pooled area-under-the-curve (AUC) value of 0.94. This figure needs to be interpreted with caution however as all but one of the included studies compared established-treated disease with non-disease (rather than testing diagnostic accuracy in those with a suspicion of disease) and the meta-analysis pooled diagnostic models which were comprised of differing VOC. In addition, a mixture of adult and paediatric studies were included; age has since been identified as a factor which should be controlled for (167).

[1] AMU = atomic mass unit

Interest in the field has continued to grow and a number of breathomic asthma studies have since been published. Neerincx et al (159) reviewed paediatric asthma breathomics, to which a systematic search has been appended (168); and recent reviews have provided an overview of metabolomics in exhaled breath (169) and across different biomediums (170, 171).

The aim of this study was to systematically review the literature on adult asthma breathomics - including studies of diagnosis and of disease characteristics - providing a comprehensive list of significant VOC identified to-date.

3.2. Methods

A study protocol was developed in line with Prisma-P guidelines and registered with the International Prospective Register of Systematic Reviews (PROSPERO) (registration number CRD42017082727). The primary objective of the review was to ascertain the classification accuracy of VOC models for asthma diagnosis, phenotyping, and disease control. The secondary objectives were to identify the study methods used and to compile a list of those VOC identified by studies as significant for use in future validation efforts. The search strategy used to identify relevant literature is outlined in table 10.

Table 10 – Asthma breathomics: search strategy

SEARCH TERMS

The following key words and MeSH terms were used - metabolomics, breathomics, exhaled breath, breath test, volatile organic compound* and asthma. The search string was optimised for each database. Below - as an example – is the search string as used in PubMed (http://www.ncbi.nlm.nih.gov/pubmed)

(("Breath Tests"[Mesh] OR "Exhalation"[Mesh] OR "exhaled"[All Fields] OR breath[All Fields]) AND ("Asthma"[Mesh] OR "asthma"[All Fields] OR "asthmatic"[All Fields]) AND ("Volatile Organic Compounds"[Mesh] OR "Volatile Organic Compound*"[All Fields])) OR (("asthma"[MeSH Terms] OR "asthma"[All Fields] OR "asthmatic"[All Fields]) AND (Breathomic*[All Fields] OR ("metabolomics"[MeSH Terms] AND ("exhalation"[MeSH Terms] OR "exhalation"[All Fields] OR "exhaled"[All Fields] OR breath[All Fields] OR "breath tests"[MeSH Terms]))))

Limits applied to results - 'humans'.

SEARCH STRATEGY

Inclusion criteria – Physician diagnosed asthma or asthma diagnosis according to recognised guidelines; clinical studies published in full; primary data; VOC in exhaled breath studied (by any collection or analytical method).

Exclusion – Reviews; editorial; secondary data; studies of exhaled breath condensate; non-asthma studies; studies published in abstract form only.

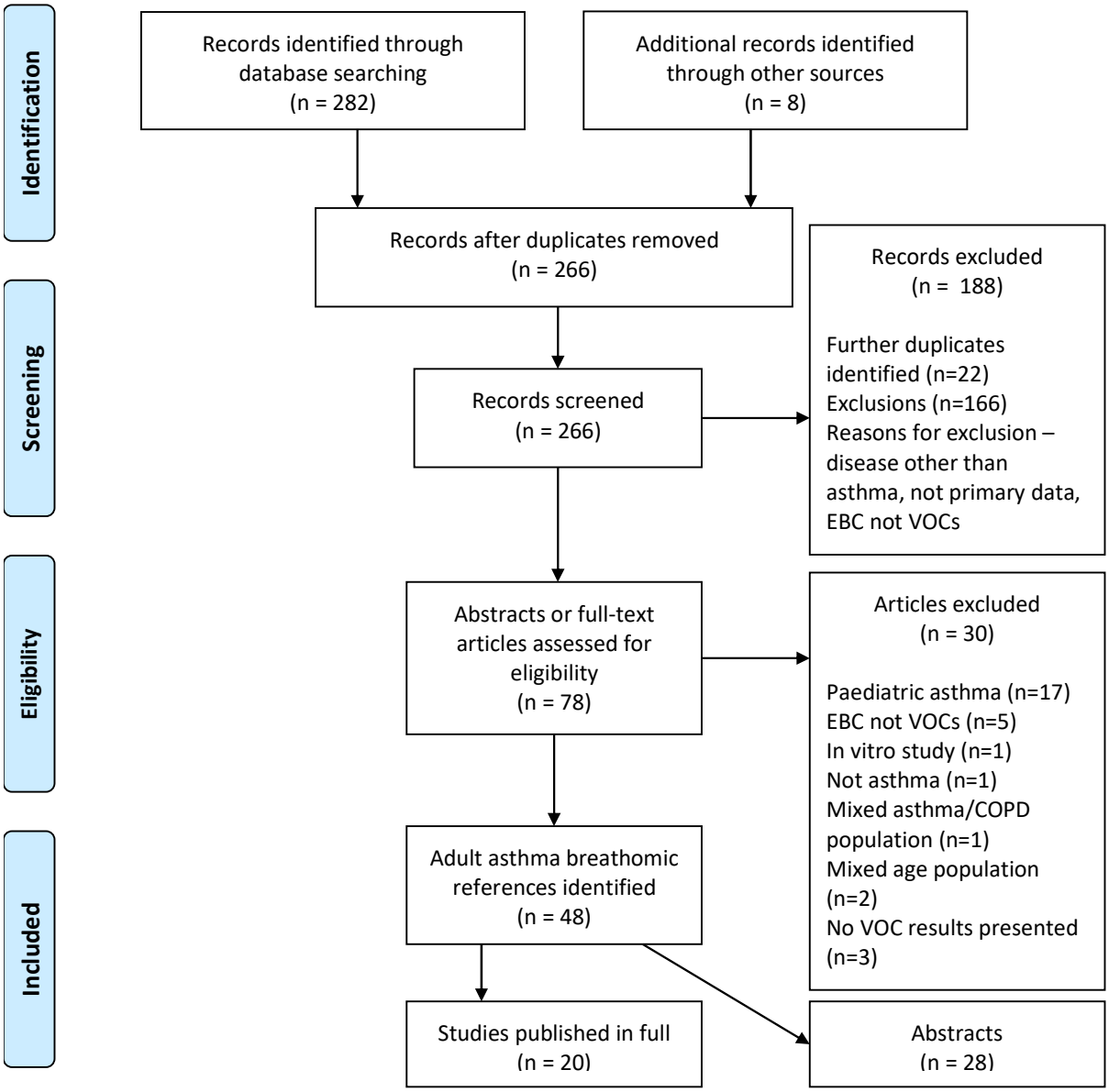
SEARCHES

PubMed; Medline (including Embase and OVID medline)

In addition, the references from Rufo et al's review; from the researchers' own reference libraries; and from the reference lists of included articles were searched. Researchers working in the field were asked to highlight papers they were aware of. The searches were conducted independently by two reviewers (AS and AP) on the 1st June 2017 and updated in November 2018.

Two reviewers (AP & AS) screened titles and abstracts for inclusion, resolving discrepancies through discussion with a third reviewer (MW). In total 290 records were identified; this was reduced to 266 after removing duplicates. On screening abstracts and/or full texts, 48 citations of adult asthma breathomic studies were identified, of which 28 were abstracts and 20 full journal articles. A PRISMA diagram (figure 7) describes the screening and selection process. Quality assessment was undertaken using the CASP diagnostic checklist (172) (see table 15). Data extraction and quality assessment was independently conducted by two researchers (AP & MW).

Figure 7 – PRISMA flow diagram: asthma breathomics review. Steps taken in the identification of studies for inclusion in review.



3.3 Results

Twenty journal articles met the criteria for inclusion (table 11). Fifteen of these compared VOC in asthma and healthy controls (70, 73, 173-184), of which ten reported diagnostic accuracy (70, 73, 174, 175, 177, 178, 181-184). Four studies reported on the ability to differentiate between asthma and COPD, one lung cancer and one allergic rhinitis (71, 175, 182-184). Seven studies examined the ability to discriminate between phenotypes (70, 71, 73, 174, 185-187) (two were cluster analyses), while three reported on levels of disease control or activity (70, 179, 185).

One paper was included (188) which failed to meet Rufo *et al*'s inclusion criteria (due to the absence of a comparator group) and one was excluded (189) due to the use of exhaled breath condensate as its sample medium. One paper reporting on volatile organosulfides was included (173) while another was excluded due to including both adults and adolescents (190, 191).

Results were typically given as accuracy rates for the correct classification of samples - the area under the curve for receiver operator characteristics (AUROC), cross-validation values (CVV) or correlation coefficients. Tables 11 and 12 display the list of full publications along with results; Table 13 summarises study design and breath sampling methods; while Table 14 details the data processing and statistical methods used. There was heterogeneity in all aspects of study methodology, from sample collection through to statistical analysis. The majority of GC-MS studies used principal component analysis (PCA) in their statistical analysis however approaches to data pre-processing, discriminatory analysis and cross-validation varied. Given the methodological heterogeneity and variety of compounds upon which breathomic models were based, meta-analysis was deemed inappropriate; instead a narrative synthesis of study findings is presented.

Table 11 – Asthma breathomics: included studies and results

Study	Country	Year	Population	Result
Awano et al (173)	Japan	2011	Asthma = 7 Non-asthma = 386 (both groups age range 60-65)	Asthma and the presence of dimethyl sulphide > 1.0nmol L^{-1} in mouth air Crude OR 7.4 (95%CI 1.4-39.0); Adjusted OR 6.9 (95% CI 1.1-44.2)
Brinkman et al (185)	Netherlands	2017	Asthma (partly controlled, mild-moderate) = 23	Baseline *vs* loss of control: eNose classification accuracy - 95%, GC-MS 68% Loss of control vs recovery: eNose classification accuracy - 86%, GC-MS 77% Significant association between exhaled metabolites and sputum eosinophils: Pearson $r \geq 0.46$, $p<0.01$
Dragonieri et al (174)	Netherlands	2007	Asthma (mild-severe) = 20 Controls = 20	Asthma vs controls: CVV 90-100%; M-distance 2.77-5.32. Mild vs severe asthma: CVV 65%; M-distance, 1.23.
Dragonieri et al (184)	Italy	2018	Asthma & allergic rhinitis (AAR): training set = 14; validation set = 7. Allergic rhinitis only (AR) and healthy controls (HC) as above	<u>Training set</u> AAR vs AR: CVA=86%, $p<0.01$; AUROC 0.93 AR vs HC: CVA=82%, $p<0.01$; AUROC 0.92 AAR vs HC: CVA=75% ,$p<0.05$; AUROC 0.87 <u>Validation set</u> AAR vs AR: CVA=83%, $p<0.01$; AUROC 0.92 AR vs HC: CVA=77%, $p<0.01$; AUROC 0.87 AAR vs HC: CVA=67 , $p<0.05$; AUROC 0.77
Fens et al (175)	Netherlands	2009	Asthma (mild-severe, persistent) = 20 COPD = 30 Controls = 40	Asthma vs COPD: accuracy 96%; $p< 0.001$ Asthma vs controls: accuracy 93 – 95%; $p< 0.001$
Fens et al (71)	Netherlands	2011	Asthma (stable) = 60 (21 w/ fixed airways) COPD = 40	Asthma vs COPD: accuracy 83-88%; $p<0.001$ AUROC 0.93-95 (95% CI 0.84–1.00); sensitivity 85-91%, specificity 90% Fixed asthma vs classic asthma: accuracy 58%; $p=0.23$; AUROC 0.68 (95% CI 0.50-0.85); sensitivity 60%, specificity 67%.

Ibrahim et al (70)	UK	2011	Asthma (mild-moderate) = 35 (sputum for phenotyping n=18) Controls = 23	Asthma vs controls: accuracy = 86% (PPV 0.85, NPV 0.89) Sputum eosinophilia: AUROC 0.98 (95% CI = 0.91-1.00; sensitivity = 0.75, specificity = 0.90). Sputum neutrophilia: AUROC 0.90 (95% CI = 0.76-1.00; sensitivity = 0.80, specificity = 0.75). Uncontrolled asthma: AUROC 0.97 (95% CI = 0.93-1.00; sensitivity = 0.89, specificity = 0.88).
Larstad et al (176)	Sweden	2007	Asthma (stable) = 13 Controls = 14	Baseline isoprene lower in asthmatic subjects (113 ppb vs 143; p = 0.03) No significant difference in baseline ethane, pentane, or nitric oxide.
Lazar et al (192)	Netherlands / Hungary	2010	Asthma (stable) = 10	Reduction in airway calibre was not associated with an altered eNose breath profile
Meyer et al (177)	Switzerland	2014	Asthma (mixed severity) = 195 Controls = 40	Asthma vs controls: accuracy 99% (sensitivity 100%, specificity 91%) Inter-cluster or cluster vs control accuracy: 82% – 95% Linear discriminant analysis (LDA) for correct classification of all clusters and controls = 43%.
Montuschi et al (178)	Italy	2010	Asthma (mild, intermittent) = 27 Controls = 24	Asthma vs controls: diagnostic accuracy 88%
Olopade et al (179)	USA	1997	Asthma (acute exacerbation) = 12 Stable asthma = 11 Controls = 17	Significantly higher exhaled pentane levels during acute exacerbation (p < 0.05). No significant difference in exhaled pentane levels between stable/controlled asthma and healthy controls (p>0.05).
Paredi et al (180)	UK	2000	Asthma (steroid naive) = 14 Asthma (steroid treated) = 12 Controls = 14	Ethane in untreated asthmatics > healthy controls or ICS treated asthma (p<0.05) In untreated asthma, exhaled ethane correlated with levels of nitric oxide exhalation (p<0.05); those with FEV_1 <60% predicted had higher levels of ethane than those >60% (p<0.05).
Plaza et al (186)	Spain	2015	Asthma (persistent) = 52	Eosinophilic vs neutrophilic: accuracy 73%; P=0.008; AUROC 0.92 Eosinophilic vs paucigranulocytic: accuracy 74%; p=0.004; AUROC 0.79 Neutrophilic vs paucigranulocytic: accuracy 89%; P=0.001; AUROC 0.88
Reynolds et al (188)	UK	2014	Asthma & controls = 17	Discriminant analysis of asthma vs controls not reported

van der Schee et al (73)	New Zealand	2013	Asthma (mild-moderate) = 25 Controls = 20	Asthma vs controls: AUROC 0.77 (95%CI = ± 0.14;*p* = 0.002) Steroid responsiveness: AUROC = 0.88 (95% CI = ± 0.16; *p* = 0.008)
van der Schee et al (181)	Europe	2013	Asthma (U-BIOPRED, severity not specified) = 10 Controls = 10	Asthma vs control: eNose AUROC = 0.77 (95% CI = 0.22), *p* = 0.050 GM-MS AUROC = 0.84 (95% CI = ± 0.17), *p* = 0.011
Timms, Thomas & Yates (182)	Australia	2012	Asthma (GINA step 1-3) = 20 COPD = 17 Controls = 7	Asthma vs controls: eNose accuracy 70%, *p*=0.047 Asthma vs COPD: eNose accuracy 70%, *p*=0.019
de Vries et al (183)	Netherlands	2015	Asthma (mild to severe) = 37 Controls = 45 COPD = 31 Lung cancer = 31	Asthma vs COPD: accuracy 81%, AUROC 0.81 (95%CI ± 0.09), *p*=0.001 Asthma vs controls: accuracy 87%, AUROC 0.94 (95%CI ± 0.15), *p*<0.001 Asthma vs lung cancer: accuracy 68%, AUROC 0.71 (95%CI ± 0.09), *p*=0.045
de Vries et al (187)	Netherlands	2018	Asthma (mild to severe)= 278 COPD = 157. Training set=321; validation set 114.	Training set Clusters differing in ethnicity (*p*=0.01); systemic eosinophilia (p=0.02); neutrophilia (*p*=0.03); BMI (*p*=0.04); FeNO (*p*<0.01), atopy (*p*<0.01); exacerbation rate (p<0.01). Regression models predictive of eosinophilia (R^2=0.58); neutrophilia (R^2=0.41) Validation set Predictive models confirmed by validation set with the exception of BMI and neutrophilia

AUROC – area under the curve for receiver operator characteristics
CVA – cross-validation accuracy
CVV – cross-validation value
LDA – linear discriminant analysis
NPV – negative predictive value (percentage of true negatives)
PPV – positive predictive value (percentage of true positives)
SD – standard deviation

Studies published only in abstract form were excluded due to the inability to fully assess inclusion criteria, study quality and risk of bias. However, the exclusion of such publications creates a vulnerability to selective dissemination bias; their results are summarised in table 12.

Table 12 – Asthma breathomics: results from abstracts

Study	Year	Title	Journal or conference	Population	Results
Brinkman et al **(193)**	2014	Electronic noses capture severe asthma phenotypes by unbiased cluster analysis	American Thoracic Society Conference	U-BIOPRED Severe asthma n = 77	Significant between-cluster differences in clinical characteristic reported but *p*-values not cited.
Brinkman et al **(194)**	2013	Unbiased cluster analysis of severe asthma based on metabolomics by the U-BIOPRED electronic nose platform	European Respiratory Society Congress	U-BIOPRED Severe asthma n = 57	*p* -values for between-cluster differences in clinical characteristics 0.001 – 0.02
Brinkman et al **(195)**	2015	Unbiased clustering of severe asthma patients based on exhaled breath profiles	European Respiratory Journal Conference	U-BIOPRED Severe asthma n= 35	*p* -values for between-cluster differences in clinical characteristics and eNose profiles *p* = 0.02-0.04
Brinkman et al **(196)**	2015	Exhaled breath volatile organic compounds can classify asthma patients with high and low sputum eosinophils	American Thoracic Society Conference	U-BIOPRED Severe asthma n = 27	Identifying sputum eosinophilia; AUROC 0.94 (95% CI, 0.85-1)
Brinkman et al **(197)**	2015	Longitudinal changes in exhaled breath GC/MS profiles during loss of asthma control by prospective steroid withdrawal	European Respiratory Society Congress	Subsequently published in full (185) (see table 1)	
Brinkman et al **(198)**	2016	Identification of exhaled volatile organic compounds (VOC) associated with loss of asthma control	European Respiratory Society Congress		

Brinkman et al **(199)**	2016	Identifying biomarkers of loss of control/exacerbations in asthma from exhaled breath	European Respiratory Society Congress		
Brinkman et al **(200)**	2018	Exhaled volatile organic compounds as markers for medication use in asthma within the U-BIOPRED cohort	American Thoracic Society Conference	U-BIOPRED Severe asthma n = 108	Identification of urinary oral corticosteroids (baseline, replication and validation) AUROCs 67 - 91; identification of urinary salbutamol AUROCs 70 – 82.
Capuano et al **(201)**	2012	Classification ability of two eletronic noses in asthma and COPD	European Respiratory Society Congress	Severe asthma n = 10 COPD n = 9 Healthy controls n = 6	Classification asthma vs COPD: Cyranose 320 = 92%, Ten2010 = 86% Classification disease vs controls: Cyranose 320 = 88%, Ten2010 = 88%
Crespo et al **(202)**	2013	Discrimination of bronchial inflammatory phenotype of asthmatic patients by using the electronic nose	European Respiratory Society Congress	Asthma n = 44 (eosinophilic = 16 neutrophilic = 8 paucigranulocytic = 20)	Eosinophilic vs neutrophilic = 100% Eosinophilic vs paucigranulocytic = 100% Neutrophilic vs paucigranulocytic = 90%
Durrington et al**(203)**	2018	An 'omics' study to investigate the mechanisms underlying circadian rhythm in asthma.	American Thoracic Society Conference	Moderate atopic asthma = 10 Healthy control = 10	Significant diurnal variability in 7 VOC including 2-undecanal (p=0.03) found in those with asthma but not controls.
Fens et al **(204)**	2011	Exhaled molecular patterns change after experimental rhinovirus 16 infection in asthma	European Respiratory Journal	Mild intermittent n = 9 Healthy controls = 14	Before and after RV16 inoculation Significant change in principal components in asthmatics p=0.01 p=0.015. No change in controls
Fens et al **(205)**	2015	Volatile organic compounds (VOC) in exhaled breath of asthma patients differ between loss of control and stable phase	American Thoracic Society Conference	n = 23	Control vs loss of control: AUROC 0.98 (95% CI 0.96-1.00)
Greulich et al **(206)**	2013	An electronic nose can distinguish between different asthma phenotypes	European Respiratory Society Congress	Eosinophilic = 9 Non-eosinophilic = 11 Controls = 10	Eosinophilic vs non-eosinophilic $p < 0.0001$ AUROC 1.0 (95% CI – 0.96 – 1.0); CVV 59.1%

Ibrahim et al **(207)**	2010	Metabolomics of breath volatile organic compounds for the diagnosis and inflammatory phenotyping of adult asthma	American Thoracic Society Conference	Subsequently published in full (70) (see table 11)	
Meyer et al **(208)**	2012	Defining adult asthma endophenotypes by clinical features and patterns of volatile organic compounds in exhaled air	European Academy or Allergy & Clinical Immunology Congress	Subsequently published in full (177) (see table 11)	
Montuschi et al **(209)**	2010	Diagnostic performance of an electronic nose, fractional exhaled nitric oxide and lung function testing	American Thoracic Society Conference	Subsequently published in full (178) (see table 11)	
Pelit et al **(210)**	2016	Breath print of severe allergic asthma with SPME-GC-MS analysis of exhaled air volatile organic compounds	European Respiratory Society Congress	Severe allergic asthma = 27 Healthy controls = 42	Asthma vs controls: classification accuracy 88.6% (sensitivity 95.6%, specificity 95.8%)
Santini et al **(211)**	2014	Discrimination between oral corticosteroid-treated and oral corticosteroid-non-treated severe asthma patients by an electronic nose platform.	European Respiratory Society Congress	U-BIOPRED Severe asthma (adult) = 73	OCS vs no OCS: accuracy 71%
Santini et al **(212)**	2015	Breathomics can differentiate between anti IgE-treated and non-treated severe asthma adults	European Respiratory Society Congress	U-BIOPRED Severe = 39 Omalizumab vs non-use	eNose: accuracy 0.85 GCMS: accuracy 0.83
van der Schee et al **(213)**	2012	Predicting steroid responsiveness in patients with asthma using the electronic nose	American Thoracic Society Conference	Subsequently published in full (73) (see table 11)	
Schleich et al **(214)**	2015	Do volatile organic compounds (VOC) discriminate between eosinophilic and neutrophilic asthma phenotype?	European Respiratory Society Congress	Asthma n= 276 (eosinophilic = 122 neutrophilic = 50 paucigranulocytic = 90)	Identification of good discriminatory VOC reported. Identity of VOC and accuracy results not reported.
de Vries et al **(215)**	2016	Exhaled breath analysis for identifying eosinophilic and neutrophilic	European Respiratory Society Congress	Subsequently published in full (187) (see table 11)	

		inflammation in a mixed population of patients with asthma or COPD			
De Vries et al **(216)**	2017	Inflammatory phenotyping of chronic airway disease (including both Asthma and COPD) by breathomics	American Thoracic Society	Subsequently published in full (187) (see table 11)	
Wagener et al **(217)**	2012	Exhaled air volatile organic compounds and eosinophilic airway inflammation in asthma	European Respiratory Society Congress	U-BIOPRED? n = 36 Mod-to-severe	Correlation coefficients - VOC & sputum eosinophilia (>3%): 0.42-0.47 VOC & sputum eosinophilia (excl. participants on OCS): 0.49-0.62
Wagener et al **(218)**	2013	Exhaled breath profiling and eosinophilic airway inflammation in asthma – results of a pilot study	American Thoracic Society Conference	U-BIOPRED N = 27 (25 severe)	Eosinophilic vs non-eosinophilic Accuracy = 85%. AUROC 99% (95% CI 0.97-1.0).
Zanella et al **(219)**	2018	Breath print for asthma phenotyping	?	n = 245	Eosinophilic, neutrophilic, paucigranulocytic, mixed granulocytic. AUROC classification 0.68-0.71.
Mixed age population (adolescents)					
Couto et al Abstract **(190)** & published in full **(191)**	2015 & 2017	Oxidative stress in asthmatic and non-asthmatic adolescent swimmers - A breathomics approach	Paediatric Allergy & Immunology Congress of the European Academy of Allergy and Clinical Immunology		No separate clustering of groups on PCA analysis Controls demonstrated a more varied response to exercise; exhibiting a more pronounced decrease in the studied metabolites post-exercise.

It should be noted that nine of the abstracts and one full paper were produced from a single large European programme of study - U-BIOPRED. These all analysed cohort sub-groups; it is not clear whether the same patients might feature as cases in more than one of these publications.

Table 13 – Research methods in asthma breathomic studies: breath sampling

Study	Year	Title	Ambient air subtraction?	Filtered air used?	Storage method	Concentrated on sorbent tubes?	Internal validation ?	External validation?	Analytical platform
Awano et al **(173)**	2011	Correlations between health status and OralChroma™-determined volatile sulfide levels in mouth air of the elderly	N	N	Syringe	N	X	X	GC (OralChroma™)
Brinkman et al **(185)**	2017	Exhaled Breath Profiles in the Monitoring of Loss of Control and Clinical Recovery in Asthma	N	Y	Tedlar bag	Y	X	X	eNose & GC-MS
Dragoneiri et al **(174)**	2007	An electronic nose in the discrimination of patients with asthma and controls	Y	Y	Tedlar bag	N	Y	X	eNose
Dragoneiri **(184)**	2018	Exhaled breath profiling by electronic nose enabled discrimination of allergic rhinitis and extrinsic asthma	N	Y	Tedlar bag	N	Y	Y	eNose
Fens et al **(175)**	2009	Exhaled breath profiling enables discrimination of chronic obstructive pulmonary disease and asthma	Y	Y	Tedlar bag	N	Y	X	eNose
Fens et al **(71)**	2011	External validation of exhaled breath profiling using an electronic nose in the discrimination of asthma with fixed airways obstruction and chronic obstructive pulmonary disease.	Y	Y	Tedlar bag	N	Y	Y	eNose

Ibrahim et al **(70)**	2011	Non-invasive phenotyping using exhaled volatile organic compounds in asthma	N	Y	X	Y	Y	X	GC-MS
Larstad et al **(176)**	2007	Determination of ethan, pentane and isporene in exhaled air – effects of breath-holding, flow rate and purified air.	Y	Y	Tedlar bag	Y	X	X	GC
Lazar et al **(192)**	2010	Electronic Nose Breathprints are independent of acute changes in airway caliber in asthma	Y	Y	Tedlar bag	N	X	X	eNose
Meyer et al **(177)**	2014	Defining adult asthma endotypes by clinical features and patterns of volatile organic compounds in exhaled air.	N	N	Tedlar bag	Y	X	X	GC-MS
Montuschi et al **(178)**	2010	Diagnostic performance of an electronic nose, fractional exhaled nitric oxide, and lung function testing in asthma.	Y	N	Tedlar bag	Sorbent gauze	Y	N	eNose & GC-MS
Olopade et al **(179)**	1997	Exhaled pentane levels in acute asthma	Y	N	Tedlar bag	N	X	X	GC
Paredi et al **(180)**	2000	Elevation of Exhaled Ethane Concentration in Asthma	Y	N	Tedlar bag	N	X	X	GC
Plaza et al **(186)**	2015	Inflammatory asthma phenotype discrimination using an electronic nose breath analyzer	N	Y	Tedlar bag	N	Y	X	eNose
Reynolds et al **(188)**	2014	Analysis of human breath samples using a modified	?	Y	?	Y	X	X	TD-SESI-MS

		thermal desorption: gas chromatography electrospray ionization interface							
van der Schee et al **(73)**	2013	Predicting steroid responsiveness in patients with asthma using exhaled breath profiling. Clinical And Experimental Allergy	N	Y	Tedlar bag	N	Y	X	eNose
van der Schee et al **(181)**	2012	Effect of transportation and storage using sorbent tubes of exhaled breath samples on diagnostic accuracy of electronic nose analysis	N	Y	Nalophan bag	Y	Y	X	eNose & GC-MS
Timms, Thomas & Yates **(182)**	2012	Detection of gastro-oesophageal reflux disease (GORD in patients with obstructive lung disease using exhaled breath profiling.	N	N	Tedlar bag	N	Y	X	eNose
de Vries et al **(183)**	2015	Integration of electronic nose technology with spirometry: validation of a new approach for exhaled breath analysis	Y	N	X	N	Y	X	eNose
de Vries et al **(187)**	2018	Clinical and inflammatory phenotyping by breathomics in chronic airway diseases irrespective of the diagnostic label	Y	N	X	N	Y	Y	eNose

		Summary:	Ambient air collected - 10 studies	VOC-filtered air -12 studies	Impermeable bags - 15 studies	Samples concentrated onto sorbent tubes - 6 studies	Internal validation conducted - 12 studies	External validation conducted - 3 studies	GC-MS / GC / TD-SESI-MS = 7 eNose = 9 Both = 4

GC-MS: Gas chromatography-mass spectrometry
TD-SESI-MS: Thermal desorption secondary electrospray ionisation mass spectrometry

Table 14 – Research methods in asthma breathomics studies: statistical analysis

Study	Data pre-processing	Compound identification	Data analysis
Awano **(173)**	Validation of gas chromatograms.	In-house	Univariate analysis chi-square and ANOVA; multivariate logistic regression.
Brinkman et al **(185)**	De-noising, peak detection & alignment, using XCMS. PCA, BoxCox power transformation, normalisation.	NIST	Univariate analysis; ANCOVA and Pearson correlation tests; FDR correction and standardised QR decomposition used. Multivariate analysis by PCA. *t*-test.
Dragonieri et al **(174)**	Savitzky-Golay filtering & baseline correction	NA	PCA & double cross-validatory implementation of linear canonical discriminant analysis. Pattern recognition algorithm & cross-validation estimate of error made.
Dragonieri et al **(184)**	?	NA	PCA, independent t-test, CDA, leave-one-out cross-validation, ROC-curve
Fens et al **(175)**	eNose sensor data reduced by PCA	NA	Linear canonical discriminant analysis & ROC. Cross-validation by leave one out method. Altman analysis with Bonferroni correction. Intra-class correlation coefficients.
Fens et al **(71)**	eNose sensor data reduced by PCA	NA	Linear canonical discriminant analysis; ROC curves, 10-fold boot strapping, combined with cross-validation.
Ibrahim et al **(70)**	**?**	**?**	Univariate logistic regression analysis, PCA, multivariate logistic regression. Discriminant function analysis with leave-one-out cross validation.
Larstad et al **(176)**	**?**	In-house	Kruskal-wallis, Wilcoxon signed rank test.
Lazar et al **(192)**	Savitzky-Golay filtering and baseline correction, sensor data reduced by PCA	NA	Mixed model analysis, paired *t*-test.
Meyer et al **(177)**	Baseline correction, peak detection, normalisation of retention times, global normalisation.	**?**	Unsupervised hierarchical 2-step cluster analysis. Linear discriminant analysis.
Montuschi et al **(178)**	?	**?**	eNose sensor data reduced by PCA. Feed-forward neural network. Unpaired *t*-test, Mann Whitney U test, Pearson coefficient.
Olopade et al **(179)**	**?**	**?**	Wilcoxon signed rank test.
Paredi et al **(180)**	**?**	**?**	One-way ANOVA with Bonferroni correction.

Plaza et al **(186)**	**?**	NA	PCA, univariate ANOVA, post-hoc least significant difference test. Linear canonical discriminant analysis. Leave-one-out validation. AUROC.
Reynolds et al **(188)**	Noise reduction, normalisation	**?**	Qualitative analysis of spectograms
Van der Schee et al **(73)**	eNose sensor data reduced by PCA	NA	Unpaired T-test, canonical discriminant analysis, cross validation by boot strapping. ROC and AUC. Pearson correlation coefficients.
Van der Schee et al **(181)**	Deconvolution, peak determination & peak alignment, background subtraction.	NIST	Principal component reduction, unpaired *t*-test, leave-one-out cross-validated linear canonical discriminant analysis. AUROC.
Timms Thomas & Dayle **(182)**	eNose sensor data reduced by PCA	NA	Canonical modelling. Cross-validation, interclass Mahalanobis distance.
de Vries et al **(183)**	Corrected for ambient VOC; normalized	NA	PCA, univariate ANOVA, internal validation by bootstrapping, linear canonical discriminant analysis, AUROC.
De Vries et al **(187)**	Corrected for ambient VOC (based on alveolar gradient), data normalised.	NA	PCA, unsupervised hierarchical clustering using Euclidean distance and ward linkage. Similarity profile analysis. 10x algorithm repetition upon sub-sets. Between-cluster comparisons by ANOVA, Kruskal-Wallis or Chi-squared tests. Validated using independent data set. Supervised analysis by multiple linear regression, Regression model validated using independent data set.

? = not reported
AUC: area under curve
COW: Correlation optimized warping
HP-SPME/GC-qMS: Headspace solid-phase extraction, gas chromatography quadrupole mass spectrometry
NIST: National Institute of Standards and Technology
PLSDA: partial least squares discriminant analysis
SPLS: sparse partial least square discriminant analysis
TD-SESI-ToFMS: thermal desorption / secondary electrospray ionisation / time-of-flight mass spectrometry
ToFMS: Time-of-flight mass spectrometry

ANOVA: analysis of variance
AUROC: Area under a receiver operating characteristic curve
GC x GCMS: 2 dimensional gas chromatography mass spectrometry
MCCV – Monte Carlo Cross Validation
PCA: Principal component analysis
ROC: Receiver operator characteristics
SPME: Solid phase microextraction
TIC: Total Ion Chromatogram
WEKA: a suite of machine learning software / algorithms hosted by the University of Waika

3.3.1 Quality assessment

Twenty studies were published in full and their quality assessed using the Critical appraisal skills programme (CASP) checklist (see table 15).

Table 15 – Asthma breathomics: CASP quality assessment

Study	1. Was there a clear question for the study to address?	2. Was there a comparison with an appropriate reference standard?	3. Did all pt's get the diagnostic test and reference standard?	4. Could the results have been influenced by the results of the reference standard?	5. Is the disease status of the tested pop. clearly described?	6. Were the methods for performing the test described in sufficient detail?	7. What are the results?	8. How sure are we about the results? Consequences and costs of alternatives performed?	9. Can the results be applied to your patient or population of interest?	11. Were all outcomes important to the individual / population considered?	12. What would be the impact of using this test on your patients / population?
Awano (173)	✗	✗	✔	✔	✗	✔	✗	✗	✗	✗	✗
Brinkman (185)	✔	✔	✔	✔	✔	✔	✔	✗	✔	✔	✗
Dragonieri 2007 (174)	✔	✔	✔	✔	✔	✔	?	✔	✔	✗	✗
Dragonieri 2018 (184)	✔	✔	✔	✔	✔	✔	✔	✔	✔	✗	✗
Fens 2009 (175)	✔	✔	✔	✔	✔	✔	✔	?	✔	✗	✗
Fens 2011 (71)	✔	✔	✔	✔	✔	✔	✔	✔	✔	✗	✗
Ibrahim (70)	✔	✔	✔	✔	✔	✔	✔	✗	✔	✗	✗
Larstad (176)	✔	?	✔	✔	✗	✔	✗	?	?	✗	✗

Lazar (192)	✔	✔	✔	✔	✔	✔	✔	✔	✔	✕	✕
Meyer (177)	✔	✔	✔	✔	✔	✔	✔	✔	✔	✕	✕
Montuschi (178)	✔	✔	✔	✔	✔	✔	✔	?	✔	✕	✕
Olopade (179)	✔	✔	✔	✔	?	✔	✔	?	?	✕	✕
Paredi (180)	✔	✔	✔	✔	✔	✕	✔	✕	✔	✕	✕
Plaza (186)	✔	✔	✔	✔	✔	✔	✔	✔	✔	✕	✕
Reynolds (188)	✕	?	✔	✔	✕	✔	✕	✕	✕	✕	✕
van der Schee 2013 (181)	✔	✔	✔	✔	✔	✔	✔	✔	?	✕	✕
van der Schee 2013 (73)	✔	✔	✔	✔	?	✔	✔	✔	?	✕	✕
Timms, Thomas & Yates (182)	✔	✔	✔	✔	✔	✔	✔	✕	?	✕	✕
de Vries 2015 (183)	✔	✔	✔	✔	✔	✔	✔	✔	?	✕	✕
de Vries 2018 (187)	✔	✔	✔	✔	✔	✔	✔	✕	✔	✕	✕

Note: CASP checklist question 10 “Can the test be applied to your patient or population of interest?” was omitted. This question refers to resource and opportunity costs for test implementation not appropriate to the field of research as it currently stands. Similarly, question 12 was answered in the negative due to the hypothesis-generating, proof-of-concept stage of the research.

Examining predictive models for their diagnostic test accuracy in asthma, there is no single valid and reliable test against which the new diagnostic can be measured. In a recent study of patients with a primary care diagnosis of asthma (220) the diagnosis could not be supported in 33% of cases; furthermore this is not a novel finding (221-223). The matter is further complicated by the heterogeneous nature of the disease; inflammation is not an essential component of the disease, thereby limiting the use of existing inflammatory biomarkers. Studies with diagnoses made by a physician or according to recognised clinical guidelines were included while accepting that, as a reference standard, this is likely to fall short of the assumed 100% accuracy. One study recruited from a severe asthma clinic with physician diagnosis inferred rather than explicitly stated (188).

Studies of diagnostic test accuracy should ideally examine the population in which the test would be employed - those with a clinical suspicion of disease or diagnostic uncertainty. The majority of studies compared healthy controls against participants with an existing asthma diagnosis (and commonly receiving treatment); such results are likely to over-estimate diagnostic accuracy and might perhaps better be characterised as hypothesis-generating or proof-of concept studies. In the majority of studies it was not clear that a random or continuous sample of patients had been used; where there is selection of participants there is risk of inclusion bias leading to over-estimation of test accuracy.

For those studies reporting on symptoms or loss of asthma control, the time between symptom reporting and VOC-sampling is important due to the potential for symptoms to vary; delay between the two could lead to inaccuracy or obfuscate a relationship. Timing in studies was frequently implied rather than explicitly stated.

In the majority of studies it was not possible to say that index tests were conducted and interpreted without knowledge of the reference standard; blinding was rarely mentioned. Nonetheless the risk of bias is low; analytical methods such as gas chromatography-mass spectrometry, and statistical methods such as PCA are hard to corrupt. Some risk nevertheless exists as storage time and conditions prior to processing have the potential to influence outcomes; in addition statistical methods for discriminant analysis are prone to over-fitting and require validation

Study participants were generally well described with the exception of body mass index (BMI) and ethnicity. BMI may affect markers of oxidative stress (224) and VOC (167, 187); and evidence exists of ethnic differences in both pulmonary function (225, 226) and breath profiles (187, 227).

3.3.1.1 Technical validity

Breath sampling

There are two key methodological issues relating to sample collection a) that of how to best deal with ambient VOC; and b) how to collect and store samples prior to analysis.

Ambient VOC

A consensus method for dealing with ambient, environmental VOC has been outlined by European Respiratory Society (ERS) technical standards (228). This recommends 1) parallel sampling of ambient air for background correction using alveolar concentration gradients, and 2) the use of VOC-filtered air. More detailed discussion of these issues can be found within the technical standards themselves (229). Of the included studies, ten measured ambient air VOC; the way these data were utilised varied.

Exogenous VOC in breath can be minimised through the use of filtered air but inhaled VOC may be retained for some time and wash-out periods vary depending on the VOC in question. Wallace et al (230) estimate that some retention times may be as long as 3 days; and breathing synthetic air for 30 minutes was found to reduce but not eliminate ambient VOC (231). If VOC analysis is to be clinically useful the period of time for which filtered air is breathed prior to assessment needs to be practicable; complete elimination of the 'exposome' is unlikely. Furthermore, ambient VOC may be absorbed trans dermally. In the case of some semi-volatile or aerosolised compounds, dermal uptake may be up to four times higher than inhalation (232, 233) however the relationship between dermally absorbed VOC and their exhalation is largely unstudied. Current recommendations offer a pragmatic rather than a perfect solution; 12 of the included studies used filtered air.

Sampling methods

Two main approaches have been taken to the collection of samples prior to analysis – 1) the use of impermeable bags, 2) the use of sorbent materials.

Numerous studies have examined the properties of gas sampling bags (234-238). Beauchamp et al (237) summarise the drawbacks of this method which include material emissions, diffusion of VOC (into or out of the bag), adsorption effects, reactive chemistry and the production of artefacts. While VOC losses have been described as within acceptable levels (236, 237) this could nonetheless result in those VOC present at very low concentrations becoming undetectable; moreover the differential decay rates reported across VOC could change relative concentrations over time.

Breath samples collected in impermeable bags can be concentrated using stainless steel tubes packed with adsorbent material. These may be stored (239, 240) before desorption and analysis; studies suggest storage at room temperature for 14 days or less results in acceptable sample retention (181). Direct sampling onto sorbents is also possible. In both cases a decision has to be made as to which adsorbent(s) to use. Tenax – a porous polymer - is used in many of the studies; its hydrophobicity is suited to humid breath samples and it can adsorb a wide range of VOC (241). Its ability to capture low mass VOC is however limited and compound breakthrough may be an issue. Dual-bed sorbents are an attempt to combat these issues while also limiting the quantity of water adsorbed. If a deductive approach is used - looking for specific compounds - the appropriate sorbent(s) need to be selected. For inductive approaches there must be recognition that sorbent selection limits the range of VOC collected; disease-related VOC permutations may go undetected if outside of this range. The stability of adsorbed samples is time and temperature dependant (242) ; of the six studies concentrating samples on sorbent tubes, two did not report the duration of storage, and three either did not report the temperature or stored samples at room temperature.

In addition to the storage of samples there is also variation in the nature of the sample. The majority of included studies using Tedlar bags collected mixed expiratory air by way of single or multiple exhalations. However, if the lung metabolome is the exclusive target of investigation there will be sample contamination from the upper respiratory tract. The importance of breath fraction to asthma breathomics is yet to be established.

Ibrahim et al (70) used a novel device with a facemask and pressure sensor to selectively sample air from the lower respiratory tract directly onto sorbent tubes. This approach has since been commercialised in a device from Owlstone (Cambridge, UK). Fifteen of the studies used a collapsible reservoir (Tedlar or Nalophan bag) while one used a syringe.

Sample analysis

A range of methods have been applied to the analysis of breath samples including various forms of mass spectrometry; some offline - such as gas chromatography-mass spectrometry (GC-MS) - and others online - such as ion mobility spectrometry (IMS), proton transfer mass spectrometry (PTR-MS), selected ion flow tube mass spectrometry (SIFT-MS) and field asymmetric ion mobility spectrometry (FAIMS). A full review of these methods may be found in Beale et al (241) and elsewhere.

Due to its sensitivity and selectivity, GC-MS has become the standard method by which to characterise the human metabolome (243), including that detectable via the breath (although alternatives such as FAIMS may be equally efficacious (244)). This approach has been complemented by the electronic nose (eNose); chemical cross-reactive sensor arrays (245) over which breath samples may be passed inducing detectable changes in the sensor material, thereby characterising the relative concentrations of VOC present (246). eNoses lack the ability of MS to identify VOC; thereby precluding their use for biomarker discovery but their ability to produce real-time data holds promise in point-of-care diagnostics. Of the studies in this review, nine used an eNose; seven mass spectrometry; and four a combination of the two.

3.3.1.2 Statistical validity

A range of statistical techniques may be used in the identification of disease-induced metabolomic permutations; these have been comprehensively reviewed elsewhere (164, 169, 241). Although strategies for avoiding false discoveries (247) and minimum reporting standards for data analysis in metabolomics (248) have been published, and applied to breathomics (228, 249) there is no standard statistical framework for analyses (250); the ERS technical standards are not prescriptive in this respect. As shown in table 14, approaches to data processing and pre-processing varied, both in the techniques used and the extent to which they were reported.

The majority of papers undertook inductive / untargeted analyses in which there was no *a priori* identification of compounds. Such analyses when applied to large data sets are prone to over-fitting and the resultant VOC models require validation; without this the performance of the model cannot purport to be accurate. Internal cross-validation is one of the methods commonly applied however the rigour this imparts may be limited by the small sample size of many of the included studies. Ten studies describe undertaking some form of internal validation such as leave one out cross-validation or boot strapping; only three studies used an external validation set (71, 184, 187).

Five studies (173, 176, 179-181) conducted targeted analyses based on compounds previous identified as associated with asthma or inflammation; a deductive approach not associated with the aforementioned statistical challenges. Although these findings provide support for the utility of certain VOC in asthma breathomics, they were not an attempt to provide external validation to any one specific model.

Furthermore, although Awano et al (173) specified compounds of interest *a priori*, their relationship with clinical variables (including asthma) was examined by way of post-hoc analyses and vulnerable to the risk of false discovery.

In studies other than those using an eNose, compound identification is possible. There are a number of databases which may be used including the Pacific Northwest National Laboratory (PNNL), the National Institute for Standards and Technology (NIST), Metlin, or in-house custom libraries constructed using reference standards. The extent to which use of different libraries might limit comparability is unclear; however, Sharpe et al (251) compared PNNL with NIST and reported that for all but one of the 12 compounds they compared, there was agreement between databases to within the level of experimental uncertainty. Few papers reported the libraries used for compound identification and none the match-percentages for compound identification. The chemical analysis working group metabolomics standards initiative (MSI) published proposed minimum reporting standards which include both data pre-processing and metabolite identification (252). Implementation of such reporting standards would allow identification of studies at risk of spurious candidate marker identification.

3.3.1.3 Clinical validity

Two potential confounders were common across studies – medication and study location. Participants with asthma were frequently taking medication such as inhaled corticosteroids (ICS) or β_2-agonists which healthy controls were not; any observed between-group difference in exhaled VOC might be due to medication metabolites rather than disease-related changes in biochemical pathways. The extent to which this was addressed in studies varied, likely due to the emergent nature of this field of research and the inclusion of small-scale, proof-of-concept studies. Evidence regarding the extent to which medication might act as a confounder is unclear (73, 174, 205, 224) but exhaled VOC have been reported to be capable of identifying those asthma patients in which oral corticosteroid and salbutamol urinary metabolites were present (200).

The second potential confounder was background bias. In many studies the site of recruitment differed between controls and those with asthma but it was unclear where breath sampling took place. de Vries et al (183) report no significant difference between samples from different medical sites ($p=0.89$); however the ambient VOC profile of hospitals may differ greatly from other locations (253) and a systematic difference in location could be the cause of sample differentiation, rather than disease metabolites.

The application of background air subtraction and use of filtered air constitute an attempt to negate this but as discussed in section 3.1.2.1 there are limitations. Other potential confounders such as smoking history, age, and gender (224) were not always well matched between groups. Asthma severity was frequently stated but where it was not, medication-use was rarely reported with sufficient detail to make an assessment of severity. Many studies contained a mixture of asthma severities; and while spirometry results were commonly presented measures of asthma control were not.

3.3.2 Qualitative synthesis

3.3.2.1 Asthma Diagnosis

The ability of breathomics to differentiate between those with asthma and healthy controls was examined by 15 studies. These models reported moderate-to-excellent discriminative ability, citing CVVs of 90-100% (174), classification accuracies of 86%(70) to 99%(177), and AUROCs of between 0.70 (182) and 0.94 (183). It should be emphasised that these accuracy rates are based on populations with diagnosed disease; the studies were examining the difference in VOC profiles between healthy controls and those with established, treated, and frequently long-standing asthma. The diagnostic performance of VOC models in a real clinical population with undiagnosed, untreated respiratory symptoms of relatively recent onset may be very different.

In many studies the risk of sampling bias was unclear; and in some studies there was a risk of confounding, for example large differences in the average age of groups (73, 183). While studies with physician diagnosed asthma were included, the standard to which this was reported and conducted varied between studies. It is also worth noting that several studies used populations of mixed asthma severity; it is unlikely that breathomic models would be applied homogenously across such a population.

Five studies conducted a targeted analysis of compounds. In the case of pentane, Olopade et al (179) report significantly higher levels during acute asthma attack but both Olopade and Larstad et al (176) report no significant difference between controlled-asthma and healthy controls. Paredi et al (180) report significantly higher levels of ethane in untreated asthma compared with treated disease or healthy controls. They do not comment on treated-asthma versus healthy controls but Larstad et al (176) found no significant difference (in ethane levels) between a largely steroid-treated controlled asthma group and healthy controls. Larstad et al do however report a significantly lower level of isoprene in those with asthma.

Awano et al cite an adjusted odds ratio of 6.9 (95% CI 1.1-44.2; $p<0.05$) for asthma in the presence of dimethyl sulphide; while van der Schee report AUCs of 0.79-0.84 ($p<0.05$) for the differentiation of asthma from controls using a five-compound model.

Ten studies performed untargeted analyses producing diagnostic models for the differentiation of asthma from healthy controls. Fewer studies aimed to differentiate between asthma and other respiratory diseases; four examined COPD and asthma reporting classification accuracies of between 70% and 96% (71, 175, 182, 183); one differentiated between asthma and allergic rhinitis reporting an AUROC of 93% (184); while another examined lung cancer and asthma, reporting a classification accuracy 68% (183). In all but the allergic rhinitis study there was a large difference in average age between the asthma and the other respiratory disease group.

3.3.2.2 Asthma Phenotypes

Eight studies examined asthma phenotypes including sputum cell type, steroid responsiveness, disease severity and airway reversibility.

Both Plaza et al (186) and Ibrahim et al (70) constructed models differentiating between eosinophilic, neutrophilic and paucigranulocytic phenotypes, with classification accuracies of 73% to 74% (186), and AUROCs of 0.79 (186) to 0.98 (70). Differentiation was likely not due to differences in ICS use (which were similar between groups in Plaza et al), but it was not reported whether there were other systematic between-group differences in treatment regime. Brinkman et al report two VOC significantly correlated with sputum eosinophilia (correlation coefficients of $r\geq0.46$ & 0.47 ($P<0.01$)) but did not find any such correlations for sputum neutrophilia (185).

de Vries et al (187) examined a combined asthma and COPD population in a large multi-centre study. They identified clusters differing in eosinophilia ($p=0.02$), neutrophilia ($p=0.03$), atopy ($p<0.01$) and exacerbation rate ($p<0.01$). Further clusters based on ethnicity ($p=0.01$) and exhaled nitric oxide ($p<0.01$) were identified.

Van der Schee et al (73) examined eNose results for the prediction of steroid responsiveness, reporting an AUROC of 0.88 and greater accuracy than either sputum eosinophil count or FeNO. For the differentiation of mild from severe asthma Dragoneiri et al (174) report a CVV of only 65% (M-distance, 1.23). Similarly Fens et al (71) report an accuracy of just 58% (AUROC 0.68) for the differentiation of fixed and classic asthma.

Meyer et al conducted a cluster analysis of both VOC data and clinical parameters. While VOC profiles were able to differentiate between some clinical clusters with good levels of accuracy, they also reported distinct clinical clusters with similar VOC profiles, and distinct VOC clusters with similar clinical characteristics.

3.3.2.3 Loss of asthma control

Four of the included studies examined some aspect of asthma control. Brinkman et al (185) conducted a prospective medication-withdrawal study. Classification accuracy for baseline versus loss of control - as measured by the asthma control questionnaire (ACQ) - was 95% using an eNose and 68% by GC-MS; loss of control versus recovery was 86% (eNose) and 77% (GC-MS). Ibrahim et al (70) using GC-MS report an AUROC of 0.96 for the identification of loss of control; and Olopade et al (179) report significantly higher levels of pentane during exacerbation compared to recovery. It is unlikely that the observed differences in breath profiles are due to changes in airway calibre - Lazar et al (192) undertook bronchial challenge testing on participants with stable asthma and reported no changes associated with bronchoconstriction.

3.3.2.4 Discriminant compounds

Nine of the included studies report on compound identities (presented in table 16). A total of 76 compounds were cited as significant. Of these, nine were reported in more than one paper - 2,4-dimethylheptane; 2,6,10-trimethyldodecane; 2,6,11-trimethyldodecane; acetone; benzene; ethane; isoprene; phenol; and toluene - and two – acetone and isoprene - were reported by three studies. The models constructed by any given study were thus comprised of compounds largely or entirely absent from the models presented by other studies. Moreover, it was not always clear in which direction the compounds differed. In the case of isoprene, Dallinga et al and van der Schee (174, 181) found it to be elevated in asthma, while Larstad et al (176) report it to be lowered. Despite the lack of concordance between studies, where attempts have been made to validate previous models the results have been positive. van der Schee (181) used five compounds previously linked to asthma (acetone, isoprene, carbon disulphide, toluene and 1-propanol) and report an AUC of 0.79-0.84 ($p<0.05$). Where compounds have not been identified but validation has been undertaken results have been similarly positive. Fens et al (71) report a phenotyping accuracy of 83-88% in an external validation exercise; Montuschi et al validated their data in a distinct test set, reporting a diagnostic accuracy of 87.5%; de Vries et al (187) found the majority of clusters identified in their training set to be confirmed in an independent validation set; and Dragonieri (184) report diagnostic AUCs of 77-92% in an external validation exercise.

Table 16 – Asthma breathomics: volatile organic compounds identified

Study	Discriminant compounds identified	Compound type	Direction of difference in asthma group (if appropriate)	Differentiated groups	Differences between case and control groups
Awano et al (173)	Dimethyl sulphide	Sulfur and nitrogen compounds	+	Asthma vs non-asthma	Not reported
Brinkman et al (185)	Acetonitrile Methanol Bicyclo[2.2.2]octan-1-ol.4-methyl	Sulfur and nitrogen Alcohol Alcohol	+ + +	Control vs loss of control	NA – longitudinal study
	Acetonitrile Bicyclo[2.2.2]octan-1-ol.4-methyl	Sulfur and nitrogen Alcohol	+ +	Sputum eosinophilia	Not reported
Dragonieri et al (174)	Isopropanol 2,3-dimethylheptane 2,4-dimethylheptane 2,6,11-trimethyldodecane 3,7-dimethylundecane 4-methyloctane Alkane Toluene Acetic acid Acetone Isoprene	Alcohol Alkane Alkane Alkane Alkane Alkane Alkane Aromatic Acids & esters Ketone Terpenoids	+ + + + + + + + + + +	Asthma vs controls	Attempts to match age and gender and disease severity. Differences in FEV_1 % predicted and FVC % predicted.
Ibrahim et al (70)	2,6,10-trimethyldodecane 2,6,11-trimethyldodecane Benzyl alcohol 3,4-Dihydroxybenzonitrile 2-methyldecane 1-methyl-4-(1-methylethylidene)cyclohexene Butanoic acid,2,2-dimethyl-3-oxo-,ethyl ester 2-butanone	Alkane Alkane Aromatic Sulfur and nitrogen Alkane Terpenoids Acids & esters Ketone	+ + + + + + + +	Asthma vs controls	Closely matched in age, gender, and BMI. Differences in FEV_1, FVC & and FEV_1/FVC.

	Allyl methyl sulphide	Sulfur and nitrogen	+		
	4-nitroso ethylester benzoic ac	Sulfur and nitrogen	-		
	2-butyl-cyclohexanol	Alcohol	-		
	5,5-Dibutylnonane	Alkane	-		
	4-ethenyl,1-,2-dimethyl benzene	Aromatic	-		
	2,5-Cyclohexadiene-1,4-dione, 2,6-bis(1,1-dimethylethyl)	Ketone	-		
	Pentadecanal	Aldehyde	-		
	Camphene	Terpenoids	-	Eosinophilic vs non-eosinophilic	Closely matched in age. Differences in FEV_1 % predicted, FVC % predicted, and in FEV_1/FVC
	1,1-Dimethylpropyl 2-Ethylhexanoate	Acids & esters	-		
	2,6,10-trimethyldodecane	Alkane	-		
	7a-Isopropenyl-4,5-dimethyloctahydroinden-4-yl) methanol	Alcohol	-		
	Cyclohexanone 3,7,7-trimethyl	Ketone	-		
	Bicyclo[4.1.0]hept-2-ene	Terpenoids	-		
	Cyclohexene-4-methylene	Alkene	-		
	Cyclopentene,1,3-dimethyl-2-(1-methylethyl)	Alkene	+	Neutrophilic vs non-neutrophilic	Differences in age, FEV_1 % predicted and FEV_1/FVC
	2,7-dimethyl naphthalene	Aromatic	+		
	3,5-dimethyl Cyclohexanol	Alcohol	+		
	Tetradecane, 4-methyl	Alkane	+		
	Decahydro-8a-ethyl-1,1,4a,6-tetramethylnaphthalene	Alkane	+		
	Benzene	Aromatic	-	Control vs loss of control	Differences in age, FEV_1 % predicted, FVC % predicted, and in FEV_1/FVC.
	Pentadecane, 1-methoxy-13-methyl	Ether	-		
	Heptanoic ac	Acids & esters	-		
	Bicyclo[3.1.0]hex-2-ene, 4-methylene-1-(1-methylethyl)	Terpenoids	+		

	O-xylene 2-4-methylene, 3-methyl/butanal, 2-methyl 2,2,4,4-Tetramethyloctane (1E)-1-(methylsulphanyl)1-propene 2,6-diisopropylnaphtalene	Aromatic Alkane Sulfur & nitrogen compounds Aromatic	+ - - -		
Larstad et al (176)	Isoprene Ethane	Terpenoids Alkane	- -	Asthma vs controls	Differences in gender, weight, FEV_1 % predicted and FVC % predicted.
Meyer et al (177)	1-Dodecanol, 3,7,11-trimethyl- Benzene 1,3-Dioxolane 2-(phenylmethyl)-4-Cyclopentene-1,3-dione, 4-phenyl- Dodecane Phenol Quinoline decahydro- 2-Propionyloxypentadecane Tetradecanoic acid Octanal 2-Butyl-2,7-octadien-1-ol 2,4-dimethylheptane 5-hexenoic acid	Alcohol Aromatic Acids & esters Acids & esters Alkane Aromatic Sulfur & nitrogen Acids & esters Acids & esters Aldehyde Alcohol Alkane Acids & esters	+ + + + - - - - - - ? ? ?	Asthma vs controls	Not reported
Olopade et al (179)	Pentane	Alkane	+	Controlled vs loss of control (acute)	NA – longitudinal study
Paredi et al (180)	Ethane	Alkane	+ (in untreated asthma)	Steroid treated vs non-steroid treated & healthy controls	Closely matched in age. Differences in gender, FEV_1 % predicted and RV/TLC % predicted

van der Schee et al (181)	Acetone Isoprene Carbon disulphide Toluene 1-propanol	Ketone Terpenoids Sulfur & nitrogen Aromatic Alcohol	+ + + + +	Asthma vs controls	Differences in age, gender, and smoking history.
Abstracts					
Fens et al (205)	Acetone; 1,2-pentadiene; 2,4,4-trimethyl-1-pentene, phenol, D-limonene 4-tert-butylcyclohexyl-acetate	Ketone Alkene Alkene Alcohol Terpenoids Exyl-acetate	Not reported	Control vs loss of control	NA – longitudinal study
Brinkman et al (198)	Pantolactone,5 Methylacetate,32 Methylcyclohexane,22 Cyclohexane-D12,50 Pinene,22 Eucalyptol,74 furan 2-methyl,70 Isopropyl alcohol	Acids & esters Acids & esters Alkane Alkane Terpenoids Terpenoids Aromatic Alcohol	Correlations	Sputum eosinophils Sputum neutrophil Blood eosinophils Blood neutrophils CRP FeNO ACQ FEV_1 % predicted	Not reported
Durrington et al (203)	2-Undecanal	Aldehyde	Diurnal variation in asthma which is not present in healthy controls		Closely matched in age. Difference in FEV_1
Mixed age group					
Couto et al (190, 254)**.**	Nonane 2,2,4,6,6-pentamethylheptane Decane, Dodecane Tetradecane Nonanal Decanal Dodecanal	Alkane Alkane Alkane Alkane Alkane Aldehyde Aldehyde Aldehyde		Asthmatic vs non-asthmatic adolescent swimmers.	No significant differences reported.

The majority of abstracts did not publish details on compounds of interest, only Brinkman et al (76), Couto et al (38), Durrington et al (203) & Fens et al (73). The abstract by Fens et al was not subsequently published in full. The study by Couto et al was, however they included study participants under the age of 18. They report that samples from asthma and healthy controls could not be separated based on distinct metabolites. Brinkman et al present a list of compounds found to correlate with clinical variables; this was a univariate analysis without the more sophisticated methods such as Bonferonni correction which would normally be applied to a large dataset; moreover the validity of compound identification is hard to determine. While the compounds of interest for these four abstracts are presented it is not possible to assess study quality, risk of bias, and full methodology.

3.4 Paediatric asthma

A review of the paediatric asthma breathomic literature was published by Neerincx et al (159) in 2017. The results were encouraging, suggesting good predictive accuracy for VOC profiles in asthma diagnosis. However, the authors reported on only those studies which they were aware of and limit their scope to the last 10 years. Given that the first study of an exhaled VOC in asthma may well have been Olopade et al in 1997 (179) it is possible that studies may have been omitted leaving the review potentially vulnerable to bias. The results of the literature search used for this chapter was screened for paediatric studies (these searches had been conducted with no date limits).

The number of references to paediatric asthma breathomic studies was 17. Six were abstracts, of which three were not published in full elsewhere (255-257); two presented identical data - early findings from a study by the review authors which is yet to be published (258, 259); and one has since been published in full (260). The list of full studies identified by this search was similar but not identical to that of Neerincx et al. Neerincx et al included two papers (261, 262) examining pre-school wheeze (not asthma) but excluded a paper published more than 10 years ago (263); this was a longitudinal study of asthmatic children living in a high pollution area (n=26). This study reported largely non-significant results, with the exception of exhaled benzene which exhibited a moderate positive association with bothersome / severe asthma symptoms. The study was limited by the small number of breath samples obtained on symptom-free days (n=6).

An absence of significant findings is of course one reason why abstracts might not progress to full publication.

While there are good reasons for excluding abstracts from a review – including the inability to assess the quality of studies and risk of bias – this does have the potential to give an unbalanced viewpoint; one which emphasises positive associations and downplays null findings. Of the abstracts not subsequently published in full - Gahleitner et al (256) did not report any results; while Brinkman et al (255) report a cluster analysis which identified groups that differed significantly in clinical parameters. Wang et al (257) found a correlation with night waking but not with asthma control test scores; whereas Vijverberg et al (258, 259) reported an area under the curve of between 0.71 and 0.97 for the identification of disease control.

It would seem then that the review by Neerincx et al (159) succeeded in capturing the majority of relevant literature; the additionally identified study adds little to their findings. The abstracts identified by this search present results largely relating to the ability of VOC to differentiate between states of disease control, the results of which were conflicting. Overall little was found to either challenge or expand the findings of their review.

3.5 Discussion

The accuracy of classification achieved by breathomic models suggests VOC-profiling in exhaled breath has potential for use in asthma diagnosis and management. The ability to discriminate between those with asthma and healthy controls has been consistently demonstrated but, to be of clinical use, these findings need to be validated in independent prospective cohort-studies undertaken in populations with only a clinical suspicion of asthma; this would enable the determination of diagnostic test accuracy. Given the high incidence of asthma misdiagnosis, development of such a test could be clinically significant and of benefit in the presence of diagnostic uncertainty.

Sputum eosinophil count has long been considered the definitive method for assessing lung inflammation, and when used to guide treatment has been shown to improve asthma outcomes (264). However, FeNO has been found to predict steroid responsiveness (265) and has now been integrated into national asthma guidelines for both management and diagnosis (266, 267).

The ease of use and rapidity of results with FeNO measuring devices has led to more widespread clinical uptake than that achieved by sputum eosinophil count.

However, VOC profiling has the potential for wider application than either including the identification of alternative sputum profiles (such as neutrophilic or paucigranulocytic); monitoring of control in non-eosinophilic phenotypes; identification of treatable traits; and the differentiation of transient pre-school wheeze and asthma.

A clinically-meaningful threshold has been determined for both sputum eosinophilia and FeNO and the reproducibility of measurements established. This is not yet the case for breathomic models. While VOC-measurements within-individuals may be reproducible and breath profiling may display good levels of accuracy, relatively few results have been replicated or externally validated. It is important to note that, in a heterogeneous disease such as asthma, findings based upon asthma populations defined by one 'gold standard' (such as sputum eosinophils) will not be accurately validated in a population based on an alternative diagnostic standard (e.g. physician diagnosed asthma) which may be composed of other or multiple phenotypes.

The inter-study variability reported in this review may in part be due to instrument variability. Between-laboratory comparisons for GC-MS data can be challenging due to the dynamic nature of the measuring equipment. However, this may be improved through the implementation of the MSI reporting standards coupled with comparative analysis of laboratory data quality. eNoses have demonstrated variability, both between manufacturers (258) and between devices of the same model (268), and sensor 'drift' can be difficult to detect. This may be, to some extent, a self-limiting problem; as potential markers are identified, study methodology may shift from inductive to deductive. With targeted studies it is possible to address calibration issues from the outset giving increased confidence in results.

Causes of inter-study variability do not lie exclusively with the instrumentation; metabolomics involves substantial inter-subject variation (241). This is not necessarily simply a result of comparing different asthma severities or phenotypes. A number of variables may have an effect on VOC profiles including the exposome (269), respiratory rate (270) and breathing route (271). In a study of healthy volunteers Philips et al (272) report the mean number of VOC per breath sample to be <350 but the number of different compounds across their studies as a whole to be >3,400. Moreover, of the total compounds identified in their study only 27 were found in the samples of all participants. However, both Fens (175) and de Vries et al (183) report a high correlation coefficient for within-day repeatability and between-day repeatability for participants.

It would seem then that breath prints are relatively stable within- but vary considerably between-individuals (229). Sterk argues that while complicating the independent validation of results this variation offers hope in terms of individual phenotyping (273) including the identification of treatable traits and implementation of personalized medicine.

Recent work in other diseases has shown that diet and lifestyle are important confounders in breath VOC analysis (274). While this may apply to many of the smaller studies included within this review, with sufficiently large patient cohorts this may not be the case. In a study of 494 participants, variables thought to be highly confounding – including age and smoking - appeared not to effect the ability of a diagnostic model to distinguish gastric cancer from healthy controls (275).

In common with other emergent fields of study (113) there is a conflict between innovation and standardisation. Due to its potential for both inductive and deductive approaches, and for both offline and online analysis, breathomics is likely to remain more heterogeneous in its methodology than some other fields. However, the arrival of technical standards for exhaled biomarkers (228), minimum reporting standards (252) and CE-marked, production-line breath capture devices, goes a long way towards addressing some of the potential sources of confounding and variation. Despite the publication of such standards there is still considerable leeway in how samples may be processed and analysed; these decisions are crucial given that the clinical relevance and wider acceptance of results hinges on the correct selection and application of these techniques. The quality of analysis amongst the included papers is inconsistent and hampered by the low numbers of participants in many of the early studies. Internal validation of results does seem however to be becoming the norm, and as participant cohorts continue to grow the risk of overfitting diagnostic models will further reduce. Whilst the determination of which features within a dataset should be included in diagnostic models has improved, compound identification remains relatively poor with few of the studies checking the putative identifications against chemical standards. Better identification will allow the biological origins of exhaled VOC to be determined; the first step in linking breathomics to other 'omics in a systems biology approach.

3.6 Conclusion

Breathomics is well suited to the age of personalised medicine; the large data sets typically produced are highly individualised and reflect a multitude of metabolomic pathways; a feature which is particularly attractive for the study of complex heterogeneous diseases such as asthma.

The potential exists not only for diagnostics, phenotyping and the identification of treatable traits but – when coupled with other 'omics – the linking of phenotypes to endotypes. Results to-date are promising but validation in independent prospective cohorts is needed; this may be challenging given the high levels of inter-individual variation. However, addressing inter-study variation through the identification of important confounders, increasing study size, and methodological and analytical standardisation will facilitate these efforts. Identification of a limited number of compounds with strong discriminative ability may decrease processing time and aid the development of point of care testing; crucial if breathomics is to make the leap into clinical application.

Chapter two presented a synthesis of the literature on exhaled breath condensate and a single potential asthma biomarker – 8-isoprostane. This chapter (**chapter three**) presented the literature on exhaled breath gases in asthma; an approach capable of capturing data on multiple potential biomarkers. Subsequent chapters present the methods used in the two studies of exhaled breath biomarkers in asthma which comprise this thesis (**chapter five**); and their results (**chapters six** and **seven**). The next chapter (**chapter four**) outlines work conducted in the development of the methods used.

Chapter 4 – Developing Methods: exhaled breath sampling and assessing patient acceptability

4.1 Introduction

In the first chapter, the heterogeneity of asthma was described and the need for clinically applicable biomarkers was outlined. Chapters two and three reviewed the literature regarding two methods of breath analysis – EBC and exhaled VOC - and their application to asthma. Later chapters presents the findings of two studies using these methods:

- *Exhaled breath biomarkers in acute asthma: a feasibility study* (chapter six).
- *Bronchial Challenge Testing in Asthma* (chapter seven).

This chapter provides a background to the methods used in capturing and processing breath samples and the development of a questionnaire used to assess these methods.

4.2 Exhaled breath condensate and 8-isoprostane

4.2.1 Background

Oxidative stress is thought to play an important role in the pathogenesis of asthma (76) and markers of oxidative stress can be found in both exhaled breath gas and EBC condensate. Amongst the many markers of oxidative stress 8-isoprostane has the advantage of being relatively stable and suitable for batch analysis. Studies have reported it to be higher in asthmatics than non-asthmatics (80-82); able to predict asthma severity (84); and responsive to treatment with corticosteroid therapy (86, 87). The results of the review presented in chapter 2 suggest that although 8-isoprostane has potential as a biomarker (in that it may be capable of both indicating an abnormal process and its response to treatment) further studies need to be undertaken to establish this. Of particular interest was the number of authors who reported an inability to measure 8-isoprostane in the majority of their samples.

4.2.2. Exhaled breath condensate study methods

Exhaled breath condensate sampling was conducted in accordance with ERS guidelines (228) using the RTube from Respiratory Research (Texas, USA)(see figure 8). This is a single use device with a one way valve which meets guideline recommendations. It works by forcing exhaled breath through a tube which is cooled through the application of a chilled metal sleeve.

As breath travels through the tube exhalate condenses on the cooled surface. The metal cooling sleeve was stored in a -80°C freezer and removed immediately prior to use.

Figure 8 – The RTube: stock image of the device in use (withour noseclips).

Participants were asked to wear nose clips and - using tidal breathing - breathe through the RTube for a period of ten minutes. Respiratory rate, time taken to collect sample and volume of condensate collected were recorded; it was not possible to record volume of breath exhaled as recommended by guidelines. Time from sampling to freezing was recorded; samples were frozen in a -80 freezer. Time-to-freezer and duration of storage prior to analysis may be important depending on the stability of the biomarker in question.

4.2.3. Methodological Issues

As alluded to in chapter 2 there are as yet unresolved methodological issues with the measurement of biomarkers in EBC and with 8-isoprostane specifically.

Ashnish Sinha – a student (Masters in Research) at the UEA - conducted a study in which he recruited patients with idiopathic pulmonary fibrosis and obtained two EBC samples of approximately 500-1,000µL from each participant. The researcher assisted in sample collection and together with members of the Norwich Medical School (Isabelle Piec and Jonathan Tang) a protocol was developed for the processing and analysis of samples. EBC was pipetted into eppendorfs containing a 0.005% solution of BHT in ethanol, an antioxidant to prevent further in-vitro formation of 8-isoprostane. Samples were frozen within 5 minutes of acquisition and assayed within 3 months of collection using an express ELISA kit from Cayman Chemicals to measure 8-isoprostane.

Of 49 samples only four had detectable 8-isoprostane levels, of which only two were above the lower limit of detection (10 pg/ml); none were above the lower limit of quantification (30pg/ml). The remaining EBC sample volume (that which was not used in the ELISA) was frozen to -80°C.

Effros et al (276, 277) published a methodology – since used in several studies (278-281) - to concentrate EBC samples through lyophilisation and calculate the relative concentration of water and airway lining fluid (ALF) within EBC samples by measuring conductivity. Effros et al suggest that, in the absence of any significant kidney dysfunction, respiratory secretions have the same osmolarity as plasma. Total ionic content as indicated by sample conductivity of EBC cannot be used to indicate how much of the sample volume is ionic-ALF and how much is water due to the presence of ammonia from the upper airways. Lyophilisation has the effect of removing ammonia thereby making this calculation possible. Using an average figure for plasma osmolarity in the healthy individual, sample conductivity can thus be used to calculate dilution rates.

This methodology was applied to the collected samples. First the conductivity of reverse osmosis (RO) water (produced 'in house' at the UEA biomedical facility) and high performance liquid chromatography (HPLC) water (deionised water commercially available from Fisher Chemical, UK) were tested. Both returned results of < 2 μS; RO water was used for all following steps.

Using a VirTis Wizard 2.0 (Genevac, UK) samples were lyophilised to 80 millitorr at minus 80°C over a period of approximately 24 hours and returned to storage at minus 80°C. Lyophilised samples were then defrosted and reconstituted in 200μL of reverse osmosis water (within 6 months of original sample collection). Due to the variety in the pre-lyophilisation volumes, reconstitution to a set volume of 200μl resulted in varying degrees of sample concentration. The concentration factor was calculated by dividing the post-ELISA, pre-lyophilisation sample weight by 200.

The reconstituted samples were placed in a heater and maintained within 1° of 25°C. A Jenway conductivity meter and micro-conductivity probe calibrated to a lower limit of 150μL of fluid volume was used to measure sample conductivity. The conductivity reading was corrected by the degree to which the sample had been concentrated.

Based on a molecular weight of 58.44g/mol, 438mg of NaCl in 50mL of RO water was used in order to produce a 150mmol/L solution of NaCl. Serial dilutions of this solution were used to produce a conductivity-concentration calibration curve. In constructing the calibration curve, the conductivity of solutions was measured in eppendorfs using a volume of 400μL. Eppendorfs containing the solution were then weighed and frozen.

The intention was to lyophilise these, reconstitute them in 200µL of RO water and measure their conductivity in order to assess the validity of conductivity measures in lyophilised and concentrated samples.

After testing the conductivity of reconstituted EBC samples, an overnight Cayman Chemicals ELISA with an 18 hour incubation period and detection limit of 2.7pg/ml was used to measure 8-isoprostane. It was hoped that concentrating the samples would raise 8-isoprostane away from the lower limit of detection thereby improving the reliability of the results; however despite these measures 8-isoprostane was universally undetectable.

Development of a liquid chromatography mass spectrometry method was posited. The conductivity measurement undertaken on the first aliquot of EBC would have allowed the approximate ratio of ALF to water within sample to be calculated. However, there was insufficient sample remaining to both set up and develop the LC-MS analysis of EBC 8-isoprostane and reserve sufficient volume for lyophilisation and conductivity testing.

Alternative methods of dilution calculation have been suggested. Plasma proteins are reported to be present in airway lining fluid in proportion to those levels in the serum. One simpler method of EBC dilution is to measure serum albumin or total protein within serum and compare this to the levels present in EBC. Unfortunately contemporaneous phlebotomy had not been conducted and so this option was not available; however this should be a consideration in future studies.

4.3 Volatile organic compounds

4.3.1 Assessing contaminants – VOC within the medical gas supply system

The ReCIVA (Owlstone, Cambridge, UK) was used to collect VOC samples (282)(see figure 9). This is a CE-marked device connected to a laptop computer; a single use mask with integral bacterial filter is used and it requires minimum patient effort (normal tidal breathing).

Figure 9 – The ReCIVA: stock image of the device. When in use the device would be connected via tubing to an air supply and attached to the participant using adjustable straps.

Air needs to be supplied at approximately 40 litres a minute to drive the device; less than this and the patient may experience resistance when breathing. Initially it was planned to use the hospital's medical air supply in conjunction with a high-rate flowmeter to achieve this. At the time of writing the study protocol the ERS technical standards for conducting exhaled breath studies (228) had not been published. As discussed in chapter 3 supplying patients with air contaminated with VOC might impact upon results; particularly if the contaminants present - and their concentration levels - vary. Background levels of contaminants might be taken into account by obtaining a sample of the gas supply contemporaneous with patient sampling; contaminants might then be accounted for by way of an alveolar concentration gradient; an approach which has been used by other research groups.

Samples from both the hospital medical air and oxygen supply were tested at a number of different locations. Samples were taken from those sites most likely to be used for patient sampling during the study, but also from the sites nearest and furthest from the source. Unlike the medical air which is manufactured (filtered) on-site, medical oxygen is obtained in compressed form from an external supplier (BOC). Results of this initial testing revealed large but consistent contaminants to be present in both. The larger contaminant peaks identified are listed in table 17.

Table 17 – Contaminant VOC identified in preliminary study

Methyl formate
Furan, 2-methyl-
1,3,5-Trifluorobenzene
Benzene
Disiloxane, hexamethyl-
Toluene
Cyclotrisiloxane, hexamethyl-
Ethylbenzene
Benzene, 1,3-dimethyl-
Styrene
Tricyclo[2.2.1.0(2,6)]heptane 1,3,3-trimethyl-
Bicyclo[2.2.1]heptane, 7,7-dimethyl-2-methylene-
Benzaldehyde
alpha.-Methylstyrene
Trisiloxane, 1,1,1,5,5,5-hexamethyl-3-[(trimethylsilyl)oxy]-
3-Carene
p-Cymene
D-Limonene
Acetophenone
Benzene, 1-methyl-3-(1-methylethenyl)-
Cyclopentasiloxane, decamethyl-

Samples were taken by running food grade tygon tubing between the hospital gas flow meter and Markes dual-bed (carbograph-5 / carbograph-2) sorbent tubes. Flow rate was set at the lowest possible calibrated level (0.5L/min) in order not to exceed the flow capacity of the sorbent tubes. Tygon tubing is used for patient sampling by the Manchester Institute of Biotechnology (MIB) breath research group in line with ReCIVA manufacturer's instructions; although it may emit VOC, levels are low and believed to have negligible impact on the chromatogram resulting from patient samples. In order to determine the source of contamination the tests were repeated with PTFE tubing. This is inflexible and not suited to patient sampling in the clinical environment but is non-emitting of VOC. In order to obtain a more consistent flow rate the following set-up was used – tubing was run between the hospital flow meter and a PTFE Y-join. To one arm a further length of PTFE tubing was attached, freely venting to the immediate environment. To the other arm of the Y-join was attached a length of PTFE tubing attached to a Markes sorbent tube.

The tube was inserted into the ReCIVA device which was set to continuous sampling at a rate of 200ml/min. A second sorbent tube was inserted into the ReCIVA and used to sample ambient air. Samples were obtained in the same fashion using Tygon tubing instead of PTFE tubing. The process was repeated on two separate occasions. On analysis of the results there was, again, a high level of contaminants in the samples obtained using tygon tubing but low levels when PTFE tubing was used. There was no apparent difference in contaminants between the oxygen samples taken using PTFE tubing on the two separate occasions. This would suggest that levels of contaminants in the hospital oxygen system are both low and consistent over time.

PTFE tubing is not practicable for sampling in the clinical situation – it is relatively inflexible and non-malleable making it difficult to attach to the Y-join and to the wall supply / flow meter. Tygon tubing is convenient, flexible, and suitable for use in the often times limited space available in a busy clinical environment. Moreover, tygon is being used by Owlstone and the MIB breathomics group; in order to make results comparable it is important to standardise procedures where as possible. The flow rate when using the tubing to supply the ReCIVA device for patient sampling is approximately 40L/min (the device then draws the supplied air through the sorbent tubes at a maximum flow rate of 0.5L/min). The rate of flow likely reduces the degree of contamination from the tubing. Given the discrepancy between flow rates used to power the ReCIVA and that which were used for sampling the hospital air, a sampling procedure was established in which oxygen was supplied to the ReCIVA at a high flow rate – in excess of 15 L/min. The high flow oxygen was directed through tygon tubing to the ReCIVA face mask which was fitted to a glass dummy-head (3D Display Ltd, Faversham, UK). The face mask had been baked at 100 degrees centigrade for 2 hours in order to remove surface VOC. Sampling was undertaken using tygon tubing which had been baked at 60 ^{o}C for 2 hours and PTFE tubing. All tubing was purged with high flow medical air (10L/min) for 10 mins prior to use. Samples were also taken at 3 different locations within the hospital using PTFE. One final set of samples was taken using the raw (unbaked) tygon). The difference between raw tygon, baked tygon and PTFE using this delivery system was minimal. This difference between oxygen samples taken at different locations using PTFE was similarly negligible.

The conclusion reached was that contaminants originating from the tygon tubing were minimised when flow rates were increased to those used in patient sampling using the ReCIVA.

Given the publication of ERS technical standards specifying a clean air supply should be used, and in light of the fact that the emergency department within the NNUH contained no supply of medical air, a CASPER clean air filter (Owlstone Medical, Cambridge) was purchased. This draws ambient air through a chamber of activated carbon pellets, scrubbing it of VOC before supplying it to the ReCIVA at the required flow rate.

It was anticipated that a proportion of those patients being treated in acute secondary care would be given supplementary oxygen as part of their initial care. In order to be most useful in informing treatment decisions at the point of care it might be necessary to undertake breath sampling while patients are in receipt of oxygen; this requires the ability to supply oxygen to the mask. Enquiries were made to the manufacturer as to the possibility of introducing oxygen to the CASPER prior to the air scrubber. The manufacturer responded that the CASPER unit was not rated to handle medical gases, it was thus able to supply VOC-scrubbed ambient air but not VOC-scrubbed medical oxygen. For those patients in receipt of supplementary oxygen NNUH oxygen was to be mixed at a rate of 15L/min with VOC-scrubbed air via the CASPER by way of a PTFE Y-join and the mixture delivered to patients via the ReCIVA. A sample of this mixture could be obtained prior to obtaining patient samples in order to allow the calculation of alveolar gradients.

4.3.2 Sample processing

VOC samples on sorbent tubes were stored in a cold room at 4-7^{o}C for a maximum of 14 days before being couriered to the MIB laboratory for dry purging, thermal desorption and GC-MS. In a study by van der Schee et al (239), 15 compounds (either common to breath samples or known to be related to asthma) were sampled, stored and transported prior to analysis by both eNose and GC-MS. They concluded that compounds were adequately stable for storage over a period of 14 days. Although stored in a cold environment, samples were shipped under normal carriage conditions (unrefrigerated); studies suggest storage at room temperature for 14 days results in acceptable sample retention (181, 240).

Sorbent tubes were dry purged with tubes weighed pre- and post-purge in order to calculate the quantity of water removed. In the most humid of samples this did not fully dry the tubes (6.8mg reduced to 2.3mg) but GC-MS results appeared to have been unaffected.

Gas chromatography is a method used to separate compounds within a sample utilising the differing rates at which they travel through a carrier medium.

An inert gas is used through which compounds travel; the larger the molecule the slower the rate of travel, thereby leading to separate of compounds based on mass. Mass spectrometry can then be used to determine the mass or particles and composition of molecules. Compounds are ionised in order to generate and separate charged molecule fragments whose mass-to-charge ratio can then be determined; compounds can be quantified by measuring daughter ions and a large proportion identified by their fragmentation pattern (60).

After analysis tubes were conditioned at MIB for 1 hour at 330°C in VOC-scrubbed dry nitrogen before they were capped, packaged and couriering to Norwich for use.

The process of analysis was as follows:

- 100µL of a calibration standard (1 ppmv, 4-Bromofluorobenzene in nitrogen, Thames Restek, UK) was loaded onto tubes as an internal standard.
- Tubes were pre-purged for 2 minutes in 50 mL min-1 He carrier gas to ensure the removal of any air or moisture.
- Tubes were desorbed at 320°C in 50 mL min-1 He carrier gas using a TD-100 (Markes International) onto a general purpose hydrophobic trap (Markes International) for cryo-focussing.
- VOC were desorbed at 330°C into the GC column capillary gas chromatograph (7890B GC, Agilent, SantaClara, CA, USA) fitted with a DB-5 ms Ultra Inert column (length 30 m × internal diameter 0.25 mm, film thickness 25 µm, (5%-Phenyl)-methylpolysiloxane, Agilent) using a temperature ramp: 40°C hold 0 min, ramp 6°C min-1 to 170°C, hold 0 min, ramp 15°C min-1 to 190°C. The total time was 23 minutes with an He carrier gas flow of 1.3 mL min-1.
- For mass spectrometry, a triple-quadrupole mass spectrometer (7010, Agilent) was used in electron ionization mode at 70 eV, with a scan range of m/z 40–500 Da at 4 Hz.

4.3.3 Sample analysis

4.3.3.1 Pre-processing

All files were converted to the open mzXML format prior to pre-processing.

Targeted analysis

Masshunter Quantitative analysis (Agilent, SantaClara, CA, USA) was used for the targeted analysis. MW at the MIB manually inspected chromatograms; selecting peaks for which a reasonably confident identification could be made. This process was repeated until continued inspection yielded no new compounds.

A target list of compounds was compiled based on those which were identified with the exclusion of known exogenous contaminants. One strength of this approach is the selection of peaks with confident identifications; unidentified compounds being of limited use to a phase one exploratory biomarker study. This target list was then used for the deconvolution of chromatograms.

Untargeted analysis

Chromatograms were screened for inclusion in the final dataset by manual appraisal and all samples deconvolved and aligned using eRah (283). This performs automated compound deconvolution and alignment across samples in GC-MS metabolomics; producing a table with the integrated area of the compound for each sample. Volatile organic compounds were identified by comparison of mass spectra and retention indices using the national institute of standards and technology (NIST) database. The results generated by this pre-processing method were then sent to the study author for analysis.

4.3.3.1 Data analysis

The internal standard – p-bromofluorobenzene – was used to normalise the data by dividing each of the columns (representing compound volume) by the internal standard value. A further data refinement was to normalise the compound intensities by the sample volume.

Correcting for exogenous compounds is a contentious issue. ERS technical standards (228) recommend calculation of an alveolar gradient – the difference between the concentration in the breath and the concentration in the air.

However, Cao and Dung (60) argue that although this provides an indication of whether a VOC is endogenous or exogenous the matter is far from straight forward, being affected by the ratio of alveolar to dead space ventilation; breathing pattern; the ventilation/perfusion ratio in the lung; and on the alveolar concentration gradients of the compounds in question. They argue that these complex interdependencies cannot be accounted for through an alveolar gradient (60). Schubert et al (284) in a study of mechanically ventilated patients measured venous, ambient air and exhaled breath concentrations. They reported that concentrations in blood and exhaled breath correlated well only for those compounds where the inspired concentration was less than 5% of the expired concentration. They advocate using uncorrected data but with the exclusion of any compound in which the inspired concentration is greater than 5% of the expired concentration. Another approach is to calculate the retention coefficient for individual VOC and then apply these in an effort to correct values according to inspiration concentrations (285). This approach might be useful once a panel of biomarkers have been identified but is impractical for use with large target lists or untargeted analysis where the retention coefficient for many compounds may not yet have been established. The data analysis plan was to follow ERS recommendations and undertake a background correction using alveolar gradients while acknowledging that this may be an imperfect solution.

4.4 Developing a questionnaire to assess patient acceptability

4.4.1 Defining Acceptability

Acceptability -

> *"the perception among implementation stakeholders that a given treatment, service, practice, or innovation is agreeable, palatable, or satisfactory" p.67,* (286).

Kaltenthaler et al (287) state that patient acceptability is a key component to consider when evaluating new technology, arguing that there is an ethical obligation to understand what treatments are most acceptable. They point out that acceptability can have important ramifications for research; if patients find a particular procedure less acceptable than others recruitment may be impacted. Moreover results may be skewed - drop-out rates may vary according to procedure received or device used; or alternatively only certain (potentially unrepresentative) patients might be willing to participate. The effect of acceptability may extend beyond research into clinical effectiveness - adherence and attendance are likely to be affected by acceptability of the procedures undertaken or devices used (287).

Proctor et al (286) published a useful review of implementation outcome taxonomy from which the above definition is taken. They differentiate 'patient satisfaction' from 'patient acceptability' suggesting that acceptability refers to a specific treatment/device, whereas satisfaction refers to the service as a whole. They also discuss the concept of appropriateness; they describe this as being similar to acceptability but defined as the perceived fit, relevance, or compatibility of the innovation for a given practice setting or to the addressing of a particular issue or problem.

4.4.2 Patient Reported Outcome Measures

Having determined that patient acceptability of devices is important and defined the terms it was necessary to find a PROM tool to capture this. The Medical Outcomes Study Visit Rating Questionnaire, or modifications of it, have been used to assess acceptability and satisfaction in the past (288, 289), however this questionnaire includes elements which were not pertinent such as length of time waiting for an appointment, and convenience of getting to the office/clinic. Specialism-specific clinical questionnaires have been developed in other areas of medicine (290, 291) but none were found assessing patient acceptability in respiratory medicine. Using the following search terms in pubmed - "acceptability AND diagnostic test AND (respiratory OR asthma OR COPD)" – two papers assessing patient acceptability were identified; one examined the acceptability of bronchial hyper-responsiveness testing and the other a new inhaler (292, 293) but neither used a validated questionnaire suitable for generic respiratory use. Treatment acceptability questionnaires have been in use for some time (294) however these tend to look at multiple areas (in addition to acceptability) such as ethics, effectiveness, side-effects, clinicians knowledge and trustworthiness (295).

Due to the lack of existing, suitable, and validated questionnaires for assessing acceptability a respiratory-specific questionnaire was developed with reference to the aforementioned existing questionnaires and in line with the NHS Health Technology Assessment guidelines (296). Questions were designed to examine the acceptability of clinical assessment methods or tools.

To be of maximum practical use the AAQ was designed to assess both acceptability and appropriateness. Five domains were identified from the literature; three relating to acceptability (appeal; comfort & ease of use; and concerns) and two to appropriateness (appropriateness to setting, appropriateness to patient/condition severity). Through feedback from members of the research team and clinical specialists an initial list was reduced to eight questions covering five domains (see table 18).

A visual analogue scale (VAS) for overall acceptability was added at the end of the questionnaire. This was to ensure that acceptability was captured not only by this non-validated questionnaire but by a second more widely used metric. This would allow the internal consistency of the test to be calculated by comparing the overall score from questionnaire with the result from this question.

Table 18 – Patient reported outcome measures: acceptability - domains & questions

	Domains				
	Appeal	Comfort and ease of use	Concerns (including truth of test result)	Appropriateness to setting	Appropriateness to condition / severity
Questions	How willing would you be to have your asthma monitored using this device in the future?	How easy / difficult did you find it to use the device? How comfortable / uncomfortable did you find the test? How bothered / embarrassed were you during the test?	How likely do you think it is that using this test could make your symptoms worse? How believable is it that this device could give useful information?	This device would be appropriate for home use?	This device would be appropriate for use in the following situations – mild attack, moderate attack, severe attack

4.4.3 Patient and Public Involvement

The Asthma UK Centre for Applied Research (AUKCAR) patient and public involvement (PPI) group were contacted and their feedback sought on draft versions of the questionnaire. Through this it was hoped to assess the face validity of the questions; their appropriateness; and the acceptability of the questionnaire. The AUKCAR PPI liaison officer (research follow Melissa Goodbourn) offered initial feedback on the AAQ-R and advice on the letter of invitation to the PPI group. The PPI group were then sent a letter of invitation to collaborate (this included a description of the study and pictures of the study devices); a draft copy of the AAQ-R upon which to comment; and a feedback sheet (see appendix 4). Four PPI group members offered to engage with the process and offered their feedback.

The feedback sheet attempted to gain input on the following three factors:

- Validity & appropriateness
    - Attempting to elicit how the participant would decide between devices; how they would weigh up a device's acceptability and by doing this determine whether the AAQ-R would capture this

- Acceptability
    - Determining whether the questionnaire is acceptable to the user and whether it makes sense to them

- Precision
    - Whether the options for responding are appropriate and give sufficient scope

The group reported that they found none of the questions difficult to understand, distressing or pointless, and felt that no key questions were missing. Via the feedback sheet, the group were asked 'if several similar breathing devices were available for monitoring asthma, what sort of things would you use to decide between them?' The answers received can be summarized as – ease of use, ability to understand the results, and suitability to the acute setting. These were all covered by questions already included in the AAQ-R. Asked for potential concerns about any new device, the group answered – the speed of getting results; confidence in the results; impact of the test on breathing; physician reliance on results; and the time taken to use the device. With the exception of time, these concerns were all covered by questions already included. The time taken to receive results was deemed not applicable – the ReCIVA and RTube being research tools currently rather than point of care devices. The acceptability of the time taken to undertake the test could be expressed by participants through the 'ease of use' question or captured in the free text box at the end of the questionnaire.

Given that one participant had expressed the opinion that the questionnaire was overly long it was decided not to add a question to assess the acceptability of time directly (see chapter seven for further discussion).

In framing and scoring the questions it was decided to use a five-point scale, as used by the majority of questionnaires found (Evidence-based Practice Attitude Scale; Behaviour Intervention Rating Scale; Patient Visit Rating Questionnaire; and the Patient Satisfaction Survey). Fitzpatrick et al (296) discuss the merits of Likert and visual analogue scales (VAS). They suggest that there is little empirical evidence that VAS are superior to Likert scales despite the apparent precision and range of responses possible with the VAS. Moreover, they cite concerns over the lower acceptability of VAS as a task. One of the PPI group suggested fewer responses could be used to simplify the questionnaire, however, a 3-point Likert scale would give a reduced amount of information and not allow for any gradation in response. The other three respondents were satisfied that the amount of choice presented in the questionnaire was about right.

In order to provide a measure to which the AAQ-R results could be compared a VAS relating to overall acceptability was included. One of the respondents suggested adding numeric gradations to this. Both Hawker et al (297) and Ferreira et al (298) when comparing pain scales found a numeric rating scale for pain to be preferable to a VAS without any gradations. Crichton (299) states that an argument can be made the VAS scales are "an attempt to produce interval/ratio data out of subjective values that are at best ordinal". However, in light of the feedback received and these comments, it was decided to change the VAS to a numerical scale.

Another suggestion from the PPI group was to add a free text box after each question. Given that another participant said they felt the questionnaire was rather long and could be simplified, this suggestion (regarding multiple text boxes) was not implemented. However, one was added to the end of the questionnaire.

To summarise the PPI feedback; all the questions were easily understandable, nothing was missing and all the criteria which they would use to choose between devices were captured in the questionnaire.

4.4.5 Assessment of Acceptability Questionnaire – Respiratory (AAQ-R)

A copy of the AAQ-R as used in the ABBA study can be found in appendix 4

4.4.6 Criteria for developing a patient reported outcome measure (PROM)

The following criteria - suggested by Fitzpatrick et al (296) and taken from the Oxford University Patient Reported Outcomes Measurement group (300) - are recommended for consideration when developing a patient reported outcome measure (PROM): appropriateness; acceptability; feasibility; interpretability; precision; reliability; validity; and responsiveness. Table 19 outlines the Oxford University PROM recommendations and how these were to be assessed in the ABBA study.

Table 19 – Assessment of patient reported outcome measures

Criteria	Explanation	Assessor	Method of assessment
Appropriateness	Is the instrument content appropriate to the questions which the application seeks to address?	PPI group Research team	Feedback from the AUKCAR PPI group and members of the respiratory research team on draft versions of questionnaire.
Acceptability	Is the instrument acceptable to patients?	PPI group	Feedback from the AUKCAR PPI group on draft versions of questionnaire. Missing data frequency and time taken to complete questionnaire.
Feasibility	Is the instrument easy to administer and process?	Research team	Assessed throughout the course of study via feedback from the research team.
Interpretability	How interpretable are the scores of the instrument?	Researcher	Does the questionnaire result in usable data that can be statistically analysed with meaningful outcomes?
Precision	How precise are the scores of the instrument?	Qualitative data from focus group	Without a gold standard to compare against evaluating this is challenging. Scores can be compared with the opinions expressed by focus group participants

Reliability	Does the instrument produce results that are reproducible and internally consistent?	Statistical analysis	Correlation between the answers on questions assessing similar aspects of acceptability (internally consistent) and the degree of concordance in results when the questionnaire is repeated in similar circumstances (reproducible). Internal consistency coefficients.
Validity	Does the instrument measure what it claims to measure?	PPI group Research team	Face validity can be assessed by members of the AUKCAR PPI groups, researchers and clinicians. Questionnaires can be compared against focus group transcripts. Drop-out rates and reasons for drop-out can be compared with questionnaire results.
Responsiveness	Does the instrument detect changes over time that matter to patients?	Statistical analysis	Descriptive and inferential statistical analysis can be used to examine the differences in questionnaire scores for the different assessment methods used (e.g. PEF, EBC, FeNO) and circumstances of use (e.g. controlled vs exacerbated states).

4.5 Conclusion

While the RTube is a commercially available, CE marked device, it can be used for a number of different purposes and a defined methodology for the biomarker of choice needs to be established. The equivocal results from the systematic literature review of asthma and 8-isoprostane (see chapter 2) coupled with a lack of success in measuring 8-isoprostane in early attempts led to the abandonment of this aspect of the study. EBC samples would still be collected in order to answer the feasibility questions; with future analysis of samples dependant on the identification of a suitable target marker.

The ReCIVA is another CE-marked commercially available device, its use is more circumscribed but choices still need to be made – from the circumstances of use and choice of sorbent through to the approach to sample analysis and data processing.

Assessment of device acceptability is an area which has received relatively little attention; this chapter outlines the steps taken in developing a tool to assess this.

The next chapter (**chapter 5**) describes how these and other methods were used in the two studies which make up this thesis - *Exhaled breath biomarkers in acute asthma: a feasibility study (ABBA)*; and the *Asthma Bronchial Challenge study (ABC).* **Chapter six** presents the results of the ABC study, while **chapter seven** presents the results of the ABBA study.

Chapter 5 – Study methods

In the first chapter, disease heterogeneity was described and the need for clinically applicable biomarkers outlined. Chapters two and three reviewed the literature regarding two methods of breath analysis and their application to asthma. Chapter four presented some of the preliminary work which was undertaken in order to ready these breath capture methods for use in studies at the NNUH. This chapter presents the methods used in the two studies which comprise this thesis - *Exhaled breath biomarkers in acute asthma: a feasibility study (ABBA)*; and *Bronchial Challenge Testing in Asthma: The Effect of Mannitol Dry Powder Inhalation on Volatile Organic Compounds in Exhaled Breath (ABC).*

5.1 Common Methods

Equipment and methods of assessment which were used in both studies are here described; the specific context of their use within the studies is detailed in sections 5.2 and 5.3.

5.1.1 Assessments

Fraction of Exhaled Nitric Oxide

The fraction of exhaled nitric oxide (FeNO) was measured using a Niox Vero nitric oxide analyser (Aerocrine, Chicago, USA) (see figure 10), with an expiratory flow rate of 50ml/sec. Measurement was conducted according to the manufacturer's instruction and National Institute for Health and Care Excellence guidelines (267, 301).

Figure 10 - The Niox Vero: stock image of the device.

Volatile Organic Compounds

Exhaled breath VOC were captured using a ReCIVA sampling device used in conjunction with a CASPER clean air filter (282) (as described in chapter four; see also fig. 8) and Markes Tenax-GR inert-coated sorbent tubes (Markes International, Pontyclun, UK).

Breath collection was gated using factory settings targeting exhalate from the lower airways. Duplicate patient-breath samples were taken in addition to a sample of the filtered air supply. Filtered air was sampled by attaching the CASPER filtered air outflow to the ReCIVA and mask which were fitted to a glass head.

Oxygen saturation level, respiratory rate and patient comfort were monitored throughout the sampling process and the test was ceased if oxygen saturation rates dropped below 92%; if respiratory rate increased markedly; or if the patient became uncomfortable, distressed or requested to stop.

Samples (adsorbed to sorbent tubes) were pseudonymised and stored securely in a cold room (5-7^{o}C) at the University of East Anglia before being shipped to the MIB and dry purged within 2 weeks; tubes were then analysed by gas chromatography-mass spectrometry. In general, VOC within breath samples are found in the mid parts-per-trillion by volume (pptV) to high parts-per-billion by volume (ppbV) range in breath. Results were supplied by the MIB - after undertaking data pre-processing - in the form of intensity counts without unit measurements. Samples consisted of patient breath replicates and CASPER filtered air samples. The geometric mean of replicate samples was calculated. Where missing values constituted one of a pair of replicates, the single present value was used in lieu of a geometric mean. Where missing values existed after this process, half the smallest value for that compound was substituted.

In the ABBA study participants wore the ReCIVA device and breath sampling was started immediately (due to the acute medical setting); whereas in the ABC study participants breathed filtered air for a period of 5 minutes in order to reduce inhalation of exogenous compounds; although background subtraction may not be accurate for all VOC - varying as it does according to factors relating to both the compound itself, duration and intensity of exposure, and the individual concerned (BMI specifically) (302) – a background subtraction was undertaken in order to generate an alveolar gradient; the results of which were then compared to those of a non-corrected analysis.

Asthma Control

The American Thoracic Society (ATS) / European Respiratory Society (ERS) recommends using a composite score for the assessment of asthma control (40). The Asthma Control Questionnaire (ACQ) is a patient-based tool for assessing asthma control based on a seven day recall (303). Recognised by the American Thoracic Society and NICE and widely used in research, the ACQ is available from http://www.qoltech.co.uk/index.htm.

Phlebotomy

Phlebotomy was undertaken by a suitably qualified person. Where a full blood count was requested as part of usual care the results were obtained and the eosinophil count noted.

General

Phlebotomy (blood eosinophil count) and FeNO were conducted in order to both characterise the study population and monitor for carry-over effect in the ABC study.

Calibration and servicing of all equipment was carried out in accordance with the manufacturers' recommendations.

5.1.2 Safety assessment

In conducting the asthma breathomic literature review (see chapter 3) the author noted no adverse events reported by previous studies using VOC capture equipment; furthermore the ReCIVA is currently in use in a large European asthma study (UBIOPRED: STRATA). Adverse events (AEs) and serious adverse events (SAEs) were recorded and reported according to NNUH Foundation Trust Protocols. Definitions of harm were taken from EU directive 2001/20/EC article 2 as operationalised in NNUH standard Operating Procedures (SOP) 206. All AEs and SAEs were considered for severity, seriousness, causality and expectedness and noted on the care report form (CRF). A serious adverse event was defined as that which results in death, is life-threatening, requires hospitalisation or prolongation of hospitalisation, results in persistent or significant disability or incapacity, or is otherwise considered medically significant by the investigator.

SAEs were noted on the CRF; entered in the SAE log; the study supervisor informed within 24 hours and the REC informed within 15 days. Participants experiencing an SAE were followed up until resolution and documented throughout; and their primary care practice informed in order that the SAE might be added to their medical record.

AEs were recorded on case report forms, patient notes and compiled in an end of study report. Unexpected AEs were also communicated to the participants' primary care practitioner.

5.1.3 Data management

Demographic data and clinical data were collected and recorded on a CRF. In order to maintain confidentiality, participant data was anonymised using a participant identification number (PIN); personal data linked to the PIN was stored securely as was data generated by the study. Paper CRFs were identified using this PIN number and locked securely; paper documents linking participant identity to the PIN number were stored separately to all CRFs and locked securely. VOC samples were securely stored at UEA laboratory facilities and pseudonymised before being transported to MIB for analysis. All data was stored securely on either the NNUH or UEA central storage server with access, security and back-up controls in place as per SOP 805. The study team then analysed study data in line with the protocol objectives and following the data analysis plans.

5.1.4 Data Analysis

After GC-MS analysis and the completion of all patient facing study activity, VOC data was examined according to the protocol developed by authors at the MIB breath research group (304), in line with minimum reporting standards for metabolomics (248). As described in chapter 4, VOC data underwent a pre-processing phase consisting of deconvolution, baseline correction and centring. Following this a target list of compounds for use in asthma metabolomics analysis was developed. Targeted analysis of VOC samples was undertaken using MassHunter (Agilent, Santa Clara, USA). This initial stage of data pre-processing was conducted by MW at MIB.

The results of this analysis were sent to the UEA as a comma separated values (CSV) excel file. Using Excel (Microsoft Office, 2016, v16.0.4954.1000), SPSS (116) (IBM, v.25) and R-Studio (version 1.2.1335) the following analytical plan was undertaken:

- Data normalisation (using the internal standard and sample volume)
- Calculation of skew and kurtosis for each compound
- Histograms of sample distribution for each compound
- Correlation plot to identify possible batch effects
- Intra-class correlation coefficient and standard deviations to assess reliability
- Background corrections and calculation of alveolar gradient
- Box plots and Mann Whitney or Wilcoxon Signed Rank test (according to the outcome being assessed)
- Multiple regression analyses: logistic regression analysis

Where background correction produced a negative values, a constant of 10 was added to the data-set before log transformation.

PCA is commonly used as the multiple regression test of choice for metabolomics however given the low numbers of samples relative to the large number of compounds logistic regression is more suited to a study of this size.

5.2 Exhaled Breath Biomarkers in Acute Asthma: A Feasibility Study

Full title: Exhaled Breath Biomarkers in Acute Asthma: A Feasibility Study

Study abbreviation: Asthma Breath Biomarker Assessment: The ABBA Study

Registered with clinicaltrials.gov Registry number NCT03084016

Research Ethics Committee (REC) Approval gained REC reference 16/LO/0639

5.2.1 Background

Asthma is a chronic disease of the airways, defined by variation in both expiratory airflow limitation and symptoms. The mainstay of asthma diagnosis and monitoring remains the assessment of these two disease aspects - symptoms through clinical history and/or validated tools such as the Asthma Control Questionnaire; and airflow limitation via spirometry or peak flow readings (305). However, as discussed in previous chapters the utility of such measures are limited by the variable nature of the disease and its heterogeneity. Non-invasive measures of assessing airway pathophysiology have been the subject of study for several decades and CE marked devices for capturing both EBC and exhaled VOC are now commercially available.

The study of exhaled VOC is still in its infancy and relatively little research has been conducted on such technology in the context of acute asthma attack. The capture and analysis of multiple VOC has the advantage of being suited to inductive analyses. Using such an approach to identify those VOC which are associated with acute asthma exacerbation could identify markers for use in identifying loss of control; while phenotyping asthma exacerbations by identifying the trigger or underlying disease phenotype might help guide treatment in the acute setting.

Before attempting to conduct any definitive study to answer such questions, it is first necessary to address issues of feasibility. These include patient toleration of breath capture devices and the acceptability of using such devices during an acute asthma attack. There are also issues of participant retention and follow-up, specifically whether the 'usual-care' post-admission follow-up appointment stipulated in UK guidelines (305) offers a suitable sampling opportunity for controlled disease, and – when targeting an acute population - what proportion of those recruited return to a controlled state during the study duration.

If attempting to phenotype exacerbations, any future study would have to establish whether the methods chosen were capable of identifying the presence or absence of common triggers; including viral and bacterial infection.

The aim of this feasibility study was to assess two alternative methods of recruiting patients and capturing exhaled breath samples during acute asthma attack. This was undertaken with a view to determining the best approach for a definitive study evaluating biomarkers in acute asthma (including but not limited to inflammatory markers and potential markers of infection). In addition to assessing the feasibility of such a study it was planned to collect data on exploratory outcomes including the ability of biomarkers to distinguish between controlled and exacerbated states and their ability to identify triggers of exacerbation.

5.2.2 Objectives

5.2.2.1 Primary objective

To determine the feasibility of a study to evaluate the utility of exhaled breath biomarkers in patients with acute asthma. This includes answering the following feasibility questions:

- What is the best method of recruiting patients into a definitive study?
- Are patients both prepared and able to provide exhaled breath condensate, exhaled breath gas and other samples in the acute asthma setting? Are patients prepared to perform repeated measures / multiple assessments?
- Are outpatients both willing and able to contact the research team and return for assessment when experiencing an exacerbation?

  Previous studies suggest that recent severe exacerbation is a strong predictor of future exacerbation (306-308); however, it is not known what percentage of such patients would contact the research team and attend a study visit during such an exacerbation.
- Are researchers able to perform the initial assessment of hospital patients early during acute exacerbation?

  In a previous study (61) patients were recruited within 24 hours of admission to hospital, however, when biomarker assessment was conducted relative to commencing systemic corticosteroid therapy is not known.
- Is it possible to assess patients who are in receipt of supplementary oxygen?
- How does collection of EBC & VOC compare with more established measurements (such as FeNO) in terms of acceptability to patients?

- Is it possible to obtain exploratory data comparing controlled and exacerbated states to power a definitive study?

- What percentage of patients experiencing an acute exacerbation have a bacterial trigger?

  Studies have found between 3 and 50% (309); further studies are required to narrow this range and to power a definitive study.

5.2.2.2 Secondary objectives

- To explore whether biomarkers are able to distinguish between stable and acutely exacerbated states

- To explore whether biomarkers have any predictive value in the event of an exacerbation
  - Do markers predict hospital attendance or admittance?

- To explore whether biomarkers are able to distinguish between viral, bacterial and allergen triggered exacerbations

5.2.3 Methods

5.2.3.1 Study design

This was a single centre, longitudinal observational study with the primary aim of assessing the feasibility of research using breath sampling devices during acute asthma attack. The ability to capture information during such an attack was assessed using two different approaches:

1) Acute arm: recruiting patients in secondary care during an acute exacerbation of their asthma; re-assessing them once their asthma was stable and controlled.

2) Outpatient arm: recruiting clinically stable outpatients at increased risk of having an exacerbation (by virtue of having had an acute exacerbation within the previous 12 months (307, 308)). These participants were to be followed for a period of up to 12 months and asked to contact the research team for assessment in the event of an exacerbation. Should they do this and undergo an acute assessment they would be invited to provide a further sample at a later date when controlled.

A recruitment target of 100 participants experiencing - or at risk of experiencing - an asthma exacerbation was set. The study included a participant focus group to obtain in-depth feedback on the breath capture devices and the study methods used.

5.2.3.2 Study setting

The study was conducted at the Norfolk and Norwich University Hospital (NNUH) (Norwich, Norfolk, UK); a regional tertiary centre for patients with difficult to control asthma. All study activities took place at the NNUH. In the year preceding study-commencement this site saw approximately 800 patients with asthma. A pragmatic / convenience sample method was used recruiting from the NNUH respiratory outpatient department; accident and emergency department (A&E); acute medical unit (AMU); and inpatient wards.

5.2.3.3 Sample size

The recruitment target of 100 participants was based on the assumption that that 20-40% of participants would experience an attack within the 12 month follow-up period (307, 310, 311) and that approximately 30% of these participants would appropriately contact the research team for assessment during an exacerbation. This would permit an estimate of the rate of individuals experiencing an exacerbation to within +/- 7.8 to 9.6% of a 95% confidence interval; and to estimate the rate at which those who experience an exacerbation attend for an assessment to within 4.7 to 6.4% of a 95% confidence interval (312). It was anticipated that recruitment in the acute study arm (during hospital attendance for acute exacerbation) would be challenging; within the overall aim of recruiting 100 participants, a target of 12 was set for recruitment in the acute arm in order to assess the feasibility of this approach (313).

Regarding the exploratory outcomes, van der Schee et al state that the inflammatory processes underlying asthma are known to provide sufficiently different VOC-based breath profiles at sample sizes of between 10-20 participants per comparator group (174, 314).

5.2.3.4 Eligibility criteria

The following inclusion criteria were applied:

1. Male or female
2. Aged 18 or above
3. Able to provide informed consent
4. A confirmed asthma diagnosis requiring treatment with inhaled bronchodilator therapy +/- inhaled corticosteroids.
5. Non-smoker (or ex-smoker of 6 months or more with a less than 10 pack year history).
6. Current exacerbation or exacerbation within the previous 12 months.
7. Within 24 hours of having presented to acute secondary care (applicable to the acute study arm only)

With no gold standard test available for the diagnosis of asthma the majority of research uses either a physician diagnosis, a diagnosis made according to recognised guidelines, or diagnosis accompanied by a measure of airway reversibility. Patients were eligible for inclusion in the acute arm if they received a diagnosis of asthma exacerbation from the acute secondary care physician assessing them. This pragmatic definition of asthma (and exacerbation) was adopted because full diagnostic evidence (such as reversibility) might not be available for all patients at the time of exacerbation. Diagnosis for all participants was confirmed retrospectively through a review of secondary care records ascertaining whether the participant's diagnosis met BTS/SIGN diagnostic guidelines.

That smoking may affect exhaled breath biomarkers is well established (315), however the extent and duration of this effect on the markers under consideration in this study is not. In line with other studies, current smokers and ex-smokers with a greater than 10 pack year history or with less than 6 months non-smoking were excluded.

Exclusion criteria:

1. Major chronic cardiorespiratory disease other than asthma
2. Significant comorbid condition
3. Receiving maintenance oral corticosteroid therapy or other immunosuppressant or immunomodulatory therapy (including biologics)
4. Pregnant
5. Participating in a clinical trial of an investigational medicinal product (CTIMP).
6. Unable to speak English.

Patients with a major chronic cardiorespiratory disease other than asthma or significant comorbid condition were excluded. Defining a 'significant comorbid condition' is problematic (316) however for the purposes of this study guidelines were developed by physician members of the research team to be used by the researcher in classifying comorbidities (see table 20). In the event of medical conditions not covered by this guidance, the opinion of the patients' acute care or respiratory physician was sought and noted. Patients receiving maintenance oral corticosteroid therapy (or other immunosuppressant therapy) or those unable to provide informed consent were also excluded. Patients found to be ineligible on screening were not entered into the trial, however reasons for ineligibility were recorded. Patients with an acute non-respiratory infection were excluded.

Patients with acute respiratory infection were included in order to capture data on infectious triggers of asthma, however patients with pneumonia were excluded. Patients who were pregnant were excluded - pregnancy increases metabolic activity and oxidative stress, and has been shown to alter exhaled breath VOC (317).

The eligibility criteria were applied as follows during the screening process:

a. Diagnosis of acute asthma exacerbation (currently or within previous 12 months).
b. Patient meets inclusion criteria
c. Patient does not meet any of the exclusion criteria.

Table 20 – Study exclusion criteria: comorbidities

1. Major chronic cardiorespiratory disease other than asthma - COPD - Bronchiectasis - Interstitial lung disease (including sarcoidosis, pulmonary fibrosis) - Pneumoconiosis - Lung cancer - Cystic fibrosis - Heart failure - Pulmonary oedema.
2. Significant comorbid condition If the comorbid condition is not listed as the primary diagnosis / presenting complaint then it will not be deemed a *significant* comorbidity unless it is one of the following: - Pneumonia - Pleural effusion - Pneumothorax - Acute infection (other than respiratory) - Septicaemia - Any cancer
Examples - An asthma attack and coexistent acute urinary tract or kidney infection would be excluded - An asthma attack with lower respiratory tract infection would be included - An asthma attack with pneumonia would be excluded

5.2.3.5 Participant identification, recruitment and retention

Participant identification

Outpatient arm:

Standard care for patients who attend secondary care with an asthma attack is to be invited to an outpatient appointment with an asthma specialist nurse 4-8 weeks later (as per BTS/SIGN guidelines(305)). In addition, outpatient clinics are populated by patients referred from primary care with difficult to control asthma who may have experienced an attack within the previous 12 months. Such clinics thus provide an opportunity to invite 'at risk' outpatients to participate. Recruitment of outpatients was undertaken by screening clinic records and sending invitations; through invitation at asthma clinic appointments; and through advertising within the NNUH and on a respiratory research microsite hosted on the NNUH website's Research and Development pages.

Acute arm:

Recruitment in acute secondary care took place at the Norfolk & Norwich University Hospital (NNUH); a number of avenues were available for patient identification. Posters were placed in accident and emergency (A&E) and the acute medical unit (AMU) asking staff to contact the research team if they had a patient they believed might be suitable for inclusion and who expressed an interest in participation. Names and presenting complaints of all patients entering A&E and AMU are logged on the hospital information technology system – Symphony - which can be used to identify potential patients for screening. In addition, the Early Supported Discharge Service (ESDS) compile a triage list each morning; this is reviewed daily by the asthma specialist nurse team who identify any patients with asthma. This team were asked to offer information to potentially suitable patients, contacting the researcher for screening should the patient wish to participate.

Participant recruitment

Acute arm:

Having identified a patient as potentially suitable for inclusion their notes were viewed and/or their clinician consulted to determine whether they were sufficiently stable to approach regarding participation. Those meeting any of the following criteria were deemed unstable and not suitable for approach:

- Transfer to intensive care (ITU), the high dependency unit (HDU) or intubation being considered.
- O_2 saturation below 94% despite receiving supplementary oxygen
- Unable to complete even short sentences
- Obvious distress and inability to complete the consent process.

If a patient was deemed insufficiently stable they were reviewed again after a period of time; if stable enough to approach they were presented with a participant information sheet. If they wanted to be considered for entry to the study they were given the opportunity to ask further questions of the researcher and time to consider their participation. Informed consent was then sought and the patient screened for eligibility.

Outpatient arm:

Potentially suitable patients were sent or given a letter of invitation and participant information sheet. If they contacted the research team and expressed interest, the researcher answered questions, outlined the eligibility criteria, and invited the person to a study visit. For those with an outpatient appointment, on arrival the patient was asked if they had received the information and whether they would like to be considered for entry to the study. This was undertaken in a location and manner which both preserved the patients' privacy and afforded them the opportunity to decline participation without pressure. Patients who wished to be considered for study inclusion were given the opportunity to ask further questions; informed consent was sought and the patient screened for eligibility.

Participant consent

The study was conducted in accordance with Good Clinical Practice (GCP) guidelines and according to the guiding principles of the Declaration of Helsinki. Consent to participate in the study was sought and obtained by an appropriately trained individual and conducted according to GCP guidelines.

Potential participants were given sufficient time to consider their inclusion into the study; informed that they were free to withdraw from the study at any time without giving a reason and that this would have no impact upon the quality of their current or future treatment; and were informed that all data collected in the study would be held confidentially. It was also explained that in the event of their withdrawal data collected up to that point could not be erased and would be used in the final analyses where appropriate. At every follow-up call and assessment it was established whether the patient still wanted to participate in the study.

5.2.3.6 Assessments

Attack severity

The GINA definition of asthma exacerbation (318) covers a broad range of severities; for this study the BTS/SIGN guidelines were used to classify the severity of exacerbation (25) (see table 21).

Table 21 – BTS/SIGN asthma guidelines: classification of severity

Moderate asthma	Increasing symptoms PEF > 50-75% best or predicted No features of acute severe asthma
Acute severe asthma	Any one of: - PEF 33-50% best or predicted - Respiratory rate > 25/min - Heart rate > 110/min - Inability to complete sentences in one breath
Life threatening asthma	Any one of the following in a patient with acute severe asthma: Clinical Signs: - Altered conscious level - Exhaustion - Arrhythmia - Hypotension - Cyanosis - Silent chest - Poor respiratory pressure Measurements: - PEF < 33% best or predicted - SpO_2 < 92% - PaO_2 < 8 kPa - Normal $PaCO_2$ (4.6-6.0 kPa)
Near fatal asthma	Raised $PaCO_2$, and/or requiring mechanical ventilation with raised inflation pressures.

Asthma exacerbation or attack was defined according to the ATS/ERS recommendations (40) as operationalised by Virchow (319):

- nocturnal awakenings due to asthma requiring short-acting beta-agonist medication (SABA) for 2 consecutive nights
- increase in occasions of SABA use on 2 consecutive days (minimum increase of 4 puffs per day)
- a decrease in PEF of 20% or more on two consecutive days or 30% at any time
- the need to increase inhaled steroids
- the need to commence oral or parenteral corticosteroid therapy or the need to access acute medical care (e.g. accident and emergency).

This was explained to study participants in the recruitment process; it was detailed on the participant information sheet; participants were given a wallet sized reminder card as well as a leaflet detailing the above threshold and giving the research teams contact details. Two members of the AUCAR PPI group reviewed the PIS and provided comments.

Demographic Data and clinical information

Contact details, general demographic information and clinical details were taken from medical notes and by direct patient enquiry.

Peak Expiratory Flow (PEF)

PEF readings taken by hospital staff were retrieved from the hospital notes and recorded. A study specific PEF was taken using a mini wright peak flow meter in order to ensure consistency and contemporaneousness with other study assessments.

Sputum

Spontaneously expectorated sputum (SES) is described by Holz et al (320) as simple, economic and non-invasive. Holz et al report that - in asthmatic subjects not experiencing acute exacerbation - approximately 75% were able to produce adequate samples. Spontaneously expectorated sputum samples were collected from patients in the acute asthma setting. For those patients producing a sample in the acute setting, a further (spontaneous) sputum sample was obtained at the time of their follow-up clinic appointment. Samples were send to the NNUH pathology laboratory for bacterial culturing.

Fraction of Exhaled Nitric Oxide

Performed as described in section 5.1.1

Exhaled Breath Condensate (EBC)

Exhaled breath condensate was collected using an RTube - a commercially available handheld device (Respiratory Research, Austin, USA). As per the manufacturer's instructions, patients were asked to breathe into the device using normal tidal breathing for a period of 10 minutes. This was conducted in the seated position. Samples were pipetted into an eppendorf, anonymized and stored securely at a temperature of -80^{o}C. Nose clips were used as per ATS/ERS guidelines (114, 321) where tolerated by the participant. Delivery of supplementary oxygen would prevent the use of nose clips for EBC, moreover it has been found to increase markers of oxidative stress in healthy volunteers (322) and those with COPD (323).

EBC assessment was not conducted until such a time as participants had their oxygen removed or were considered stable enough to do so. The planned analysis of EBC samples from the ABBA study was not undertaken due to the equivocal findings of the systematic review of EBC 8-isoprostane and an inability to detect 8-isoprostane using a commercially available ELISA kit (a result which the review findings suggest is perhaps not uncommon (see chapter 2)). The samples remain in storage at -80^{o}C pending the establishment of a reliable and sensitive method of 8-isoprostane detection or selection of alternative biomarker. An alternative method of sample dilution calculation (such as serum albumin) would need to be used unless it were to be established that there is little inter-and intra-individual variation in EBC dilution.

Volatile Organic Compounds

VOC sampling was conducted as describe in section 5.1.1. For those patients receiving supplementary oxygen, the patients' oxygen mask / nasal cannulae were substituted with the ReCIVA device; this was used to supply an air-oxygen mixture for a period of less than 10 minutes, before returning them to their original oxygen supply (see chapter four). Patients in receipt of > 50% oxygen or saturating at less than 94% were not assessed.

Nasal swabs

Nasal swabs were taken from patients experiencing an exacerbation and underwent PCR analysis for viral DNA at the NNUH pathology laboratory.

Assessment Acceptability Questionnaire – Respiratory (AAQ-R)

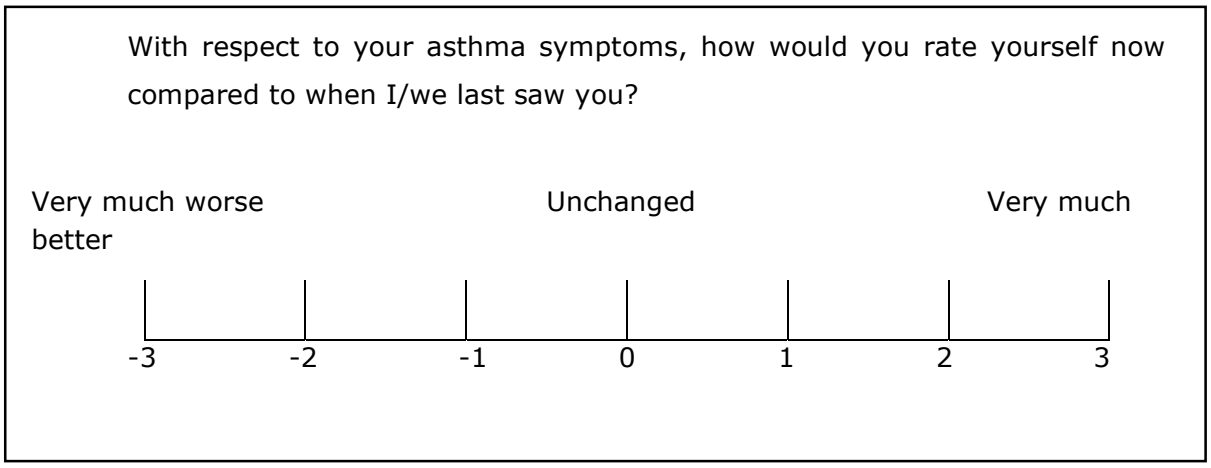
After use of each of breath assessment / capture device – RTube, ReCIVA, Niox Vero - patients were asked to complete the AAQ-R questionnaire investigating its acceptability.

A global rating of change (GRC)

A GRC measure was used to capture the change in asthma symptoms between exacerbation and follow-up. Kamper et al (2009) in their review suggest that GRC's are high in face validity and correlate well with other self-report measures (e.g. disability or pain scales) and clinician rated measures of change. Kamper et al recommend a scale of 7-9 points suggesting that this offers the best compromise between patient preference, adequate discriminative ability and test-retest reliability.

The following GRC was used for this study:

Figure 11 – The Global Rating of Change scale as used in the ABBA study.



Phlebotomy

Phlebotomy was performed as detailed in section 5.1.1. An additional vacutainer was taken for study purposes; permitting future analysis of samples once a marker of interest has been identified. Samples were left for 30-60 minutes to clot, centrifuged at 2,000 revolutions per minute for ten minutes at room temp (21^{o}C) before being transferred to eppendorfs, pseudonymised and placed in a -80^{o}C freezer.

Table 22 – ABBA study assessments

Test	**Marker**
ACQ	Symptomology
AAQ-R	Acceptability
Asthma severity assessment	Physiological signs & symptoms
Wrights Mini Peak Flow	Respiratory function - expiratory airflow obstruction
RTube – Exhaled breath condensate	Analysed for 8-isoprostane (+/- other novel markers)
Niox Vero	Fraction of exhaled nitric oxide
ReCIVA	VOC
Spontaneously expectorated sputum	Bacterial culturing
Phlebotomy	Blood eosinophils Serum stored for potential analysis of other markers
Nasal swab	Viral DNA

Qualitative Assessments of Feasibility

A focus group was undertaken in order to obtain more in depth, qualitative data on the study methods used, and for the purpose of assessing the validity and utility of the AAQ-R. Views on the acceptability of trial methods including recruitment strategy and assessment procedures/devices were captured. The Krueger & Casey (324) approach to running a focus group was used, with the discussion scheduled for approximately one hour. A bank of questions / prompts for use in facilitating the discussion was compiled (see appendix 2). Prompts were designed to move from the general to the more specific, although participants were encouraged to discuss the issues that are/were important to them rather than following a pre-determined schema. The discussions were recorded on a digital tape recorder, and the recording transcribed and pseudonymised.

5.2.3.7 Safety assessment

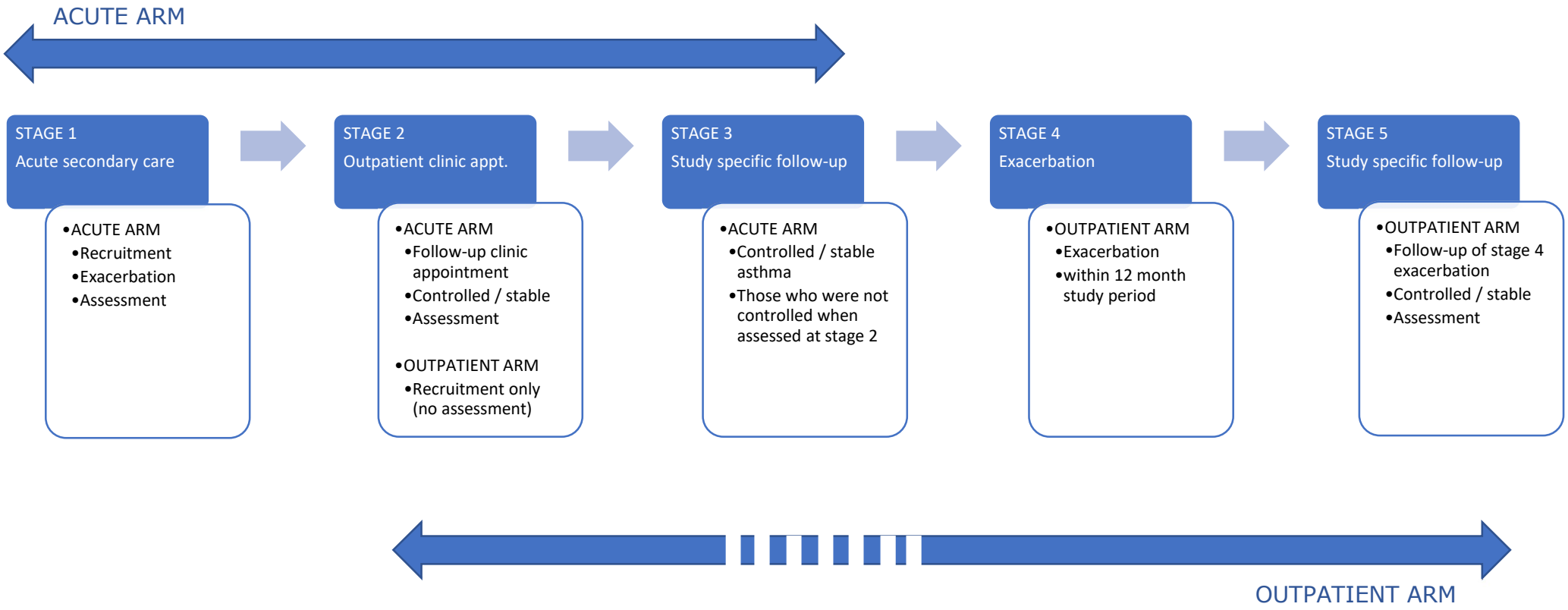
The protocols regarding adverse events are documented in section 5.1.1. Only procedure-related adverse events (AEs) and procedure-related serious adverse events (SAEs) were recorded in the ABBA study. Participants experiencing a procedure-related SAE were to be followed up until resolution and documented throughout. An annual report was submitted to the REC detailing any SAEs.

5.2.3.8 Schedule

Participants who were recruited in the acute arm were asked if they wished to continue in the outpatient study arm (and contact the research team in the event of experiencing another asthma attack). By combining the two study arms, five possible stages of assessment were created.

Those patients recruited during acute exacerbation (stage one) whose asthma was not controlled at the time of their follow-up outpatient appointment (stage two) were invited back for a study-specific assessment at a later date, thereby allowing more time to regain asthma control (stage three). Those patients enrolled in the outpatient study arm who contacted the study team and attended for assessment during an asthma attack (stage four) were invited back for a further assessment once they had regained asthma control (stage 5). The flow of participants through the study is outlined in figure 12.

Figure 12 - Participant flow in the ABBA study



In the acute arm patients underwent study assessment after completing the recruitment and consent processes. Following discharge, information on tests, therapies received and duration of hospital stay was extracted from patient notes and/or hospital IT systems. Upon their follow-up outpatient appointment being booked the researcher contacted the participants asking them if they consented to continued participation and arranging a study visit. In addition to those tests performed as standard care for outpatient appointments (such as spirometry) a repeat set of study assessments was conducted. A measure of global record of change (GRC) was recorded in addition to ACQ. Those participants not meeting the ACQ threshold for disease control (1.5 in the case of the study) were invited to return for a further assessment at a later date.

Those participants in the outpatient arm who contacted the researcher in the event of an exacerbation were asked to attend the NNUH for a full study assessment. Participants were contacted every three months to remind them about study participation and to ask for details of any unreported exacerbations; this contact was by telephone or mail. Those outpatients who were admitted to hospital for an exacerbation were approached as inpatients and asked if they were happy to undergo a study assessment. All those outpatients who underwent assessment were invited to attend a follow-up appointment at a later date when symptom control had been achieved.

Participants were withdrawn from the study for any of the following reasons:

a. Removal of consent - participant choosing to withdraw.
b. Diagnosis of major cardiorespiratory condition other than asthma.
c. Development of a significant comorbidity.

Study participants all received standard care in both the secondary and primary healthcare settings. Participants in the outpatient arm were followed up for a period of 12 months or until the 31st December 2018; whichever was sooner.

5.2.4 Outcomes

5.2.4.1 Feasibility outcomes

Primary outcomes were chosen to provide feasibility data informing the design of future acute asthma studies using exhaled breath capture devices.

Table 23 – ABBA study feasibility outcomes

Feasibility – recruitment	
Recruitment rate (participants recruited/time)	Time required to recruit to study
Number of invited patients that agree to participate.	How many patients with asthma are willing to participate
Number of participants excluded on screening	How many patients with asthma are eligible
Numbers recruited and in which arm	Which recruitment method results in the greatest number of assessments
Reasons for exclusion	Are the eligibility criteria appropriate
Acceptability of recruitment process	Is the recruitment process appropriate
Number of patients recruited in the acute arm who fail to attend their outpatient follow-up appointment?	Is the usual-care outpatient appointment a suitable opportunity for capturing follow-up data?
Number of participants who are lost to follow-up or withdraw consent	Sample size calculation - how many patients will be required to account for the study drop-out rate.
Number of patients recruited in the outpatient arm that experience an exacerbation	Planning future study
Number of patients experiencing an exacerbation, appropriately contacting the research team and attending for assessment	Planning future study
Severity of exacerbations captured in outpatient and acute study arms	Are a range of attack severities captured by both study arms?
Number of potentially eligible patients in the acute arm that were approached	Staffing resources
Number of acute-arm participants who were controlled at their follow-up outpatient appointment	Does the follow-up outpatient clinic appointment provide an opportunity for assessing clinically stable patients?

Number of outpatient arm participants who were controlled at their follow-up appointment	Planning future study – how many participants are required to obtain a sufficiently large number of controlled samples?
Feasibility – assessment	
Time taken to collect samples and/or complete tests.	Planning of future study
Sample size / volume	Determining future sampling methodology appropriate to biomarker chosen and required analysis.
The number of patients able to produce spontaneously expectorated sputum sample	Planning of future study methods
The number of participants completing tests successfully (yielding a sample sufficient for use).	Sample size calculation – how many participants are required to obtain the required number of samples
Reasons for incomplete tests	Planning of future study methods
Reasons for unusable samples	Planning of future study methods
Number of patients who successfully complete all tests at all visits.	Future study design – delivering on target recruitment numbers
Acceptability of collection processes - obtaining sample, timing of sampling, time taken to obtain sample, acceptability of devices used.	Future study design
Adverse events – number, nature, context, severity, action taken.	Future study design
Time between study assessment and: - Admission to hospital - Commencement of systemic corticosteroids.	Future study design
Ability to obtain data on exacerbation from patient	Feasibility
Acceptability of study methods to participants	Patient perception of study; do any design features deter participation

5.2.4.2 Secondary outcomes

In addition to the feasibility outcomes described above, an exploratory analysis of exhaled breath biomarkers was planned.

Exploratory data

Is there a significant difference in VOC concentration levels between exacerbated and stable states?

Are there distinct breath profiles associated with exacerbated and stable states?

What are the ranges for inflammatory markers in the acute state and in the stable state?

What is the variability of the difference in inflammatory marker levels between the acute and stable state?

Do exhaled breath biomarkers predict hospital admission?

Does the questionnaire developed to capture patient acceptability of clinical assessment devices have validity & reliability?

5.2.5 Data analysis

5.2.5.1 Primary outcomes

Primary Outcome: To collect sufficient data to determine whether a larger trial investigating biomarkers in acute asthma is feasible to undertake.

Measures used to determine this outcome include:

- Screening and recruitment rates, drop-out rate, and exacerbation rate.
- Summary data on the use of time and patient feedback will also be produced.

A screening log was kept in order to record reasons for exclusion. The analysis of primary outcomes was conducted using SPSS version 23.0 (116). Analysis of qualitative data was to be undertaken using framework analysis supported by use of NVivo software with the credibility of thematic analysis checked by a second reader. Results were to be presented both quantitatively (e.g. keyword frequency) and qualitatively (e.g. vignette's or anonymised quotations).

Both qualitative and quantitative data on recruitment and assessment methods were to be used to assess study feasibility.

5.2.5.2 Secondary outcomes

Exploratory outcomes were to be assessed by comparing acute VOC data with that obtained when controlled; with analysis conducted according to the protocol developed by authors at the MIB breath research group (304) and outlined in section 5.1.4.

Data from all stages of the study was to be summarised; differences between stages/groups estimated; the ability of biomarkers to differentiate exacerbated from controlled asthma; infective from non-infective triggers; and their ability to predict clinical outcomes (such as severity of exacerbation and therapy dose/duration) estimated. The degree of concordance between novel biomarkers and existing asthma markers (such as FeNO, blood eosinophils) and respiratory function tests was also to be evaluated.

5.2.6 Study approvals

The ABBA study was registered with the clinicaltrials.gov database (registration number NCT03084016). Research ethics committee (REC) approval was gained from London-Fulham REC on 1st July 2016 (reference number 16/LO/0639). The study was registered with the NIHR clinical research network portfolio (central portfolio management system ID 33202).The study was approved for adoption to the NIHR portfolio on 2nd March 2017.

5.2.7 Study amendments

Amendment 1 (protocol v1.2)

During the course of the study it became apparent (see chapter 4, developing a methodology) that it would be possible to deliver oxygen to patients via the ReCIVA. This meant that, contrary to the original protocol, patients would not need to wait until they were stable enough to be removed from oxygen before undergoing breath capture with the ReCIVA. In light of this a request was made to assess patients while still in receipt of supplementary oxygen and then to add a further additional assessment visits for these patients in which the effect of supplementary oxygen on exhaled VOC could be assessed.

An offer was made to the researcher to conduct genomic analysis (16S rRNA and/or whole genome shotgun analysis) on any sputum samples obtained. In light of this a request was made to undertake induced-sputum sampling at the follow-up appointment for those participants who were able to provide a sample in the acute setting. Amendment 1 was approved by London-Fulham REC on the 1st September 2016 and by the HRA on the 3rd November 2016.

Amendment 2 (protocol v1.3)

A number of patients booked into outpatient clinics did not have any electronic letters available to view nor notes available for screening. As a result it was challenging for the researcher to screen and ascertain whether to send a study invitation. In light of this a request was made for permission to approach patients who had not received advance information, providing this at their clinic appointment. Other requests in this amendment were to push the study end date back to July 2018.

Due to difficulties in obtaining appropriate training in sputum laboratory processing techniques and ongoing quality assessment, the analysis of sputum was limited to bacterial culturing at the NNUH pathology laboratory (for spontaneously generated sputum samples only). The addition of butylated hydroxytoluene (BHT) solution to EBC samples was removed; the originally proposed EBC analyte – 8-isoprostane – had been shown to be an unreliable measure of oxidative stress using current methods. Tests for alternative markers in EBC could be adversely affected by the presence of BHT, thus storage without this additive is preferable. While an ACQ score of 1 was selected for asthma control, initial screening revealed that many patients attending the asthma outpatient clinic had an ACQ consistently > 1. This population with largely moderate-to-severe or difficult-to-control asthma may rarely score < 1. As a result a cut point of 1.5 was proposed, the threshold recommended by the ACQ authors for use in clinical trials.

Both pneumonia and acute infection were listed as 'significant comorbid condition's which would result in study exclusion. It was realised that this guidance was not sufficiently specific; patients who presented to secondary care with both acute asthma attack and respiratory infection were proposed for inclusion. Any other type of acute infection would continue to be excluded. This would allow data capture for participants presenting to secondary care with an asthma exacerbation triggered by bacterial or viral infection. Patients with a physician diagnosis of pneumonia or septicaemia would be excluded. A CASPER clean air supply pump for the ReCIVA was purchased from Owlstone Medical Ltd (Cambridge, UK); use of this rather than hospital air was proposed. Amendment number 2 (protocol v1.3) was approved by London-Fulham REC on 24th April 2017.

Amendment 3 (non-substantial) (protocol v1.4)

After the REC approved protocol 1.3, HRA requested minor changes leading to amendment 3 (protocol 1.4). This was comprised of minor changes to some study documents; it was deemed to be a non-substantial amendment and did not require REC approval. Amendment number 3 (protocol v1.4) received HRA approval on the 3rd July 2017.

Amendment 4 (non-substantial) (protocol v1.5)

A fourth amendment request was submitted requesting a number of small changes - permission to advertise the study on a study specific website hosted by NNUH or UEA; permission to take an additional vacutainer of blood; permission to recruit a PIC site if required to boost recruitment; and permission to issue participants a universal container for collection of a sputum sample. Amendment number 4 (v1.5) was approved by the HRA on the 3rd October 2017.

Amendment 5 (protocol v1.6)

Due to slower than anticipated recruitment a request was made to extend the study end date to the 31st December 2018; with participants being followed up for a period of 12 months or until December 2018, whichever came first.

In the original study protocol a follow-up assessment for a small cohort of participants was suggested in order to assess the effect of oxygen therapy on exhaled breath profiles. Given a lack of participants assessed while in receipt of oxygen the need for this assessment was obviated. An exit questionnaire was drafted to collect data on unreported / unassessed exacerbations; the degree of control attained; and whether any exclusion criteria had developed during the study period.

Amendment number 5 – was approved by London–Fulham REC on the 20th July 2018 and by the HRA on 31st July 2018.

5.3 Bronchial Challenge Testing in Asthma: The Effect of Mannitol Dry Powder Inhalation on Volatile Organic Compounds in Exhaled Breath

Study Abbreviation - Asthma Bronchial Challenge: The ABC Study

Registered with clinicaltrials.gov - Registry number NCT03575663

Research Ethics Committee (REC) Approval gained - REC reference 17/EE/0430

5.3.1 Background

As discussed in previous chapters, asthma is defined by variation in both expiratory airflow limitation and symptoms. Bronchial hyperresponsiveness (BHR) - as a cause of airflow limitation - is a key pathophysiological feature of asthma; although the extent to which its presence depends on the inflammatory phenotype is debated (325, 326). BHR is defined by Joos et al as an abnormal increase in airflow limitation following exposure to non-allergic stimulus (44). Bronchial challenge testing attempts to identify BHR through the inhalation of a stimulus at doses which cause no significant reaction in healthy subjects but provokes bronchoconstriction in those with hyperresponsiveness. It is recommended as one possible method of assessing airflow variability for asthma diagnosis (39, 327).

Bronchial challenge tests can be classified as direct or indirect. Direct testing with an agent such as histamine or methacholine stimulates the smooth muscle of airway walls thereby causing bronchoconstriction.

Indirect testing works by provoking inflammatory cells (chiefly mast cells and eosinophils) to cause the release of inflammatory mediators including prostaglandins, leukotrienes, and histamine (328); mannitol achieves this through its osmotic effect, dehydrating the airway lining. These inflammatory mediators act upon airway smooth muscle to cause bronchoconstriction. Indirect testing should only generate this response if inflammatory cells are present in the airways; a notion supported by its strong correlation with other measures of inflammation such as the fraction of exhaled nitric oxide (FeNO) (329, 330) and sputum eosinophils (331). This may be the reason for its greater specificity - as an asthma diagnostic - than methacholine (332, 333), but it may also limit the test's utility to the Th2 inflammatory phenotype - those with eosinophilic asthma have the strongest response to mannitol (334) and those with neutrophilic asthma the lowest (326).

Whether bronchial challenge testing has a role outside of diagnosis remains to be seen; the STAMINA trial (335) compared management according to a standard strategy (involving symptoms and lung function tests) with a strategy based on dose-response to mannitol. The group whose inhaled corticosteroids (ICS) was titrated according to mannitol remained on a greater dose of ICS but had fewer mild exacerbations, and there were significant differences in the fraction of exhaled nitric oxide (FeNO), reliever inhaler use and BHR (as measured by methacholine challenge). Overall the results were equivocal and the mainstay of disease monitoring remains the assessment of symptoms and airflow limitation (305).

The pathology of asthma exacerbation or attack is still insufficiently understood (12). Investigating changes in VOC profiles occurring during acute asthma attack may help elucidate mechanisms at work; moreover, statistical associations with clinical outcomes offer a potential route for disease monitoring and/or treatment guidance even when the pathophysiological pathway responsible for the given metabolite remains unclear. Conducting research into acute asthma is, however, challenging (see chapter 7). This is particularly the case for breathomic studies which may be confounded by exogenous VOC in the hospital environment and elsewhere (the exposome) and by treatment. Prospective medication withdrawal-studies constitute a valid approach but given the effect of medication metabolites on VOC profiles (336), differences in medication status needs to be accounted for; as does the environmental confounding which may result from the time between sampling points (baseline and loss of control).

Lazar et al (192) studied the effect of direct bronchial challenge (methacholine) on exhaled breath using an electronic nose (eNose). Lazar et al (192) concluded that acute bronchoconstriction in response to methacholine did not affect exhaled breath profiles as measured by the eNose. However the challenge procedure itself – irrespective of whether methacholine or sham - altered the VOC profile relative to baseline. The use of the eNose precluded the ability to identify individual analytes; while the use of direct bronchial challenge assessed the metabolomics of airway constriction rather than inflammatory mediator release.

5.3.2 Objectives

The study aimed to determine the effect of indirect bronchial challenge testing upon volatile organic compounds (VOC) in the exhaled breath of adults with well-controlled, mild-to-moderate asthma.

5.2.3.1 Primary objective

To determine the effect of indirect bronchial challenge testing on VOC profiles in patients with asthma.

a. The effect of indirect bronchial challenge testing with mannitol dry powder
b. The effect of a sham indirect bronchial challenge test

5.2.3.2 Secondary objectives

a. To explore the variability of biomarkers in patients with asthma
    i. What is the inter-individual and intra-individual variability in exhaled breath VOC in a cohort of patients with asthma

b. To explore whether biomarkers are able to predict outcomes
    i. Are VOC profiles able to predict response to bronchial challenge testing?

5.3.3 Methods

5.3.3.1 Study design

This was a placebo-controlled study of exhaled breath metabolomics. Participants underwent sampling of exhaled breath VOC before and after undertaking an indirect bronchial challenge test using mannitol dry powder (MDP). Those participants exhibiting a positive mannitol challenge response returned for a sham bronchial challenge and further breath sampling.

The release of inflammatory mediators was provoked through the use of a mannitol challenge; taking breath samples before and after the bronchial challenge offers a route by which changes in exhaled VOC may be assessed while minimising potential confounders such as time, medication and the exposome.

The study was conducted over one consent visit and one-to-two assessment visits. At each assessment visit a breath sample was obtained before and after undertaking either an indirect bronchial challenge test or a sham challenge. The bronchial challenge test used Osmohale (Pharmaxis, Sydney, Australia), a MDP inhalation. This has an elimination half-life of 4.7 hours. The sham challenge consisted of performing the required inspiratory effort through the Osmohale delivery device using a 0mg capsule (containing no mannitol).

The study assessments occurred at intervals of no sooner than two weeks from the previous assessment, in order to permit post-challenge normalisation of airways.

Only those who exhibited a positive bronchial challenge result at the first visit were invited for a second, sham-challenge visit. Where possible the two assessment visits were completed within one month (with a two week tolerance) in order to minimise the potential for dropout or changes in disease activity. Study visits were postponed for participants reporting a recent asthma exacerbation, respiratory tract infection (RTI), change in treatment or significant change in either asthma control, FeNO or FEV_1 within the previous four weeks. A 'significant change' was defined as the minimum clinically important difference in the parameters of interest – for asthma control this was a change of 0.5 in ACQ score (337); for spirometry a decline in FEV_1 of greater than 0.25L (338); and for FeNO an increase of 10 parts per billion. This was to ensure that results are not influenced by a carry-over effect from the first visit or a change in the underlying disease activity or treatment.

At the study visit participants undertook breath capture and VOC sampling using a ReCIVA device to selectively sample late phase expiratory breath. The laboratory staff undertaking the analysis of breath samples were blinded through use of a randomly generated sample number. It was not possible to blind the researcher or the participant as it became apparent during the testing process whether the mannitol capsules were full or empty.

5.3.3.2 Study setting

All study assessments were conducted within the Respiratory Outpatients Clinic at the Norfolk & Norwich University Hospital (NNUH). Test results for all participants not under the care of the respiratory department at the NNUH were communicated to their GP in order that they form part of their primary care record.

The risk of participant drop-out was minimised by conducting the study over a relatively short (one month) period for each participant. Lazar et al (192) used a two visit cross-over study design; they recruited 18 participants in total and reported no drop-outs.

5.3.3.3 Sample size

A formal power calculation was not possible due to a lack of information on the number of VOC which might be of interest and the degree of variance which might occur. However, van der Schee et al state that "The inflammatory process underlying this disease is known to provide a sufficiently differential VOC-based breath profile at the current sample size" and they reference two studies (174, 314) with sample sizes of between 10 and 20 participants in each group.

An approximate number was calculated using MetSizeR, a publicly available application constructed by statisticians at University College Dublin for Principal Component Analysis (PCA) sample size calculations in metabolomics. For an untargeted analysis using nuclear magnetic resonance (NMR) spectroscopy - and based on 300 spectral bins of which an assumed 0.1 were significant and a target false discovery rate (FDR) of 0.05 - a sample size of 38 was required (19 in each group). This is roughly in agreement with those sample numbers used by previous breathomic studies (70, 192).

The study aimed to obtain pre- and post-MDP challenge samples from 20 participants with positive mannitol challenge results. Both Porsbjerg et al (339) and Brannan et al (340) report MDP challenge to have a sensitivity of approximately 60% for the identification of those with asthma. However, it should be noted that the sensitivity of bronchial challenge tests may be lower in populations of asymptomatic participants (341); hyper-responsiveness may be attenuated by treatment (342) with some patients becoming as non-responsive as non-asthmatics (343). In contrast, Leuppi et al (342) report negative mannitol challenges in only 16% of asthmatics assessed at their clinic (the majority of which had FEV_1 >80% predicted). If a positive bronchial challenge result in 38% of participants is assumed, a total participant number of 55 would yield 20 participants with a positive response upon which pre- and post-test analysis could be performed.

The end of the study was deemed to have been reached once 60 participants had been recruited; once 20 participants had a positive bronchial challenge result (and sham follow-up), or by the 28th February 2019.

5.3.3.4 Eligibility criteria

Inclusion criteria

- Male or female
- Aged 18 or over
- Able to provide informed consent
- Self-report of asthma diagnosis from health professional
    - Diagnostic confirmation meeting BTS guidelines will be sought from primary care
    - Suspected asthma being investigated by way of mannitol challenge
- Non-smokers; or ex-smokers of at least two years duration with less than a ten pack year history
- Asthma treated according to level-1 to level-4 of BTS treatment guidelines

Exclusion Criteria:

- Respiratory tract infection, asthma exacerbation or change in treatment step within the previous four weeks
- Major chronic cardiorespiratory disease other than asthma
- Significant comorbid condition
- Condition that may be compromised by repeated spirometry manoeuvres or induced bronchospasm (see appendix, eligibility guidelines)
- Asthma treated at level 5 of the treatment guidelines or higher
- Pregnant or nursing mothers
- Current smokers or 'vapers'
- Ex-smokers of < 2 years duration or > 10 pack years.
- Participating in a clinical trial of an investigational medicinal product (CTIMP).
- Unable to speak English.
- Low baseline lung function (FEV_1 <1.50 litres or < 70% predicted value)
- Known hypersensitivity to mannitol, gelatin or strong anaphylactic response in the past.

Patients with asthma treated at level-one to level-four of the BTS/SIGN treatment guidelines were assessed. This excluded any patients receiving oral corticosteroids or biologic drugs (such as omalizumab or mepolizumab).

Patients were eligible for inclusion if they reported a diagnosis of asthma from their primary care physician; from their secondary care physician, or if they were being investigated (by way of mannitol challenge) in secondary care for possible asthma. If the participant was recruited from primary care, diagnostic confirmation - meeting BTS standards - was be sought from their primary care practice. This included a clinical history suggestive of asthma and diagnostic tests confirming variability in airflow over time (such as spirometry or reversibility testing).

Participants were all never smokers or non-smokers of at least 2 years duration with less than a 10 pack year history. Participants had no other serious cardiorespiratory disease or significant comorbidities, and had not experienced an asthma attack, chest infection or changed their asthma medication within the preceding 4 weeks. Those with conditions which might be compromised by repeated spirometry manoeuvres, or low baseline lung function (FEV_1 <1.50 litres or < 70% predicted value) were excluded. A questionnaire was sent to the general practice of all participants to establish the extent to which their diagnosis could be supported (with respect to BTS/SIGN guidelines). For those recruited from the NNUH this information was extracted from their outpatient records.

All participants gave their written informed consent. The study was approved by the Cambridge South NHS Ethics Committee and registered with ClinicalTrial.org under the identifier NCT03575663.

5.3.3.5 Participant identification, recruitment and retention

The study was conducted in accordance with Good Clinical Practice guidelines and according to the guiding principles of the Declaration of Helsinki; the researcher maintained all relevant participant privacy requirements; ensured that participant consent was fully informed and that potential participants had sufficient time to consider their inclusion in the study. All participants were informed that they were able to withdraw from the study at any point in time should they choose to, and that this would not prejudice the quality of their current or future treatment. Participants were reassured that all data collected in the study would be held confidentially.

In order to maintain confidentiality, participants' data was anonymised using a case reference number; personal data linked to the case reference number was be stored securely as was data generated by the study.

Two urban general practices were recruited to act as participant identification centres; their patient registers screened; and those with a diagnosis of asthma who had been prescribed a short-acting β_2-agonist and/or low dose of inhaled corticosteroids were sent a study invitation. All those who contacted the study centre (Norfolk & Norwich University Hospital Foundation Trust (NNUH)) were invited to attend a screening / consent visit; those who passed the inclusion criteria were recruited. In addition, electronic letters from NNUH outpatient asthma clinics were screened and invitations sent to patients with diagnosed asthma, treated at or below level 4 of the British Thoracic Society asthma guidelines; advertisements were also placed at the NNUH and local university (University of East Anglia). Recruitment ceased on 28th February 2019.

A flexible recruitment strategy was employed

Advertising (poster, leaflet or electronic)

- Advertisements in secondary care services (including respiratory outpatient clinics) at the NNUH
- Advertisements on the UEA and NNUH intranet (noticeboard and email bulletins), and print advertisements on the UEA campus.
- Advertisement on a respiratory research microsite within the NNUH website's Research and Development pages.

- Screening of NNUH asthma clinic lists, clinic letters, physiology laboratory records and/or the NNUH respiratory research database.
- The study was accepted onto the National Institute of Health Research (NIHR) portfolio and two local GP surgeries agreed to act as Participant Identification Centres. Participating surgeries searched their clinical management systems and invite potentially suitable patients to contact the study team. A patient information sheet was sent by the GP surgery along with the letter of invitation.
- Patients being referred for a mannitol challenge test as part of usual investigatory care or clinical indication were also eligible for study participation.

Advertisements featured contact details for the respiratory research team. Upon contact from a potential participant an information sheet and letter of invitation was sent and a follow-up phone call made to ensure the potential participant has received the information.

Those who were given study information in face-to-face contact received a participant information sheet and letter of invitation; they were given an explanation of the study and the inclusion criteria, and time to ask questions. Those who wished to offer their consent on the spot this were accepted only after confirming that they had fully understood the study. They were encouraged to take the information away, to think about it and discuss it with others, and contact to us with any further questions before deciding if they wished to participate.

For those who were sent the study information (either in the post or electronically) a follow-up phone call was made in order to confirm they have received the requested information. If at this time the recipient expressed an interest in participation, or if they subsequently contact the research team and expressed interest, the researcher answered any questions they had, outlined the eligibility criteria, and invited the person to a study visit. In line with good clinical practice full, formal assessment of inclusion/exclusion criteria was not undertaken prior to obtaining informed consent, however, in order to avoid ineligible members of the public attending a study appointment (and thereby wasting their time) the researcher outlined the inclusion criteria to all potential participants before inviting them to a study visit.

Informed consent was undertaken at the first study appointment, after which the participant was assessed as to whether they met the study eligibility criteria.

Participants or potential participants scoring >1.25 on the ACQ were encouraged to refer to their personalised asthma management plan and consider making an appointment with their care team should they feel they need help in achieving greater control. Assessment of patients was grouped into study assessment days in order to maximise time efficiency and patients sent appointment reminders by mail, email or phone.

For those participants who had not taken any of the restricted medications it was possible to undertake the first bronchial challenge assessment at their initial consent visit. For example, patients using only a short-acting bronchodilator (SABA) and who had not used it in the prior 8 hours. Participants who had taken one of the restricted medications were invited back for the first study assessment at a future date when they had sufficient time to withhold their medications. Communication with participants in order to arrange and remind about upcoming study assessments included contact by telephone, text, email and/or letter.

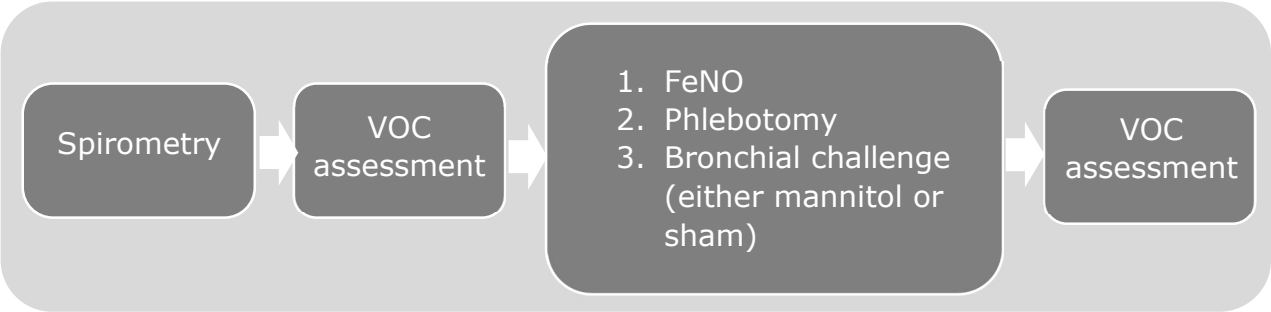
As a thank you for participation, participants received a voucher to the value of £20 for each assessment visit (excluding the consent visit if this was on a separate occasion).

Patients who were found to be ineligible at the recruitment / consent visit were invited to return in the future if the reason for ineligibility was subject to change – for example if they had experienced a chest infection in the last few weeks.

5.3.3.6 Assessments

Study visits consisted of capturing demographic details, clinical history, degree of asthma control (using the ACQ), and asthma severity. Disease severity was based on the level of treatment required to achieve symptom control according to the BTS/SIGN guidelines. Following this, assessments were conducted sequentially as described in figure 13.

Figure 13 - Participant assessments in the ABC study



Those with a positive bronchial challenge response were invited for a second visit with a sham bronchial challenge.

Participants' asthma diagnoses were categorised as being of high, intermediate or low probability according to BTS/SIGN guidelines. For those recruited from GP surgeries, if the GP questionnaire was not returned and there was a negative bronchial challenge test, normal FeNO and no evidence of airway obstruction, the likelihood of asthma was graded as low.

Mannitol & placebo challenges

Bronchial challenge tests are recommended by both BTS/SIGN and GINA guidelines as a method for confirming variability in airflow limitation. Tests were undertaken by trained staff according to both manufacturer and clinical guidelines. This included post-test monitoring for 15 minutes and ensuring that the FEV_1 had returned to within 5% of pre-challenge level. Testing took place in the NNUH hospital with appropriate access to staff, medication and resuscitation equipment as per guidelines. Challenges were undertaken using an Osmohale test kit containing mannitol dry powder. Doubling doses of mannitol dry powder (MDP) were inhaled with two FEV_1 measurements between each dose. The MDP used was Osmohale (Pharmaxis, Sydney, Australia). Manufacturer guidelines suggest a single, between-dose fall in FEV_1 of 10%, or a cumulative fall of 15% (relative to baseline) constitutes a positive test result. Sham challenges using empty capsules were performed in an identical manner with the same number of inhalations and spirometric manoeuvres as during the participant's MDP challenge. As per the manufacturer guidelines patients withheld medications for a period of time prior to assessment, including short-acting beta-2 agonists (8 hours), inhaled corticosteroids (12 hours), long acting beta-agonists (24 hours), antihistamines (72 hours), leukotriene-receptor antagonists (4 days). It was not possible to blind the researcher or participant due to the ease of detecting the presence of absence of mannitol during the test procedure.

Exhaled breath collection

VOC were captured in a non-invasive test using the ReCIVA (see section 5.1.1.). Patients were asked to refrain from vigorous exercise for 24 hours and caffeine on the morning of the test. Diet, last alcohol intake, use of mouthwash, and home location were recorded to look for potential confounding factors. Participants breathed filtered air for 5 minutes before sampling commenced; the aim of this five minute period was to normalise the exhaled breath milieu post spirometry and to washout out VOC inhaled from the hospital environment as much as was practicably possible. In addition to duplicate patient-breath samples and a filtered air sample; a sample of room air was also obtained.

For this the ReCIVA unit was set to collect 500mls of room air and the unit left running (without a mask attached) in the area in which patient sampling was to take place

Asthma Control Questionnaire (ACQ)

Performed as described in section 5.1.1

Fraction of Exhaled Nitric Oxide

Performed as described in section 5.1.1

Phlebotomy

Performed as described in section 5.1.1

Spirometry

Spirometry was undertaken according to best practice guidelines (344) which included spirometry both before and after the bronchial challenge test, and measurements of FEV_1 during the test as per guidelines (345).

5.3.3.7 Safety assessment

Adverse events were recorded as described in section 5.1.1. The risk of adverse events was deemed to be low. The study included only those with well controlled, mild-to-moderate asthma and satisfactory baseline lung function; using methods recommended by international guidelines (GINA & BTS/SIGN) and a CE-marked collection device already used in other clinical trials.

It was anticipated that undertaking the tests had the potential to cause anxiety; participants were continually accompanied by staff and advised that they might withdraw consent at any time without their care being compromised. The aim of bronchoprovocation testing is to cause a bronchoconstriction reaction, this carries with it a known risk of bronchospasm. This risk was managed by following guidelines for test administration which include the exclusion of anyone with a condition that may be aggravated by bronchoprovocation; a step-wise increase in provocation dosage; close monitoring of spirometry; delivery of a post-test bronchodilator, and post-procedural monitoring for a minimum of 15 minutes. Undertaking the post-test VOC sampling delayed the administration of the post-test bronchodilator by up to 10-15 minutes, however in doing so it also had the effect of extending the overall post-test observation period. Tests were conducted within the NNUH, in an area where a physician trained to treat acute bronchospasm was available; and where nebulised bronchodilators, oxygen, subcutaneous adrenalin, and resuscitation equipment were also available. Participants were advised to take their post-test salbutamol prior to the post-challenge VOC capture if they felt a clinical need to do so.

As part of the preparation for the test, participants were required to withhold certain medications, including asthma medications, for between six hours and four days (as per guidelines). Inhaled corticosteroids were withheld for 12 hours; a full list of medications and the times required for withholding can be found in the appendix. This forms part of the guidelines for this procedure (346) and is considered appropriate for a population of participants with asthma. Patients were advised to take their medication should they experience any symptoms; to prioritise their care over study participation and to follow their existing personalised asthma action plan (as agreed with their GP, nurse or respiratory clinician). It was also suggested that if they have any concerns over this they may wish to have someone with them for the period during which they are withholding medications. The safety profile for Osmohale (available from http://www.aridol.info/assets/pdf/20151014_Osmohale_spc_uk.pdf) states that in a study of 1,046 subjects - which included both healthy individuals and those with asthma - no serious adverse effects were reported and most adverse events were mild and transient.

5.3.3.8 Schedule

This was a two to three visit study depending on - a) whether the first bronchial challenge could be conducted at the time of consent and, b) whether the participant exhibited a positive bronchial challenge result. Participants received an indirect (MDP) bronchial challenge test first. Those exhibiting a positive response were invited back for a second study visit at which they received a sham MDP challenge. The two bronchial challenge visits occurred within 4 weeks of one another (with a two week tolerance either way).

While there is an extensive discussion of exhaled breath confounders in chapters 2 and 3, it is worth here mentioning physiological determinants of exhaled breath VOC profiles outside of disease processes. It is likely that the expiratory manoeuvres undertaken in spirometry and as part of the FeNO and bronchial challenge tests would impact on exhaled VOC. Use of a sham mannitol challenge permits this to be assessed and differentiate these effects from those of the physiological response to mannitol. In a trio of papers by Sukul et al (347-349), breath holding, forced expiratory manoeuvres and body positioning were investigated for their effect on VOC. All were found to have an effect on breath VOC profiles however they returned to baseline within 10-20 seconds in the case of breath holding. The authors conclude that reliability of breath profiles is dependent upon the avoidance of forced breathing. However, it was essential to conduct spirometry in order to ascertain a baseline FEV_1 against which to judge airway response to the bronchial challenge.

5.3.4 Outcomes

5.3.4.1 Primary outcomes

To determine the effect of indirect bronchial challenge testing on VOC profiles in patients with asthma.

1. Is there a significant difference in VOC levels between breath samples taken before and after an indirect bronchial challenge using mannitol dry powder?

2. Is there a significant difference in VOC levels between breath samples taken before and after a sham mannitol challenge?

3. Are there any compounds which differ significantly before and after a mannitol challenge which do not also differ before and after a sham bronchial challenge?

5.3.4.2 Secondary outcomes

What is the inter-individual and intra-individual variability in exhaled breath VOC in a cohort of patients with mild-to-moderate asthma?

Is there a significant difference in VOC concentrations at baseline between those who have a positive mannitol challenge and those who have a negative result?

5.3.4 Data Analysis

5.3.4.1 Data management

Data was managed as described previously (section 5.1.3). In addition, after pseudonymisation VOC samples were labelled with a randomly generated sample ID number before being securely transported to MIB for analysis. Those laboratory staff processing the sorbent tubes were blinded as to the nature of the sample until all samples had been processed.

5.3.4.2 Data analysis

Data analysis followed the plan outlined in section 5.1.4.

Primary outcomes

Data analysis was conducted in order to assess whether a significant difference in the concentration of exhaled VOC exists between pre- and post- positive mannitol challenge samples; between pre- and post- negative mannitol challenge samples; and between pre- and post- sham challenge samples. Those compounds identified as significantly differing across pre- and post- samples were then compared to determine if any were unique to the positive mannitol challenge group.

Secondary outcomes

Inter and intra-individual variability was characterised by standard deviation and by calculating the intraclass correlation coefficient

Baseline samples were divided into those belonging to those with a positive bronchial challenge and those with a negative bronchial challenge and compound intensities tested for significant difference.

5.3.5 Study approvals

The ABC study was registered with the clinicaltrials.gov database (registration number NCT03575663). Research ethics committee (REC) approval was gained from Cambridge South REC on 12th December 2017 (reference number 17/EE/0430) and from the Health Research Authority (HRA) on the 21st December 2017. The study was registered with the NIHR clinical research network portfolio (central portfolio management system ID 35754).

Amendment number one (protocol version 1.2; 10th May 2018) was approved by Cambridge South REC on the 8th June 2018 and by the HRA on 16th June 2018.

Amendment number two (protocol version 1.3; 5th November 2018) was approved by Cambridge South REC on 30th November 2018 and by HRA on 3rd December 2018.

5.3.6 Amendments

Amendment One (protocol version 1.2)

On initial application, the REC requested the exclusion of those with moderate disease, limiting recruits to those with mild disease (British Thoracic Society treatment levels 1-2). However, a limited response was received to the mailshot from the first PIC. It is possible that those with very mild disease may be less motivated to participate in research and that recruitment in this patient population may therefore be challenging. Furthermore, those participants with well controlled, mild asthma are less likely to experience a positive mannitol challenge response pushing up the overall number of participants required in order to achieve 20 positive responses (the number required by the sample calculation).

A request was made seeking permission for the recruitment of those treated at up to level 4 of the British Thoracic Society guidelines without a threshold Asthma Control Questionnaire score. Such patients may be more motivated to participate in research and are more likely to exhibit a positive bronchial challenge result.

This treatment level includes those on a high dose of inhaled steroids and/or additional treatment such as an oral medication; but excludes those on oral steroids or biologics.

The initial study protocol specified a randomised cross-over study design. Given that it was possible to blind neither the research participant nor the researcher, a cross-over design was used solely to account for any carry-over effect from the bronchial challenge. However, given the short half-life of Mannitol (4.7 hours) and one-month interval between study visits it was anticipated this washout period would obviate the need for randomisation. Furthermore, washout was assessed using measures of pulmonary function and asthma control including spirometry, the ACQ, and FeNO. Conducting the real mannitol challenge first for all patients would allow the researcher to invite only those participants exhibiting a positive response to return for a sham challenge; thereby reducing the study burden on those participants with negative bronchial challenge results.

Amendment Two (protocol version 1.3)

Initially a recruitment target of 40 participants had been set. The literature regarding the number of participants likely to have a bronchial hyperreactivity (BHR) response to the mannitol challenge was varied and an estimate of 55% was made. After recruiting 40 participants and conducting 29 bronchial challenges it became apparent that this was an over-estimate; there was a BHR response rate of 38% at this juncture. The researcher requested the recruitment ceiling be increased to a maximum of 60. In order to facilitate the recruitment and assessment of these additional participants the study end-date was pushed back to the 28th February 2019. As per the original protocol, recruitment would cease once 20 positive challenge tests with sham-controlled follow-up were completed.

5.4 Omissions

Several elements of the ABBA study were omitted at the sample and data analysis stages; table 24 details these omissions and their reasons.

Table 24 – ABBA study omissions

EBC analysis	The systematic review of EBC 8-isoprostane (chapter two) threw doubt on the utility of 8-isoprostane as an asthma biomarker; and methodological issues (detailed in chapter 4) dissuaded us from pursuing this analysis. Samples were stored for analysis pending identification of a suitable marker and methodology for its analysis.
VOC data	The results of ABC data analysis (detailed in the following chapter) revealed methodological issues which undermined the reliability of the data analysis. Furthermore, difficulty in deconvolving the data at the pre-processing stage meant the supplied data were not of sufficient quality to enable statistical analysis within the available time frame.
Nvivo analysis of focus group data	Due to a limited number of participants in the focus group insufficient data was generated for a thematic analysis. Data instead was used for illustrative vignettes and in an assessment of the AAQ-R questionnaire.

5.5 Conclusion

The identification or confirmation of markers of disease activity may provide opportunities for more effective asthma management, while alternative tests for airway hyper-reactivity would have the potential to save time and patient discomfort. Biomarkers appropriate for disease monitoring, phenotyping and stratification are key in the development of personalised medicine and targeted therapeutics and have the potential to create more effective management of exacerbations, reduced medication usage (21-24), and reduced hospital admissions. In this chapter the methods and rationale for both the ABBA and ABC studies have been described, and the history of amendments recorded to make clear those changes which took place over the course of the study.

Assessing the feasibility of such research in the acute setting is an important part of the translational pipeline in which basic science research finds a real-world clinical application. Findings from these studies may aid in the identification of asthma biomarkers and inform the design of future studies. The studies have been peer reviewed and deemed by members of the AUKCAR to meet the aims of their Core Research Programme. The next chapter (**six**) presents the results of the ABC study; while chapter **seven** presents those of the ABBA study.

Chapter 6 - Bronchial Challenge Testing in Asthma: The Effect of Mannitol Dry Powder Inhalation on Volatile Organic Compounds in Exhaled Breath

The study aimed to determine the effect of indirect bronchial challenge testing upon VOC in the exhaled breath of adults with well-controlled, mild-to-moderate asthma. The release of inflammatory mediators was provoked through the use of a mannitol challenge; breath samples were obtained before and after the challenge and analysed by GC-MS (as described in chapter 5).

6.1 Participants

6.1.1 Recruitment

Forty six adult participants with mild-to-moderate asthma were recruited, treated at level-one to level-four of the BTS/SIGN treatment guidelines(39). Two different recruitment strategies were used. In the first, two urban general practices were selected to act as participant identification centres; their patient registers screened; and those with a diagnosis of asthma who had been prescribed a short-acting β_2-agonist and/or low dose of inhaled corticosteroids were sent a study invitation. All those who contacted the study centre (NNUH) were invited to attend a screening / consent visit; of these 25 passed the inclusion criteria and were recruited. In the second recruitment strategy, electronic letters from NNUH outpatient asthma clinics were screened and invitations sent to patients with diagnosed asthma, treated at or below level 4 of the British Thoracic Society asthma guidelines; advertisements were also placed at the NNUH and local university (University of East Anglia). A further 21 participants were recruited via this route.

6.1.2 Participation

All patients attended a baseline visit at which they provided a breath sample before and after a bronchial challenge using mannitol dry powder. Those participants (n = 16) who exhibited a BHR response (a decrease in FEV1 of $\geq$ 15%) were invited to return and give further breath samples before and after a sham bronchial challenge. 15 participants undertook a follow-up placebo challenge; one participant with a positive bronchial challenge was excluded due to asthma exacerbation in the intervening period. Follow-up sham-challenges occurred no sooner than two weeks from the bronchial challenge in order to permit post-challenge normalisation of airways.

Baseline characteristics of participants are described in table 25. The mean age was 52; 61% of participants were female; 11% were on the lowest level of treatment, prescribed only a SABA inhaler; mean baseline FEV_1 was 2.98 litres; and the mean ACQ score was 0.52 (indicating good control).

Between group difference was tested using the Chi-squared and independent-*t* tests; where ordinal data was skewed the Mann-Whitney test was used. The FEV_1% predicted was significantly lower in those with a positive mannitol challenge (93.3% $\pm$16.5) when compared to those with a negative challenge (102% $\pm$ 12.1), $t(44)=2.04$, $p=0.047$. The number of participants who - when classified according to BTS/SIGN diagnostic guidelines - had a high likelihood of asthma, was significantly larger in the positive mannitol challenge group ($p=0.005$). There were no other significant between-group differences in participant baseline characteristics.

Table 25 – ABC study: participant baseline characteristics

Subjects, *n*	46	Mannitol +ve (16)	Mannitol -ve (30)	Chi-square	Independent-*t* test	Mann whitney
Age years mean ± SD	51.8 ± 16.4	50.5 ± 15.8	52.4 ± 17.0			*p*=0.60
Gender; female *n* (%)	28 (61%)	10 (63%)	18 (60%)	*p*=0.87		
BMI (kg/m^2) mean± SD	27.7 ±6.4	27.3 ± 5.5	28.0 ± 7.0		*p*=0.74	
Ethnicity; identifying as white British *n* (%)	40 (87%)	15 (94%)	25 (83%)	*p*=0.32		
Home environment; urban, suburban, rural *n* (%)	30 (65%) 5 (11%) 11 (24%)	11 (69%) 1 (6%) 4 (25%)	19 (63%) 4 (13%) 7 (23%)	*p*=0.76		
Ex-smokers *n* (%)	12 (26%)	5 (11%)	7 (15%)	*p*=0.56		
Pack years mean ± SD	1.15 ± 2.42	1.63 ± 3.0	0.9 ± 2.1			*p*=0.46
Probability of asthma (BTS/SIGN) high¦medium¦low *n* (%)	32¦7¦7 70¦15¦15%	16¦0¦0 100¦0¦0%	16¦7¦7 53¦23¦23%	*p*=0.005		
Diagnosed in childhood [%]	18 (39%)	7 (44%)	11 (37%)	*p*=0.64		
Years since diagnosis mean ± SD	18.8 ± 18.6	23.7 ± 16.8	16.3 ± 19.2			*p*=0.09
Personal or family history of atopy	19 (41%)	4 (25%)	15 (50%)	*p*=0.10		
Blood eosinophils 10^9L median (IQR)	0.21 (0.27)	0.235 (0.39)	0.185 (0.25)			*p*=0.72
Blood eosinophilia (≥0.3 10^9L) n (%)	14 (33%)	9 (56%)	5 (36%)	*p*=0.82		
Blood neutrophils 10^9L median (IQR)	3.62 (1.84)	3.695 (1.89)	3.615 (1.98)		*p*=0.72	
Blood neutrophilia (>7.50 10^9L) *n* (%)	2 (5%)	0 (0%)	2 (7%)	*p*=0.33		
ACQ score [median & IQR]	0.35 (0.9)	0.4 (0.7)	0.4 (0.7)			*p*=0.22
FeNO (PPB) [mean± SD]	33.7 ± 32.1	45.3 ± 45	27.04 ± 19.6			*p*=0.40

FeNO elevated *n* (%)	11 (25%)	6 (38%)	5 (18%)	*p*=0.15		
BTS treatment level - 0¦1¦2¦3¦4 *n* (%)	6-17-14-7-2 (13-37-30-15-4%)	2-8-4-1-1 (13-50-25-6-6%)	4-9-10-6-1 (13-30-33-20-3%)	*p*=0.58		
ICS use [%]	40 (87%)	14 (30%)	26 (57%)	*p*=0.94		
LABA use [%]	18 (39%)	5 (11%)	13 (28%)	*p*=0.42		
Leukotriene receptor antagonist use *n* (%)	9 (20%)	1 (2%)	8 (17%)	*p*=0.10		
FEV_1 Median (IQR)	2.79L (1.32)	2.58L (1.32)	2.80L (1.33)			*p*=0.40
FEV_1 %predicted [mean± SD]	99% ± 14.2	93.3% ± 16.5	102% ± 12.1		*p*=0.047	
FVC %predicted [mean± SD]	114% ± 14.98	112.1% ± 15.3	114.5% ± 15		*p*=0.61	
FEV_1/FVC ratio [mean± SD]	0.73 ± 0.09	0.70 ± 0.11	0.74 ± 0.08		*p*=0.17	
Evidence of obstruction (FEV_1/FVC <70%) [%]	18 (39%)	9 (56%)	9 (30%)	*p*=0.82		
Previous positive histamine challenge [%]	10 (22%)	3 (7%)	7 (16%)	*p*=0.72		
Symptoms triggered by exercise [%]	25 (54%)	9 (20%)	16 (35%)	*p*=0.85		

Change in disease activity and carry-over effect is presented in table 26; the mean time between visits was 36 days and there were no significant differences in participants between the two study visits in respect of spirometry, FeNO, ACQ and blood markers. Normally distributed data was analysed by the paired *t*-test, while for non-normally distributed data the Wilcoxon signed rank test was used.

Table 26 – ABC study: disease activity and carry-over effect

	Bronchial challenge visit	Placebo challenge visit	Wilcoxon signed rank test	Paired *t*-test
Days between challenge and placebo; days. Mean (range)	36 (106)			
Blood eosinophils 10^9L median [IQR]	0.24 (0.4)	0.21 (0.36)	z = -0.18 p = 0.86	
Blood eosinophilia (>0.3 10^9L) n [%]	5 (33%)	4 (27%)	z = 0.00 p = 1.0	
Blood neutrophils 10^9L median [IQR]	3.46 (1.48)	3.11 (1.41)	z = -0.39 p = 0.70	
Blood neutrophilia (>7.5 10^9L) n [%]	0 (0%)	0 (0%)	z = 0.00 p = 1.0	
ACQ score Median (IQR)	0.3 (0.7)	0.4 (0.3)	z = -0.71 p = 0.48	
FeNO Median (IQR)	24.0 (62)	25.0 (38)	z = -1.65 p = 0.10	
FEV_1 %predicted [mean± SD]	94.4% (± 16.4)	95.2% (± 15.4)		p=0.65
FVC %predicted median (IQR)	108 (17)	112 (28)	z = -0.16 p = 0.88	
FEV_1/FVC ratio [mean± SD]	0.72 (± 0.10)	0.69 (± 0.13)		p=0.33

6.2 GC-MS results – targeted approach

6.2.1 Data analysis – target list

Chromatograms were inspected and compounds identified; this process was repeated with subsequent chromatograms to a point of saturation. This list was then reduced by excluding common exogenous contaminants, resulting in 58 compounds. Using this target list the remaining chromatograms were deconvolved in Masshunter Quantitative (Agilent, Santa Clara, USA). Data processing resulted in a data set consisting of 381 samples with a result for each of 58 VOC.

Table 27 – List of VOC for targeted analysis

1. Sulfur dioxide
2. Acetone
3. Isoprene
4. Dimethyl selenide
5. Ammonium acetate
6. Furan, 2-methyl
7. 1,3,5-trifluorobenzene
8. Benzene
9. Propanoic acid
10. Heptane
11. Trichloroethylene
12. Urea, ethyl-
13. 3,5-dihydroxybenzamide
14. Toluene
15. Hexanal
16. Tetrachloroethylene
17. ß-Methylhistamine
18. Dimethylsulfoxonium formylmethylide
19. Maleic anhydride
20. Ethylbenzene
21. Benzene, 1,3-dimethyl-
22. Phenylethyne
23. Oxime-, methoxy-phenyl-
24. Styrene
25. Heptanal
26. Methanesulfonylacetic acid
27. Tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl-
28. Benzaldehyde
29. Pentanoic acid
30. 2-vinylfuran
31. Benzonitrile
32. Heptane, 2,2,4,6,6-pentamethyl-
33. Benzofuran
34. Decane
35. Octanal
36. 3-Carene
37. Benzyl chloride
38. o-Cymene
39. 2,2,4,4-tetramethyloctane
40. d-Limonene
41. 1,2-butanediol, 1-phenyl-
42. Benzenemethanamine, N,N-dimethyl-
43. Benzene, (methoxymethyl)-
44. Octane, 2,6,6-trimethyl-
45. Acetophenone
46. Benzene, 4-ethenyl-1,2-dimethyl-
47. Benzene, (bromomethyl)-
48. Nonanal
49. Benzoic acid
50. 1-decen-3-one
51. Decanal
52. Ethanol, 2-phenoxy-
53. Phthalic anhydride
54. Tetradecane
55. 5,9-undecadien-2-one, 6,10-dimethyl-, (E)-
56. Phenylmaleic anhydride
57. Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester
58. Benzophenone

This target list includes acetone and isoprene; the two compounds which the systematic review of asthma breathomics (chapter four) found to have been reported as significant in three or more publications. From the list of nine compounds to have featured in two publications (chapter four), two - toluene and benzene – also feature in the above target list.

6.2.2 Technical consistency and baseline characterisation

6.2.2.1 Skew & kurtosis

Patient sample data was log-transformed and the skew and kurtosis calculated. Despite transforming the data, many of the compounds exhibited high levels of skew and kurtosis. This is can be seen in the histograms of sample distribution (fig 14). This is relatively common in breathomic data which is typically heavy tailed. Of note is the apparently bimodal distribution of dimethyl selenide; ammonium acetate; heptane, 2,2,4,6,6-pentamethyl-; and nonanal, which could reflect differing levels of the compound in pre- and post-bronchial challenge samples.

The results of skew and kurtosis calculations were divided by their own standard error; using a threshold of 1.96 to indicate normal distribution, 25 of the compounds were found to be normally distributed while 33 were above this threshold. These results are presented in table 28. Those compounds without high levels of skew and/or kurtosis are highlighted. Of the compounds reported to be of interest by previous studies, isoprene, acetone and benzene were skewed and kurtosed.

Table 28 – ABC study VOC results: skew and kurtosis

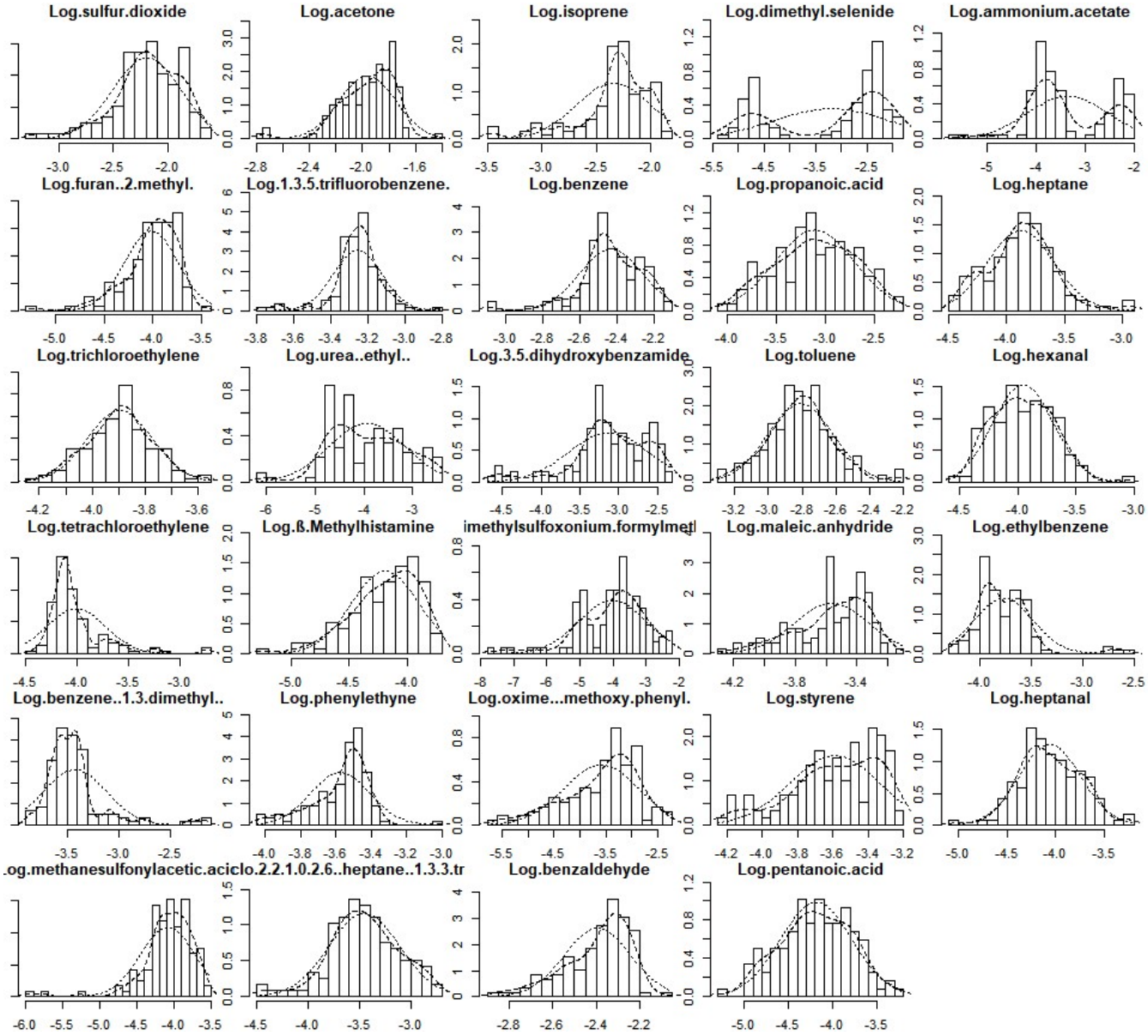
Skewness		Kurtosis		Statistic / std. error <1.96	Compound
Statistic	Std. error	Statistic	Std. error		
-0.879	0.223	1.062	0.442	N	Log sulfur dioxide
-1.227	0.223	2.927	0.442	N	Log acetone
-1.367	0.223	1.963	0.442	N	Log isoprene
-0.610	0.223	-1.438	0.442	N	Log dimethyl selenide
0.082	0.223	-0.571	0.442	Y	Log ammonium acetate
-1.291	0.223	2.672	0.442	N	Log furan, 2-methyl
-1.008	0.223	4.272	0.442	N	Log 1,3,5-trifluorobenzene
-0.979	0.223	2.675	0.442	N	Log benzene
-0.134	0.223	-0.703	0.442	Y	Log propanoic acid
0.404	0.223	0.947	0.442	N	Log heptane
0.027	0.223	0.017	0.442	Y	Log trichloroethylene
-0.083	0.223	-0.210	0.442	Y	Log urea, ethyl-
-0.973	0.223	1.124	0.442	N	Log 3,5-dihydroxybenzamide
0.308	0.223	0.835	0.442	Y	Log toluene
0.272	0.223	0.001	0.442	Y	Log hexanal
2.231	0.223	6.399	0.442	N	Log tetrachloroethylene
-0.851	0.223	0.742	0.442	N	Log ß-Methylhistamine
-1.040	0.223	1.887	0.442	N	Log dimethylsulfoxonium formylmethylide
-0.839	0.223	0.156	0.442	N	Log maleic anhydride
1.815	0.223	5.747	0.442	N	Log ethylbenzene
2.226	0.223	6.261	0.442	N	Log benzene, 1,3-dimethyl-
-0.710	0.223	1.338	0.442	N	Log phenylethyne
-0.783	0.223	0.155	0.442	N	Log oxime-, methoxy-phenyl-
-0.698	0.223	-0.208	0.442	N	Log styrene
0.056	0.223	0.170	0.442	Y	Log heptanal
-2.282	0.223	8.753	0.442	N	Log methanesulfonylacetic acid
-0.275	0.223	0.374	0.442	Y	Log tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl-
-0.935	0.223	0.589	0.442	N	Log benzaldehyde
-0.119	0.223	-0.389	0.442	Y	Log pentanoic acid
-0.239	0.223	1.305	0.442	N	Log 2-vinylfuran
-1.388	0.223	3.752	0.442	N	Log benzonitrile
-0.696	0.223	-0.540	0.442	N	Log heptane, 2,2,4,6,6-pentamethyl-
-0.634	0.223	-0.368	0.442	N	Log benzofuran
-0.468	0.223	0.643	0.442	N	Log decane
-0.148	0.223	-0.533	0.442	Y	Log octanal
-0.212	0.223	-0.494	0.442	Y	Log 3-Carene
-0.231	0.223	-0.493	0.442	Y	Log benzyl chloride
0.419	0.223	-0.177	0.442	Y	Log o-Cymene
-0.020	0.223	-0.089	0.442	Y	Log 2,2,4,4-tetramethyloctane
0.100	0.223	-0.204	0.442	Y	Log d-Limonene
-0.211	0.223	-0.158	0.442	Y	Log 1,2-butanediol, 1-phenyl-
-0.361	0.223	-0.462	0.442	Y	Log benzenemethanamine, N,N-dimethyl-
-0.698	0.223	0.201	0.442	N	Log benzene, (methoxymethyl)-
-0.579	0.223	-0.151	0.442	N	Log octane, 2,6,6-trimethyl-

-0.971	0.223	0.525	0.442	N	Log acetophenone
-0.571	0.223	1.167	0.442	N	Log benzene, 4-ethenyl-1,2-dimethyl-
-0.714	0.223	0.630	0.442	N	Log benzene, (bromomethyl)-
0.221	0.223	-0.655	0.442	Y	Log nonanal
0.161	0.223	1.494	0.442	N	Log benzoic acid
0.273	0.223	0.353	0.442	Y	Log 1-decen-3-one
0.295	0.223	-0.459	0.442	Y	Log decanal
0.258	0.223	-0.144	0.442	Y	Log ethanol, 2-phenoxy-
-1.553	0.223	3.882	0.442	N	Log phthalic anhydride
-0.094	0.223	0.346	0.442	Y	Log tetradecane
-0.216	0.223	0.407	0.442	Y	Log 5,9-undecadien-2-one, 6,10-dimethyl-, (E)-
-0.590	0.223	-0.720	0.442	N	Log phenylmaleic anhydride
0.022	0.223	0.399	0.442	Y	Log pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester
-0.281	0.223	0.084	0.442	Y	Log benzophenone

The results of skew and kurtosis calculations were divided by their own standard error using a threshold of 1.96 to indicate normal distribution; those compounds without high levels of skew and/or kurtosis are highlighted in grey.

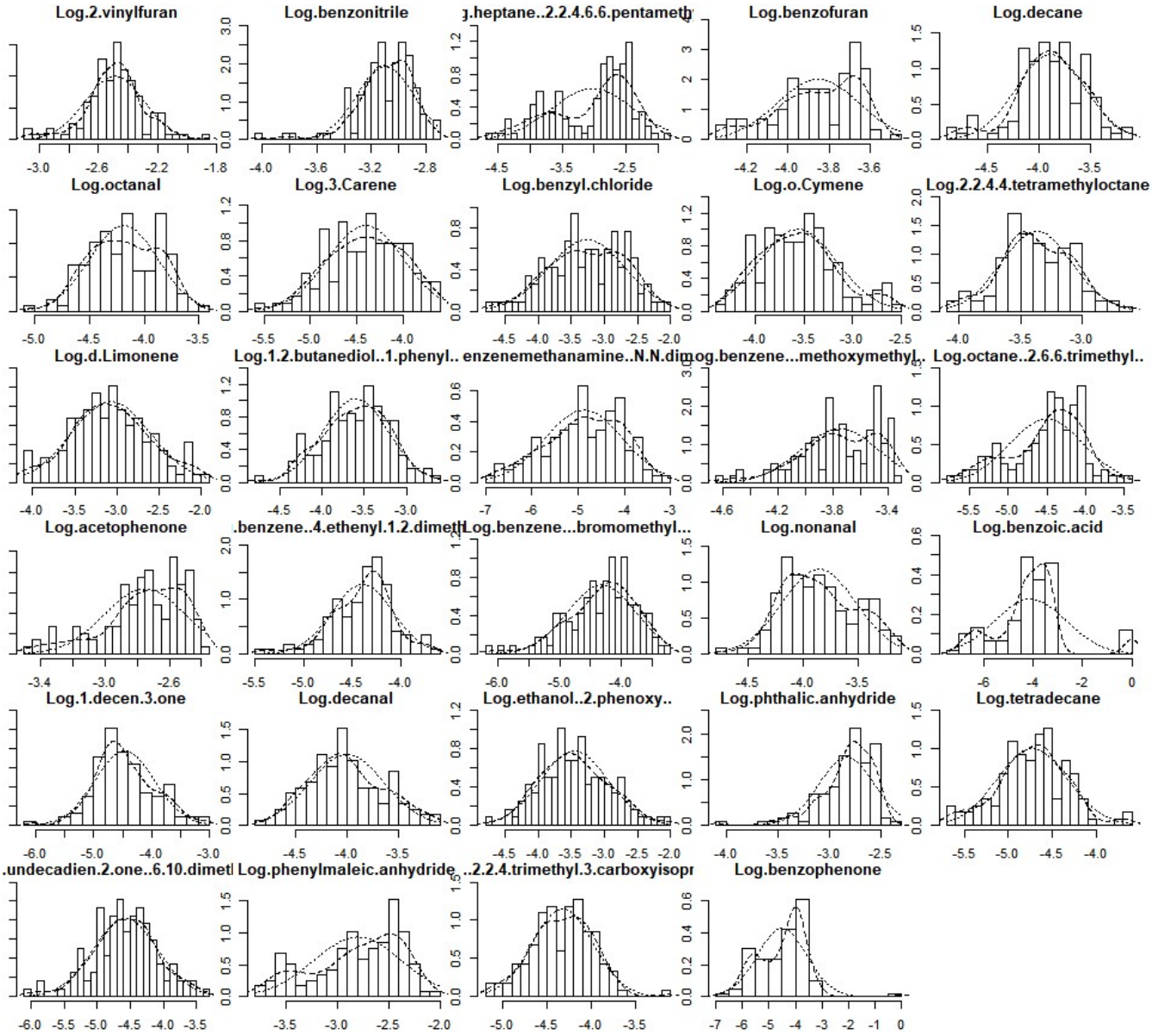
9.2.2.2 Sample distribution

Figure 14 – ABC study: VOC distribution histograms showing a range of skew and kurtosis and a bimodal distribution in some compounds. These histograms present the combined data from pre- and post-challenge breath samples, in both mannitol and sham challenges. In many cases data transformation has not resulted in a normal distribution.



Key: Distribution histograms were produced using the log-transformed geometric mean of replicate patient samples. Raw data for compounds were in the form of intensity counts; a semi-quantitative method that provides relative quantification without a unit of measurement represented on the X-axis.

Figure 14 – ABC study: VOC distribution histograms showing a range of skew and kurtosis and a bimodal distribution in some compounds. These histograms present the combined data in pre- and post-challenge breath samples, from both mannitol and sham challenges. In many cases data transformation has not resulted in a normal distribution

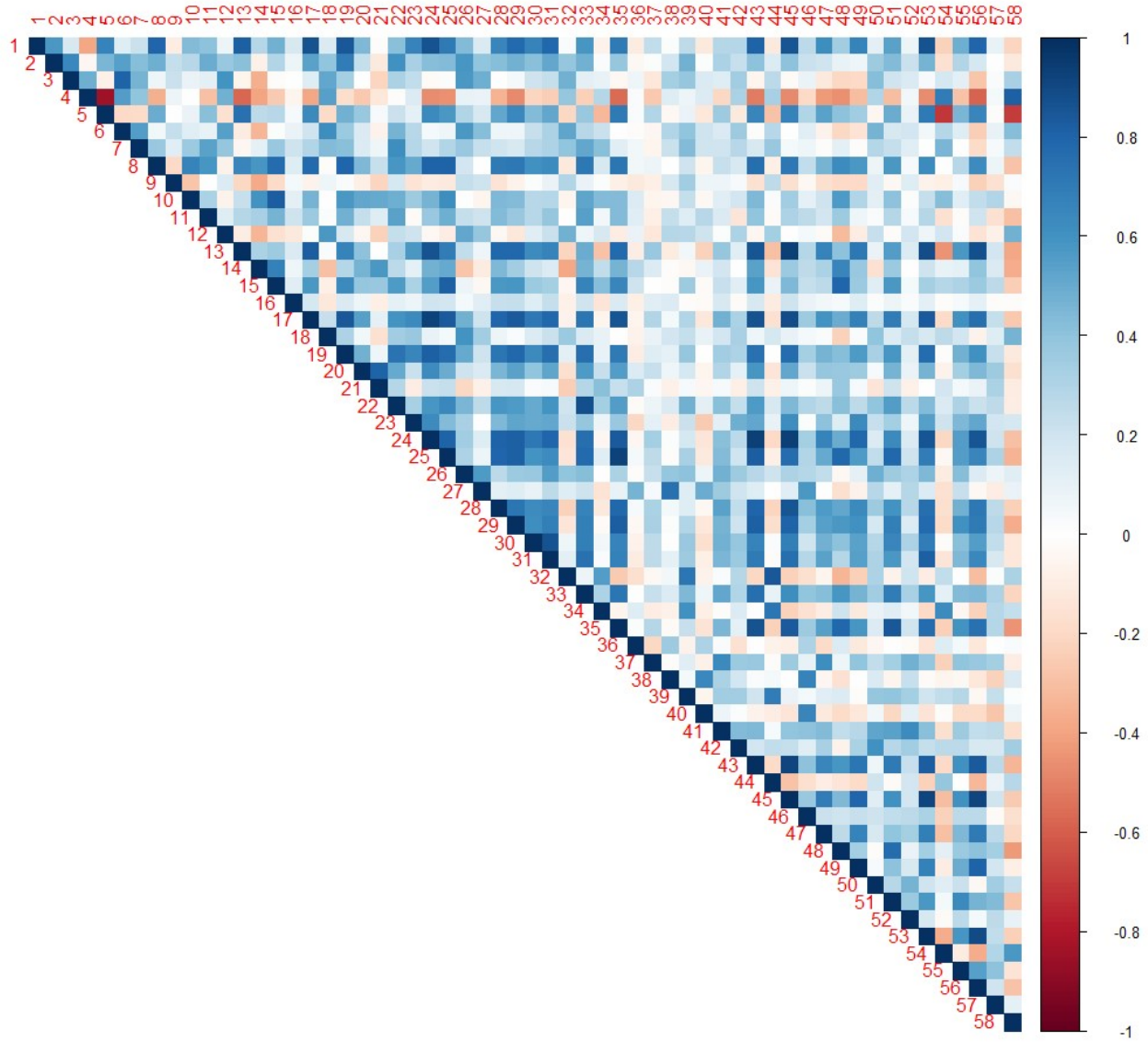


Key: Distribution histograms were produced using the log-transformed geometric mean of replicate patient samples. Raw data for compounds were in the form of intensity counts; a semi-quantitative method that provides relative quantification without a unit of measurement represented on the X-axis.

9.2.2.3 Correlation plot

Non-biological variation between sample analyses in GC-MS is possible due to differences in the analytical equipment and instrumentation over time – the so called 'batch effect' (350). Batch effects lead to increased variability and decreased power to detect significant differences (351). Attempts to remove non-biological variation are made by normalising data; in this study by normalising VOC data against an internal standard. In order to screen for correlations between compounds which might indicate the persistence of a batch effect despite data normalisation, a correlation plot was produced (see figure 15). The lack of highly correlated results between compounds suggests such an effect is unlikely.

Figure 15 – ABC study: VOC correlation plot showing few highly correlated compounds. This is suggestive of the absence of a batch effect in sample analysis.



Key Correlation coefficients are colour coded according to the degree of positive or negative correlation, with darker / stronger colours indicating an increasing degree of correlation. VOC are numbered as follows:

1. Sulfur dioxide
2. Acetone
3. Isoprene
4. Dimethyl selenide
5. Ammonium acetate
6. Furan, 2-methyl
7. 1,3,5-trifluorobenzene
8. Benzene
9. Propanoic acid
10. Heptane
11. Trichloroethylene
12. Urea, ethyl-
13. 3,5-dihydroxybenzamide
14. Toluene
15. Hexanal
16. Tetrachloroethylene
17. ß-Methylhistamine
18. Dimethylsulfoxonium formylmethylide
19. Maleic anhydride
20. Ethylbenzene
21. Benzene, 1,3-dimethyl-
22. Phenylethyne
23. Oxime-, methoxy-phenyl-
24. Styrene
25. Heptanal
26. Methanesulfonylacetic acid
27. Tricyclo[2.2.1.0(2,6)]heptane , 1,3,3-trimethyl-
28. Benzaldehyde
29. Pentanoic acid
30. 2-vinylfuran
31. Benzonitrile
32. Heptane, 2,2,4,6,6-pentamethyl-
33. Benzofuran
34. Decane
35. Octanal
36. 3-Carene
37. Benzyl chloride
38. o-Cymene
39. 2,2,4,4-tetramethyloctane
40. d-Limonene
41. 1,2-butanediol, 1-phenyl-
42. Benzenemethanamine, N,N-dimethyl-
43. Benzene, (methoxymethyl)-
44. Octane, 2,6,6-trimethyl-
45. Acetophenone
46. Benzene, 4-ethenyl-1,2-dimethyl-
47. Benzene, (bromomethyl)-
48. Nonanal
49. Benzoic acid
50. 1-decen-3-one
51. Decanal
52. Ethanol, 2-phenoxy-
53. Phthalic anhydride
54. Tetradecane
55. 5,9-undecadien-2-one, 6,10-dimethyl-, (E)-
56. Phenylmaleic anhydride
57. Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester
58. Benzophenone

9.2.2.4 Reliability

Reliability of results can be assessed by calculating the intraclass correlation coefficient for compounds (352); this describes the degree of correlation for a given VOC between replicate samples. In addition - using the geometric mean of replicates – the standard deviation of VOC values was calculated; this represents the degree of variation between participants. This was compared with the standard deviation *within* participants (between replicate samples). Results are present in table 29 below. Those compounds exhibiting a greater variation between-replicates than between-participants (suggesting unreliability) have been highlighted in grey. Similarly, intraclass correlation coefficients of less than 0.7 suggest unreliability; these compounds have also been highlighted.

Table 29 – ABC study VOC results: intraclass correlation coefficient and standard deviation in baseline (pre-challenge) samples

Compound	Standard deviation		Intraclass correlation coefficient
	Between participants **Data used:** **Baseline (pre-challenge) samples only; geometric mean of replicates, log-transformed.**	**Within participant (between replicates)** **Data used:** **all samples; log-transformed.**	
Log sulfur dioxide	0.35	0.18	0.92
Log acetone	0.25	0.09	0.95
Log isoprene	0.39	0.11	0.97
Log dimethyl selenide	1.14	0.26	0.99
Log ammonium acetate	0.88	0.10	1.00
Log furan, 2-methyl	0.30	0.17	0.90
Log 1,3,5-trifluorobenzene	0.13	0.08	0.91
Log benzene	0.17	0.10	0.92
Log propanoic acid	0.44	0.56	0.53
Log heptane	0.29	0.18	0.90
Log trichloroethylene	0.12	0.04	0.98
Log urea, ethyl-	0.84	0.17	0.99

Log 3,5-dihydroxybenzamide	0.56	0.16	0.98
Log toluene	0.22	0.15	0.86
Log hexanal	0.27	0.18	0.88
Log tetrachloroethylene	0.28	0.06	0.99
Log ß-Methylhistamine	0.34	0.14	0.94
Log dimethylsulfoxonium formylmethylide	1.11	0.34	0.96
Log maleic anhydride	0.26	0.10	0.96
Log ethylbenzene	0.29	0.16	0.92
Log benzene, 1,3-dimethyl-	0.33	0.18	0.91
Log phenylethyne	0.18	0.08	0.95
Log oxime-, methoxy-phenyl-	0.78	0.30	0.96
Log styrene	0.27	0.13	0.93
Log heptanal	0.34	0.15	0.95
Log methanesulfonylacetic acid	0.44	0.24	0.89
Log tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl-	0.37	0.29	0.82
Log benzaldehyde	0.16	0.08	0.94
Log pentanoic acid	0.43	0.26	0.89
Log 2-vinylfuran	0.21	0.10	0.94
Log benzonitrile	0.24	0.12	0.91
Log heptane, 2,2,4,6,6-pentamethyl-	0.68	0.50	0.85
Log benzofuran	0.21	0.07	0.97
Log decane	0.35	0.20	0.91
Log octanal	0.34	0.20	0.90
Log 3-Carene	0.43	0.23	0.92
Log benzyl chloride	0.63	0.75	0.58
Log o-Cymene	0.40	0.11	0.98
Log 2,2,4,4-tetramethyloctane	0.29	0.33	0.67
Log d-Limonene	0.47	0.12	0.98
Log 1,2-butanediol, 1-phenyl-	0.42	0.44	0.68
Log benzenemethanamine, N,N-dimethyl-	0.88	0.73	0.83
Log benzene, (methoxymethyl)-	0.30	0.20	0.87
Log octane, 2,6,6-trimethyl-	0.48	0.49	0.74
Log acetophenone	0.28	0.08	0.98

Log benzene, 4-ethenyl-1,2-dimethyl-	0.32	0.14	0.95
Log benzene, (bromomethyl)-	0.61	0.59	0.70
Log nonanal	0.37	0.21	0.91
Log benzoic acid	1.25	0.98	0.87
Log 1-decen-3-one	0.55	0.42	0.83
Log decanal	0.38	0.30	0.82
Log ethanol, 2-phenoxy-	0.54	0.56	0.69
Log phthalic anhydride	0.32	0.18	0.88
Log tetradecane	0.43	0.26	0.90
Log 5,9-undecadien-2-one, 6,10-dimethyl-, (E)-	0.52	0.45	0.81
Log phenylmaleic anhydride	0.45	0.14	0.97
Log pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	0.35	0.40	0.66
Log benzophenone	0.91	0.31	0.97

Compounds highlighted in grey have a greater variation between-replicates than between-participants and/or intraclass correlation coefficients of less than 0.7 suggestive of unreliability.

9.2.2.5 Correcting for exogenous VOC

Correcting for exogenous compounds has been a contentious issue and a number of different approaches have historically been used (see chapter 3). A consensus method was outlined by the ERS in their technical standards for exhaled breath analysis (228). These recommend a) parallel sampling of ambient air for background correction using alveolar concentration gradients, and b) the use of VOC-filtered air. Calculating an alveolar gradient through background subtraction may not be accurate for all VOC, varying as it does according to factors relating to both the compound itself, duration and intensity of exposure, and the individual concerned (for example BMI) (302). It is possible to calculate the retention coefficient for individual VOC and then apply these in an effort to correct values according to inspiration concentrations (285). This approach might be useful once a panel of biomarkers have been identified however the retention coefficient for the 58 compounds making up the target list have not yet been established.

Following the ERS' technical standards study participants breathed VOC-scrubbed air for a period of 5min in order to reduce inhalation of exogenous compounds and an alveolar gradient was calculated. Using normalised data, the geometric mean of replicate background air samples was subtracted from the geometric mean of patient samples. While the majority of samples yielded a positive result after this calculation was performed (i.e. in the majority of cases the patient exhaled VOC in greater quantities than were present in the air), for a significant minority of samples this produced a negative value (concentrations in the air were higher than those in the patient breath sample). There was no VOC for which all values were positive after performing this calculation. Overall, after background correction 71% of values were positive.

The calculation was repeated subtracting the value obtained from a sample of the CASPER filtered air. Again a sizeable number of negatives were present; for only one compound – isoprene – were negative values entirely absent. Overall 64% of values were positive.

An argument can be made against background subtraction (284) but in order to maintain rigour it is recommended that any compound occurring in background samples at greater than 5% of the level found in breath samples should be excluded. (302, 353). Were such a rigorous approach to be applied the majority of the data set would be excluded; only 33 of the 58 compounds had any background samples where VOC levels were less than 5% of that found in the patient samples; and there were no compounds where this was the case for all of the samples.

The percentage of samples in which background VOC were found to be less than 5% of patient VOC ranged from 0% to 66% of samples for any given VOC.

An alternative approach to background contaminants was trialled in which the mean level of each VOC in background samples was calculated; the mean plus three standard deviations was set as a threshold for inclusion. There were no VOC for which all values were above this threshold, indeed the majority of samples were below this threshold. Only one compound had greater than 75% of results above this threshold (acetone, 85%); and only two compounds had 50% of results above this threshold (acetone and isoprene).

It is possible that levels of some compounds might be equal or higher in the background air than in the patient samples, particularly if this exogenous VOC was metabolised in the lungs, reducing the quantity exhaled; furthermore some compounds may be at the lower limit of detection, affecting reliability of the results.

Filtered air samples were compared directly to the background air samples; when subtracting the filtered air values from those of the background air the results were frequently negative indicating that VOC / contaminants existed at higher levels in the filtered air than in the background air. The percentage of positive results for each VOC ranged from 2 to 84% with a mean of 53%.

Given that participants underwent a five minute washout period in which they were breathing filtered air this was deemed the most appropriate data to use for background subtraction. The background calculations were undertaken on all patient samples – both pre and post-challenge. It is possible that some VOC might be present at equal or greater levels in background air (producing a negative value) when sampled at baseline but present at lower levels (producing a positive value) in post-challenge patient samples due to the test procedure or inflammatory response. For this reason 50% was selected as the negative value threshold for compound exclusion.

9.2.2.6 Exclusions

Those compounds with negative values in greater than 50% of samples after background subtraction had been conducted were excluded from further analysis; as were those compounds with poor reliability (as indicated by a low ICC and a within-participant SD greater than between participants). This resulted in a compound list of 38 VOC. Compounds excluded and the reasons are summarised in table 30.

Table 30 – ABC study VOC results: excluded compounds

COMPOUND	REASON FOR EXCLUSION
1,3,5-TRIFLUOROBENZENE	BG>PT
PROPANOIC ACID	WP>BP ICC <0.7 BG>PT
TRICHLOROETHYLENE	BG>PT
TOLUENE	BG>PT
HEXANAL	BG>PT
ETHYLBENZENE	BG>PT
BENZENE, 1,3-DIMETHYL-	BG>PT
PHENYLETHYNE	BG>PT
HEPTANE, 2,2,4,6,6-PENTAMETHYL-	BG>PT
DECANE	BG>PT
BENZYL CHLORIDE	WP>BP ICC <0.7 BG>PT
2,2,4,4-TETRAMETHYLOCTANE	WP>BP ICC <0.7 BG>PT
1,2-BUTANEDIOL, 1-PHENYL-	WP>BP ICC <0.7
BENZENE, (METHOXYMETHYL)-	BG>PT
OCTANE, 2,6,6-TRIMETHYL-	WP>BP BG>PT
BENZENE, (BROMOMETHYL)-	BG>PT
NONANAL	BG>PT
DECANAL	BG>PT
ETHANOL, 2-PHENOXY-	WP>BP ICC <0.7 BG>PT
PENTANOIC ACID, 2,2,4-TRIMETHYL-3-CARBOXYISOPROPYL, ISOBUTYL ESTER	WP>BP ICC <0.7 BG>PT

Key

WP>BP = within-participant standard deviation greater than between-participant
ICC<0.7 = intraclass correlation coefficient less than 0.7
BG>PT = VOC present in background air in greater quantity than in patient breath sample in more than 50% of cases

6.2.3 Comparative data – the association of VOC with patient status

The geometric mean of patient replicate samples, corrected by the values obtained from filtered air samples were used. Given that up to 50% of these values were negative, in order to log transform the data a constant of 10 was added to the values before transformation. R Studio was used for log transformation and production of boxplots. Excel was used for the constant addition and log transformation prior to conducting Mann Whitney and Wilcoxon-signed rank tests in SPSS.

6.2.3.1 Baseline VOC associations with mannitol challenge outcome

6.2.3.1.1 Boxplots

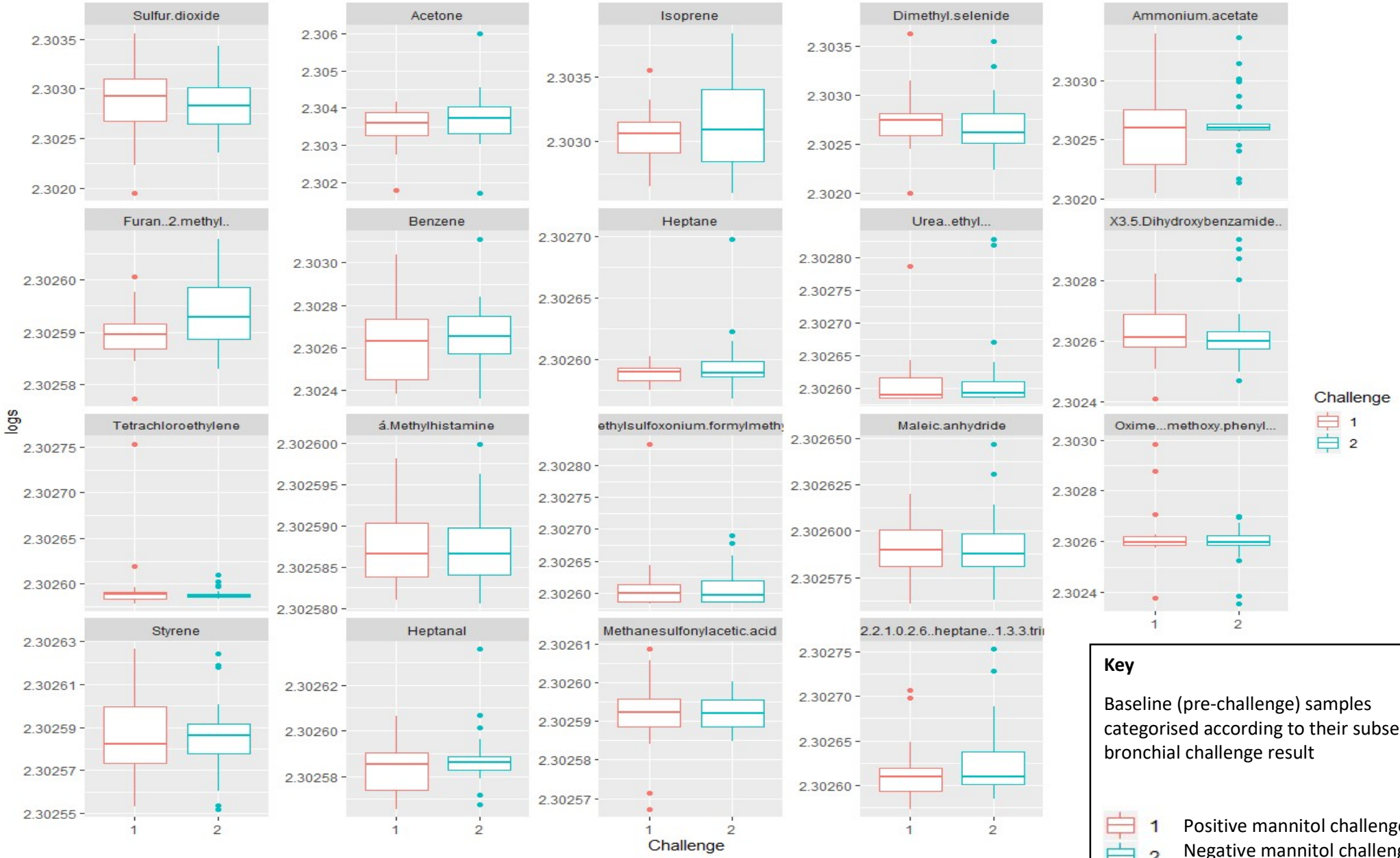
Baseline (pre-challenge bronchial challenge) samples were categorised into those who had a positive response to mannitol challenge and those who had a negative response. Box plots were produced for each VOC (fig. 16) and a Mann Whitney test conducted (table 31).

Some results were widely spread with a large inter-quartile range (IQR) and range (for example benzene); whereas others were closely grouped with only a small degree of variance (for example tetrachloroethylene).

In the case of ammonium acetate and benzoic acid, results exhibited a large range in the baseline positive samples but a far smaller range in the baseline negative samples. Conversely, for both isoprene, furan,2-methyl and 3-carene results were more tightly grouped in the baseline positive and more widely spread in the baseline negative samples.

While the degree of variance in the results appears to differ between baseline positive and negative samples, with the exception of furan, 2-methyl there is not an obvious difference in the median compound level between the two patient groups.

Figure 16 – ABC study boxplots: a comparison of baseline samples categorised according to subsequent mannitol challenge result (positive vs negative). While some differences in distribution are apparent there is little obvious bertween-group difference in median VOC levels

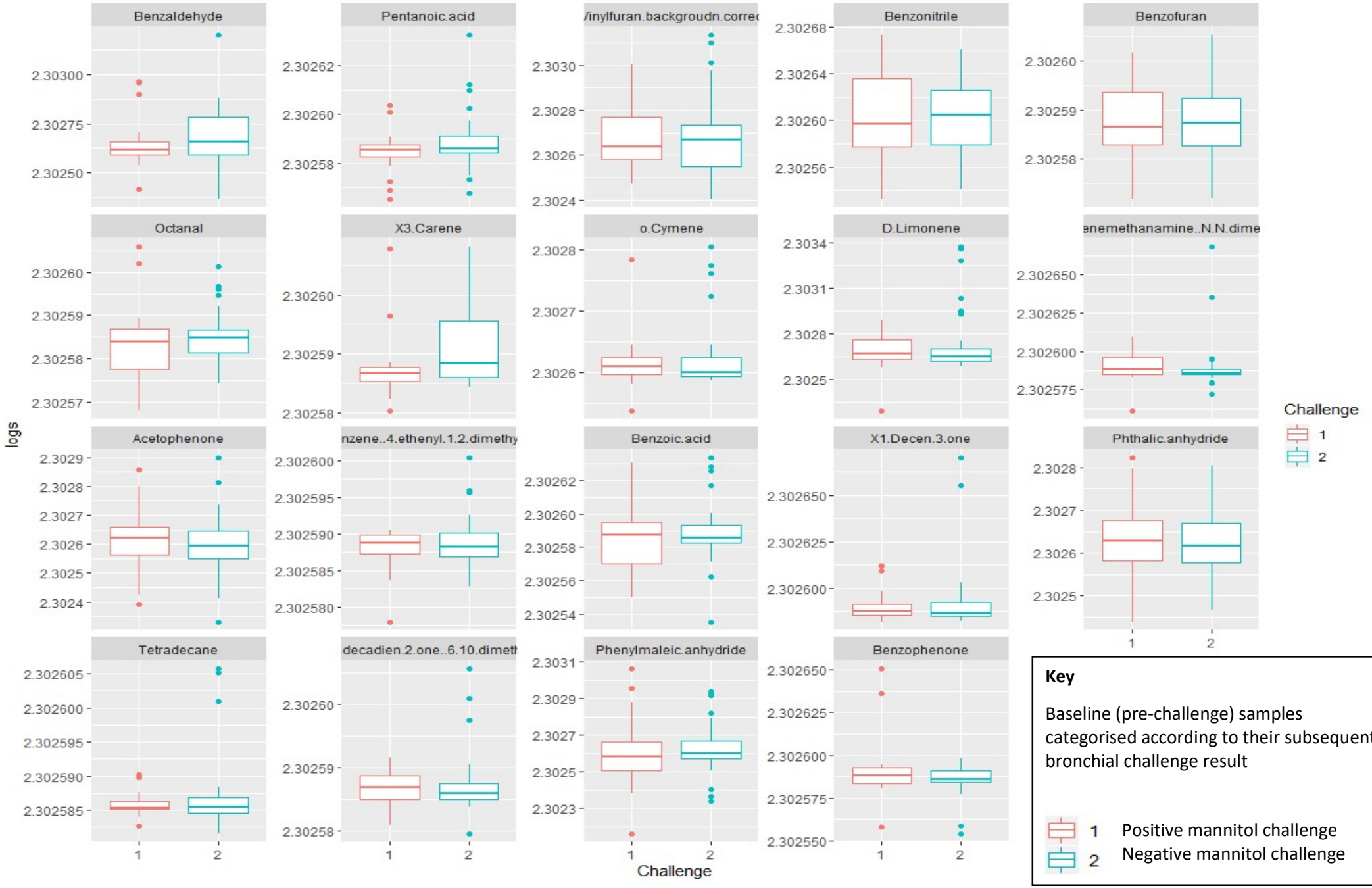


Key

Baseline (pre-challenge) samples categorised according to their subsequent bronchial challenge result

1 Positive mannitol challenge

2 Negative mannitol challenge


Benzaldehyde
Pentanoic.acid
/inylfuran.backgroudn.correc
Benzonitrile
Benzofuran
Octanal
X3.Carene
o.Cymene
D.Limonene
enemethanamine..N.N.dime
Acetophenone
nzene..4.ethenyl.1.2.dimethy
Benzoic.acid
X1.Decen.3.one
Phthalic.anhydride
Tetradecane
decadien.2.one..6.10.dimeth
Phenylmaleic.anhydride
Benzophenone
logs
Challenge
1
2
Key
Baseline (pre-challenge) samples categorised according to their subsequent bronchial challenge result
1 Positive mannitol challenge
2 Negative mannitol challenge

9.2.3.1.2 Mann Whitney test

Table 31 – ABC study: baseline association with mannitol challenge outcome (Mann Whitney test)

Compound	***p*-value**
Sulfur dioxide	0.580
Acetone	0.393
Isoprene	0.612
Dimethyl selenide	0.310
Ammonium acetate	0.836
Furan, 2-methyl	0.032
Benzene	0.747
Heptane	0.393
Urea, ethyl-	0.695
3,5-dihydroxybenzamide	0.580
Tetrachloroethylene	0.890
ß-Methylhistamine	0.854
Dimethylsulfoxonium formylmethylide	0.800
Maleic anhydride	0.963
Oxime-, methoxy-phenyl-	0.628
Styrene	0.747
Heptanal	0.549
Methanesulfonylacetic acid	0.982
Tricyclo[2.2.1.0(2,6)] heptane, 1,3,3-trimethyl-	0.433
Benzaldehyde	0.580
Pentanoic acid	0.333
2-vinylfuran	0.890
Benzonitrile	0.836
Benzofuran	0.818
Octanal	0.518
3-Carene	0.059
o-Cymene	0.645
d-Limonene	0.854
Benzenemethanamine, N,N-dimethyl-	0.181
Acetophenone	0.420
Benzene, 4-ethenyl-1,2-dimethyl-	0.982
Benzoic acid	0.926
1-decen-3-one	0.645
Phthalic anhydride	0.926
Tetradecane	0.836
5,9-undecadien-2-one, 6,10-dimethyl-, (E)-	0.678
Phenylmaleic anhydride	0.504
Benzophenone	0.678

For only one compound was there a significant difference at baseline between those who went on to have a positive bronchial challenge test and those who had a negative one – Furan, 2-methyl. This was found to be higher in those who went on to have a positive challenge. Using a *p*-value of 0.05 is likely to result in false discoveries given that 38 compounds were being investigated. A Benjamini-

Hochberg false discovery rate (FDR) calculation was conducted in Excel with a threshold of 0.05; all results were found non-significant.

9.2.3.1.3 Logistic regression analysis

A logistic regression was performed to ascertain the effect of VOC levels on the likelihood of a positive or negative challenge. Sulfur dioxide and acetone contributed the most to the model with significance values of 0.290 and 0.251 respectively; nether meeting the 0.05 threshold for significance. The model explained 10.2% (Nagelkerke R^2) of the variance in mannitol challenge result. It correctly classified 71.7% of cases but with an accuracy of 25% in classifying positive challenges; producing a very large number of false negatives.

Table 32 – ABC study: baseline association with mannitol challenge outcome (logistic regression)

		PREDICTED		
		Positive challenge	Negative challenge	Percentage correct
OBSERVED	Positive challenge	4	12	25.0
	Negative challenge	1	29	96.7
	Overall percentage			71.7

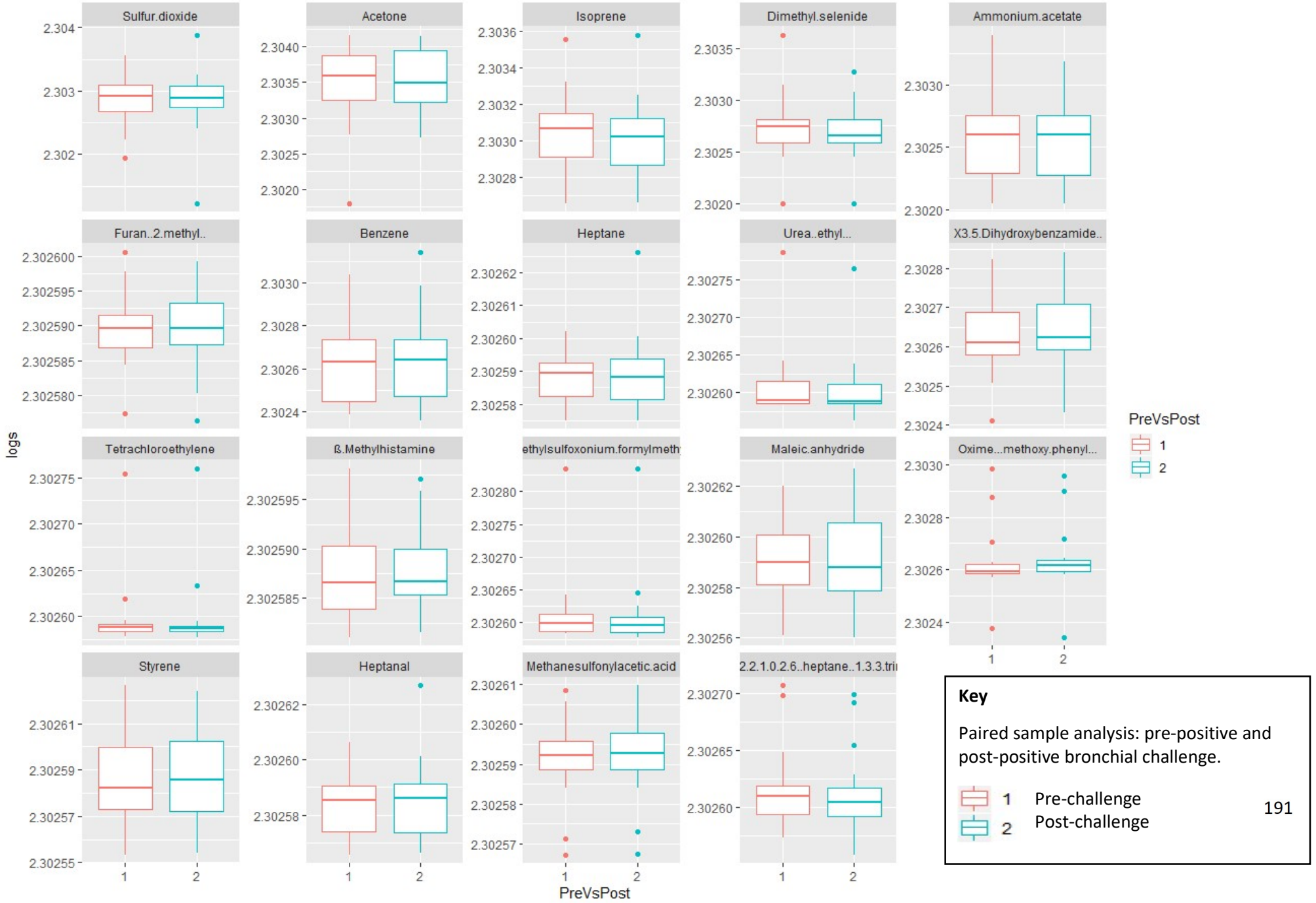
(cut value = 0.500)

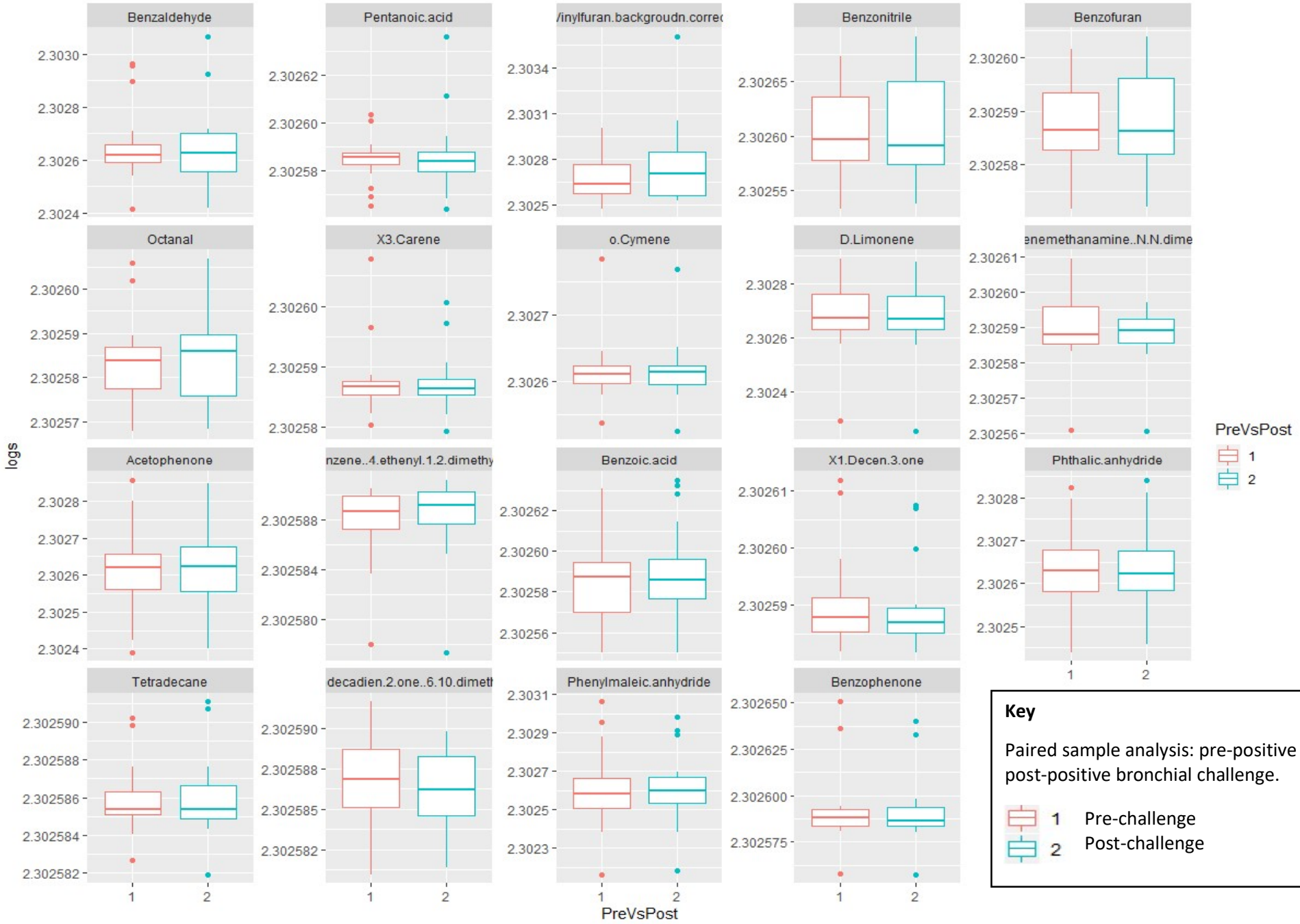
6.2.3.2 Differentiating between pre and post-challenge samples (positive challenges only)

6.2.3.3.1 Boxplots

Pre-challenge and post-challenge samples were compared for those participants who experienced a positive mannitol challenge. The resultant box plots (figure 17) show no marked differences between the two sets of samples with median results appearing quite similar. Unlike the previous comparison there appears to be relatively little difference between groups in the degree of variance. However, on conducting a Wilcoxon-signed ranks test three compounds exhibited a significant difference between baseline and post-challenge – isoprene; urea ethyl; and 5,9-undecadien-2-one, 6,10-dimethyl-, (E)-.

Figure 17 – ABC study boxplots: paired sample analysis of participants with a positive mannitol challenge – pre- vs post-challenge samples. For the majority of compounds there is little apparent difference in median VOC levels or distribution across the two groups.




Benzaldehyde
Pentanoic.acid
/inylfuran.backgroudn.correc
Benzonitrile
Benzofuran
Octanal
X3.Carene
o.Cymene
D.Limonene
enemethanamine..N.N.dime
Acetophenone
nzene..4.ethenyl.1.2.dimethy
Benzoic.acid
X1.Decen.3.one
Phthalic.anhydride
Tetradecane
decadien.2.one..6.10.dimeth
Phenylmaleic.anhydride
Benzophenone
logs
PreVsPost
PreVsPost
1
2
Key
Paired sample analysis: pre-positive and post-positive bronchial challenge.
1 Pre-challenge
2 Post-challenge

6.2.3.3.2 Wilcoxon-Signed Rank test

Table 33 – ABC study VOC results: pre- vs post- positive mannitol challenge

Compound	***p*-value**
Sulfur dioxide	0.605
Acetone	0.605
Isoprene	0.017
Dimethyl selenide	0.134
Ammonium acetate	0.079
Furan, 2-methyl	0.605
Benzene	1.000
Heptane	1.000
Urea, ethyl-	0.002
3,5-dihydroxybenzamide	0.438
Tetrachloroethylene	0.088
ß-Methylhistamine	0.352
Dimethylsulfoxonium formylmethylide	0.234
Maleic anhydride	0.326
Oxime-, methoxy-phenyl-	0.109
Styrene	0.679
Heptanal	0.796
Methanesulfonylacetic acid	0.134
Tricyclo[2.2.1.0(2,6)] heptane, 1,3,3-trimethyl-	0.079
Benzaldehyde	0.642
Pentanoic acid	0.605
2-vinylfuran	0.079
Benzonitrile	0.501
Benzofuran	0.339
Octanal	0.438
3-Carene	0.733
o-Cymene	0.717
d-Limonene	0.098
Benzenemethanamine, N,N-dimethyl-	0.063
Acetophenone	0.301
Benzene, 4-ethenyl-1,2-dimethyl-	0.079
Benzoic acid	0.221
1-decen-3-one	0.179
Phthalic anhydride	0.088
Tetradecane	0.642
5,9-undecadien-2-one, 6,10-dimethyl-, (E)-	0.039
Phenylmaleic anhydride	0.326
Benzophenone	0.756

On conducting a Benjamini-Hochberg FDR calculation (with a threshold of 0.05) all results were found non-significant. In order to determine if these changes (the statistical significance of which were not verified after FDR) were likely to have occurred due to the release of inflammatory mediators caused by the mannitol challenge or whether these were changes induced by the testing process – repeated spirometry - a Wilcoxon-signed rank test was performed.

This was conducted on the pre- and post- challenge data from the negative mannitol and sham mannitol challenges (see table 34).

Table 34 – ABC study VOC results: pre- vs post- sham & negative challenges

Compound	Wilcoxon-signed rank test *p*-value	
	Pre- versus post- mannitol challenge (negative challenge)	**Pre- versus post- sham challenge**
Sulfur dioxide	0.441	0.875
Acetone	0.006	0.008
Isoprene	0.614	0.583
Dimethyl selenide	0.063	1.000
Ammonium acetate	0.428	0.158
Furan, 2-methyl	0.975	0.937
Benzene	0.299	0.272
Heptane	0.199	0136
Urea, ethyl-	0.199	0.004
3,5-dihydroxybenzamide	0.229	0.028
Tetrachloroethylene	0.035	0.004
ß-Methylhistamine	0.428	0.875
Dimethylsulfoxonium formylmethylide	0.428	0.041
Maleic anhydride	0.719	0.530
Oxime-, methoxy-phenyl-	0.082	0.005
Styrene	0.237	0.638
Heptanal	0.766	1.000
Methanesulfonylacetic acid	0.254	0.117
Tricyclo[2.2.1.0(2,6)] heptane, 1,3,3-trimethyl-	0.111	0.028
Benzaldehyde	0.558	0.272
Pentanoic acid	0.309	0.209
2-vinylfuran	0.178	0.754
Benzonitrile	0.229	0.583
Benzofuran	0.021	0.530
Octanal	0.280	1.000
3-Carene	0.015	0.182
o-Cymene	0.517	0.239
d-Limonene	0.041	0.008
Benzenemethanamine, N,N-dimethyl-	0.393	0.638
Acetophenone	0.192	0.071
Benzene, 4-ethenyl-1,2-dimethyl-	0.766	0.136
Benzoic acid	0.289	0.424
1-decen-3-one	0.975	0.638
Phthalic anhydride	0.339	0.973
Tetradecane	0.894	0.308
5,9-undecadien-2-one, 6,10-dimethyl-, (E)-	0.009	0.814
Phenylmaleic anhydride	0.339	0.814
Benzophenone	0.043	0.308

p-values <0.05 are highlighted in grey.

Acetone and d-limonene show a significant difference pre- and post-challenge on both the negative mannitol challenge and the sham bronchial challenges. This would suggest that the changes are due to the challenge procedure (repeat spirometry) rather than the presence of MDP or release of inflammatory mediators.

Those compounds where there is a significant differene with the negative mannitol challenge but not with the sham suggest the change might be due to the presence in the lungs of MDP rather than an inflammatory reaction to it; a non-inflammatory pathway such as the suppression of VOC exhalates by the presence of powder; or altered breath profiles due to coughing. Alternatively it is possible that these are compounds related to inflammation that were present in those negative mannitol challenges which were borderline (those which saw some fall in FEV_1 but not sufficient to meet the threshold for a positive test).

That there are a number of compounds in which a significant difference occurred with the sham challenge but not with the negative mannitol challenges raises questions. These may be spurious results or it may be that changes induced by spirometry in the sham challenge are obscured or counteracted by changes which occur in the presence of inhaled MDP.

Comparing pre- and post-challenge samples from those who had a positive response to the mannitol challenge revealed three compounds exhibiting a significant difference – isoprene; urea, ethyl-; and 5,9-undecadien-2-one, 6,10-dimethyl-, (E). Isoprene did not change significantly in those participants having a sham challenge nor those who experienced a negative mannitol challenge; the difference in observed levels may therefore be due to inflammatory changes induced by the positive mannitol challenge. That urea, ethyl- exhibited a significant change in the sham challenge, throws into doubt its utility as a marker of inflammatory activity. 5,9-undecadien-2-one, 6,10-dimethyl-, (E) exhibited a significant change in the negative mannitol challenge however this was in the opposing direction (see table 35); this suggests that the effect of a positive challenge may be quite marked.

Table 35 – ABC study: pre vs post-positive mannitol challenge samples - list of VOC which differed significantly (before the application of FDR)

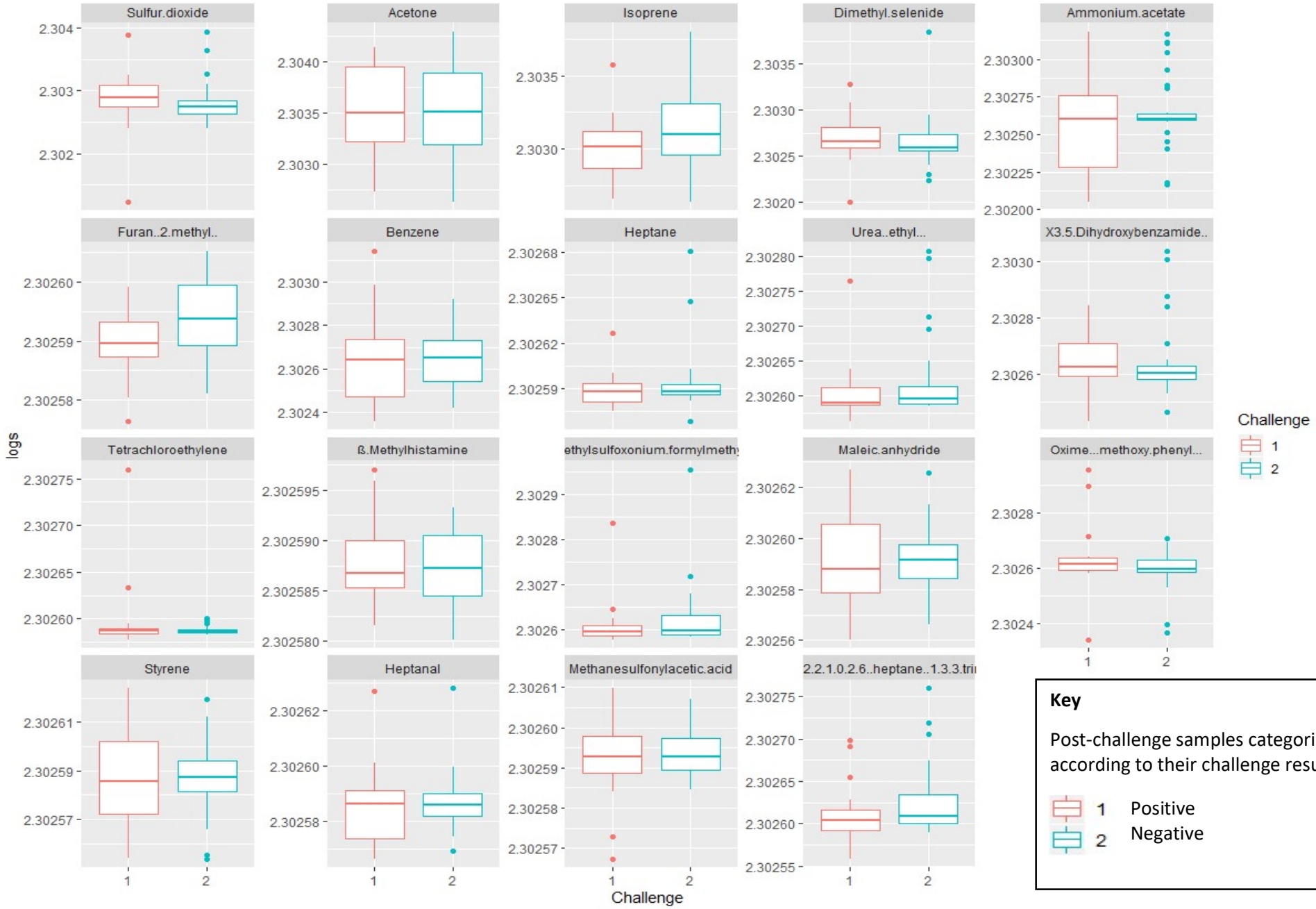
Compound	Direction of change in positive mannitol challenge	Sham or negative challenge
Isoprene	Decreased post-challenge	No significant change
Urea, ethyl-	Decreased post-challenge	Decreased in post-sham challenge samples
5,9-undecadien-2-one, 6,10-dimethyl-, (E)-	Decreased post-challenge	Increased in post-negative mannitol challenge

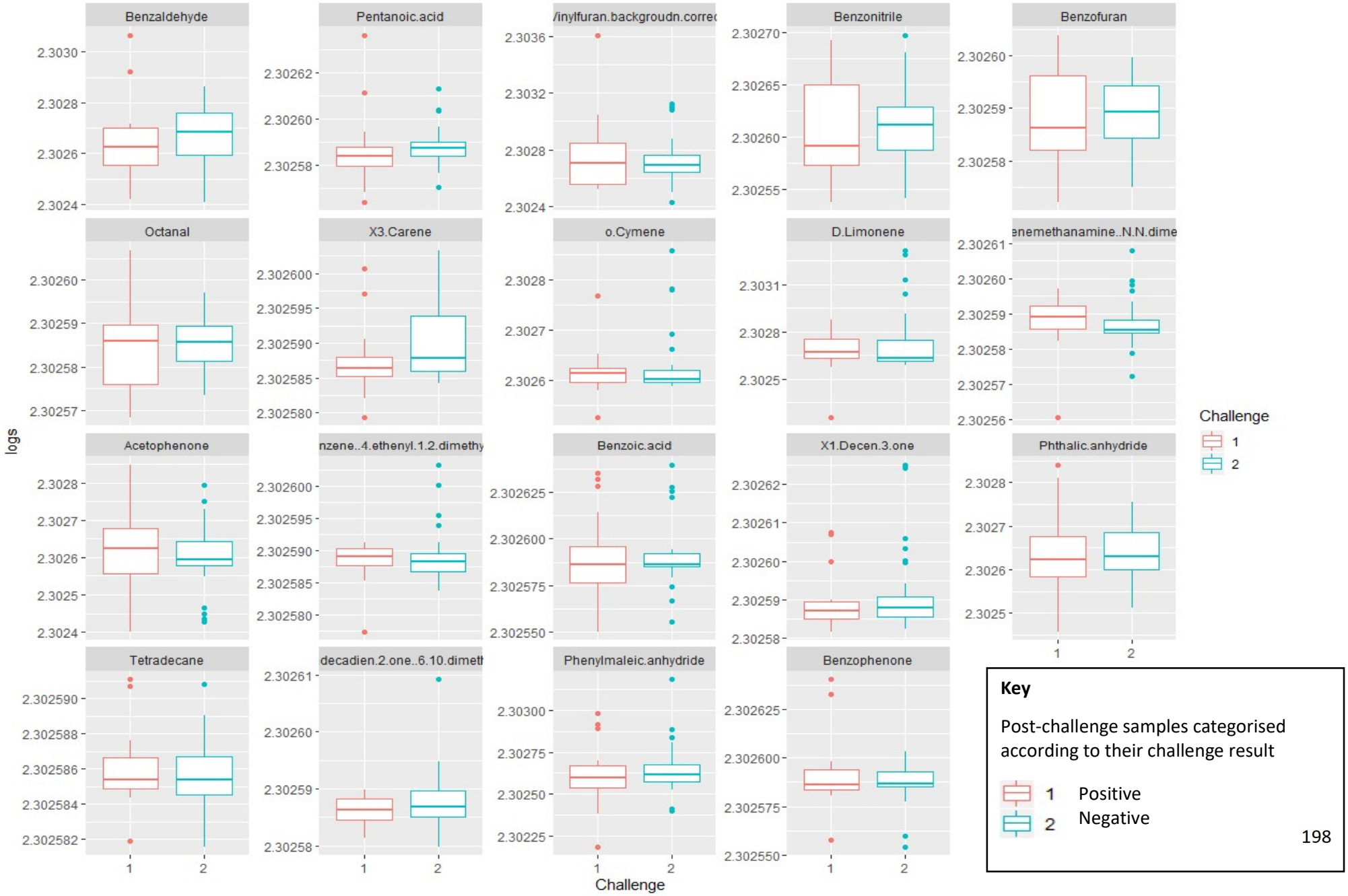
6.2.3.3 Differentiating between positive and negative post-challenge breath samples

6.2.3.3.1 Boxplots

The following box plots (figure 18) compare the post-challenge breath samples from two groups - those who exhibited a positive mannitol challenge and those with a negative one.

Figure 18 – ABC study box plots: a comparison of post-challenge samples categorised according to their challenge result (positive vs negative). For the majority of compounds there is little apparent difference in median VOC levels or distribution across the two groups.




Benzaldehyde
Pentanoic.acid
/inylfuran.backgroudn.corre
Benzonitrile
Benzofuran
Octanal
X3.Carene
o.Cymene
D.Limonene
enemethanamine..N.N.dime
Acetophenone
nzene..4.ethenyl.1.2.dimethy
Benzoic.acid
X1.Decen.3.one
Phthalic.anhydride
Tetradecane
decadien.2.one..6.10.dimeth
Phenylmaleic.anhydride
Benzophenone
logs
Challenge
1
2
Key
Post-challenge samples categorised according to their challenge result
1 Positive
2 Negative

There appears to be relatively little difference between the two groups for the majority of VOC; an observation borne out by the lack of significant values produced by the Mann Whitney test conducted (see table 36).

6.2.3.3.2 Mann Whitney test

Table 36 – ABC study VOC results: positive vs negative post challenge samples (Mann Whitney test).

Compound	*p*-value
Sulfur dioxide	0.134
Acetone	0.890
Isoprene	0.189
Dimethyl selenide	0.420
Ammonium acetate	0.764
Furan, 2-methyl	0.059
Benzene	0.695
Heptane	0.580
Urea, ethyl-	0.159
3,5-dihydroxybenzamide	0.310
Tetrachloroethylene	0.908
ß-Methylhistamine	0.982
Dimethylsulfoxonium formylmethylide	0.310
Maleic anhydride	0.818
Oxime-, methoxy-phenyl-	0.189
Styrene	0.872
Heptanal	0.628
Methanesulfonylacetic acid	0.926
Tricyclo[2.2.1.0(2,6)] heptane, 1,3,3-trimethyl-	0.258
Benzaldehyde	0.460
Pentanoic acid	0.159
2-vinylfuran	0.890
Benzonitrile	0.356
Benzofuran	0.695
Octanal	0.800
3-Carene	0.134
o-Cymene	0.628
d-Limonene	0.712
Benzenemethanamine, N,N-dimethyl-	0.159
Acetophenone	0.645
Benzene, 4-ethenyl-1,2-dimethyl-	0.393
Benzoic acid	0.773
1-decen-3-one	0.612
Phthalic anhydride	0.729
Tetradecane	0.926
5,9-undecadien-2-one, 6,10-dimethyl-, (E)-	0.205
Phenylmaleic anhydride	0.612
Benzophenone	0.982

The Mann Whitney *p*-value for Furan 2-methyl was 0.004 when comparing baseline (pre-challenge) samples of positive and negative mannitol challenge groups; and was 0.059 when comparing the post-challenge samples of those same groups. However, the Wilcoxon-signed ranks *p*-value for this compound was 0.605 in a paired-samples analysis comparing pre- with post-positive mannitol challenge samples. This might suggest that Furan 2-methyl is a product of an underlying mechanism related to positive mannitol response but that it in itself does not change during the release of inflammatory mediators triggered by the mannitol challenge.

Isoprene was the only VOC which differed significantly between the pre- and post-positive mannitol challenge samples and did not also differ in pre- and post-comparisons for negative or sham challenges

6.2.3.3.3 Logistic regression analysis

A logistic regression was performed (see table 37) to ascertain the effects of VOC on the likelihood that participant breath samples were from a positive mannitol challenge. The logistic regression model was not statistically significant. The model explained 6% (Nagelkerke R^2) of the variance in bronchial challenge response. Isoprene was the compound more closely associated with an increased likelihood of a positive challenge but at 0.187 fell short of significance. The model correctly classified 65% of cases but - with a large number of false negatives - had a sensitivity of only 13%.

Table 37 – ABC study VOC results: logistic regression analysis of post-challenge samples.

		PREDICTED		
		Positive challenge	Negative challenge	Percentage correct
OBSERVED	Positive challenge	2	14	12.5
	Negative challenge	2	28	93.3
	Overall percentage			65.2

(cut value = 0.500)

6.2.4 Analysis without background correction

6.2.4.1 Per compound analysis

Given the contention regarding the best way of dealing with exogenous VOC and background samples; further analysis was conducted with uncorrected data. Compounds were not excluded based on the level present in background samples however exclusions based on reliability (ICC and SD) were still enforced. Data was normalised by the internal standard and sample volume without any correction for VOC found in the background air or filtered air samples. The logged geometric mean of replicates was used and the following analyses applied:

A) Baseline (pre-challenge) samples were categorised into those which had a positive result to mannitol challenge and those which were negative. A Mann Whitney test was performed.

B) All baseline (pre-challenge) samples were compared to post-positive challenge samples. A Mann Whitney test was performed.

C) Post-challenge samples were categorised into those from a positive challenge and those from a negative challenge. A Mann Whitney test was performed.

D) Baseline (pre-challenge) samples were compared to post-mannitol challenge samples. A paired analysis was conducted using samples from those participants who experienced a positive bronchial challenge only. A Wilcoxon signed rank test was conducted.

The results are outlined in table 38.

Table 38 – ABC study VOC results based on analysis of data without correction for background contaminants

	Mann Whitney test *p*-value			Wilcoxon Signed Rank test *p*-value		
Compound	**A** **Pre-challenge samples: positive vs negative challenge**	**B** **All pre-challenge samples versus positive post-challenge samples**	**C** **Post-challenge samples: positive vs negative**	**D** **Paired analysis Pre- vs post-challenge (positive only)**	**E** **Paired analysis Pre vs post challenge (negative only)**	Background VOC greater than patient VOC in >50% of samples
Log sulfur dioxide	0.159	0.537	0.122	0.717	0.339	
Log acetone	0.504	0.537	0.800	0.679	0.010	
Log isoprene	0.112	0.282	0.174	0.501	0.339	
Log dimethyl selenide	0.047	0.212	0.146	0.679	0.043	
Log ammonium acetate	0.268	0.386	0.181	0.756	0.017	
Log furan, 2-methyl	0.174	0.439	0.222	0.717	0.829	
Log 1,3,5-trifluorobenzene	0.549	0.555	0.854	0.717	0.213	Y
Log benzene	0.181	0.958	0.489	0.196	0.544	
Log heptane	0.268	0.864	0.344	1.000	0.299	
Log trichloroethylene	0.628	0.684	0.174	0.044	0.000	Y
Log urea, ethyl-	0.764	0.969	0.963	0.278	0.016	
Log 3,5-dihydroxybenzamide	0.533	0.325	0.489	0.015	0.063	
Log toluene	0.381	0.990	0.230	0.379	0.030	Y
Log hexanal	0.174	0.854	0.580	0.163	0.959	Y
Log tetrachloroethylene	1.000	0.854	0.564	0.163	0.005	
Log ß-Methylhistamine	0.393	0.344	0.213	0.234	0.141	
Log dimethylsulfoxonium formylmethylide	0.112	0.618	0.289	0.049	0.943	
Log maleic anhydride	0.818	0.655	0.475	0.469	0.992	
Log ethylbenzene	0.518	0.338	0.249	0.756	0.781	Y
Log benzene, 1,3-dimethyl-	0.122	0.834	0.258	0.049	0.329	Y
Log phenylethyne	0.645	0.763	0.800	0.023	0.090	Y
Log oxime-, methoxy-phenyl-	0.299	0.854	0.310	0.020	0.009	

Log styrene	0.420	0.358	0.278	0.148	0.544	
Log heptanal	0.344	0.694	0.800	0.756	0.111	
Log methanesulfonylacetic acid	0.764	0.763	0.982	0.098	0.026	
Log tricyclo [2.2.1.0(2,6)]heptane, 1,3,3-trimethyl-	0.580	0.783	0.278	0.215	0.020	
Log benzaldehyde	0.549	0.393	0.299	0.642	0.673	
Log pentanoic acid	0.645	0.937	0.764	0.959	0.057	
Log 2-vinylfuran	0.278	0.145	0.166	0.215	0.147	
Log benzonitrile	0.333	0.121	0.072	0.326	0.221	
Log heptane, 2,2,4,6,6-pentamethyl-	0.084	0.093	0.053	0.063	0.028	Y
Log benzofuran	0.533	0.431	0.747	0.070	0.020	
Log decane	0.628	0.050	0.065	0.001	0.185	Y
Log octanal	0.645	0.478	0.645	0.679	0.043	
Log 3-Carene	0.092	0.958	0.518	0.044	0.614	N
Log o-Cymene	0.764	0.823	0.872	0.642	0.229	
Log d-Limonene	0.393	0.783	0.504	0.109	0.006	
Log benzenemethanamine, N,N-dimethyl-	0.289	0.462	0.289	0.569	0.734	
Log benzene, (methoxymethyl)-	0.433	0.416	0.356	0.278	0.318	Y
Log acetophenone	0.368	0.294	0.344	0.163	0.037	
Log benzene, 4-ethenyl-1,2-dimethyl-	0.533	0.237	0.222	0.679	0.943	
Log benzene, (bromomethyl)-	0.982	0.684	0.406	0.877	0.318	Y
Log nonanal	0.213	0.834	0.890	0.049	0.057	Y
Log benzoic acid	0.426	0.365	0.963	0.776	0.048	
Log 1-decen-3-one	0.420	0.823	0.205	0.918	0.478	
Log decanal	0.963	0.674	0.612	0.326	0.041	Y
Log phthalic anhydride	0.166	0.180	0.134	0.796	0.082	
Log tetradecane	0.854	0.288	0.356	0.352	0.178	

Log 5,9-undecadien-2-one, 6,10-dimethyl-, (E)-	0.368	0.906	0.433	0.196	0.006
Log phenylmaleic anhydride	0.153	0.160	0.213	0.918	0.045
Log benzophenone	0.368	0.264	0.166	0.535	0.012

p-values <0.05 are highlighted in grey

The data with background correction applied revealed three compounds which differed significantly (prior to the application of an FDR calculation) between pre- and post-positive mannitol challenge - isoprene; urea, ethyl-; and 5,9-undecadien-2-one, 6,10-dimethyl-, (E)-). In contrast, the uncorrected data revealed nine compounds; a list in which none of the above three feature. Of the nine compounds reported to significantly differ, five were excluded from the previous analysis due to background VOC exceeding exhaled VOC in more than 50% of samples. The remaining four compounds were not excluded from the previous analysis, but were not found to be significant when background correction was applied.

Comparisons A and B - comparing positive with negative challenges in both baseline and post-challenge samples – identified two compounds which differed significantly; dimethyl selenide and decane; neither of which featured significantly in the results of the background-corrected analyses.

None of the results from the uncorrected-data analyses were found to be significant after applying a Benjamini-Hochberg FDR calculation (threshold of 0.05).

Table 39 – ABC study results: pre vs post-challenge samples - statistically significant results using uncorrected data (without FDR)

Compound	Direction of change in response to challenge	Negative challenge
Trichloroethylene	Decrease	Decrease
3,5-dihydroxybenzamide	Increase	No significant change
Dimethylsulfoxonium formylmethylide	Increase	No significant change
Benzene, 1,3-dimethyl-	Decrease	No significant change
Phenylethyne	Increase	No significant change
Oxime-, methoxy-phenyl-	Increase	Increase
Decane	Decrease	No significant change
3-Carene	Decrease	No significant change
Nonanal	Decrease	No significant change

Table 40 – ABC study results: positive vs negative pre-challenge samples - statistically significant results using uncorrected data (without FDR)

Baseline positive vs baseline negative	
Dimethyl selenide	Lower in those who went on to have a positive challenge
Decane	Lower in those who went on to have a positive challenge

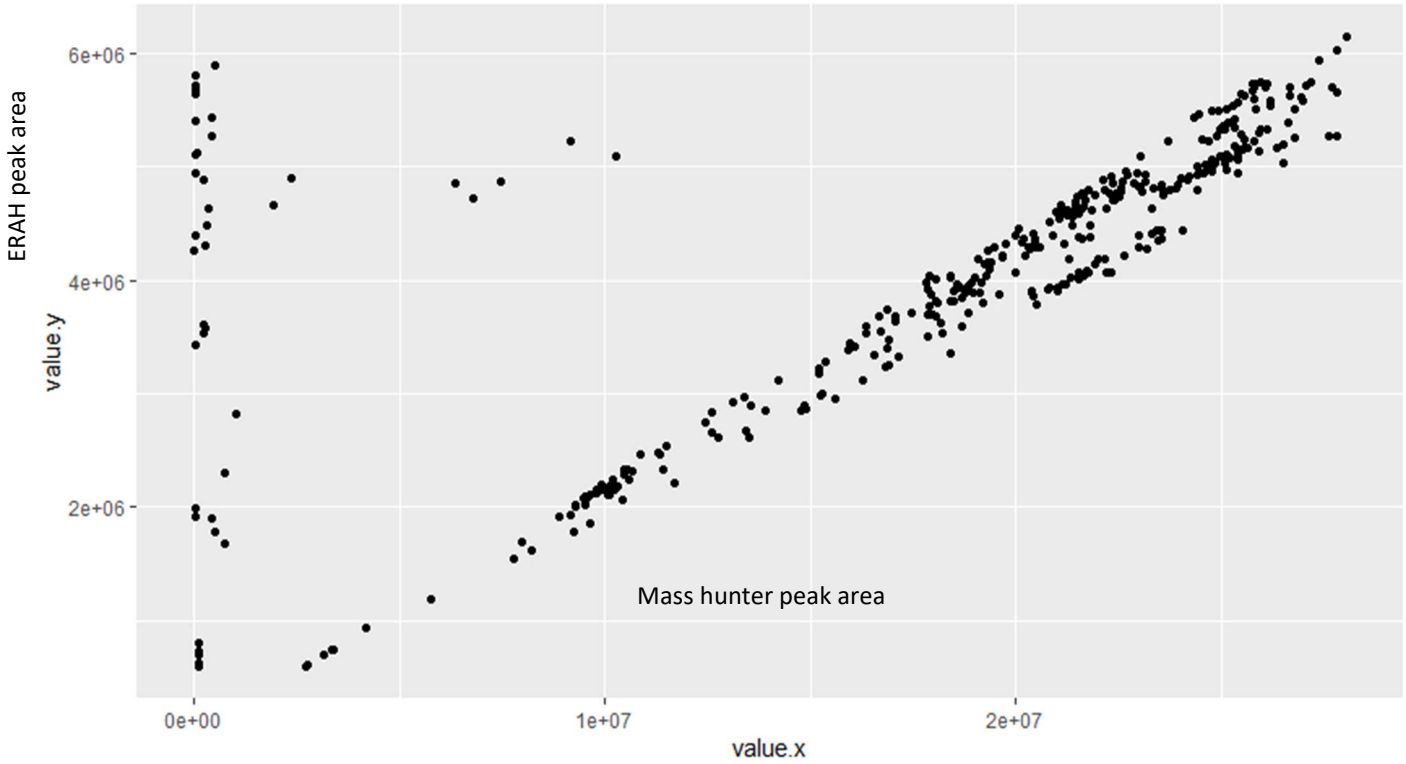
9.2.4.2 Logistic regression analysis

Logistic regression analysis was attempted for both pre- and post-challenge associations with challenge outcome however a model could not be fitted.

6.3 GC-MS results – untargeted approach

Untargeted deconvolution using the eRah package (283) within R Studio was conducted by MW at the MIB. The data proved problematic to deconvolve; integrations for the internal standard peak did not seem to accurately reflect what was present and there was a high likelihood of phantom peaks where background 'noise' had been integrated. A plot of the masshunter peak area (x axis) against the eRah peak area (y axis) (see figure 19) shows 40 samples for which the integration was poor. With further work the data may prove possible to deconvolve in a more reliable fashion however this has not proved possible within the timeframe of this thesis.

Figure 19 – ABC study: a correlation plot displaying problematic deconvolution of untargeted data. When examining the internal standard it is clear that for a number of samples there is a poor correlation between the relative abundance suggested by the mass hunter intensity count and that resulting from untargeted data deconvolution.



Key

The raw data used for the analysis was comprised of intensity counts with the area under the peak reflecting the relative abundance of the compound. The X-axis represents that generated by Mass Hunter, while the Y-axis represents that produced after untargeted data deconvolution using ERAH.

6.4 Power calculation

Powering future studies appropriately is of paramount importance. For this purpose acetone and isoprene were selected – two compounds identified both in this study and previously published research, and absent from the list of contaminants related to tygon tubing (see chapter 4). Using the compound intensity data normalised by the internal standard and sample volume, the mean and standard deviation for pre and post-challenge were calculated (see table 41).

Table 41 – ABC study results: Acetone & isoprene - intensity counts

		Acetone	*Isoprene*
Positive challenge samples	Baseline	0.0110 (mean) 0.0051 (SD)	0.0044 (mean) 0.0028 (SD)
	Post challenge	0.0116 (mean) 0.0044 (SD)	0.0046 (mean) 0.0022 (SD)
Negative challenge samples	Baseline	0.0128 (mean) 0.0063 (SD)	0.0062 (mean) 0.0037 (SD)
	Post challenge	0.0112 (mean) 0.0042 (SD)	0.0060 (mean) 0.0031 (SD)

These were used to conduct a power calculation for detecting a significant difference between positive and negative mannitol challenge samples at a threshold of 0.05 with 80% power (see table 42).

Table 42 – Power calculations based on acetone & isoprene

	Number of participants required	
	Acetone	Isoprene
Baseline samples	256	76
Post challenge samples	3,798	78

6.5 Discussion

The methods used in this study captured data on several VOC previously identified as significant in asthma breathomic studies, and indicators of sampling reliability were positive for the majority of VOC recorded. On initial inspection of distribution histograms, the majority of compounds were skewed and kurtosed despite log-transformation, however this is not uncommon in breathomic data. The following compounds appeared to have a bimodal distribution - dimethyl selenide; ammonium acetate; heptane, 2,2,4,6,6-pentamethyl-, and nonanal. While this could reflect differing levels of the compound in pre- and post-bronchial challenge samples none of these were found to be significantly different when paired analysis of samples was conducted. A total of twelve VOC were identified as differing significantly after a positive mannitol challenge. However, the VOC differed depending on whether the dataset used an alveolar gradient. This is not entirely surprising given that levels of exogenous VOC in the background samples were high; frequently rivalling or exceeding that found in patient samples. That this occurred despite using filtered air is surprising particularly given that common contaminants were excluded from the target compound list. The clean air filter was used as per manufacturer's instructions using tygon tubing to connect to the ReCIVA sampling device. It is worth noting that twelve of the contaminants thought to be related to the use of tygon tubing (see chapter 4) also feature in the target compound list for this study. However, high levels of tygon contaminants were not found at the higher flow rates used for this study; moreover, high VOC levels in filtered air were reported for the majority of the 58 target compounds and not limited to those 12 linked to tygon. It is therefore not thought likely that the tubing played a critical role in generating contaminants.

Numerous studies have examined VOC in exhaled breath and their potential as markers of respiratory disease; these studies have been the subject of several reviews (159, 171) the conclusions of which suggest metabolomic profiles have a high discriminative ability for asthma identification. The evidence regarding their ability to differentiate between disease control states is less clear (168), however, a recent longitudinal, medication withdrawal study by Brinkman et al (185) reported classification accuracies of 68% (GC-MS) and 95% (eNose) when comparing baseline breath profiles with loss of control. Despite powering the study for paired analyses based on prior research, and for PCA using a metabolomics sample size calculator - it is likely that the study was underpowered. Significant differences noted on univariate analysis were not sustained after the application of a FDR calculation.

Nevertheless, the results highlight those compounds which might be prioritised in future biomarker research - changing in response to a positive mannitol challenge but failing to exceed the threshold set when accounting for false discoveries. Powering future studies appropriately is of paramount importance; sample size based on the power calculation reported in section 6.4 is significantly higher than that reported in previous studies; unpaired analyses such as these require a greater sample size than paired analyses. While the results of the paired analyses highlight markers that could be prioritised in the search for biomarkers; the results of unpaired analyses are likely to be of greater clinical utility – for example the ability differentiate between VOC samples generated in the presence inflammatory mediators and those without. The reliability of this sample size calculation is compromised by the uncertainty regarding the size of difference in compounds due to the high degree of background contaminants.

Chapter 7 – Exhaled Breath Biomarkers in Acute Asthma: a Feasibility Study

7.1 Primary Objective – feasibility assessment

7.1.1 Study participation

7.1.1.1 Screening and recruitment

7.1.1.1.1 Outpatient arm

There were 1,095 independent screening episodes – in which paper or electronic notes were screened for suitability – of 764 patients; the difference being due to repeat or duplicate screening. 565 patients were excluded on screening electronic records. Information was sent or given to the remaining 199 patients. A further 23 were excluded after disclosures made in response to receiving information or on being approached in clinic.

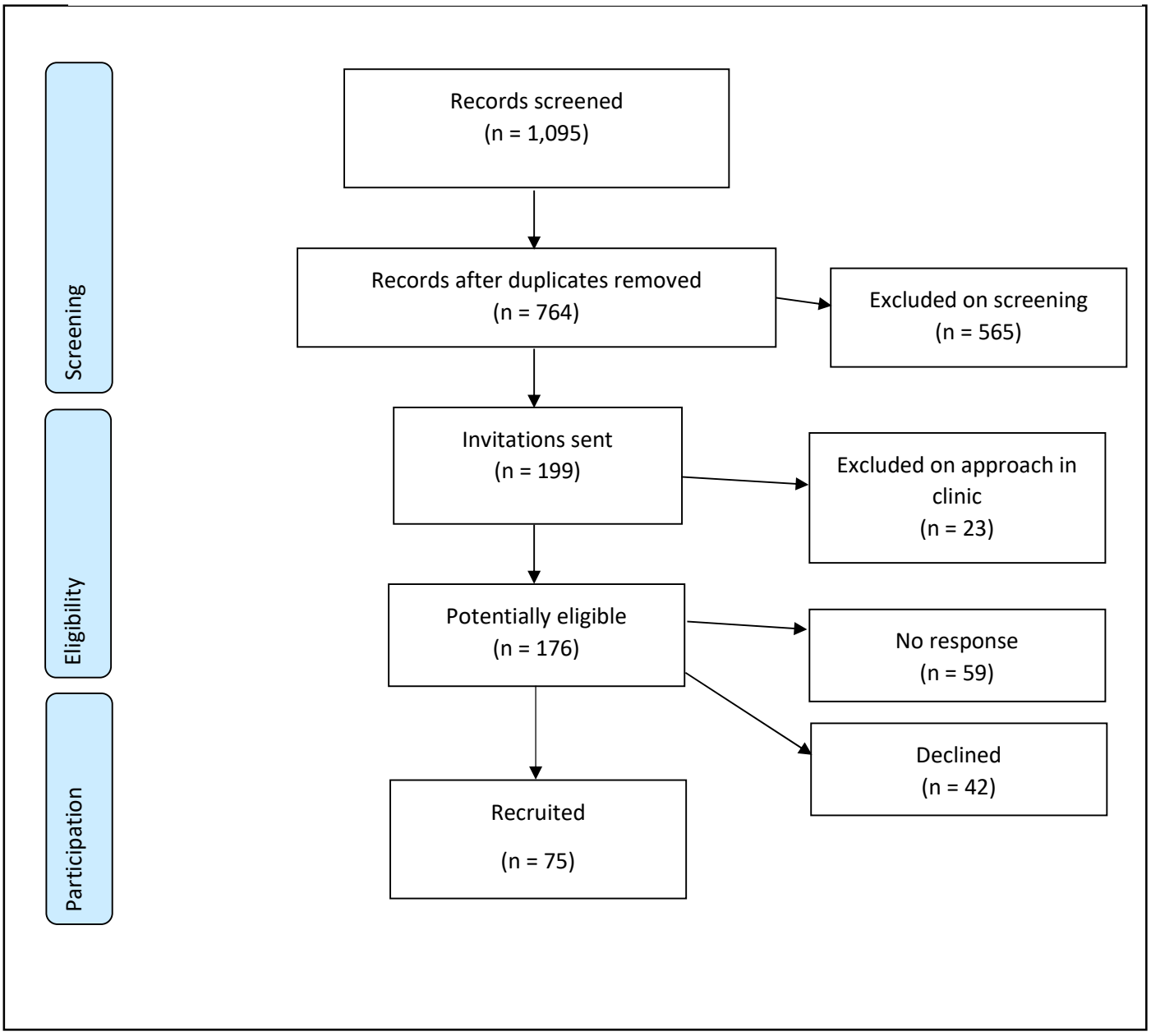
In total, 588 (77%) of the 764 screened patients were excluded. Of the 176 patients who were eligible or potentially eligible for inclusion, 59 were missed in clinic and did not otherwise respond to the invitation – either they did not attend their appointment, there was no available room in which to see the patient, the researcher was unable to attend the clinic or the researcher was with another patient. Of those who were invited to participate and who were seen in clinic 42 declined to participate, while 75 were recruited (64% of those who were approached (95% CI (55,77), and 43% of those who were potentially eligible (95% CI (36, 50)).

Reasons for exclusions were as follows: comorbidity (n=103); receiving maintenance oral corticosteroids (OCS) or biologic medication (n=37); smoker (n=58); greater than a 10 pack year smoking history (n=67); either diagnosis other than asthma or no diagnosis yet made (n=301); no asthma attack/exacerbation within the preceding 12 months (n=33); other (n=43). The category 'other' includes those for whom there were no clinic letters available to screen; those who were pregnant; under 18 years of age; non-English speaker; and those already approached via the acute arm of the study. The number of reasons for exclusion totals more than the 588 persons excluded as many patients met more than one exclusion criteria.

Recruitment rate

During the period in which the researcher was working full time on the project and recruitment was open (1st March 2017 to the 3rd November 2017) 64 participants were recruited; this represents approximately seven participants per month, or 7.2 recruits per 28 days.

Figure 20 - ABBA study screening and recruitment diagram (outpatient arm). The screening of 764 patient records resulted 75 participants recruited.



7.1.1.1.2 Acute arm

Acute screening – there were 192 patients screened a total of 205 times; 11 were recruited into the acute study arm. Of the 192 who were screened, 138 (72%) were excluded while 54 (28%) were potentially eligible. Eleven were recruited (6% of those screened); 12 declined, of whom two subsequently joined the outpatient study arm.

Of the 138 patients who were excluded, 56 were excluded due to a comorbidity; 13 were on maintenance oral corticosteroids or a biologic medication; 24 were current smokers; 16 were ex-smokers with a history in excess of 10 pack years; 31 did not have asthma, or had asthma but were not presenting with an asthma attack; 15 were 'other'. The 'other' category included inability to offer informed consent, pregnancy, being in critical care, living out of area (in the region on holiday), and/or being distressed.

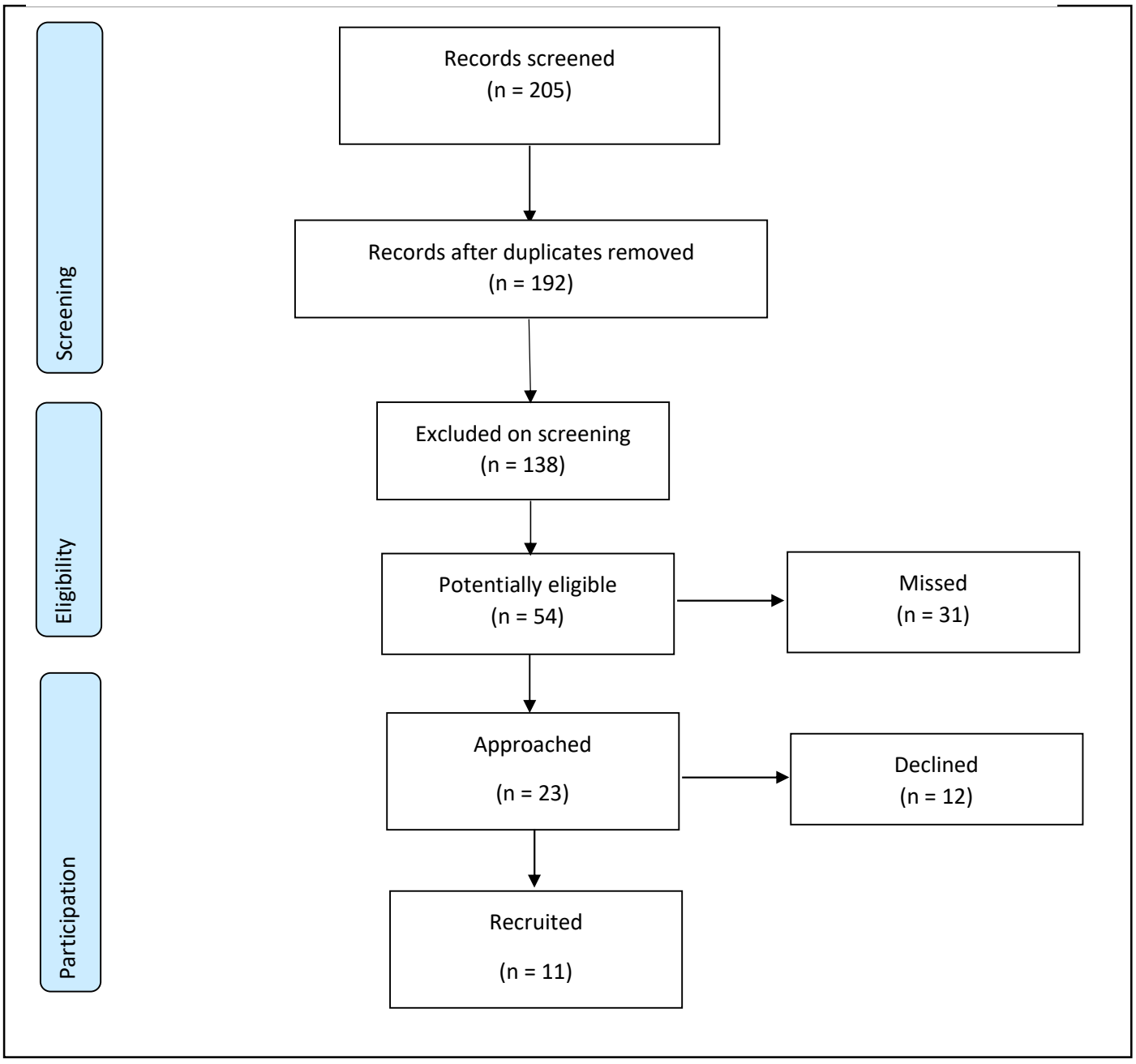
Of the 54 potentially eligible patients, 31 (57%) were 'missed' or not approached for the study; these participants were discharged prior to being approached, or were either identified outside of the 24 hours-of-presentation period or with insufficient time for them to consider participation and complete the assessment process within the 24 hour period. Other reasons included the researcher being occupied recruiting another participant or the patient being too poorly to approach when first identified and either remaining too poorly to approach or being improved and discharged before being re-approached.

Of the 23 patients who were potentially eligible and approached, 11 (48%) agreed to participate.

Recruitment rate

During the period in which the researcher was working full time on the project and recruitment was open (1st March 2017 to the 3rd November 2017) eight participants were recruited; this represents a recruitment rate of one per month.

Figure 21 - ABBA study screening and recruitment diagram (acute arm). The screening of 192 patient records resulted in 11 participants recruited.



Accident and Emergency

An audit of patients presenting to A&E between the 6th March 2016 and the 5th October 2017 was conducted identifying those for whom 'asthma attack' was listed as a possible, probable or confirmed diagnosis. There were a total of 1,521 patient presentations during this period (including paediatric cases); the number of patients – as opposed to presentations - may have been lower than this figure due to repeat presentations. Difficulty breathing was the most commonly recorded presenting complaint to which an asthma diagnosis was attached, however, others listed included the following - unwell, abdominal pain, unspecified chest pain, collapse, smoke inhalation, re-attendance, requesting prescription, inhalation of chemical, chest pain likely to be cardiac, sepsis, injury to face, unspecified bleeding, injury to elbow, accidental ingestion of tablets, back pain, injury to ribs, cardiac arrest, rash, request to see doctor, painful lower leg, headache, personal problem, post-operative problem, and pleuritic chest pain.

899 (59%) of these patients were discharged home; 10 went to critical care; 4 did not wait; 219 (14%) were paediatric patients; 323 (21%) were transferred to AMU; 16 were admitted directly onto respiratory wards.

Acute Medical Unit

An audit of patients presenting to the acute medical unit between 6th March 2016 and the 5th October 2017 revealed 459 patients for whom 'asthma' was listed as the presenting complaint. Of these 459 patients, 342 were admitted, while 116 were discharged home. Of those admitted, two went to critical care and 200 were admitted onto respiratory wards.

Acute screening considerations

Taking into account those patients admitted from A&E onto AMU, the two audits identify a total of 1,657 patients presenting to secondary care in the above time frame. Of these, 1,015 (61%) were discharged home. As can be seen by the variety of 'presenting complaint' labels with which asthma patients were initially recorded on the acute care IT systems, it is difficult to identify acute asthma exacerbations within the hospital on presentation. A diagnosis was typically added at the point of admission/discharge, this meant that for 1,015 potential patients, by the time an asthma diagnosis appeared on the system they were in the process of being discharged and leaving the hospital. The alternative would be to screen every patient presenting with 'difficulty breathing' as their presenting complaint, this would necessitate an almost constant presence in the acute hospital departments; while also resulting in a huge number of unsuitable patients (for example breathing difficulties due to other lung disease, infection or cardiac causes).

Furthermore it would not identify all asthma patients as many other alternative presenting complaints were associated with a later asthma diagnosis. It was not possible for the researcher to take this approach, however, for a future study with sufficient staffing and resources this might be a fruitful – if inefficient - method of recruiting.

7.1.1.2 Consent

7.1.1.2.1 Acute arm

Of the 11 participants, three (27%) had questions about the study after reading the participant information sheet. These were 1) whether it was compulsory to continue on in the outpatient arm of the study after completing the inpatient arm; 2) whether the blood test was compulsory (this question was asked by two patients); and 3) whether there was any flexibility regarding the date/time of the follow-up study appointment.

All participants agreed that they had been given sufficient time in which to contemplate study participation. Time taken to consider participation and complete the consent process was a mean 98 minutes (median 72).

7.1.1.2.2 Outpatient arm

Of the 75 participants recruited at stage 2 (in the outpatient clinic), 11 (15%) had questions about participation.

Questions were as follows:

- The amount of time and number of visits involved
- Whether a blood test is absolutely necessary for participation
- How long the study lasts for
- How much and what sort of information study participants will receive on completion of the study and publication of results
- Further detail on the anonymization process and measures to ensure confidentiality
- Whether it is permissible – in the event of an asthma attack - to take antibiotics or steroids prior to undertaking the study assessment.
- Whether infective exacerbations of asthma were included in the study definition of asthma attack
- Whether the hospital / GP would be informed if the experimental breath tests (VOC, EBC) revealed anything of interest or unusual
- More information on the aims of the study and what we hope to discover

The proportion of participants asking questions was lower than that in the acute arm; the reasons for which may be speculated upon.

The participants in the outpatient arm commonly had the information posted to them in advance of their clinic appointment allowing a greater time to read and digest the material; however, it was the researchers impression that outpatient recruits were not necessarily better informed; during the consent process it was frequently necessary to discuss the study in some detail again. This impression is supported by the fact that many of the questions were already covered in the participant information sheet. Those questions above which were not covered by the PIS would warrant inclusion in the PIS of a future study.

The average time to complete consent and recruitment in the outpatient arm was a mean 19 minutes (median 14).

7.1.1.2.3 Focus group

Four participants were recruited to a focus group in which the study methods were discussed in detail; three were recruited from the outpatient arm and one from the acute. All those from the outpatient arm had experienced an asthma attack, contacted the researcher and attended a study assessment.

Initially it had been planned to undertake thematic analysis of the focus group data, however, despite invitations being sent to all study participants there were only four who were willing and able to attend on the dates / times available. Thematic analysis of such a small group was deemed inappropriate; instead a combination of summary and vignettes is used.

Focus group participants were asked how they felt about being approached in the two different settings (acute and outpatient), whether they thought it was appropriate and whether they felt that they'd been put on the spot by being asked. Responses were:

- P1 - *No not at all*
- P2 - *No*
- P3 - *No. I was a bit intrigued about why you* [the patient] *were picked… what knowledge was gone before… somebody approached you.*
- P4 - *Well I just thought that was part of it* [usual clinical care] *to begin with until you said about it* [research project]

P3's comment suggests uncertainty about how participants are identified and the extent to which their medical records are examined. The letter of invitation to the study came from a respiratory consultant at the participant's hospital, however, it may have been a name with which they were unfamiliar.

In the participant information sheet was a section entitled 'why have I been chosen' in which it was explained that those with asthma who had experienced an exacerbation within the previous 12 months were being invited. However, it could be made clear during the consent process that – outside of their asthma status - their general medical history had not been scrutinised.

Next, the focus group were asked if they felt they had been given sufficient information on which to base a decision about whether to participate. As part of the consent process participants were asked if they felt that they'd had sufficient time to consider participation. However, asking this question of the focus group participants allowed them to answer this question after completing the assessments and having had time to reflect.

- P1 - *Yes definitely*
- P2 - *I think people who have got asthma quite badly could always be keen to do something that will help, improve don't you? you're so used to it and it goes on but any little thing that looks as if it's going to improve life, go for it.*
- P3 - *Yes*
- P4 - *Yes*

Participants were also asked whether they thought it was appropriate being approached and asked to do undertake breath sampling while having an acute asthma attack.

- P1- *Yes, true readings isn't it?* (P2 - *yes,* P4 – *yes*)
- P2 - *I thought it was very interesting; makes you think*
- P4 - *Can't do it unless you're ill can you? the whole point of it is to do it then and you were quite, it was quite plain that should it cause you discomfort or you found all of a sudden you could not do it that you could stop; it wasn't that you've got to have this on you know; and you said how much more you've got to put up with it.*

7.1.1.3 Study participation

7.1.1.3.1 Acute arm

Assessment of acute exacerbation while an inpatient - time from completion-of-consent to completion-of-assessment - took a mean 137 minutes (median 116). Follow-up controlled assessments - undertaken as an outpatient - took a mean 59 minutes (median 60). This information may inform the participant information sheet for any future study.

However, this time does not include that required for the researcher to process samples (including centrifuging blood samples and freezing samples) or to clean and tidy facilities and equipment. These would need to be factored in when planning staffing and resources.

Time between exacerbation and follow-up assessment was a mean 65 days (median 28). Seven out of the eight patients had their follow-up within 6 weeks of their exacerbation; with the earliest follow-up being just 15 days after their exacerbation (this participant was still uncontrolled at the time of their outpatient appointment and so was invited to return for a further, stage 3, study-specific assessment appointment).

One of the 11 participants recruited at stage one withdrew their consent, declining follow-up. All ten of the remaining participants attended their follow-up outpatient appointment in the respiratory clinic. However, two had to be excluded - one was put forward for a biologic while the other was diagnosed with a comorbidity. Of the remaining eight participants who completed a follow-up, five reported feeling back to 'normal' but only three had an ACQ score of less than 1.25 (which would indicate good control). This does throw into doubt the feasibility of using the routine post-admission outpatient appointment as an opportunity for obtaining a controlled follow-up sample. It was planned that - for those not controlled at their follow-up appointment - a second study-specific follow-up would be undertaken once they had regained disease control. This was not possible for the five participants who remained uncontrolled at their outpatient appointment; reasons were as follows – diagnosis of a comorbidity; failure to return any contact; commencement of a biologic; and did not wish to re-attend solely for a study visit. It was possible to arrange an additional follow-up assessment for one participant but their ACQ score remained greater than 1.25.

Nine (82%) of the 11 acute recruits agreed to continue in the outpatient arm of the study. Two patients who declined participation when approached at stage one indicated that they would be happy to consider participation in the outpatient arm; these were approached in outpatient clinic and consented to participation.

Prior to their exacerbation six of the participants were being treated at level-4 of the BTS treatment guidelines, four at level-3, and one at level-1. All but one participant had experienced an exacerbation in the previous 12 months; with the mean number being six prior exacerbations (median four).

Based on the earliest peak expiratory flow recorded in the hospital notes, two participants were in the 'life-threatening' category when they first presented to the hospital (due to a PEF of less than 33% predicted). Four were severe (33-50%) and five were moderate (PEF 50-75%). See table 43.

Table 43 – ABBA study: exacerbation severity (inpatient arm)

Severity of attack on initial presentation	Number of participants
Moderate	5
Severe	4
Life-threatening	2

7.1.1.3.2 Outpatient arm

There were 84 participants in total – 75 participants recruited in outpatient clinics, and nine recruited from the acute study arm.

Post-study questionnaires were sent to 84 participants, 20 (24%) were received back, of which 14 (70%) reported having experienced an exacerbation during the course of the study (two (14%) of those reporting an exacerbation in their exit questionnaire had attended a study assessment).

Including returned questionnaires and participant contact during the course of the study, exacerbations were reported by 34 participants in the outpatient arm. The total number of exacerbations reported was 82; this figure was contributed to by one outlier who reported 26 attacks; the mean number of attacks reported to have occurred during the study duration was two (median one).

Outpatient exacerbation assessment was undertaken in 18 participants, this was 21% of the 84 participants, and 53% of those participants who reported an exacerbation. The researcher was unable to assess while still symptomatic four patients who made contact; it was not possible to offer a convenient appointment in a sufficiently timely manner. The other exacerbations which were not assessed were those reported after the event by way of the 3-monthly follow-up enquiry letter or by completing the exit questionnaire.

Method of contact for those 18 participants for whom it was possible to arrange an assessment was as follows: email (n=3); telephone (n=12); exacerbation noted at outpatient clinic visit (n=1); hospital admission noted on symphony (n=2); and in one case a follow-up visit for a stage 1 acute exacerbation was used as outpatient exacerbation due to the participant remaining uncontrolled (n=1).

On two occasions the participant did not contact us but they were detected when screening acute asthma admissions for the acute study arm; when approached these participants were happy to participate. One participant underwent acute assessment twice (for two separate exacerbations).

Of the 14 participants reporting an exacerbation in their exit questionnaire, seven stated that they'd managed the exacerbation themselves, three that they managed it via primary care only, two that they'd managed it via secondary care only; and four that they'd been managed by both primary and secondary care. Of the 20 questionnaires returned, none reported starting a biologic but two reported having commenced maintenance oral corticosteroids. None reported a change in their asthma diagnosis. Eighteen reported returning to baseline or obtaining full control at some point during the study.

The range of severity of exacerbations amongst those who attended for assessment in the outpatient arm ranged from mild/early to severe (see table 44). The severe attacks were from those patients noted on acute screening (n=2) or under management at asthma outpatient clinic appointment (n=1). The other two had a respiratory rate of 25 (meeting the criteria for a severe asthma attack) but had PEF rates of greater 90% predicted and no other features of severe attack.

Table 44 – ABBA study: exacerbation severity (outpatient arm)

Severity of asthma attack	**Number of participants**
Mild / early (PEF > 75% predicted, respiratory rate < 25)	6
Moderate	7
Severe	5
Life-threatening	0

Participants in the outpatient arm reported their exacerbation to have started a median 8 days prior to assessment, with symptoms peaking 4 days prior to assessment (both mean and median 4). Eight participants were receiving oral steroids at the time of assessment.

Of the 18 exacerbations captured from the 17 participants who were recruited in the outpatient arm the time between exacerbation and follow-up assessment was a median 67 days. All but one of the follow-up assessments were conducted within 6 weeks of the exacerbation assessment.

7.1.1.3.3 Acute and outpatient arms

Table 45 – ABBA study: patient characteristics - Inpatient vs outpatient arm

		Acute arm	**Outpatient arm**
Days between exacerbation and follow-up assessment	Median (IQR)	28 (88.8)	65 (76)
Participants controlled (ACQ <1.25) at follow-up	n (%)	3 (38%)	5 (36%)
Days between onset of symptoms and exacerbation assessment	Mean (± SD) Median (IQR)	10.2 (± 8.3) 7 (11)	24.3 (± 27.8) 8 (40)
Receiving OCS at time of assessment	n (%)	8 (100%) [data missing on three]	9 (64%) [data missing on five]
Severity of exacerbation Mild-Mod-Severe-Life threatening	n (%)	0-5-4-2 (0-45-36-18%)	6-8-5-0 (32-42-26-0%)

In the outpatient arm, all but one of the 14 participants who attended follow-up assessment reported feeling back to normal again, but only five (36%) gained a score on the ACQ which would indicate good control (< 1.25).

Table 46 – ABBA study: significant differences at follow-up (acute arm)

Measure	Exacerbation assessment	Follow-up assessment	Paired samples T-test	Wilcoxon Signed-Ranks Test
ACQ7 Mean (±SD)	4.4 (± 0.4)	1.8 (± 1.2)	P = 0.001	
PEF Mean (±SD)	273.8 (± 52.3)	375.8 (± 165.3)	P = 0.079	
PEF (% Predicted) Median (IQR)	63.0 (20.1)	72.5 (26.9)		P = 0.017
FeNO Median (IQR)	48 (58.5)	25 (28.0)		P = 0.237
Respiratory rate Mean (±SD)	19.2 (± 4.4)	16.4 (± 3.8)	P = 0.021	
Heart rate Median (IQR)	78 (17)	73 (13)		P = 0.017
SPO2 Median (IQR)	96 (2)	98 (2.8)		P = 0.202

There was a significant difference in ACQ score, PEF percent predicted, respiratory rate, and heart rate between acute and follow-up assessments. While there appears to be a difference between acute and follow-up FeNO scores this did not reach significance; the mean difference between the two visits was 31ppb but the 95% confidence interval for this figure was -20 to +82. Similarly, the mean difference in PEF between the two visits was -102 but the 95% confidence interval for this figure was -219 to +15.

Table 47 – ABBA study: significant differences at follow-up (outpatient arm)

Measure	Exacerbation assessment	Follow-up assessment	Paired samples *t*-test	Wilcoxon Signed-Ranks Test
ACQ7 Mean (±SD)	3.4 (± 1.0)	1.3 (± 0.9)	P = 0.000	
PEF Mean (±SD)	276.7 (± 87.3)	348.3 (± 91.3)	P = 0.000	
PEF (% Predicted) Median (IQR)	75 (30.3)	94 (9)		P = 0.001
FeNO Median (IQR)	28 (44.5)	21 (6)		P = 0.207
Respiratory rate Median (IQR)	17 (10)	17 (4)		P = 0.293
Heart rate Mean (±SD)	90.1 (± 10.2)	84.3 (± 13.3)	P = 0.06	
SPO2 Median (IQR)	98 (3)	97 (2)		P = 0.887

As in the acute arm, there was a significant difference in ACQ score and PEF percent predicted between the acute / exacerbated state and the follow-up assessment. Like the acute arm there was no significant difference between the two assessments in FeNO. In contrast to the acute arm, there was no significant difference in respiratory rate or heart rate; this may be a reflection of the less severe nature of the exacerbations being captured. Unlike the acute arm, there was a significant difference in the raw PEF rate between the two assessments.

A more flexible follow-up schedule - not tied to the post-admission clinic appointment - might lead to a greater number of samples from participants in a controlled state. However, this did not prove to be the case in the outpatient arm of which only five scored under 1.25 on the ACQ at follow-up. That the majority reported a return to full control scored despite this score throws some doubt on the ability to use the results of the exit questionnaire as an indicator of the number of participants from whom it would - theoretically - be possible to obtain a controlled sample.

In the acute arm two participants became ineligible and were excluded from follow-up (one commenced a biologic while the other was diagnosed with a comorbidity). This compared with 13 participants in the outpatient arm. Of these 13, nine had not contacted us for an exacerbation or assessment; three completed both an exacerbation assessment and follow-up before becoming ineligible; and one completed an exacerbation assessment but was excluded before follow-up.

7.1.1.3.4 Adverse Events

Only one study-related adverse event occurred. This was in the acute study arm during assessment of an exacerbation. While using the Niox Vero to undertake a measurement of FeNO the participant developed a cough which necessitated the use of their salbutamol inhaler. The participant was using their inhaler frequently for symptomatic relief; the causality with the study assessment was not clearly established but nonetheless was reported as an adverse event in the study end report to the study site (NNUH) and REC. The cough rapidly settled and the participant was comfortable for the remainder of the study visit. This was documented in the patient's hospital notes, on their study CRF and no further attempts at obtaining a FeNO result were undertaken.

7.1.1.4 Sampling

Across both acute and outpatient arms of the study there were 86 participants, from whom 30 acute asthma exacerbations were sampled. These 30 exacerbations were captured from 27 study participants (35%, 95% CI (26, 45)); three participants provided samples during two separate exacerbations; 11 exacerbations were captured in the acute arm of the study and 19 in the outpatient arm of the study.

Follow-up samples were obtained for 20 of the 27 participants; reasons for loss to follow-up were:

- Development of exclusion criteria; this affected three participants (one commenced a biologic medication, while two were diagnosed with a comorbidity)
- Three participants dropped out (one withdrew of consent, one failed to return any contact, while a third did not attend appointments)
- Insufficient time to complete follow-up before study end

94% of participants managed to complete all three breath assessments (ReCVA, Niox Vero and RTube) at each assessment. All participants, irrespective of the severity of their asthma attack were able to provide an exhaled breath sample using the ReCIVA. During an attack 90% of participants managed the Niox Vero, compared with 100% when assessed in a more controlled state at follow-up. Sampling issues were as follows:

7.1.1.4.1 Acute arm

RTube – Five of those who attempted a sample needed to remove the nose clip before completing the test. One participant was not able to provide a sample of EBC; they were unable to tolerate breathing through the tube.

At the follow-up assessment all participants were able to complete the test but one attempt yielded no condensate (query manufacturing fault with tube).

Niox Vero – all participants were able to complete a least one successful attempt on the Niox Vero bar one; this patient was unable generate sufficient exhalatory effort to complete the test. The aim was to undertake three readings in order to assess reliability within the acute setting. Two patients took six and seven attempts in the course of their assessment and stopped before managing to obtain all three readings. At follow-up all participants managed to complete the test (with the exception of one participant for whom the test was not attempted due to practical / resourcing reasons).

7.1.1.4.2 Outpatient arm

RTube – all participants completed the test when attempted during their exacerbation, although eight (27%) had to remove their nose clips during the test. In two cases there was very little sample collected (one was too small to pipette). There was also an equipment failure with one tube – a sample had been collected but leaked during the harvesting process. At follow-up all participants completed the test and only three (15%) had to remove their nose clips part way through.

Niox Vero – During asthma exacerbation, two patients were unable to complete the test. One was unable to sustain their exhalation for the sufficient length of time, the other was already familiar with the test and declined to attempt it such was her certainty that she would not be able to manage it. At follow-up all completed the test successfully.

Table 48 – ABBA study: sampling method success

SAMPLING METHOD	ACUTE AND OUTPATIENT ARMS COMBINED: NUMBER OF PARTICIPANTS PROVIDING A SAMPLE	
	Acute exacerbations n = 30	Follow-up n = 20
VOC SAMPLE	30 (100%)	20 (100%)
EBC SAMPLE	27 (90%)	19 (95%)
FENO RESULT	27 (90%)	19 (95%)
SPUTUM SAMPLE	11 (37%)	3 (15%)
SERUM SAMPLE	22 (73%)	16 (80%)
FBC	26 (87%)	15 (75%)

A serum sample was obtained in 73% of exacerbations. Reasons for a lack of serum sample and absence of FBC include failed phlebotomy attempts, needle phobia or patient declining this aspect of study participation (for example due to having had multiple blood tests or cannula insertion as part of their standard clinical care). The figure for FBC is slightly higher than that for serum due to those participants who had phlebotomy as part of their standard care but declined further phlebotomy for the study.

Sputum samples were obtained at only 37% of exacerbation assessments and 15% of follow-ups. A low yield of spontaneously expectorated sputum samples at the follow-up visits is not entirely surprising given that participants' asthma was largely controlled at this point. Reasons for a lack of sample in the exacerbated state included an inability to produce a sample but also patients declining to attempt active cycle breathing techniques in pursuit of a sample.

7.1.1.4.3 Sampling considerations

Sputum and the metabolomics of respiratory infection

One potential application of exhaled breath metabolomics in asthma might be the identification of infectious exacerbations; in particular identifying those with a bacterial infection whom antibiotics would benefit. Based on the current study design this would be challenging and would require large participant numbers in order to generate sufficient sample numbers. There are a number of modifications which might address this. Yield at the follow-up visit might be increased by undertaking induced sputum sampling. This method requires the inhalation of nebulised saline to provoke cough and increase mucous clearance. This could be undertaken for those participants who were successful in producing a sample during the acute stage. However, it would be necessary to first ascertain whether spontaneous sputum samples differ from induced sputum samples in terms of identification of infection, and also whether the induced sputum process has any effect upon exhaled breath VOC. A study looking at infection would not necessarily need to be longitudinal; a cross-sectional design might be more appropriate in which case the question would be how to increase the number of sputum samples in the acute stage. One option would be to use induced sputum sampling during asthma attack, this could be done only for those who were not able to provide a sample (in which case differences between induced and spontaneous sputum would need to be taken into account in addition to the effect – if any – upon exhaled VOC). Undertaking induced sputum sampling during asthma exacerbation may be challenging; patient recruitment might be negatively impacted; and it may not be appropriate for those experiencing a more severe asthma attack.

It may be that with multiple breath assessments patient effort was diluted and participants were less motivated to provide a sputum sample. If the study was designed solely with infection in mind and the participant understood this from the patient information sheet and consent process a larger number of participants might be willing to go through the active cycle breathing techniques and provide maximum effort in attempting to provide such a sample. That this would increase the percentage of participants providing a sputum sample is speculation.

Exhaled breath condensate

The European Asthma research and Innovation Partnership (EARIP) reached similar conclusions to those in chapter 2 of this thesis; they state that although EBC methods have the potential for use in asthma diagnosis / monitoring there needs to be increased standardisation in both sample collection and analysis (101). While the EBC analysis of 8-isoprostane has not proceeded, other potential markers could be sought. Moreover, a metabolomics approach such as that which is being applied to the exhaled breath gases could also be applied to EBC (or serum) by way of LC-MS; such analyses have differentiated between samples from healthy controls and those with asthma (101). Ibrahim et al used EBC as a biomedium for a metabolomics analysis; their feasibility study suggests it would be possible to collect both simultaneously for use in a systems biology approach, to study the whole of the exhaled breath metabolome (both volatile and non-volatile) (354).

Table 49 – ABBA study: sampling success rates

		ALL ASSESSMENTS (N=50)	EXACERBATION ASSESSMENT (N=30)	CONTROLLED / FOLLOW-UP ASSESSMENT (N=20)	ACUTE ARM (N=19)	OUTPATIENT ARM (N=33)
EBC	Sample successfully collected N (%)	48 (96%)	27 (90%)	19 (95%)	17 (89%)	31 (94%)
	Volume collected (ml) Mean (± SD)	1.1 (0.3)	1.1 (0.4)	1.1 (0.3)	1.1 (0.3)	1.1 (0.3)
	Time to freezer (minutes) Mean (± SD)	49 (16)	47 (18)	51 (14)	46 (15)	51 (17)
RECIVA	Sampling time, minutes & seconds Mean (± SD)	6m58s (102s)	7m4s (105)	6m51s (100)	7m38s (94s)	6m36s (101)
NIOX VERO	Time taken for first successful attempt (minutes) Mean (± SD)	3.0 (2.1)	3.5 (2.3)	2.1 (1.4)	3.6 (2.6)	2.6 (1.5)
	No. of attempts required Mean (± SD)	2 (1.5)	2.3 (1.7)	1.9 (1.1)	2.4 (1.6)	2.1 (1.4)

The RTube collection period was a standardised 10 minutes; it took a mean seven minutes to obtain a breath VOC sample of 500ml using the ReCIVA; while it took a mean three minutes and two attempts to obtain a successful reading using the Niox Vero.

It took a mean 49 minutes to get EBC samples into the -80°C freezer. This time was comprised of the remainder of the study assessment; saying goodbye to the participant; completing paperwork; cleaning the room / equipment; preparing it for transport; and transporting the sample to the freezer room. This time would likely be reduced if a future study was taking fewer other samples or could be reduced to approximately 5 minutes were additional staff available for the transport and processing of samples. Based on the current processes, selection of a target analyte for EBC sample analysis would have to take into account the sampling-to-freezing time; a time during which biochemical processes would continue (for example oxidisation). Similarly, if the samples from this study are to be analysed the analyte would need to be stable in storage for the period which has elapsed since the first samples were collected.

7.1.1.5 Data extraction

Data extraction relating to PEF during admissions was frequently incomplete. It was hoped that it would be possible to use inpatient peak flow charts to track when the patient achieved 75% of predicted or previous best PEF. This could potentially be used in an analysis of VOC or EBC marker; looking for a correlation between an exhaled breath compound and the length of time required to regain lung function. However, it was frequently difficult to find peak flow charts in the patient notes and when present these tended not to be completed with a frequency that would permit identification of this moment with any accuracy. Data extraction conducted retrospectively was frequently challenging due to the size, complexity and organisation of some patient records. If the study were to be repeated, even though it is frequently difficult (due to time, demands on the paperwork from other professionals, and a lack of physical resources such as desk space) it would be prudent to undertake the fullest possible data extraction at the initial visit, and return to extract data from the notes over the course of the participant's admission rather than relying on requesting notes post-discharge.

7.1.1.6 Sample processing

7.1.1.6.1 Exhaled breath condensate

The planned analysis of EBC samples from the ABBA study was not conducted due to the equivocal findings of the systematic review of EBC 8-isoprostane (see chapter 2) and the inability to detect 8-isoprostane using a commercially available ELISA kit (see chapter 4); a result which the review findings suggest is perhaps not uncommon. The samples remain in storage at -80°C pending the establishment of a reliable and sensitive method of 8-isoprostane detection or selection of alternative biomarker. Unless it were to be established that there is little inter-and intra-individual variation in EBC dilution a method of sample dilution calculation is required.

Whether sample conductivity can be used will depend on the stability of the marker of choice when the sample is subjected to lyophilisation. An alternative method of sample dilution calculation (such as serum albumin) could be used as the majority of participants have contemporaneous blood results. Given the duration of storage, the marker of choice would need to be stable for extended duration at -80^{o}C.

7.1.1.6.2 Volatile organic compounds

Isoprene is ubiquitous in exhaled breath samples and was used as an indicator of successful sampling. Similarly it was anticipated that breath humidity would result in water retention; the presence of water was therefore used as another indicator of successful sampling (with a threshold of 1.0mg used). Mean water retention was 2.7mg (SD 1.45mg).

Three tubes were not effectively dry purged and retained too much water for successful GC-MS analysis. Eleven samples had water quantities < 1.0mg; while seven samples were associated with low or absent levels of isoprene. Three of the seven had both low isoprene and low water retention levels. These levels may be a reflection of a wide distribution of isoprene concentration and water retention across the data set, alternatively they may indicate an issue with sampling. Table 50 presents those samples which failed to meet the above thresholds. As a percentage of the total patient samples taken these make up 15%.

Table 50 – ABBA study: sampling and analysis issues

Participant identification number and sampling stage		*Sampling comments*
Water retention < 1.0mg	*Low or absent isoprene levels*	
PIN 28 Acute exacerbation		No issues noted at time of sampling. No device data available
PIN 28 Acute exacerbation		
PIN 17 Acute exacerbation	PIN 17 Acute exacerbation	No issues noted at time of sampling Device data suggests sampling successful
	PIN 17 Acute exacerbation	
	PID 36 Acute exacerbation	No issues noted at time of sampling Device data suggests sampling successful
PIN 36 Acute exacerbation	PID 36 Acute exacerbation	
PIN 60 Acute exacerbation		No issues noted at time of sampling Device data suggests sampling successful
PIN 60 Acute exacerbation		
	PIN 17 Controlled follow-up	Patient had low isoprene levels in their previous visit; this is a consistent result across study visits. No comments made by researcher but device data shows sample volume of just 220ml.
	PIN 17 Controlled follow-up	
PIN 56 Controlled follow-up	PIN 56 Controlled follow-up	No issues noted at time of sampling Device data suggests sampling successful
PIN 68 Acute exacerbation		No issues noted at time of sampling Device data suggests sampling successful
PIN 73 Controlled follow-up		No issues noted at time of sampling Device data suggests sampling successful

PIN 53 Acute exacerbation		Only 20% of the sample volume / 180ml collected in the 10 minute sampling window. Reason unclear - fit of mask and head strap adjusted (good seal achieved); slower breathing encouraged in order to improve tracking and sample gating. Patient was bearded. There is a mis-match in respiratory rate with the researcher recording 24-30 and the device data suggesting 12.
PIN 80 Acute exacerbation		Sample capture was slow, took the full 10 minute sampling window. The respiratory rate as recorded by the researcher was 24 breath per minute and shallow. In contrast to this, device data reports a respiratory rate of 13 and a 540ml sample taken.

When undertaking sampling the ReCIVA allows parameters such as sampling volume and collection rate to be modified, it then reports when the specified sample volume has been collected and the time taken to do so. All other data recorded by the ReCIVA during sampling is saved in a proprietary format and is not displayed by the user interface on the laptop. MW from the MIB developed a programme using R to read these files. While the use of such a programme might have alerted to the researcher to certain sampling issues, in the majority of cases above the device data appears to suggest that sampling was successful. In two cases the respiratory rate as recorded by the researcher was at odds with that recorded by the device. The rate recorded by the researcher was simply a snapshot of respiratory rate at a midway point in collection whereas the rate presented by the device represents an average over the collection period. The latter should be more accurate but a mismatch between the two could indicate difficulties in detecting the respiratory rate and a subsequent sample-gating issue. Of note is the fact that only 3 of the 10 samples where issues were noted occurred in the controlled / follow-up assessment, with the majority occurring during the acute assessment. This might suggest that the frequently rapid breathing rate of the acute patient poses problems for sample gating.

7.1.2 Acceptability of study methods

The acceptability of the three breath capture devices used in the study was assessed by way of a visual analogue scale (VAS) and the assessment of acceptability questionnaire (AAQ); a participant focus group was used to explore feelings about study participation more generally.

The VAS was presented with a range of 0 ('very acceptable') to 10 ('not at all acceptable'). The AAQ was scored in the same direction with a higher AAQ score indicating increased unacceptability of the device. Possible scores range from 7 (most acceptable) to 40 (most unacceptable).

Across both the acute and outpatient study arms, the AAQ and VAS were completed 153 times. VAS scores ranged from 0 to 9; while AAQ scores ranged from 7 to 27. Results are summarised in table 51. The average VAS score was less than three for all devices suggesting they were generally acceptable to the majority of participants.

Table 51 – ABBA study: acceptability of breath capture devices

	RECIVA	Niox Vero	RTUBE	RECIVA		Niox Vero		RTUBE	
	Total across visits			Exacerbation	Follow-up	Exacerbation	Follow-up	Exacerbation	Follow-up
VAS SCORE									
MEAN (± SD)	1.3 (1.8)	1.0 (1.3)	2.6 (2.6)	1.2 (1.9)	1.3 (1.6)	1.0 (1.5)	1.0 (1.2)	2.6 (2.7)	2.6 (2.7)
MEDIAN (IQR)	1.0 (2.0)	0.0 (2.0)	2.0 (3.0)	0.0 (1.5)	1.0 (2.0)	0.0 (1.75)	0.5 (2.0)	2.0 (4.3)	2.0 (5.0)
AAQ SCORE									
MEAN (± SD)	13.0 (4.1)	13.1 (4.6)	16.5 (5.4)	12.6 (4.2)	13.6 (4.1)	13.6 (4.9)	12.4 (4.1)	16.6 (5.6)	16.3 (5.4)
MEDIAN (IQR)	13.0 (8.0)	13.0 (8.3)	16.0 (8.0)	11.5 (9.0)	13.5 (7.0)	13.0 (9.0)	12.0 (6.8)	16.0 (8.3)	16.0 (9.5)

The ReCIVA and the Niox Vero appear to be similarly acceptable, with the RTube the least acceptable of the three devices used.

Whether considering the VAS score or the AAQ-R, the Niox Vero was deemed marginally more acceptable when the measurement was undertaken at the controlled, follow-up appointment than when it was undertaken during acute exacerbation. In contrast the ReCIVA was deemed slightly less acceptable at follow-up when compared to the acute setting. Acceptability of the RTube was consistent across both settings. The difference in scores between the assessment settings were not found to be statistically significant when assessed using a paired samples t-test.

Although the differences between assessment settings were not found to be significant it is interesting to note that the results of the VAS score also differed; and although the direction in which the AAQ-R score differed was different for the ReCIVA (less acceptable at follow-up) and the Niox Vero (more acceptable at follow-up), the VAS score differences were also in the same directions. If the setting did indeed have some small effect on attitudes towards acceptability, it is not clear why this would be in opposite directions for the two devices.

The AAQ-R, like the VAS score, does not have an established threshold for whether a device should be used in a study; it is merely data to inform the decision making process of researchers. Despite grading a device as poor in terms of acceptability a participant may nonetheless be very happy to participate in research; deeming the discomfort (or whatever the reason for a low acceptability level) worthwhile for the stated research aim. The results may also inform the design of a future participant information sheet. The acceptability scores may give some indication of the number of participants likely to say yes to study participation, likely to complete the assessment, or likely to drop-out if asked for a repeat measurement. However, given that the study has generated these actual figures – the number declining participation; the number dropping out – attempting to imply this from acceptability data adds little value. It may help to make informed decisions about device design, and whether a device, if rolled out for - for example for home use - would actually be used.

Table 52 – ABBA study: focus group feedback on device acceptability

• Focus group comments in grey • CRF comments in white	RTube	Niox Vero (frequently referred to by patients during the focus group as 'the cloud')	ReCIVA
P1	I struggled with that one and the nose clips. That one is my worst. Dried my throat out a little bit. Probably wouldn't do it at home. Those two [ReCIVA and RTube] are kind of almost like, not suffocating but… it's covering my face and I feel a bit claustrophobic. I found that one [RTube]… because your nose is blocked and then you're dry as well and it's almost like you're afraid to swallow because everything is dry, and that kind of puts a … well, a slight panic. I wouldn't panic while I was doing it but it can make you feel a bit uncomfortable.	I love it. The cloud is my favourite. I'd do the cloud at home, I probably wouldn't do the others. That one is just blowing into a machine and playing around with a cloud which is fun, plus I don't feel so claustrophobic. I love technical things, I love gadgets and stuff so that [the Niox Vero] I really like	Probably wouldn't do at home. Facilitator: anything at home where you had to wear a mask - even if it was say for 10 minutes - that might put you off? Yeah I think so Those two [ReCIVA and RTube] are kind of almost like, not suffocating but… it's covering my face and I feel a bit claustrophobic. Least favourite. Would do it at GP surgery if asked [and in preference to the RTube]
P1		Fun and easy to use	The device was comfortable and easy to use
P2	No real preference [between RTube and the Niox Vero] Agreed with the following statement from another participant - because your nose is blocked and then you're	I still have trouble with it just keeping it at that level, and to keep it there. I say 'not the cloud, not the cloud' The cloud and I didn't get on very well.	Would do it at GP surgery if asked

	dry as well and it's almost like you're afraid to swallow because everything is dry, and that kind of puts…well a slight panic Would do it at GP surgery if asked	I remember being cross with myself because I couldn't get it to work. No real preference [between RTube and the Niox Vero] I'd probably get to grips and I'd argue with myself until I got it right. If you were doing it yourself that would probably be the easiest one to cope with. I'd prefer to do that [the Niox Vero, when compared with ReCIVA or something with a mask] if you could get to grips with it and got practices at it Yes, once you'd got to grips with it	
P2	I don't know if some people would be able to use the device	Once you get the hang of it then OK	If this device will give accurate information it could be a good thing.
P3	I didn't mind the clip [nose clip] and doing that [RTube]. Now I tolerated that and that one but when I've had anaesthetic I can't have a mask over me Would do it at GP surgery if asked	I had great difficulty a couple of months ago trying to do that. I can't, I haven't got much 'out', I can breathe in but it's the 'out'. No the cloud and I didn't get on very well. It didn't really matter but I was annoyed with myself that I couldn't do that. You'd have to practice with it.	I think this was the hardest thing. Would do it at GP surgery if asked
P3	Seemed a long time; nose clip a little uncomfortable, but still very useful.	All OK; found it fun to watch	Did not find it unpleasant at all
P4	Agreed with the following statement from P1 - Those two [ReCIVA and RTube] are kind of almost like, not suffocating but… it's covering my face and I feel a bit claustrophobic	Cloud is my favourite I couldn't quite grasp what I was meant to be doing with the cloud to start with, it took two goes to actually realise what it was about.	That one is my worst Agreed with the following statement from P1 - Those two [ReCIVA and RTube] are kind of almost like, not suffocating but… it's covering my face and I feel a bit claustrophobic

7.1.3 Acceptability of Assessment Questionnaire

7.1.3.1 Evaluation

As discussed in chapter four (developing a methodology) the AAQ-R was designed with the following eight criteria in mind - appropriateness; acceptability; feasibility; interpretability, precision, reliability, validity and responsiveness (296, 300).

7.1.3.1.1 Appropriateness

Is the instrument content appropriate to the questions which the application seeks to address

Both appropriateness (is the instrument content appropriate to the questions) and face validity (does the instrument measure what it claims to measure) were assessed during the questionnaire design stage through the input of health professional colleagues and the AUKCAR PPI group.

7.1.3.1.2 Acceptability

Is the instrument acceptable to patients?

Feedback from members of the PPI suggested that its use would be acceptable to them. The time taken to complete the questionnaire - a mean 2.7 minutes (median 2.0) - seems likely to be an acceptably small amount of time, adding little to the study burden for either participant or researcher. Furthermore, the absence of incomplete or not attempted questionnaires again suggests a level of acceptability to participants.

Four study participants attended a focus group; among the topics discussed were their opinions on the AAQ-R. Little usable feedback was generated with regards to the AAQ-R – one participant stated that the questions were really clear and that they thought it was good; another expressed an aversion to questionnaires in general but observed that the use of statements accompanied by a range of emoticons such as are used on pain scales (smiley face to grimace) might simplify the format and make it more accessible, particularly to those with a language barrier or learning difficulty. This would not however address the fact that the questionnaire relies on statements to elicit a reaction on acceptability.

7.1.3.1.3. Feasibility

Is the instrument easy to administer and process?

The experience of the researcher was that the questionnaire was easy to administer. The only issue noted was that the direction of scoring for the VAS was opposite to that of the participants expectations – in one or two cases the score on the VAS was in sharp contrast to the participants other responses and apparent

experience, on questioning they stated that they had made an error and had not read the scoring instructions.

It is possible that other errors in this question went unnoticed and that the correlation between VAS score and AAQ would have been stronger had the scoring on the VAS scale been reversed in its orientation. Data entry and processing was straight-forward.

7.1.3.1.4 Interpretability and precision

How interpretable and how precise are the scores of the instrument?

Scores on the AAQ-R can range from a minimum of 7 (very happy with use, very comfortable, and suitable for use during any severity of asthma attack) to a maximum of 40 (very uncomfortable, very unhappy to use, and not suitable for use during even a mild attack).

Most questions having a range of 1-5 allows for a degree of precision; should the researcher wish to look at any individual question, a five point scale is large enough to capture meaningful differentiation. However, an overall score of 7-40 results in a large data range; the question can be asked as to what the minimum clinically significant or meaningful difference in score might be (whether a score of 27 is significantly less happy with the device than a score of 25; and whether this is likely to translate into any clinically significant action such as cessation of device usage). Without a gold standard to compare against it is challenging to quantify the precision of the instrument scores. The question posed by the Oxford University Patient Reported Outcomes Measurement group (300) is whether the questionnaire results in usable data that can be statistically analysed with meaningful outcomes. The data has certainly shown that the RTube was deemed less acceptable to participants than either of the other devices. In and of itself this does not tell us any more than the VAS results; however, the AAQ allows us to see what elements contributed to this unacceptability.

Participant scores on the VAS ranged from 0 to 9; while AAQ-R scores ranged from 7 to 27. It is interesting to note that participants were happy to score a 9 on the VAS (the 2nd highest possible score for unacceptability), whereas the maximum score on the AAQ-R was 27 out of 40, some way short of the maximum score for unacceptability. This suggests that the AAQ-R is a more discriminative test and is capturing some elements of the device which the patient deems to be acceptable.

7.1.3.1.5 Reliability

Does the instrument produce results that are reproducible and internally consistent?

Across both the acute and outpatient study arms, the AAQ-R was completed 153 times.

It is possible that the perceived acceptability of a device might change depending on the context in which a patient is asked. Asking whether a device might be suitable to use during a severe attack may elicit a different answer if the question is asked during such an attack as compared to when it is being posed as a hypothetical question during a period of control. While the average scores in the exacerbated and controlled states do differ, the direction of difference is not consistent (for example, at follow-up the ReCIVA scores more highly than during an exacerbation, whereas the Niox Vero scores slightly lower). The difference is however small; it was not found to be statistically significant when tested using a paired samples *t*-test; and there is little-to-no difference in scores for the RTube across settings. This would suggest an encouraging degree of reproducibility in test results.

7.1.3.1.6 Responsiveness

Does the instrument detect changes over time that matter to patients?

This question is perhaps less relevant to the AAQ-R. The acceptability of a device depends not only on the device related factors such as comfort, time required to complete the assessment but also perhaps upon contextual factors such as the location of assessment, familiarity with the device (likely to improve if enters clinical practice), current health status and belief in the potential benefit of using the device. One might anticipate attitudes towards the acceptability of a device to depend upon on the severity of a patient's condition and the potential benefit a device might offer. Moreover, attitudes to self-care, and the wider patient-disease relationship in chronic conditions may be subject to change over time; change in these broader health attitudes may result in a change in the threshold of what an individual might consider acceptable. Without having undertaken contemporaneous assessment of broader health attitudes it is not possible to comment on the extent to which the AAQ-R is sensitive to such changes over time. The more pertinent questionnaire for the AAQ-R is not whether it detects change over time but whether it detects difference between devices. As can be seen from table 51 the results of the AAQ-R suggest there is a difference in acceptability between the RTube and other devices. This result is corroborated both by the VAS score and the researchers own experience of conducting the tests – informal feedback from participants on the RTube (primarily the use of the nose clips) was more consistently negative than that of the other devices.

7.1.3.1.7 Validity

Does the instrument measure what it claims to measure?

Face validity was established through feedback from health professionals and the Asthma UK PPI group.

The is no gold standard PROM against which to compare the results of this study, however, the degree of correlation between AAQ result and VAS score was calculated using Pearsons correlation co-efficient; r=0.732, p=0.00 suggesting a strong positive correlation. The relationship can be seen in the scattergraph below (figure 22), which exhibits a linear relationship between the two measures of acceptability.

Figure 22 – ABBA study scattergraph of VAS versus AAQ score indicating a linear relationship between the two. The coefficient of determination (R^2) = 0.54 suggests this linear regression model explains approximately 50% of the data variance.

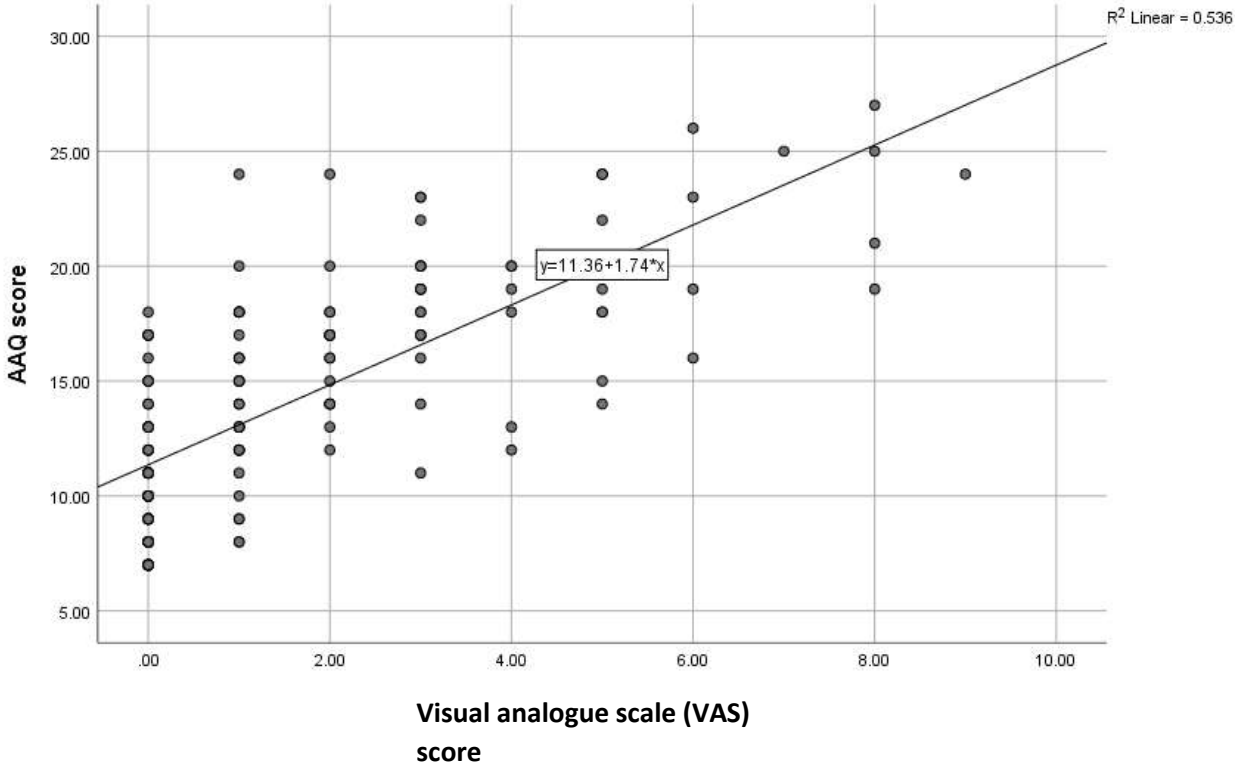


Table 53 shows comments from the free text section of the AAQ-R and the overall score they gave to the device (comments are taken from all study stages and all devices). It can be seen that the overtly negative comments are generally associated with a higher score and vice versa. This subjective assessment does appear to lend support to the questionnaire validity; scores appear to represent patients overall views on device acceptability. Table 54 presents a summary of focus group participants' views on the assessment devices alongside their scores on the acceptability assessments (VAS and AAQ).

Table 53 – ABBA study: comments and AAQ-R scores

Comment	Score
I like the design, it's a fun way to get a constant breath at an appropriate rate	7
All OK	7
The device was very comfortable and did not make me feel claustrophobic like some do. Also it was much quieter. I would say that I felt relaxed.	7
Very simple piece of kit; comfortable and easy to use	7
I am able to use this machine quite easily	7
Did not find it unpleasant at all	8
Unsure if this device could be used during a major attack, for the timescale it would be used. Other than this, very comfy to use	8
Happy to use while in hospital during a severe attack but not at home alone	8
Mouthpiece a little uncomfortable on the lips	8
The device was comfortable and easy to use	8
The equipment may need to be simplified for home use. My lips touched the tubes. Maybe remember to say if patient may shut mouth and breathe through the nose during the test. Fine.	8
Absolutely fine	8
The headset is comfy and easy to use, I just needed to hold the nose area of the mask to get a good seal.	9
It would be helpful if we had one at home for when symptoms are worse, to be able to compare / monitor. I always feel comfortable with this test.	9
I have no worries about the device but do think that an old person might feel a bit anxious	10
All fine	10
Easy to do, so long as you can breathe at the beginning of the test	11
If the device was made to operate easier by myself I would be comfortable to use it at home. I found the whole experience very acceptable	11
Only thing with the test is the fact of wearing a nose clip, really hampers breathing.	12
More difficult to use as I am a natural nose-breather; left me feeling a little dizzy at the end.	12
Feel much better	14
The mask could feel a little claustrophobic when having a moderate-to-severe asthma attack; having anything over ones face can be quite upsetting. The airflow made my mouth dry.	14
Nose plug always slightly uncomfortable, generally easy to use though if I was having a flare up I feel it may aggravate things.	14
Found it fun to watch	15

Having had some difficult exacerbations this week as an inpatient I was a little bit hesitant to accept the test, however I found it went very well	15
A little hard to draw breath but, all told, very good	17
Seemed a long time; nose clip a little uncomfortable, but still very useful.	18
OK once you get the hang of it	18
I felt the nose clip uncomfortable as I realised I couldn't breathe out very well; overall easy to use.	19
Main issue that bothered me was the feeling of an almost inability to get a breath in. As a whole relatively comfortable and straight forward	19
I don't know if some people would be able to use the device	19
Very hard to keep the flow rate up	20
I found this very difficult to do however my asthma is pretty bad; it would be much easier if I could breathe	20
Fix the noisy valve!	21
Just one flaw, it feels more difficult to breathe. Harder to get breath in. All in all not overly comfortable.	22
Would not be able to use this whilst having an attack, made me feel breathless today	23
I just couldn't breathe	24
I did struggle on this test needing a few breaks	25
Uncomfortable, solely the nose clip, very tight.	26

Table 54 – ABBA study: focus group summaries and acceptability scores

Participant	Summary of focus group transcript	VAS score			AAQ-R scores		
		RTube	Niox Vero	ReCIVA	RTube	Niox Vero	ReCIVA
P1	Niox Vero was a clear favourite. Stated that the RTube was the least favourite.	0 + 0	0 + 0	0 + 0	11 + 12	7 + 8	8 + 10
	VAS score failed to differentiate; correlation with AAQ score.						
P2	Reported struggling with the Niox Vero but also stated that if able to practice and get the hang of it, this would the preferred device.	3 + 3	6 + 3	4 + 3	19 + 20	18 + 23	17 + 19
	No consistent pattern						
P3	Slight preference for the RTube; reported struggling with the Niox Vero and not liking the face mask with the ReCIVA	1 + 0	0 + 0	0 + 0	12 + 18	7 + 15	8 + 10
	Does not correlate with AAQ scores						
P4	Niox Vero was clear favourite; ReCIVA the least preferred.	4 + 2	1 + 1	1 + 1	20 + 24	12 + 13	14 + 15
	VAS and AAQ scores correlate; AAQ score in agreement with focus group comments on Niox Vero but not ReCIVA.						

The focus group transcript excerpts (see table 52) and the comments made contemporaneously with sampling (see table 53) were at odds with one another in the case of one participant. At the time of assessment they reported the RTube to be slightly uncomfortable and taking a long time, whereas the ReCIVA was not unpleasant at all. In the focus group the opinions were reversed with the ReCIVA being reported as the hardest of the three devices. This may have to do with the length of time between undertaking the assessment and participating in the focus group; and the perception of a problem (e.g. wearing a mask) looming large in the participants mind when in actuality - in the clinical environment with close supervision and support - it had not bothered the patient. The scores on the AAQ-R support the comments made at the time of the assessment, and conflict with the opinions expressed as the time of the focus group. In future, focus groups should be undertaken in a more timely manner.

In the focus group one participant expressed a very strong preference for the Niox Vero *"I love it… the cloud is my favourite"* scoring the device 7 and 8 in their two visits; however, despite describing the RTube as their worst they scored this 11 and 12 offering only a relatively small score difference between the two devices. One participant expressed a strong opinion that the RTube was the least acceptable device to them; they scored this device 20 and 24 in their two visits, compared to 12 and 13 for their favourite, the Niox Vero. Another participant expressed no preference between the Niox Vero and the RTube; the scores from the AAQ were roughly in agreement - 18 & 23 for the Niox and 19 & 20 for the RTube. This limited and subjective assessment - linking qualitative statements with the AAQ score – does nonetheless supports the notion that the questionnaire is measuring what it sets out to measure.

7.1.3.2 AAQ-R Discussion

The key question is whether the AAQ-R offers any benefit over and above a simple VAS.

Because acceptability is a subjective construct any measure is going to have limited value in comparing between people – for example one participant might never score something at the positive extreme of a VAS whereas another person might score everything there unless really awful. It is therefore difficult to establish a threshold score for acceptability as this may differ between individuals. Moreover, if such a score were established it would need to be validated in terms of clinical impact – whether the score correlated with, for example, device adherence. More useful might be the minimum clinically significant difference; this would be useful in determining preference between devices, or determining improvement in acceptability with design refinement. The advantage this offers over a VAS is that the researcher can look at the individual domains, digging into the reason behind the score. Moreover, participants tended to use a very limited spread of scores on the VAS; the focus group participant P1 (see table 54) gave all devices a score of 0 on the VAS, whereas in the focus group expressed clear preferences which were captured in their responses on the AAQ.

One member of the PPI group who offered feedback on the draft AAQ-R listed the time taken to complete the test as one of their concerns when considering any new device for the assessment of asthma. Having conducted the research with participants, it became apparent that the pleasantness / unpleasantness of the test was often magnified by the time required to complete it (particularly the case for those participants bothered by the nose clip). This emerged in some of the focus group comments with some participants suggesting that they'd prefer the test they struggled to complete – the Niox Vero – because, with practice, they could imagine being able to complete it more easily, and they would prefer this quick test (with no face mask or nose clip) to one of the more time consuming devices. Future modification of the questionnaire might directly assess the acceptability of time taken to complete the test.

Although face validity of the questionnaire was considered by the research team and members of the AUKCAR PPI group, an assessment of content validity was not undertaken. This refers to the extent to which the elements of an assessment tool reflect the domain and operational definition of a construct. To be done properly Almanasreh et al (355) state that a panel of experts is required to evaluate the instrument; their judgements being quantified in a content validity ratio.

This process lies outside the scope of this thesis but would form an important part of validation were the questionnaire to be developed for use in a future study.

7.2 Secondary objectives - VOC analysis

The analysis of targeted VOC data from the ABC study (see chapter 6) was shown to have levels of background contaminants despite the use of a clean air filter; and the untargeted deconvolution of ABC data revealed poor sample integration. Given the degree of background contamination and lack of a consensus method for dealing with such; the conflicting results yielded when comparing results from background corrected and non-corrected data; and the lack of significant results after the application of a post-hoc false discovery rate – and having achieved the primary study objectives – it was decided that the pursuit of the ABBA study's secondary objectives through VOC analysis was not warranted.

7.3 Conclusion

The primary study objective was to determine the feasibility of conducting a study of exhaled breath biomarkers in patients with acute asthma.

What is the best method of recruiting patients into a definitive study?

Both the acute and outpatient arms were successful in recruiting participants while falling short of the initial target. The outpatient arm succeeded in capturing a greater number of exacerbations however only the acute arm captured participants who had experienced a life-threatening attack. With the exception of those participants with missing data, all those in the acute arm were receiving oral corticosteroids at the time of their assessment compared with 65% of those in the outpatient arm. Depending on the research question and the population of interest, these two factors may be important considerations when determining the recruitment method of any future study.

Are patients both prepared and able to provide EBC, VOC and other samples in the acute asthma setting? Are patients prepared to perform repeated measures / multiple assessments?

In the acute arm, 67% of patients screened were excluded; of those who were approached regarding the study 48% agreed to participate. Two participants were lost to follow-up, one from each study arm (acute and outpatient). Two participants withdrew their consent - again one from each study arm - constituting 2.3% of the total participants.

The outpatient who dropped-out moved out of the area, stating that they would not be able to travel the required distance to be assessed should they experience an exacerbation. The acute recruit did not give a reason; they completed an acute assessment but when later offered a follow-up appointment declined. This acute drop-out constitutes 3.3% of those who underwent exacerbation assessments (in either study arm) or 9% of acute arm participants.

Are patients both willing and able to contact the research team and return for assessment when experiencing an exacerbation?

Including those identified by the exit questionnaire, a total of 34 participants reported having an exacerbation during the course of the study. Of these, 21 (62%) made contact to arrange an exacerbation assessment (95% CI (45, 76); 17 participants attended providing 18 acute samples. With staffing and availability of rooms being no object a greater number of assessments would have been possible. Furthermore, with greater resources study reminders could have been undertaken by phone, email, text in addition to by post which may have increased awareness and numbers of contact.

Are researchers able to perform the initial assessment of hospital patients early during the acute exacerbation?

No; this will be discussed further in chapter 10 (discussion) but the study failed to identify and recruit participants in A&E prior to their admission; 59% of patients presenting to A&E with asthma listed as a possible, probable or confirmed diagnosis were discharged home. The vast majority of these patients were not approached for study participation as coding of their complaint with 'asthma' was done at the point of discharge. This resulted in the systematic exclusion of those patients who were not admitted to hospital. Of those who were admitted and for whom a full data-set exists, 100% had received oral corticosteroids prior to assessment; it did not prove possible to recruit and assess patients in the acute arm before this had occurred.

Is it possible to assess patients who are in receipt of supplementary oxygen?

Chapter 4 (developing a methodology) details the work undertaken to make this possible. However, surprisingly, only one participant was on supplementary oxygen during their assessment. There were no adverse events reported in this assessment and a sample was successfully collected.

How does collection of EBC & VOC compare with more established measurements (such as FeNO) in terms of acceptability to patients?

There is relatively little difference in acceptability between the three devices. The RTube was perhaps the least preferred but this seemed to be largely due to the wearing of nose clips. This was done in accordance with EBC sampling guidelines however the necessity of this measure may depend on the biomarker of interest and whether nasal contamination could act as a confounder. The ReCIVA was least favoured by some due to the use of a face mask. Some participants expressed real fondness for the Niox Vero while others were more frustrated by it; however, irrespective of preference, three participants were not able to manage the breath control required to generate a result on the Niox Vero when in the acute stage; all managed it at their follow-up appointment. Only one participant was unable to tolerate the RTube, this was in the acute stage. All participants managed to tolerate the ReCIVA and complete the sampling process, irrespective of stage.

Is it possible to obtain exploratory data comparing controlled and exacerbated states to power a definitive study?

The recruitment rate in the acute arm was one patient per month and in the outpatient arm seven per month. Recruitment percentage was 64% of those approached in the outpatient arm and 48% of those approached in the acute arm. These figures will help inform the planning of future acute studies. However, in terms of sample size, a definitive study will be one with adequate power to assess pre-specified outcome measurements. To conduct such a power calculation requires knowledge of the anticipated difference in the biomarker of interest. It was not possible to provide this information; EBC sample analysis was not conducted due equivocal results in a review of the proposed analyte and lack of success in the analytical process; while the reliability of VOC data was negatively affected by methodological issues and difficulties with data deconvolution.

What percentage of patients experiencing an acute exacerbation have a bacterial trigger?

It was not possible to answer this question due to the lack of spontaneous sputum samples provided by study participants. Any future study would likely require induced sputum to be part of the research protocol. Nasal swabs identified only four viral respiratory infections in the exacerbation assessment (representing 13% of all exacerbations) and two at follow-up. It is not clear whether this represents the true number of viral exacerbations or whether throat swab or cough swab might have reliably identified a greater number of infections.

Conclusion

This chapter has reported on the feasibility outcomes from the ABBA study. The qualitative data may be useful in the design of future acute asthma studies using breath capture devices; while the quantitative data may be used to help in study design and recruitment calculations. The following chapter discusses the results reflecting on their implications for future research.

Chapter 8 – Discussion

8.1 Introduction

The work presented in this thesis has illustrated the complex, heterogeneous nature of asthma and the need for biomarkers to improve both diagnosis and management; it has reviewed the literature on two different approaches to exhaled breath analysis; undertaken research to determine whether changes induced by indirect bronchial challenge are detectable using exhaled VOC; and undertaken research to determine the feasibility of conducting exhaled breath studies in the acute setting. This chapter concludes the thesis by considering the implications of the results presented and discussing study limitations.

8.2 ABC study

8.2.1 A summary of results

Results were presented on the effect of indirect bronchial challenge testing on VOC profiles in patients with asthma; identifying those VOC whose intensity changed after positive mannitol challenge; and comparing these results with those from negative and sham challenges. In addition, those compounds exhibiting significant differences - at baseline or in the post-challenge samples - between those who tested positive and those who tested negative were identified. With the exception of those compounds excluded due to a lack of reliability, 15 significant differences were identified in 14 compounds (see table 55; further detail can be found in chapter 6 sections 6.2.3 and 6.2.4).

Table 55 – ABC study: compounds identified as statistically significant (before application of FDR calculation) across all analyses

Compound	Analysis in which significant difference was noted
Background corrected data	
Furan, 2-methyl	Baseline, pre-challenge samples Significant difference between those who went on to have a positive challenge and those with a negative challenge
Isoprene	Significant difference between pre- and post-challenge samples in those experiencing a positive bronchial challenge
Urea, ethyl-	
5,9-undecadien-2-one, 6,10-dimethyl-, (E)-	
Uncorrected data	
Trichloroethylene	Significant difference between pre- and post-challenge samples in those experiencing a positive bronchial challenge
3,5-dihydroxybenzamide	
Dimethylsulfoxonium formylmethylide	
Benzene, 1,3-dimethyl-	
Phenylethyne	
Oxime-, methoxy-phenyl-	
3-Carene	
Nonanal	
Decane	Significant difference between pre- and post-challenge samples in those experiencing a positive bronchial challenge + Significant difference in baseline samples between those who went on to have a positive challenge and those with a negative challenge
Dimethyl selenide	Baseline, pre-challenge samples Significant difference between those who went on to have a positive challenge and those with a negative challenge

Of these compounds, five have been previously reported in the adult asthma literature (see chapter three, table 16) - Furan 2-methyl; Isoprene; Benzene, Decane; and Nonanal. Furthermore, evidence of 5,9-undecadien-2-one, 6,10-dimethyl-, (E)- has been published in the paediatric (356) and adult asthma literature (357) since the completion of this thesis.

The fact that several previously identified compounds featured in the results serves to validate the methodology used (to a limited extent). These compounds may be considered to be potential biomarkers warranting further investigation; possible targets for the future development of point of care tests; and markers of a metabolic, pathophysiological pathway worthy of elucidation.

Although statistically significant results are reported for pairwise comparisons of individual VOC before and after mannitol challenge, these results were not found to be significant once a FDR calculation (Benjamini Hochberg) was applied.

Differentiating positive from negative samples - being able to identify breathprints associated with the release of inflammatory mediators - has potential use in disease monitoring. Unpaired analysis comparing samples from the positive mannitol challenge group with those of the negative challenge group revealed three statistically significant results in the comparison of pre-challenge samples. These were furan 2-methyl (in the background corrected data), and dimethyl selenide and decane (in the uncorrected data). These results were not found to be significant once a FDR calculation (Benjamini Hochberg) was applied.

The compounds identified in these analysis varied depending on whether the data used accounted for background contaminants; and for both data sets the statistical significance was not maintained after application of a FDR calculation. Similarly, binary logistic regression analysis failed to reveal any results of statistical significance and the resultant model displayed a high number of false negatives, indicating poor sensitivity.

The paired-sample analysis was powered based on the limited prior results available, but the study was not powered appropriately for non-paired analyses. A sample size calculation for independent samples was conducted; for this purpose acetone and isoprene were selected - compounds found by systematic review (chapter 3) to be the most frequently named as significant in asthma breathomic studies. In the case of isoprene, a sample size in the region of 80 participants would be required to have sufficient power to detect significant differences in either baseline or post-challenge samples between those who tested positive and those who tested negative. In the case of acetone a much larger samples size – 260 – would be required. For any future study investigating this subject such a sample size would lend additional weight to the paired analysis. Given that the size of difference across clinical groups for many compounds has not been reported, larger samples sizes are likely required, particularly for those studies taking an untargeted inductive approach to analysis.

While the values produced by this study may help researchers to plan sample size in future studies (depending on the compound of interest and clinical question), it should be stressed that the calculations are dependent on the accuracy and reliability of the data upon which they were based.

Before further studies are undertaken methodological issues need to be addressed in order to improve reliability. On analysis of the clean air supply used in the study, exogenous VOC were detected in similar quantities to those found in the unfiltered background air; quantities which frequently rivalled or exceeded the levels found in patient breath samples. Methods for addressing background contaminants suggest that compounds with such high levels in background air should be excluded from analysis. The results of the analyses conducted in this thesis point to the importance of addressing this issue – those compounds identified as significant when analysing data uncorrected data set differed from those identified when using the background-corrected data-set with exclusions based on contaminant levels.

8.2.2 Compounds of interest

Furan 2-methyl

Exposure to environmental 2-methyl furan - which is released by the degradation of biomatter including the combustion of fossil fuels - has been linked to occupational asthma (358). However, it has also been found in the faeces, urine, exhaled breath, and skin of healthy individuals (359); a ubiquity which may be advantageous in a potential biomarker. Furans have been studied and reported most frequently in the context of smoking where they have been consistently found to be elevated in the exhaled breath of smokers (360, 361). In an asthmatic population Caruso et al (362) reported significantly higher levels of 2-methyl furan in *ex*-smokers when compared to never-smokers, suggesting it may be a product of long term airway changes associated with past smoke exposure. In a non-smoking population, Brinkman et al (198) reported a significant correlation between exhaled 2-methyl furan and asthma control test scores; and van Vliet et al produced a seven-compound model predictive of asthma exacerbation (356) which included 2-methyl furan. Waqar et al (363) point out that such findings might be due to epiphenomena such as differences in diet or exposome. However, Zanella et al (364) stimulated lung inflammation *in vitro* using epithelial cells exposed to oxidative stress (using hydrogen peroxide) and biological stress (sputum supernatant from patients with asthma). They report increased 2-methyl furan production in response to both stressors.

Mochalski et al (365) suggest that one possible endogenous source of furans might be the degradation of isoprene by alkoxy radicals in a pro-oxidant state. With

previous studies reporting an association with asthma; *in vitro* evidence suggesting endogenous production; and the identification of a possible biological production pathway, 2-methyl furan warrants further investigation as a potential asthma biomarker. It is clear however that smoking status / history is a potential confounder which will need to be controlled for.

Isoprene

An unsaturated hydrocarbon, isoprene is produced in large quantities by plant matter (366). It is also a by-product of cholesterol synthesis and one of the most abundant VOC found in exhaled breath. Although part of the mevalonate pathway, its exact biochemical origin and physiological actions are not yet fully understood (367).

Studies have reported isoprene to be elevated in asthma when compared to healthy controls (174, 181) and it is one of five VOC in van der Schee et al's (181) model for the differentiation of asthma and healthy control samples. Isoprene has also been shown to have a circadian rhythm in asthma that is absent in health (368). However, exhaled isoprene has been found to be elevated in other inflammatory states including myocardial infarctions, post-cardiac surgery, and high cardiac indices (369). In a study monitoring ozone exposure (370) it was found that FeNO was inversely associated with isoprene leading to the suggestion that endogenous isoprene may play a protective, ozone-scavenging, antioxidant role. In particular it may react with H_2O_2 and peroxynitrite (products of the oxidation of nitric oxide) in the lungs. This, they suggest, may explain the correlation between exhaled isoprene and acute inflammation or inflammatory states.

The ubiquity of isoprene in exhaled breath samples; its typically positive alveolar gradient (272); and its reporting in previous asthma studies suggest it warrants further investigation as a potential biomarker. However, highly variable levels in the breath - influenced by age, gender, ventilatory effort and respiratory effort (371-373) – and a lack of specificity to asthma are likely to complicate efforts to confirm its utility.

Urea, ethyl-

Urea is both the name of a compound and a functional group (which may be found as a part of other compounds). While the compound urea has been used in EBC studies as a maker of sample dilution, ethylurea does not feature in the existing breathomics literature.

5,9-undecadien-2-one, 6,10-dimethyl-, (E)-

5,9-undecadien-2-one, 6,10-dimethyl-, (E)- is a ketone. These are typically produced by lipolysis in the liver, with production being increased during fasting or prolonged exercise. Similar compounds are produced as a result of the peroxidation of unsaturated fatty acids (374) however detail of an endogenous production pathway for this particular ketone could not be found in the literature.

van Vliet et al (356) report that exhaled 5,9-undecadien-2-one, 6,10-dimethyl-, (E)- levels differed significantly between exacerbated and non-exacerbated asthma states in a paediatric population; while Stefanuto et al (375) developed a 10 compound model (which included 5,9-undecadien-2-one, 6,10-dimethyl) for the differentiation of eosinophilic asthma from other phenotypes. Although its biogenesis and relation to asthma is unclear, given its recurrence in asthma breathomic studies this compounds warrants further investigation.

Trichloroethylene

A halocarbon and industrial pollutant, the effect of exogenous trichloroethylene exposure on asthma and bronchial hyperreactivity has been studied (376, 377) but it does not feature in the asthma breathomics literature and little information is available regarding endogenous production pathways.

3,5-dihydroxybenzamide

An aromatic compound previously unreported in breathomics literature.

Dimethylsulfoxonium formylmethylide

An organosulfur, Paudel et al (378) posit possible antimicrobial properties for this compound which is present in some plant-based traditional Chinese medicines. It is absent from the breathomics literature and an understanding of endogenous metabolic pathways is lacking.

Benzene, 1,3-dimethyl-

An aromatic compound. While benzene occurs in the asthma literature with some frequency (70, 177, 379) 1,3-dimethyl-benzene is absent. Benzene was included in several breathomic models; these include the differentiation of asthma and controls (70, 177) and the prediction of exacerbation (376). It is a common environmental (carcinogenic) contaminant and its use as a biomarker could stem from the way in which asthma differentially affects the metabolism of inhaled, ambient benzene rather than its endogenous production.

Refinement of the approach for dealing with negative alveolar gradients may therefore be required to successfully evaluate the utility of this compound.

Phenylethyne

An aromatic hydrocarbon. Such compounds in particulate matter / pollution have been examined in terms of their relation to asthma severity and exacerbation but they are absent from the breathomics literature.

Oxime-, methoxy-phenyl-

The origin of many methoxy esters is not known (359). Methoxy-phenyl-oxime has been found to be associated with the ulcerative colitis (380), and to be decreased in those with severe pulmonary arterial hypertension when compared to healthy controls (381). Significant differences in both lung and inflammatory conditions suggest this marker might warrant further investigation however it is absent from the asthma breathomics literature to-date.

3-Carene

3-carene is a terpenoid, a group which have been found to have anti-inflammatory effects - inhibiting the production of reactive oxygen species; increasing anti-oxidant enzyme production; and stimulating autophagy (382). 3-carene itself is a common exogenous VOC being emitted by both living and dried wood. Like other terpenoids anti-inflammatory effects have been reported (382-384), however, it may also be an irritant (385) and has been found to cause bronchoconstriction on inhalation (386). While the biotransformation of exogenous 3-carene has been elucidated (387) little has been published on endogenous production (386). It was identified in exhaled breath samples by Ibrahim et al (70) and formed part of their model differentiating eosinophilic from non-eosinophilic asthma.

Nonanal

A saturated aldehyde, nonanal is one of many products formed in the oxidisation of arachidonic acid (388). It has previously been reported to differ between exacerbated and non-exacerbated asthma (356); between allergic asthma and healthy controls (389); and between neutrophilic and eosinophilic phenotypes (390). It has also been found to be elevated in lung cancer (391). These observations are lent further credence by Zanella et al (364) who found nonanal to be under-expressed following chemically induced oxidative stress but over-expressed following inflammatory stress. The frequency of its occurrence in asthma breathomic studies coupled with a known endogenous production pathway and *in vitro* evidence makes nonanal a good candidate for further study.

Decane

Decane was one of six alkanes reported by Caldeira et al (389) in a model differentiating paediatric asthma from healthy controls. They describe leukocyte activation in asthma leading to the release of inflammatory mediators and free radicals, with alkanes subsequently being produced through the lipid peroxidation of fats (389). Other alkanes were also reported as significant by Ibrahim et al (70) and Meyer et al (177) in the differentiation of asthma from controls. Zanella et al (364) report numerous alkanes differentially expressed as a result of exposure to inflammatory or oxidative stress; with decane altered by both. Decane was found in all exhaled breath samples in a study by Jalali et al (392) but was significantly higher in those exposed to crystalline silica dust – a cause of oxidative stress and silicosis. The prevalence of decane in breath samples; the frequency with which it is reported in asthma breathomics; and its plausibility as a marker of both inflammation and oxidative stress marks this as a potential candidate for further investigation

Dimethyl selenide

An organoselenium which can be found in the exhaled breath of healthy volunteers (393) originating from the metabolism of ingested selenium and responsible for a garlic-like breath odour. Associations have been drawn between exhaled dimethyl selenide and liver disease (394); it is not clear if or how dimethyl selenide might relate to the pathophysiology of asthma.

A summary of compounds

Alkanes, aldehydes, and ketones have all come up frequently in the asthma literature; these are identified as being associated with inflammation and oxidative stress via lipid peroxidation (363). Aromatic hydrocarbons have been studied in terms of exogenous sources and their potential to trigger exacerbation, rather than as markers of disease. The *in vitro* study by Zanella et al also reported altered levels of aldehydes, alkanes and ketone (364) in response to inflammatory sputum supernatant and/or hydrogen peroxide, lending support to the notion that the altered levels of these compounds in asthma breathomic studies are the result of inflammatory cell activity and oxidative stress.

Many of the compounds identified above also feature as significant compounds in the identification of other disease, for example nonanal and dimethylbenzene in colorectal cancer (395); decane in lung cancer (396); dimethyl selenide in liver disease (397) and furan 2-methyl in COPD (398, 399) and lung cancer (398).

This provides further impetus to the argument that biomarker panels rather than individual compounds will be required to produce accurate diagnostic or phenotyping performance.

8.2.3 Secondary study objectives

The first of the secondary objectives for the ABC study was to determine whether VOC profiles could predict bronchial challenge response. The ability to predict a positive mannitol challenge - to identify bronchial hyperreactivity from baseline samples – would save the time and unpleasantness of undertaking a mannitol challenge. However, upon viewing the results; noting the lack of compounds identified as significant in the non-paired analysis; and performing a post-hoc power calculation; it was clear that modelling the data in this way was not appropriate. A further secondary objective related to variability in breath VOC; when analysed on a per VOC basis, the variance between participants (standard deviation from the mean) was compared with the standard deviation *within* participants (between replicate samples); for only seven of the 58 compounds was the between-replicates difference greater than that between participants. Coupled with a low intraclass correlation coefficient this suggests reliability in the sampling process.

In summary, the results fell short of significance once a false discovery rate was applied; a larger sample size will be required to tackle research questions based on differentiating between positive and negative challenges. While a power calculation was completed for the paired analysis, given the scarcity of data on the size of difference which might be expected, it is not possible to say with certainty that the lack of significant results (after FDR) is a robust conclusion. Future studies powered according to the size of difference reported in this and other studies - for the compound/s of interest - will be required to confirm the absence of any significant differences. However, before this can be undertaken methods need to be refined in order to address the issue of background contaminants.

8.2.2 ABC study design strengths

One of the strengths of this study is the control of confounders. Attempts to capture a breath sample during an acute exacerbation and then at a future, controlled time suffer potential confounding from changes in treatment introduced as a result of the exacerbation (whether a change of medication or simply increased adherence); exogenous VOC exposure; changes in sampling environment; and changes in the participants' general health. Similar confounders are present in medication withdrawal studies.

By taking samples before and after a bronchial challenge such confounders are kept to a minimum. Furthermore, if looking solely at changes occurring in a positive mannitol challenge the certainty of diagnosis (frequently an issue in studies of asthma) is confirmed in the process of participation. The key limitation of this study design is that a bronchial challenge is being used to provoke an inflammatory response and the extent to which this might differ - in terms of inflammatory pathways and VOC produced – to that which might occur 'naturally' has not been established. Alongside other asthma breathomic studies this approach does however provides an important means of verifying findings.

8.2.4 ABC study limitations

8.2.4.1 Study design

A randomised cross-over study design would typically be considered for this type of study and indeed this was the initial approach used. However, it was decided to conduct mannitol challenges first and invite only those with a positive mannitol challenge to return for a placebo challenge. Had a cross-over design been maintained, some participants would have had a placebo challenge first, returned, and experienced a negative mannitol challenge. This would, in effect, have given us two 'control' results and resulted in a number of unnecessary challenges. Furthermore, in attempting to account for the effect of the challenge process, it is necessary to match the number of inhalatory efforts and spirometries conducted in the sham challenge with that conducted in the positive challenge. This would not be possible if some participants were randomised to have the sham challenge first. A non-randomised, sequential design was deemed acceptable due to the transient effect of mannitol; the lengthy washout period used; and the use of FeNO and other measures of disease activity to ascertain whether any carry-over effect was occurring.

It was not possible to blind the researcher or participant due to the ease of detecting the presence or absence of mannitol during the test procedure. When undertaking spirometry, normality of the flow volume loop trace, good peak expiratory flow rates and maximum apparent effort was looked for in order to safeguard against any participant bias in spirometric effort. Laboratory staff processing the samples were blinded in order to prevent any bias in application of processing procedures such as time-to-dry purging.

As discussed in section 8.2 the mannitol challenge was used as a proxy for real exacerbation. Given the mechanism of action, the pathophysiological activity and resultant VOC profile may be comparable to that of an exercise induced-asthma attack, however, the extent to which is might serve as a proxy for other types of exacerbation such as viral or allergy triggered exacerbation is not clear.

Results may be limited to those asthma phenotypes which are associated with a positive mannitol challenge; given the high number of false negatives associated with mannitol challenge this may limit the wider applicability of results.

The initial sample size calculation (see chapter 5) was necessarily imprecise. It had been hoped to obtain samples totalling 19 in each group (positive and negative); however there were only 16 positive challenges and a resultant excess of negative challenges. It is possible that the paired (pre- and post-) analyses were underpowered to detect statistically significant differences, nevertheless the results provide target compounds for future investigation.

A number of participants showed some reaction to the MDP – dropping their FEV_1 by 10-14.9% - but did not meet the threshold for a positive challenge. In order to maximise the difference between positive and negative challenges in an initial analysis such participants could be excluded; however, the sample size used was not sufficiently large to permit such an exclusion of data. Future studies might consider this when calculating sample size.

8.2.4.2 Disease activity

Several participants who believed themselves to have mild, well-controlled asthma had elevated FeNO results and/or poor spirometry. Upon being informed of this some participants engaged with their asthma management plan, became more adherent in their use of preventative medication and returned with a lowered FeNO and improved ACT score. This has the potential to affect comparisons of pre-challenge or post-challenge samples across the two study visits.

The mannitol challenge has high specificity but relatively poor sensitivity for asthma; this may be due, in part, to its inherent inability to provoke non-inflammatory asthma phenotypes. It is nonetheless likely that some of those with a negative mannitol challenge did not in fact have asthma. An attempt was made to ascertain the diagnostic certainty by extracting data from participants' primary care notes. Due to the low negative predictive value of mannitol challenge (333) a negative mannitol challenge cannot be used to identify those with a misdiagnosis of asthma. Moreover, several participants failed to abstain from caffeine or exercise prior to the challenge. The bronchodilator effect of caffeine has been established (400) but evidence regarding its or bronchoprotective effect and confounding of bronchial challenge tests is equivocal for methacholine (401) and unstudied for mannitol. It is likely that - in comparisons between the positive and negative challenge groups – the negative challenge group is likely to include a mixture of those with and without asthma.

That some participants had a low likelihood of asthma when assessed according to BTS/SIGN guidelines is likely to be relatively unimportant. These participants had negative bronchial challenge tests and their data was used to account for the changes in breath profile induced by repeated spirometry and mannitol inhalation only. There is, however, a possibility that even in a negative challenge, the changes which occur in exhaled VOC profiles after spirometry may differ between those with asthma and those without.

8.2.4.3 Sample processing factors

The most significant limitation was the high degree of background contamination present in the filtered air samples. Key to breathomic studies is reducing the impact of exogenous compounds on exhaled breath results.

This is typically tackled in two ways – reducing the inhaled VOC by supplying filtered air and accounting for those contaminants present through the calculation of alveolar concentration gradients or eliminating from analysis those compounds present at high levels. The high levels of contaminants present in the filtered air supply impacted on both of these elements of sampling and analytical rigour.

8.2.4.4 Methodological Recommendations

A reliable clean air supply needs to be established and monitored during any future study. Given the growing number of published studies of both targeted and untargeted approaches, future studies should aim to validate previous findings with studies powered according to reported compound differences.

8.3 ABBA study

The primary objective was to determine the feasibility of a study to evaluate the utility of exhaled breath biomarkers in patients with acute asthma. It was established that patients are able and willing to provide breath samples using multiple collection methods in the acute setting taking a mean of approximately two hours for a study visit; it was found that outpatients are willing to contact researchers and attend for a study visit when experiencing an exacerbation. While falling short of the overall recruitment target, both the inpatient and outpatient study arms were successful in recruiting and sampling suggesting either method is possible for future studies. The shortfall in recruitment is discussed further in section 8.4.1 and will have implications for the resourcing of any future study. A method was devised for sampling patients in receipt of oxygen permitting the assessment of those more acute patients in receipt of such treatment. It was established that assessing patients before receiving systemic steroids in the acute population was not possible; while this may frustrate

the study of certain research questions it is reassuring and in keeping with clinical guidelines that acutely unwell asthma patients are receiving systemic steroids at the earliest possible juncture. The collection of both EBC and VOC was deemed acceptable by patients; although EBC was less favourably reviewed – likely due to the closing off of the nasal airways with a nose clip.

Performing FeNO proved challenging for some acutely unwell patients; and while some patients in the focus group expressed a degree of exasperation with the test, they also expressed confidence in it and a belief that once they'd practiced the technique it was an acceptable device. It was not possible to establish with certainty the percentage of participants with a bacterial trigger due to the limited number of participants found capable of producing a sputum sample.

This could be addressed through the use of induced sputum sampling – a process which is not contra-indicated in the acute population - however it would first be necessary to establish how many patients in an acute setting would be willing to undergo this process. It was not possible to obtain reliable exploratory data from the ABBA study for assessing the secondary study objectives and powering a future VOC study; the current methodology resulted in a high level of background contaminants and reliable figures could not be obtained.

8.3.1 ABBA study strengths

The ABBA study captured data on real-life acute asthma exacerbations in patients receiving usual care; it did not rely upon an exacerbation proxy (such as bronchial challenge), nor a medication withdrawal design. As such the results can be generalised more readily to real asthma populations. The acute study setting is appropriate to tests of diagnostic accuracy (for exacerbation phenotyping) or to attempts to validate findings from controlled settings in a real life clinical environment. In trialling two different recruitment approaches it was possible to provide data on both methods allowing future researchers to determine which might be more appropriate to the clinical question under investigation and estimate recruitment rates accordingly.

8.3.2 ABBA study limitations

8.3.2.1 Clinical factors

Patients approached in the acute arm tended to be recruited towards the end of the 24 hour window. This was either due to their having been admitted in the afternoon / evening and not being approached until the following day, or due to declining the initial invitation and requesting the researcher return later when they felt better. This will have led to an increased exposure to the hospital environment and increased treatment duration.

Exhaled VOC have been reported to be capable of identifying those asthma patients in which oral corticosteroid and salbutamol urinary metabolites were present (200); a possible confounder. Known drug metabolites could be excluded from analysis and the use of filtered air and alveolar gradients aim to minimise the impact of the exposome but – as previously discussed – this is an imperfect solution; particularly given the apparently limited filtration achieved with the current equipment.

The initial intention had been to capture breath samples during an acute exacerbation and then again at a future time-point when the patient was controlled and stable. This was often not the case; although participants frequently returned to their prior 'normal' state this could not necessarily be called stable and controlled; this can be seen from ACQ scores.

A comparison can therefore be made between acute attack and a less acute state but it cannot be claimed that the comparison is with that of a controlled condition. In a steroid withdrawal study Fens et al (205) reported that VOC present in the breath at the point of loss of control often had not returned to baseline values by the time of recovery (four weeks after completing a course of steroids).

This does raise concerns as to whether follow-up appointments at a post-exacerbation asthma clinic would represent a true baseline.

Although diagnosed with an acute asthma attack the diagnosis in many cases was unclear and potentially confounded. Seven participants were removed before study completion due to a change of diagnosis or diagnosis of a comorbidity; these included sarcoidosis, tachycardia, heart failure, atelectasis and bronchiectasis. The extent to which new diagnoses were considered to have replaced the prior diagnosis (asthma) varied, in some cases the new diagnosis did not alter the view that the presentation was an acute asthma attack; in other cases the prior diagnosis was considered to have been a misdiagnosis. In any future study an *a priori* decision as to how such samples will be used in the analysis should be made, and a decision tool for the process leading to sample exclusion detailed.

A surprising (to the researcher) number of patients admitted with acute asthma attack were current or recent smokers. This reduced opportunities for recruitment but in addition suggests that future studies of diagnostic test accuracy or biomarker use would need to include smokers if the equipment is to be clinically useful.

There were a number of incomplete data sets; the reasons included recruitment late in the study with insufficient time for follow-up; subsequent diagnosis leading to exclusion; lack of recovery and commencement on a biologic; withdrawal of consent;

and moving out of area. Relatively few participants were willing or able to provide a spontaneous sputum sample thereby limiting the ability to identify bacterially triggered exacerbation.

8.3.2.2 Processing factors

Due to the location of facilities within the hospital and the researcher working alone, the time between obtaining EBC samples and their freezing was longer than desirable (a mean of 49 min). The extent to which this might affect samples will depend upon the analyte being examined. The time between the first samples being obtained and the end of the study meant EBC samples were frozen for up to 18 months; the extent to which this might compromise any subsequent analysis will again depend to some extent on the analyte in question.

A future study of similar design would need to balance the twin issues of processing samples in batches within a timeframe designed to minimise sample degradation, and accounting for possible batch difference. The analyte under consideration will also determine whether any chemical additive is required – for example the antioxidant butylated hydroxytoluene (BHT) is suggested for the storage of EBC prior to analysis of 8-isoprostane.

The equivocal results of the systematic review of 8-isoprostane in EBC (chapter 2), coupled with the lack of success in analysing previously obtained EBC samples for 8-isoprostane (chapter 4) led to abandonment of the planned analysis of EBC samples for 8-isoprostane. Having commenced the collection of EBC samples from study participants this was continued in order to obtain data on the feasibility of obtaining such samples in the acute setting and of participants undergoing multiple sampling methods at one acute visit. Data was also obtained on the acceptability of such devices with the use of the RTube providing useful contrast to the ReCIVA. It is a limitation of this thesis that no alternative analyte was identified in order to permit swift processing and analysis of samples upon study completion.

Sample transport, processing and tracking was improved for the ABC study but was not optimised for the ABBA study. The unpredictable nature of the study design meant that sampling tubes which were prepared by MIB were shipped to the UEA and stored until use, with the period between preparation and usage being variable.

Conducting study visits for inpatients proved to be time consuming and frequently disrupted. On occasion participants had completed a nebulized therapy just prior to a study visit; moved wards part way through an assessment; or received other medications or a meal part way through the assessment.

While this may not have affected the VOC sampling process it did mean that – on occasion - there was a time delay between for example completing FeNO sampling and undertaking VOC sampling.

Samples were stored for up to 2 weeks before they were shipped to MIB. In the case of one sample insufficient purging resulted in excess humidity and an inability to analyse the sample. For three participants sampling appeared to be slow and in ten minutes less than the target 0.5L was collected; in two of the cases this occurred during the acute exacerbation sampling rather than the controlled follow-up. The reasons for this under-sampling are unclear – none of the participants complained of air leak nor was there any audible indicator of air egress; and the respiratory rates of the participants varied.

In one case there was a significant deficit with only 180ml collected; in this case the participant had a beard and on the CRF was recorded as breathing rapidly (24-30 breaths per minute) when using the ReCIVA. On the sampling log subsequently obtained from the ReCIVA data files, the respiratory rate was recorded at 12 – a significant mismatch between observed and recorded breath rate. It is likely that there was a gating issue. The MIB breathomics group used R to create a file reader capable of presenting the data files created by the ReCIVA in a user-friendly format. This allowed sampling volumes to be checked on completion of the sampling process rather than at a later date. While this provides useful data it would not allow resolution of gating problems such as those experienced on this occasion. Gating settings are modifiable but would require input from the manufacturer to develop a troubleshooting guide. This would also require goodwill and additional time from the participant to repeat sampling processes.

While pre-study tests were conducted to determine the presence of contaminants in the hospital oxygen supply and to determine the contribution of tygon tubing to contaminants, a direct comparison wasn't made between contaminants in room air and filtered air; nor was a comparison made between VOC in the filtered air supply and those in exhaled breath.

This was largely because there was no *a priori* target compound list and also because the GC-MS analysis was not being conducted in-house. The external processing of samples and pre-processing of sample data proved lengthy, with the first data set not available until a significant time after study-completion. In-house GC-MS analysis might facilitate more timely analysis of data and permit interim quality control analysis; alternatively commercial agreement with agreed timescales for sample processing including quality assurance procedures might be explored.

8.3.2.3 Organisation factors

It is likely that those experiencing milder asthma attacks or those which were more responsive to treatment will have been excluded from the acute arm of recruitment. For patients presenting to the emergency department, 'shortness of breath' or 'chest pain' were frequently recorded. Of these, patients with asthma were a minority; cardiac and other respiratory conditions such as infection and COPD exacerbation were commonly recorded under these codes. It tended to be only be at the point of either admitting the patient or discharging them that a diagnosis – for example asthma - would be entered onto the IT system. Screening all patients presenting with chest symptoms would have given a relatively low yield of suitable patients and would have required a permanent presence in the emergency department; this was not possible.

Screening for an asthma diagnosis tended to yield patients who had been or were in the process of being discharged (and did not want to stay an additional 2 hours for study participation) or who had been or were in the process of being admitted. The NNUH has launched a 12-month pilot project in which an asthma specialist nurse is based in A&E; a referral pathway has been established and the asthma service is being contacted with the details of asthma presentations soon after admission.

Were the study to run now it is likely that this group of patients would not be excluded. However, the feasibility outcomes may differ – given the mean time taken to complete an assessment with multiple methods of breath assessment, it is not a surety that the participation rate and number of participants completing the assessment would be the same. It is likely that a shorter, more focussed study visit would be required to assess such a patient group.

Extracting data relating to inhaler use was difficult; patients were often – understandably - unclear as to what treatments they had received when, so patient reports of time-since-last-inhaler/nebuliser were often unclear.

Closer contact with study participants might have resulted in a greater number of stage four (outpatient exacerbation) visits. Collection of email addresses - along with the appropriate consent - could have been used to issue a newsletter; this could have contained short articles on asthma research along with a reminder on how to participate and when to contact us. Alternatively text reminders or a WhatsApp group could be used. In a similar vein, a greater number of staff trained on the study protocol would have resulted in greater flexibility in offering convenient appointments for those contacting the study team for sampling during an exacerbation.

8.3.2.4 Methodological recommendations

Future studies investigating acute asthma exacerbation and capturing data on triggers - including infection - will need to improve the number of sputum samples yielded. It is conceivable that an experienced respiratory physiotherapist coaching participants through active cycle breathing could increase the yield; also a study focussed more specifically on infective markers might exclude those participants not willing to provide a sample and/or reduce the number declining to attempt sputum production. Induced sputum is not contra-indicated in this population but including this in a study protocol might reduce participation. Given the growing number of published studies of both targeted and untargeted approaches, future studies should aim to validate previous findings with studies powered according to reported compound differences.

8.4 Common limitations – contaminants

In preparing for the studies, the levels of contaminants present in hospital oxygen and air supplies was measured and the effect of the tubing used upon contaminant levels examined. It was determined that contaminants with the hospital oxygen supply were low and consistent however this proved not to be pertinent to the study outcome as it did not prove possible to recruit and assess participants early in their admission while still in receipt of oxygen.

Hospital air could not be used as it was not supplied at sufficient flow rates to power the ReCIVA breath capture device and was not available in all clinical areas. A CASPER clean air supply device was purchased; this contains Desotec activated carbon pellets in a cylindrical chamber. With the CASPER in commercial use and used by the MIB breath research group we did not conduct tests to ascertain its filtration efficacy. Moreover, such efforts are challenging to perform in advance; until patient samples are analysed; a target list of compounds generated; and chromatograms deconvolved, it is not possible to determine the compounds of interest and the levels of such within filtered air. Moreover, the absolute level is less important than the concentration relative to patient samples; the range of which may not be known in advance. This provides a salutary lesson; if undertaking a targeted analysis compounds of interest should be identified *a priori* and filtration systems assessed with regard to these compounds; alternatively if undertaking an inductive analysis checks should be undertaken at the earliest opportunity to determine possible compounds, their levels within patient samples and their levels in filtered air.

Future research will need to assess clean air filtration systems and their performance particularly on compounds of interest in order to determine the best clean air source for breathomic studies.

Additionally, predictive models based on data generated by different approaches (alveolar gradient; exclusions; correction according to retention indices) might be tested in clinical settings to evaluate their validity.

8.5 Difficulties in study completion – organisational difficulties

The study was the result of a matched funding bid between the UEA and AUKCAR. Initially the study author was the UEA funded candidate; the funding stream was later switched to that of AUKCAR; this had the unintended effect of making the project eligible for registration on the NIHR clinical portfolio. That this happened at a late stage meant that the author was not able to take advantage of all the benefits this entailed and did not receive any staffing support.

Running the two studies single-handedly was challenging but gave the author a good appreciation of all aspects of research work from study design, through recruitment and assessment to data analysis.

The progress of the study was hampered by an inter-organisation debate on the definition of 'direct patient care' status. Although the researcher's role – as outlined in the study protocol and ethics application – was presented to and approved by the study sponsor (UEA), research site (NNUH), research ethics committee (REC), and Health Research Authority (HRA) the question arose as to whether the researcher was eligible to screen hospital records for potential participants. Continued recruitment would have been a possible protocol breach so the study was placed on hold while the study sponsor and research site reached an agreement. This process took four months to complete.

In order to minimise the potential impact of this 4 month hiatus and offer participants an appropriate length follow-up period the PhD was converted to a part-time programme (50% full time equivalent). At the same time the researcher was attempting to launch the ABC study. The reduced hours available to run all aspects of both studies necessarily impacted on activities such as screening; recruitment; assessment of those experiencing an asthma attack; and keeping in touch with participants in the outpatient arm. The veracity of the feasibility conclusions must be tempered by this fact. While the intended numbers of participant recruits was not achieved, the feasibility data gives a good indication of what might be possible with increased resourcing. An assessment of organisational capacity is required to ensure successful delivery with particular reference being paid to staffing and the nature of employment contracts.

8.6 Conclusion

In conclusion, this thesis has demonstrated that an acute asthma breath study is feasible but methods are not yet fully established and further developmental work is required. That statistically significant changes in VOC levels after a positive mannitol challenge were detectable, and that these included some of the compounds identified by previous studies is encouraging. However, the reliability of the data is compromised by the high exogenous VOC levels present in background samples; and the statistical significance of the results is undermined by the results of FDR calculations. Results of the ABC study should therefore be interpreted with caution.

While the ABBA study is concerned with feasibility, the ABC study can best be characterised as a phase one, pre-clinical exploratory study identifying compounds with potential relevance to asthma for prioritisation in future research. A more appropriately powered study may determine whether there are any baseline markers predictive of response to mannitol challenge, while also validating those compounds highlighted by the pairwise analysis as potential markers of early inflammatory activity. Based on the two most frequently identified VOC in asthma breathomics a future trial would need to enrol approximately 80 participants to have sufficient power to detect significant differences in isoprene between positive and negative bronchial challenges (in either baseline or post-challenge samples). If acetone were the focus then a much larger sample size - running into hundreds or even thousands of participants - might be needed. While these numbers may be helpful, it is clear that making such results available from other breathomic studies would aid the design of appropriately powered studies for alternative compounds of interest.

The implication for future research is that sample size needs to be much greater if untargeted analysis or targeted analysis with a large compound list is attempted. While a large study with untargeted methods is perfectly appropriate, given the number of research papers published on asthma, and the range of clinical questions these have addressed, targeted studies validating previously published results is perhaps the next step. Isoprene is amongst the compounds most frequently reported in the literature; it was found to differ in this study as a result of a positive bronchial challenge and should be at the front of any list of compounds to be validated as potential asthma biomarkers.

For industry - those developing equipment to capture and analyse VOC - there are major issues about technical performance and the ability to monitor this over time. Addressing issues such as contaminants will be more easily achieved when analysing a limited number of compounds, however, attempts to deal with this in inductive studies with large data sets are required; along with methods for monitoring the success of any such attempts.

Cost and resources have not been explicitly considered in this thesis but conducting breath research is an expensive undertaking. The RTube is relatively cheap to purchase but processing the samples requires technical expertise and access to laboratory equipment. The ReCIVA, CASPER clean air filter, and sorbent tubes used for sample capture are more expensive and again require specialised equipment and expert staff to undertake sample analysis. Furthermore, data obtained from such analysis requires pre-processing which is a time consuming and specialised undertaking. As such these methods are unlikely to be widely available to clinicians in their current form. However, using these methods to identify and subsequently validate disease biomarkers would narrow the number of target compounds opening the way for more focussed methods of analysis appropriate to online or point of care testing. The development of point of care tests measuring markers of disease activity would provide opportunities for the development of personalised medicine and the targeted use of therapeutics.

In summary, this thesis has found that disease heterogeneity is well matched by heterogeneity in approaches to breath analysis and compounds identified as potential biomarkers. VOC analysis has been the methodology of choice in recent years; it is well suited to identifying panels of markers reflective of multiple underlying disease processes. The researcher has shown that such a study is possible in the acute setting, although there are a number of major limitations in the research presented herein. Methodological refinement is essential, not least clean air filtering and it's monitoring; and an appropriately tolerated method for obtaining sputum samples in the acute setting. Changes in exhaled VOC as a result of mannitol challenge are detectable but determining their significance relies upon the generation of reliable data. While VOC analysis holds great promise it still has a long way to go before it becomes a clinically useful tool.

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Appendices

Appendix 1 - Research Ethics Committee and Health Research Authority approvals for Bronchial challenge Testing in Asthma: The Effect of Mannitol Dry Powder Inhalation on Volatile Organic Compounds in Exhaled Breath

Appendix 2 - Research Ethics Committee and Health Research Authority approvals for Exhaled Breath Biomarkers in Acute Asthma: A Feasibility Study

Appendix 3 - ABC study: patient information sheets and questionnaires

Appendix 4 - ABBA study: patient information sheets and questionnaires

Appendix 5 - Documents relating to the development of the AAQ-R questionnaire

Appendix 1 – Research Ethics Committee and Health Research Authority approvals for Bronchial Challenge Testing in Asthma: The Effect of Mannitol Dry Powder Inhalation on Volatile Organic Compounds in Exhaled Breath

NHS
Health Research Authority
East of England - Cambridge South Research Ethics Committee
The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

12 December 2017

Mr Adam M Peel
Norwich Medical School
University of East Anglia
Norwich Research Park, Norwich
NR4 7TJ

Dear Mr Peel

Study title:	**Bronchial Challenge Testing in Asthma: The Effect of Mannitol Dry Powder Inhalation on Volatile Organic Compounds in Exhaled Breath**
REC reference:	**17/EE/0430**
IRAS project ID:	**229225**

Thank you for your letter responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair. .

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact hra.studyregistration@nhs.net outlining the reasons for your request.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Non-NHS sites

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Copies of advertisement materials for research participants [eBulletin advertisement]	1.1	09 November 2017
Copies of advertisement materials for research participants [ABC recruitment advert 1]	1.1	09 November 2017
Copies of advertisement materials for research participants [ABC recruitment advert 2]	1.1	09 November 2017
Copies of advertisement materials for research participants [Text for NNUH webpage]	1.1	09 November 2017
Copies of advertisement materials for research participants [ABC Social Media Text]	1.1	09 November 2017
Copies of advertisement materials for research participants [ABC academic poster]	1.1	09 November 2017
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [I & I letter]	1.0	27 September 2017
GP/consultant information sheets or letters [GP letter (non-PIC site)]	1.1	09 November 2017
GP/consultant information sheets or letters [GP letter (PIC site)]	1.1	09 November 2017
GP/consultant information sheets or letters [GP letter - patient results]	1.1	09 November 2017
GP/consultant information sheets or letters [Primary care data extraction questionnaire]	1.1	09 November 2017
Instructions for use of medical device [Osmohale (mannitol challenge) product information sheet]	3.0	
Instructions for use of medical device [CASPER Air Supply - Instructions for Use]	1	02 February 2016
Instructions for use of medical device [ReCIVA Breath Sample - Instructions for Use]	5b	13 October 2016
IRAS Application Form [IRAS_Form_06102017]		06 October 2017
Letter from funder [Letter of financial commitment; NNUH]	v2.0	16 August 2017
Letters of invitation to participant [Letter of invitation with PIS]	1.1	09 November 2017
Letters of invitation to participant [Letter of invitation without PIS]	1.1	09 November 2017
Letters of invitation to participant [Letter of invitation from NNUH]	1.1	09 November 2017

Letters of invitation to participant [ABC letter of invitation to potential participants_ Mailout reminder]	1.1	09 November 2017
Letters of invitation to participant [Appointment 1 letter]	1.1	09 November 2017
Letters of invitation to participant [Appointment 2 letter]	1.1	09 November 2017
Letters of invitation to participant [Appointment 3 letter]	1.1	09 November 2017
Letters of invitation to participant [Appointment reminder letter]	1.1	09 November 2017
Letters of invitation to participant [Missed appointment letter]	1.1	09 November 2017
Letters of invitation to participant [Response to enquiry follow-up letter]	1.1	09 November 2017
Non-validated questionnaire [ABC waiting room questionnaire_ pre-assessment v1.0]	v1.0	26 July 2017
Non-validated questionnaire [Baseline visit, waiting room questionnaire]	1.1	09 November 2017
Non-validated questionnaire [ABC waiting room questionnaire_ pre-assessment v1.0]	1.1	09 November 2017
Non-validated questionnaire [ABC Pre-assessment safety checks]	1.1	09 November 2017
Other [Database search_ Inclusion-exclusion criteria]	1	26 July 2017
Other [Primary Care Search Guidelines]	1	26 July 2017
Other [PhD funding letter]	v1.0	03 February 2017
Other [Database search_ Inclusion-exclusion criteria]	1.1	09 November 2017
Other [Primary Care Search Guidelines]	1.1	09 November 2017
Participant consent form [Consent form]	1.1	09 November 2017
Participant information sheet (PIS) [Participant information sheet]	1.1	09 November 2017
Participant information sheet (PIS) [Preparing for your study visit - information sheet]	1.1	09 November 2017
Research protocol or project proposal [Protocol v1]	1.1	09 November 2017
Response to Request for Further Information		
Summary CV for Chief Investigator (CI) [Adam Peel CV]	1	21 July 2017
Summary CV for student [Adam Peel CV]	1	21 July 2017
Summary CV for supervisor (student research) [Andrew Wilson CV]	1	06 February 2014
Summary CV for supervisor (student research) [Supervisior CV Yoon Loke]	2	13 December 2015
Validated questionnaire [Asthma Control Questionnaire (ACQ)]		08 September 2010

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at
http://www.hra.nhs.uk/hra-training/

17/EE/0430	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

Dr Leslie Gelling
Chair

Email:nrescommittee.eastofengland-cambridgesouth@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Ms Sarah Green*
Ms Laura Harper, Norfolk & Norwich University Hospital NHS Trust

Appendices

LYLE, Isobel (HEALTH RESEARCH AUTHORITY) <isobel.lyle@nhs.net>

Today, 11:05

Adam Peel (MED - Student)

Reply

IRAS ID 229225 ABC s... 542 KB | IRAS ID 229225 ABC s... 537 KB | IRAS ID 229225 ABC s... 94 KB | IRAS ID 229225 ABC s... 95 KB

4 attachments (1 MB) Download all Save all to OneDrive - University of East Anglia

Dear Adam

Thank you for your response to the Initial Assessment. I can confirm that your study now meets HRA standards with the following outstanding –

Evidence of insurance which you have confirmed you will send to me when available from the Sponsor. This can be sent to me by email. There is no need to also upload to IRAS.

CRN support

I note your confirmation that PIC activity is reliant on CRN support and will record this in the outcome of your application for HRA Approval.

Statement of Activities and Schedule of Events

-

I have made some adjustments to each of these. In the Statement of Activities for both the research site and the PIC sites, I have clarified that there is no separate Agreement and adjusted Schedule 1 on Funding for the PIC sites for clarity.

In both of the Schedule of Events, I have clarified where a member of the direct care team is required to identify and introduce the study to a potential participant which you have advised in the case of the research site, may or may not be yourself.

I have attached these documents which can now be used as an engagement tool with participating NHS organisations

Regulatory approval

I note the changes that REC has requested. HRA Approval consists of an assessment against HRA standards and the relevant regulatory approval(s). I will, therefore, be in a position to provide you with an outcome to your application for HRA Approval when I have evidence of insurance and the REC opinion is known.

If you have any queries, please don't hesitate to contact me.

Kind regards

Isobel Lyle | Senior Assessor
Health Research Authority
Room 002, TEDCO Business Centre, Rolling Mill Rd, Jarrow NE32 3DT
T: 0207 972 2496
Hra.approval@nhs.net or Isobel.lyle@nhs.net

Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

Adam Pool
Norwich Medical School
Floor 2, Bob Champion Research and Education Building
James Watson Road
University of East Anglia
Norwich Research Park
Norwich, NR4 7UQ

Please reply to: Research and Development Department
Level 3, East Block, Room 352
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Colney Lane
Norwich
Norfolk
NR4 7UY
Direct Dial: 01603 286806
Internal: 5806
e-mail: rodoffice@nnuh.nhs.uk
Website: www.nnuh.nhs.uk

12/03/2018

Dear Mr.Pee ,

Confirmation of Capacity and Capability

RE: 229226(19-02-18)

Study Title: Bronchial Challenge Testing in Asthma: The Effect of Mannitol Dry Powder Inhalation on Volatile Organic Compounds in Exhaled Breath

This letter confirms that **Norfolk and Norwich University Hospitals NHS Foundation Trust** has the capacity and capability to deliver the above referenced study. Please find attached our Statement of Activities as confirmation.

We agree to start this study on a date to be agreed when the sponsor gives the green light to begin.

If you wish to discuss further, please do not hesitate to contact me.

Kind regards

Professor Alastair Forbes
Chief of Research and Innovation

Cc. Professor Andrew Wilson,Susan Robinson, Chris Atkins, Dayle Terrington

Appendices

MAJSAK-NEWMAN, Gosia (NHS SOUTH NORFOLK CCG) <g.majsak-newman@nhs.net>

Reply

Wed 03/01, 15:35

Adam Peel (MED - Student); Sarah Green (RIN - Staff); MCCLOSKEY, Kate (NORFOLK COMMUNITY HEALTH AND CARE NHS TRUST) <kate.mccloskey@nhs.net>; +2 more

Dear Adam,

Arrangements to support the below study at GP practices within CRN: Eastern.

Re: IRAS ID 229225 Bronchial Challenge Testing in Asthma: The Effect of Mannitol Dry Powder Inhalation on Volatile Organic Compounds in Exhaled Breath (The ABC Study)

This email confirms that the following arrangements are in place to support the study:

- HRA Approval is in place
- Service support costs have been agreed
- Agreements are drafted and acceptable
  - SOAs will be used as agreement with Practices
- Human Resources arrangements are not required for Primary Care (research activities will be placed at the NNUH)

Practices will need to confirm their capacity and capability to deliver the research by returning

- **Email confirmation of SOA or signed RISP**

Once your study has completed, we would be grateful if you could forward a copy of the final report, a one page lay summary and any publications associated with the study to snccg.randdoffice@nhs.net or CAPCCG.RandDoffice@nhs.net

May we take this opportunity to wish you well with your research and we look forward to hearing the outcomes for the study. Please note the reference number for this study is **Ref: IRAS ID 229225** and this should be quoted on all correspondence.

Kind regards

Vivienne Shaw
Cambridge Office
Research Governance Manager

Clare Symms
Norfolk and Suffolk Office
Research Management and Finance Lead

Gosia Majsak-Newman
Research & Development Officer/ NIHR GCP Facilitator

Norfolk and Suffolk Primary and Community Care Research Office | Hosted by South Norfolk CCG | Lakeside 400, Broadland Business Park | Norwich NR7 0WG |

Switchboard: 01603 257 000
Direct dial: 01603 257 283
Mobile: 07552 008271

E-mail: g.majsak-newman@nhs.net
Team email: snccg.RandDoffice@nhs.net

NHS
Health Research Authority

East of England - Cambridge South Research Ethics Committee

The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

08 June 2018

Mr Adam M Peel
Norwich Medical School
University of East Anglia
Norwich Research Park, Norwich
NR4 7TJ

Dear Mr Peel,

Study title:	**Bronchial Challenge Testing in Asthma: The Effect of Mannitol Dry Powder Inhalation on Volatile Organic Compounds in Exhaled Breath**
REC reference:	**17/EE/0430**
Amendment number:	**Version 1.2 10/05/2018**
Amendment date:	**10 May 2018**
IRAS project ID:	**229225**

The above amendment was reviewed on 01 June 2018 by the Sub-Committee in correspondence.

Ethical opinion

The researchers were contacted via to respond to requests made by the Sub-Committee as follows.

The Sub-Committee noted a lack of clarity in one Participant Information Sheet about the receipt of gift vouchers by the participant after each study visit, and requested that this be revised for clarity. The Sub-Committee members also noted a spelling error in the Participant Information Sheet "Preparing for your study visit" and requested this be corrected.

The applicant responded, providing the amended documents as requested.

The members of the Sub-Committee were satisfied with the response and amended documents submitted by the researcher and were content to issue a Favourable Opinion for the Amendment.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Copies of advertisement materials for research participants [ABC recruitment advert 1 - Clean]	1.2	
Copies of advertisement materials for research participants [ABC recruitment advert_grey - Tracked Changes]	1.2	
Copies of advertisement materials for research participants [ABC recruitment advert 1_peach - Clean]	1.2	
Copies of advertisement materials for research participants [ABC recruitment advert 1_peach - Tracked Changes]	1.2	
Copies of advertisement materials for research participants [ABC recruitment advert 3]	1.1	
Copies of advertisement materials for research participants [ABC recruitment flyer 1]	1.1	
Copies of advertisement materials for research participants [ABC recruitment flyer 2]	1.1	
Copies of advertisement materials for research participants [ABC Social Media Text - Clean]	1.2	
Copies of advertisement materials for research participants [ABC Social Media Text - Tracked Changes]	1.2	
Covering letter on headed paper		16 May 2018
GP/consultant information sheets or letters [ABC NNUH Bronch Chal_GP letter]	1.1	10 May 2018
Letters of invitation to participant [ABC Appointment 1 letter _Consent visit - Clean]	1.2	10 May 2018
Letters of invitation to participant [ABC Appointment 1 letter _Consent visit - Tracked Changes]	1.2	10 May 2018
Letters of invitation to participant [ABC NNUH Bronch Chal_f.up appt letter]	1.1	10 May 2018
Letters of invitation to participant [ABC NNUH Bronch Chal_Letter of invitation]	1.1	10 May 2018
Notice of Substantial Amendment (non-CTIMP)	Version 1.2 10/05/2018	10 May 2018
Other [Osmohale_spc_uk datasheet]		
Participant consent form [ABC consent form - Clean]	1.2	10 May 2018
Participant consent form [ABC consent form - Tracked Changes]	1.2	10 May 2018
Participant information sheet (PIS) [ABC NNUH Bronch Chal_Participant Info Sheet - Tracked Changes]	1.2	10 May 2018
Participant information sheet (PIS) [ABC NNUH Bronch Chal_Preparing for your study visit]	1.1	10 May 2018
Participant information sheet (PIS) [ABC Preparing for your study visit info sheet]	1.2	10 May 2018
Participant information sheet (PIS) [ABC Participant Info Sheet]	1.2	10 May 2018
Research protocol or project proposal [Clean]	1.2	10 May 2018
Research protocol or project proposal [Tracked Changes]	1.2	10 May 2018

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

17/EE/0430:	**Please quote this number on all correspondence**

Yours sincerely,

P.P.

Dr Leslie Gelling
Chair

E-mail: nrescommittee.eastofengland-cambridgesouth@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Ms Laura Harper, Norfolk & Norwich University Hospital NHS Trust*
Mr Adam M Peel

Appendices

IRAS Project ID 229225. HRA Approval for the Amendment

H hra.amendments@nhs.net

Today, 12:50

Adam Peel (MED - Student); Sarah Green (Adapt - Staff); Sarah Green (Adapt - Staff); hra.amendments@nhs.net; l.harper@nnuh.nhs.uk; l.harper@nnuh.nhs.uk

Reply

Deleted Items

Dear Mr Peel,

IRAS Project ID:	229225
Short Study Title:	Asthma Bronchial Challenge: The ABC Study
Amendment No./Sponsor Ref:	Version 1.2 10/05/2018
Amendment Date:	10 May 2018
Amendment Type:	Substantial Non-CTIMP

I am pleased to confirm **HRA and HCRW Approval** for the above referenced amendment.

You should implement this amendment at NHS organisations in England and Wales, in line with the conditions outlined in your categorisation email.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/.

Please contact [hra.amendments@nhs.net]hra.amendments@nhs.net for any queries relating to the assessment of this amendment.

Kind regards

Andrea Bell
Health Research Authority
Ground Floor | Skipton House | 80 London Road | London | SE1 6LH
E.hra.amendments@nhs.net
W. www.hra.nhs.uk

From: hra.amendments@nhs.net [mailto:hra.amendments@nhs.net]
Sent: Monday, June 11, 2018 12:50 PM
To: a.peel@uea.ac.uk; sarah.green@uea.ac.uk; sarah.green@uea.ac.uk
Cc: hra.amendments@nhs.net; l.harper@nnuh.nhs.uk; l.harper@nnuh.nhs.uk
Subject: IRAS Project ID 229225. HRA Approval for the Amendment

Dear Mr Peel,

IRAS Project ID:	229225
Short Study Title:	Asthma Bronchial Challenge: The ABC Study
Amendment No./Sponsor Ref:	Version 1.2 10/05/2018
Amendment Date:	10 May 2018
Amendment Type:	Substantial Non-CTIMP

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You should implement this amendment at NHS organisations in England and Wales, in line with the conditions outlined in your categorisation email.

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Please contact hra.amendments@nhs.net for any queries relating to the assessment of this amendment.

Kind regards

Andrea Bell
Health Research Authority
Ground Floor | Skipton House | 80 London Road | London | SE1 6LH
E.hra.amendments@nhs.net
W. www.hra.nhs.uk

NHS
Health Research Authority

East of England - Cambridge South Research Ethics Committee

The Old Chapel
Royal Standard Place
Nottingham
NG1 6FS

Please note: This is the favourable opinion of the REC only and does not allow the amendment to be implemented at NHS sites in England until the outcome of the HRA assessment has been confirmed.

30 November 2018

Mr Adam M Peel
Norwich Medical School
University of East Anglia
Norwich Research Park, Norwich
NR4 7TJ

Dear Mr Peel,

Study title:	**Bronchial Challenge Testing in Asthma: The Effect of Mannitol Dry Powder Inhalation on Volatile Organic Compounds in Exhaled Breath**
REC reference:	**17/EE/0430**
Amendment number:	**Version 1.3**
Amendment date:	**05 November 2018**
IRAS project ID:	**229225**

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

The members of the Sub-Committee were in agreement that the Substantial Amendment did not raise any material ethical issues.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering letter on headed paper		05 November 2018
GP/consultant information sheets or letters [ABC GP participant results letter v1.2 - Clean]	1.2	05 November 2018

GP/consultant information sheets or letters [ABC GP participant results letter v1.2 - Tracked Changes]	1.2	05 November 2018
GP/consultant information sheets or letters [ABC GP letter for non-PIC surgeries v1.2 - Clean]	1.2	05 November 2018
GP/consultant information sheets or letters [ABC GP letter for non-PIC surgeries v1.2 - Tracked Changes]	1.2	05 November 2018
GP/consultant information sheets or letters [ABC GP letter for PIC surgeries v1.2 - Clean]	1.2	05 November 2018
GP/consultant information sheets or letters [ABC GP letter for PIC surgeries v1.2 - Tracked Changes]	1.2	05 November 2018
Notice of Substantial Amendment (non-CTIMP)	Version 1.3	05 November 2018
Research protocol or project proposal [ABC protocol v1.3 05.11.18 - Clean]	1.3	05 November 2018
Research protocol or project proposal [ABC protocol v1.3 05.11.18 - Tracked Changes]	1.3	05 November 2018

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

17/EE/0430: Please quote this number on all correspondence

Yours sincerely,

P.P.

Dr Leslie Gelling
Chair

E-mail: nrescommittee.eastofengland-cambridgesouth@nhs.net

Enclosures: *List of names and professions of members who took part in the review*

Copy to: *Ms Laura Harper, Norfolk & Norwich University Hospital NHS Trust*
Mr Adam M Peel

East of England - Cambridge South Research Ethics Committee

Attendance at Sub-Committee of the REC meeting on 30 November 2018

Committee Members:

Name	*Profession*	*Present*	*Notes*
Dr Leslie Gelling	Reader in Research Ethics	Yes	
Mrs Nikki Phillimore	Locum Pharmacist	Yes	

Also in attendance:

Name	*Position (or reason for attending)*
Kate Loven	REC Assistant

hra.amendments@nhs.net <noreply@harp.org.uk>
Mon 03/12, 07:03
Adam Peel (MED - Postgraduate Researcher); Sarah Green (Adapt - Staff); l.harper@nnuh.nhs.uk

You forwarded this message on 03/12/2018 07:16

Dear Mr Peel

IRAS Project ID:	229225
Short Study Title:	Asthma Bronchial Challenge: The ABC Study
Amendment No./Sponsor Ref:	Version 1.3
Amendment Date:	05 November 2018
Amendment Type:	Substantial Non-CTIMP

I am pleased to confirm **HRA and HCRW Approval** for the above referenced amendment.

You should implement this amendment at NHS organisations in England and Wales, in line with the conditions outlined in your categorisation email.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/.

Please contact hra.amendments@nhs.net for any queries relating to the assessment of this amendment.

Kind regards

Mrs Kirsten Peck
HRA Approval Amendment Coordinator
Health Research Authority
Ground Floor | Skipton House | 80 London Road | London | SE1 6LH
E.hra.amendments@nhs.net
W. www.hra.nhs.uk

Appendix 2 – Research Ethics Committee and Health Research Authority approvals for Exhaled Breath Biomarkers in Acute Asthma: A Feasibility Study

NHS
Health Research Authority
London - Fulham Research Ethics Committee
Barlow House
3rd Floor, 4 Minshull Street
Manchester
M1 3DZ

Telephone: 0207 104 8021

01 July 2016

Mr Adam M Peel
Norwich Medical School
University of East Anglia
Norwich Research Park, Norwich
NR4 7TJ

Dear Mr Peel

Study title:	**Exhaled Breath Biomarkers in Acute Asthma: A Feasibility Study**
REC reference:	**16/LO/0639**
Protocol number:	**AsthmaEBC2016**
IRAS project ID:	**197935**

Thank you for your submission, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager, Ms Anna Bannister, nrescommittee.london-fulham@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for NHS permission for research is available in the Integrated Research

Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Copies of advertisement materials for research participants [ABBA primary care recruitment poster v1.0]	1	08 March 2016
Covering letter on headed paper [Re-submission cover letter]		08 June 2016
GP/consultant information sheets or letters [ABBA GP letter v1.0]	1	08 March 2016
Instructions for use of medical device [Niox Mino User Manual v.9]	9	01 December 2012
Instructions for use of medical device [RTUbe User Instructions]	1	08 March 2016
Instructions for use of medical device [ReCIVA Instructions]	1	17 December 2015
Interview schedules or topic guides for participants [ABBA Exacerbation card v1.0]	1	08 March 2016
Interview schedules or topic guides for participants [ABBA Exacerbation Flyer v1.0]	1	08 March 2016

IRAS Checklist XML [Checklist_21032016]		21 March 2016
Letters of invitation to participant [ABBA letter of invitation to focus group v1.0]	1	08 March 2016
Letters of invitation to participant [ABBA Letter of invitation to potential participants]	1	08 March 2016
Non-validated questionnaire [AAQ-R Questionnaire v1.0]	1	08 March 2016
Other [ReCIVA Summary]	No version number and not dated	
Other [CASPER air supply for ReCIVA (if required) - Instructions]	a	02 February 2016
Participant consent form [ABBA consent form v1.0]	1	08 March 2016
Participant consent form [ABBA Focus group consent form v1.0]	1	08 March 2016
Participant information sheet (PIS) [ABBA Patient Information Sheet A - Acute]	1.1	06 May 2016
Participant information sheet (PIS) [ABBA Patient Information Sheet B - Outpatient]	1.1	06 May 2016
REC Application Form [REC_Form_14062016]		14 June 2016
Research protocol or project proposal [ABBA protocol v1.0]	v1.1	06 June 2016
Summary CV for Chief Investigator (CI) [Adam Peel CV]	v1	15 December 2015
Summary CV for supervisor (student research) [Andrew Wilson CV]		
Summary CV for supervisor (student research) [Yoon Kong LOKE]		
Validated questionnaire [Asthma Control Questionnaire]	1	08 March 2016

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form

available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

16/LO/0639	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

Signed on behalf of:
The Rev'd Nigel Griffin
Chair
Email:nrescommittee.london-fulham@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: *Ms Hannah Ings*
Miss Laura Harper, Norfolk & Norwich University Hospital NHS Trust

Our Vision
To provide every patient with the care we want for those we love, every time

Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

Mr Adam Peel
University of East Anglia
NR4 7TJ

Research & Development Office
Level 3 East
Norfolk & Norwich University Hospitals NHS Foundation Trust
Colney Lane
Norwich
NR4 7UY

Direct dial: 01603 289808
Internal: 5808
E-mail: rdoffice@nnuh.nhs.uk
Website: www.nnuh.nhs.uk

01/11/2016

Dear Mr Peel

Re: **IRAS Reference Number:** 197935
R&D Reference Number: 197935 (206-12-15)
Project Title: The Utility of Biomarkers in the Management of Acute Asthma: A Feasibility Study Examining Assessments of Severity, Phenotypes and Triggers
Sponsor: University of East Anglia

I am pleased to inform you that the above Non CTIMP project has been given full NHS permission for research at Norfolk & Norwich University Hospitals NHS Foundation Trust.

This NHS permission for research has been granted on the basis described in the application form, protocol and supporting documentation as listed below:

Document	Version	Date
Protocol	1.2	21/07/2016
Letter of Invitation (ABBA Invite to Potential Participants)	1	08/03/2016
Letter of Invitation (ABBA Invite to Focus Group)	1	08/03/2016
ABBA Primary Care Recruitment Poster	1	08/03/2016
ABBA Patient Information Sheet A - Acute	1.2	21/07/2016
ABBA Patient Information Sheet B - Outpatient	1.2	21/07/2016
ABBA Participant Consent Form	1.1	21/07/2016
ABBA Focus Group Consent Form	1	08/03/2016
ABBA GP Letter	1	08/03/2016
AAQ-R Questionnaire	1	08/03/2016
Asthma Control Questionnaire	1	08/03/2016 08 Sept 2010 – on document
CASPER Air Supply for ReCIVA - Instructions	a	02/02/2016
Niox Mino User Manual	9	01/12/2014
RTUbe User Instructions	1	
ReCIVA Instructions	1	17/12/2015

NHS
Health Research Authority

London - Fulham Research Ethics Committee
Barlow House
3rd Floor, 4 Minshull Street
Manchester
M1 3DZ

Tel: 0207 104 8021

01 September 2016

Mr Adam M Peel
Norwich Medical School
University of East Anglia
Norwich Research Park, Norwich
NR4 7TJ

Dear Mr Peel

Study title:	**Exhaled Breath Biomarkers in Acute Asthma: A Feasibility Study**
REC reference:	**16/LO/0639**
Protocol number:	**AsthmaEBC2016**
Amendment number:	**1**
Amendment date:	**21 July 2016**
IRAS project ID:	**197935**

Two protocol amendments

1. It has become apparent that the ReCIVA can be used to deliver oxygen to patients thereby permitting breath sampling while simultaneously supplying oxygen support. In light of this the below is proposed the following amendment to the protocol:
    a. For those patients receiving supplementary oxygen, we would not wait for their oxygen to be removed prior to assessment (as per the original protocol), rather we would substitute their oxygen mask / nasal cannulae with the ReCIVA device, using the ReCIVA to supply their oxygen for a period of less than 10 minutes (while simultaneously obtaining a breath sample).
    b. We would also like to ask patients who are admitted to hospital if they would be willing to undertake a further assessment. We propose only approaching patients who have been admitted (i.e. who are no longer in A&E); who have been in the hospital for 8 hours or more; who are not in receipt of oxygen; who are saturating at oxygen levels > 96% on air, speaking in full sentences and report being comfortable. In these patients we propose taking a further four samples – two samples on one occasion and two on another (both while still admitted to hospital)
2. An offer from the microbiology research team at the University of East Anglia to conduct genomic analysis (16S rRNA and/or whole genome shotgun analysis) on sputum samples. In light of this the below is proposed the following amendment to the protocol:
    a) For those patients who produce a spontaneous sputum sample when assessed in the acute setting, we will obtain a further sputum sample at the time of their follow-up clinic appointment using the commonly used sputum induction technique.
    b) Sputum will be used for bacterial culturing +/- viral analysis (at the NNUH pathology laboratory) as per routine care; but sputum eosinophil count and genomic analysis will also be conducted (at the UEA laboratory).

The above amendment was reviewed by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering letter on headed paper		08 August 2016
Notice of Substantial Amendment (non-CTIMP)	1	21 July 2016
Participant consent form	1.1	21 July 2016
Participant information sheet (PIS) [Acute]	1.2	21 July 2016
Participant information sheet (PIS) [Outpatient]	1.2	21 July 2016
Research protocol or project proposal	1.2	21 July 2016

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

16/LO/0639:	**Please quote this number on all correspondence**

Yours sincerely

Dr Shaun Griffin
Alternate Vice Chair

NHS
Health Research Authority

London - Fulham Research Ethics Committee
Barlow House
3rd Floor, 4 Minshull Street
Manchester
M1 3DZ

Tel: 0207 104 8021

04 April 2017

Adam Peel
Norwich Medical School
Faculty of Medicine & Health Sciences
University of East Anglia
Norwich Research Park
Norwich, NR4 7TJ

Dear Adam

Study title:	**Exhaled Breath Biomarkers in Acute Asthma: A Feasibility Study**
REC reference:	**16/LO/0639**
Protocol number:	**AsthmaEBC2016**
Amendment number:	**2**
Amendment date:	**29 March 2017**
IRAS project ID:	**197935**

- Change to the recruiting and consenting process
- Extend the study until 31st July 2018
- Change to analysis and storage of samples
- Change to inclusion/exclusion criteria for asthma control group
- Change of oxygen used from hospital to ReCIVA

Thank you for submitting the above amendment, which was received on 04 April 2017. I can confirm that this is a valid notice of a substantial amendment and will be reviewed by the Sub-Committee of the REC at its next meeting.

Documents received

The documents to be reviewed are as follows:

Document	*Version*	*Date*
Covering letter on headed paper		31 March 2017
Letters of invitation to participant	1	31 March 2017
Notice of Substantial Amendment (non-CTIMP)	2	29 March 2017
Participant consent form	1.2	29 March 2017
Participant information sheet (PIS) [Sheet A Acute]	1.3	29 March 2017
Participant information sheet (PIS) [Sheet B OutPatient]	1.3	29 March 2017
Research protocol or project proposal	1.3	29 March 2017

IRAS 197935. Confirmation of Amendment Assessment

GS GRIMSHAW, Sarah (HEALTH RESEARCH AUTHORITY) <sarah.grimshaw1@nhs.net>

Reply all

To: Adam Peel (MED); persephone.magdelene@uea.ac.uk
Cc: 'l.harper@nnuh.nhs.uk'; Andrew Wilson (MED); Yoon Loke (MED)

Dear Adam,

Further to the below, I am pleased to confirm that HRA Approval has been issued for the referenced amendment, following assessment against the HRA criteria and standards. During the Assessment, a non-substantial change was made to the Protocol. This was after the REC Favourable Opinion was issued but further REC review was not required due to the non-substantial nature of the amendment made. The final documents approved as part of this amendment are therefore:

Document	*Version*	*Date*
Covering letter on headed paper		31 March 2017
Letters of invitation to participant	1	31 March 2017
Notice of Substantial Amendment (non-CTIMP)	2	29 March 2017
Participant consent form	1.2	29 March 2017
Participant information sheet (PIS) [Sheet A Acute]	1.3	29 March 2017
Participant information sheet (PIS) [Sheet B OutPatient]	1.3	29 March 2017
Research protocol or project proposal	1.4	15 May 2017

The sponsor should now work collaboratively with participating NHS organisations in England to implement the amendment as per the below categorisation information. This email may be provided by the sponsor to participating organisations in England to evidence that the amendment has HRA Approval.

Please contact hra.amendments@nhs.net for any queries relating to the assessment of this amendment.

Kind regards,
Sarah

Miss Sarah Grimshaw | Senior Technical Assurances Officer
Health Research Authority
Jarrow HRA Centre, Room 001, Jarrow Business Centre, Rolling Mill Road, Jarrow, Tyne and Wear. NE32 3DT
E: sarah.grimshaw1@nhs.net | T: 0207 104 8062

Dear Mr Peel,

Thank you for submitting an amendment to your project.

If you have participating NHS/HSC organisations in any other UK nations we will forward the information to the relevant national coordinating function(s).

Please note that you may only implement changes described in the amendment notice.

What Happens Next?

When available, please forward any other regulatory approvals that are expected for this amendment to hra.amendments@nhs.net.

Information Specific to Participating NHS Organisations in England

1. You should now share details of the amendment and, if applicable, amended documents, together with this email, with all participating NHS organisations in England. In doing so, you should include the NHS R&D Office, LCRN (where applicable) as well as the local research team. A template email to notify participating NHS organisations in England is provided on the HRA website.
2. The participating NHS organisations in England should prepare to implement this amendment.
3. Your amendment will be assessed against HRA standards.
4. Once the HRA assessment has been successfully completed, you will receive an email confirming that your amendment has HRA Approval.
5. You may implement your amendment at all participating NHS organisations in England 35 calendar days from the day on which you provide the organisations with this email and your amended documents (or as soon as the participating NHS organisation confirm that you may implement, if sooner), so long as you have HRA Approval for your amendment by this date. **NHS organisations do not have to confirm they are happy with the amendment.** If HRA Approval is issued subsequent to this date, you may implement following HRA Approval.
6. You may not implement the amendment at any participating NHS organisations in England that requests additional time to assess, until it confirms that it has concluded its assessment.
7. You may not implement at any participating NHS organisation in England that declines to implement the amendment.

IRAS Project ID:	197935
Short Study Title:	Exhaled Breath Biomarkers in Acute Asthma: A Feasibility Study
Date complete amendment submission received:	25/09/2017
Sponsor Amendment Reference Number:	Amendment III
Sponsor Amendment Date:	30/05/2017
Amendment Type	Non Substantial
Outcome of HRA Assessment	**HRA Approval for the amendment is pending.** The HRA will separately confirm HRA Approval for the amendment by email.
Implementation date in NHS organisations in England	35 days from date amendment information together with this email, is supplied to participating organisations **(provided HRA Approval is in place and conditions above are met)**
For NHS/HSC R&D Office information	
Amendment Category	**A**

IRAS Project ID: 197935. HRA Approval for the Amendment

AH AMENDMENTS, Hra (HEALTH RESEARCH AUTHORITY) <hra.amendments@nhs.net>
Yesterday, 21:53
Adam Peel (MED - Student); persephone.magdelene@uea.ac.uk; l.harper@nnuh.nhs.uk

Reply

Dear Mr Peel,
Further to the below, I am pleased to confirm **HRA Approval** for the referenced amendment.

You should implement this amendment at NHS organisations in England, in line with the conditions outlined in your categorisation email.

Please contact hra.amendments@nhs.net for any queries relating to the assessment of this amendment.

Kind Regards

Beverley

Beverley Mashegede
Assessor
Health Research Authority
The Old Chapel | Royal Standard Place | Nottingham | NG1 6FS
T. 02071048065
E. beverleymashegede@nhs.net
W. www.hra.nhs.uk

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From: AMENDMENTS, Hra (HEALTH RESEARCH AUTHORITY)
Sent: 02 October 2017 15:11
To: 'Adam Peel (MED - Student)'
Cc: 'persephone.magdelene@uea.ac.uk'; 'l.harper@nnuh.nhs.uk'
Subject: Amendment 16/LO/0639/AM03 , IRAS Project ID: 197935 - Amendment Categorisation and Implementation Information

Amendment Categorisation and Implementation Information

NHS
Health Research Authority

London - Fulham Research Ethics Committee
Barlow House
3rd Floor, 4 Minshull Street
Manchester
M1 3DZ

Tel: 0207 104 8021

20 July 2018

Mr Adam M Peel
Norwich Medical School
University of East Anglia
Norwich Research Park, Norwich
NR4 7TJ

Dear Mr Peel

Study title:	**Exhaled Breath Biomarkers in Acute Asthma: A Feasibility Study**
REC reference:	**16/LO/0639**
Protocol number:	**AsthmaEBC2016**
Amendment number:	**Protocol version 1.6 (20/06/2018)**
Amendment date:	**20 June 2018**
IRAS project ID:	**197935**

- Study end date to the 31st December 2018
- Add end of study questionnaire and thank you letter
- Remove follow-up assessment (stage 1A)

The above amendment was reviewed at the meeting of the Sub-Committee held on 04 July 2018.

Ethical opinion

The Sub-Committee reviewed the amendment and queried whose initials would be used at the top of the study end questionnaire and noted that acronyms were not defined on first use in the questionnaire.

You explained that participant's initials would be used however with the participant ID number on the document there wasn't a need to have both so you removed the initials box. You further removed the reference to AMU but kept A&E as this was a common phrasing.

The Sub-Committee were satisfied with the clarification and amended questionnaire and had no further issues.

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	*Version*	*Date*
Covering letter on headed paper		20 June 2018
GP/consultant information sheets or letters [GP letter - Clean]	1.2	20 June 2018
GP/consultant information sheets or letters [GP letter - Tracked]	1.2	20 June 2018
Non-validated questionnaire [Study End Questionnaire - Clean]	1.1	19 July 2018
Non-validated questionnaire [Study End Questionnaire - Tracked]	1.1	19 July 2018
Notice of Substantial Amendment (non-CTIMP)	Protocol version 1.6 (20/06/2018)	20 June 2018
Other [Study end letter]	1.0	20 June 2018
Participant consent form [ABBA consent form - Clean]	1.4	20 June 2018
Participant consent form [ABBA consent form - Tracked]	1.4	20 June 2018
Participant information sheet (PIS) [ABBA patient information sheet A - Acute - Clean]	1.5	20 June 2018
Participant information sheet (PIS) [ABBA patient information sheet A - Acute - Tracked]	1.5	20 June 2018
Participant information sheet (PIS) [ABBA patient information sheet B - Outpatient - Clean]	1.5	20 June 2018
Participant information sheet (PIS) [ABBA patient information sheet B - Outpatient - Tracked]	1.5	20 June 2018
Research protocol or project proposal [Tracked]	1.6	20 June 2018

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

Working with NHS Care Organisations

Sponsors should ensure that they notify the R&D office for the relevant NHS care organisation of this amendment in line with the terms detailed in the categorisation email issued by the lead nation for the study.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

We are pleased to welcome researchers and R & D staff at our Research Ethics Committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

16/LO/0639:	**Please quote this number on all correspondence**

Yours sincerely

The Rev'd Nigel Griffin
Chair

IRAS Project ID 197935. HRA Approval for the Amendment

You forwarded this message on Wed 08/08/2018 11:50

H hra.amendments@nhs.net <noreply@harp.org.uk>
Tue 31/07/2018 20:08
Adam Peel (MED - Postgraduate Researcher); persephone.magdelene@uea.ac.uk; hra.amendments@nhs.net; l.harper@nnuh.nhs.uk

Dear Mr Peel,

IRAS Project ID:	197935
Short Study Title:	Exhaled Breath Biomarkers in Acute Asthma: A Feasibility Study
Amendment No./Sponsor Ref:	Protocol version 1.6 (20/06/2018)
Amendment Date:	20 June 2018
Amendment Type:	Substantial Non-CTIMP

I am pleased to confirm **HRA and HCRW Approval** for the above referenced amendment.

You should implement this amendment at NHS organisations in England and Wales, in line with the conditions outlined in your categorisation email.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/.

Please contact [hra.amendments@nhs.net]hra.amendments@nhs.net for any queries relating to the assessment of this amendment.

Kind regards

Aliki Sifostratoudaki
HRA Assessor
Health Research Authority
Ground Floor | Skipton House | 80 London Road | London | SE1 6LH
E.hra.amendments@nhs.net
W. www.hra.nhs.uk

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Appendix 3 – ABC study: patient information sheets and questionnaires

University of East Anglia

Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

Bronchial Challenge Tests in Asthma:

Their Effect on Volatile Organic Compounds in Exhaled Breath

Asthma Bronchial Challenge – The ABC Study

Patient Information Leaflet

We would like to invite you to take part in a research study looking at markers of inflammation in the breath of patients with asthma.

Before you decide whether you would like to take part, it is important for you to know why this study is being done and what it would involve for you if you do take part. Please take time to read this information and to discuss it with others if you wish. Please talk to us if anything is unclear or if you would like more information.

What is the purpose of this study?

Every time a person exhales, they breathe out water vapour and various gases. These contain many different substances that come from the lining of the airways. We hope that by capturing and examining these we can discover which ones provide the most reliable and useful information about asthma and airways inflammation.

Measuring inflammation might give us useful information about how severe an attack is (or how severe it might become) and what treatments might be most effective.

Bronchial challenge tests – commonly used in the assessment of difficult-to-diagnose asthma – cause the cells responsible for inflammation to release chemical messages. By capturing exhaled breath samples before and after such tests we can assess our ability to detect subtle inflammatory changes.

Why me?

We are seeking participants with asthma which is controlled without the need for oral steroid tablets. Participants may use reliever and preventer inhalers and other asthma medications. Participants must be non-smokers (this includes 'vaping') without any other serious lung or heart disease.

Do I have to take part?

No. The decision to take part is entirely up to you. If you decide that you would like to take part in the study you remain free to withdraw at any time. You do not need to give a reason if you decide to withdraw. If you decide to withdraw from the study or that you do not wish to take part, it will not affect the standard of care that you receive.

What will happen to me if I take part?

We would invite you to attend a study appointment at the hospital. We will answer any questions you might have before seeking your consent for participation. We will then ask some questions to ensure you are eligible for the study and complete an asthma control questionnaire. We will ask permission to contact your GP to inform them of your participation and to ask for details of how your asthma was diagnosed.

We may be able to undertake the first study assessment at this visit. If, however, we need you to withhold any medications prior to the tests we will invite you back at a future date with adequate time for preparation.

During the study assessment we would ask you to complete an asthma control questionnaire and to provide a breath sample using the ReCIVA Breath Analyser. This requires you to wear a face mask and breathe normally for 10-15 minutes. The machine captures the gases you breathe out which can then be analysed. After this we would undertake spirometry and measure FeNO - both routine breathing tests commonly used in asthma clinics – and take a sample of blood (less than 10ml or two teaspoons).

The ReCIVA Breath Analyser

Note: people with severe claustrophobia may have difficulty using the device.

We would then perform a bronchial challenge test. This involves inhaling increasing concentrations of mannitol, a substance delivered as a powder from an inhaler device. We would perform regular spirometry as you undertake the test. The test finishes as soon as you experience a fall in your spirometry readings or once you have completed all the inhalations. You may experience some chest tightness or coughing. Other less common side effects of the test may include light-headedness, dizziness, headache, breathlessness, wheezing, runny nose, itchy eyes, nausea/vomiting, or sore throat.

If you do experience a side-effect these are only temporary and should stop before you leave the appointment.

We would ask you to bring a reliever inhaler and spacer with you for use after the test (although these will be provided if you do not have them).

Following this we would ask you to provide another breath sample, taking 10-15 minutes. There would be nothing further at this visit. The whole process should take 60-90 minutes in total.

If you have a positive result in the bronchial challenge test (ie if your lungs react to the mannitol) then we would invite you back for a second assessment visit. This would consist of the same tests as the first except that the bronchial challenge would be a placebo / 'mock' challenge in which the challenge ingredient – Mannitol - is left out.

In some cases it may be possible to undertake the first assessment at the recruitment visit.

In order to undertake the test successfully you would need to withhold certain medications before the assessment including any preventer (steroid) inhaler for 12 hours; short-acting reliever inhaler for 8 hours; long acting reliever inhaler for 24 hours and antihistamines for 72 hours. The bronchial challenge test (including the withholding of medications) is recommended by both national and international asthma management guidelines and is considered safe. Nonetheless there is the possibility of experiencing an asthma attack during the period in which medications are withheld. In the event of you experiencing any asthma symptoms we would ask you to ignore these instructions, take your medications as usual and inform the study team.

What do I have to do?

If you agree to take part in this trial we will ask you to:

1. Complete the consent and recruitment process
2. Attend the hospital for assessment
3. Perform the breathing tests as part of this assessment
4. Return for a follow-up assessment

What is the investigation being tested?

Collecting exhaled breath gas is a way of measuring substances which tell us about conditions within the lung. We would like to know whether these tests can detect changes taking place during bronchial challenge testing and predict the results of such testing.

What happens to my samples?

The exhaled breath and other samples will be used exclusively for research and will be treated as a gift. They will be stored anonymously and labelled with a unique code i.e. not your name or personal details. Breath gas samples will be analysed by the University of Manchester. Please see section *Will my taking part in the study be kept confidential?* below for more details.

What are the possible disadvantages of taking part?

We will ask you to attend a consent visit and two study assessment visits (although it may be possible to conduct the first assessment at the consent visit). We will reimburse your transport costs for these visits but they are additional appointments which you would not normally have as part of 'usual care'. The bronchial challenge tests can cause coughing and chest tightness; this can be relieved by using a reliever inhaler on completion of the assessment.

What are the potential benefits of taking part?

The results from this study may help us to develop better ways of assessing and monitoring asthma, and better ways of directing treatment.

There is no payment for participation however we will provide you with a £20 gift voucher by way of a thank you at the two study assessment visits.

What happens when the research study stops?

Once the data has been collected from all participants the results will be compiled. We will send you a copy of these results so that you know what the outcome of the study was.

What if there is a problem?

The contact details for the research team are shown at the end of this sheet. If you have any concerns please contact the research team. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints mechanisms. Contact details can be obtained from the Patient Advice and Liaison Service (PALS) 01603 289036.

What will happen if I don't want to carry on in the study?

You have the right to withdraw your consent at any time. If you wish to withdraw from the study you do not have to give a reason for this. Withdrawing from this study will not affect your present or future treatment in any way.

Once data has been entered onto the database it is not be possible to remove it; this means that although you are free to withdraw from the study at any point,

once your results have been included in our database we cannot remove this data.

Will my taking part in this study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled with the strictest confidence. All information which is collected about you during the research will be held securely in accordance with the Data Protection Act. It will be kept for 10 years after which it will be disposed of securely. All of your data (your questionnaire information, samples and clinical details) will be labelled by a code and will not have your name or any other details about you on it. We refer to this as linked anonymised data as, although your sample is anonymous, it can be linked to you by a code. The code will only be known by members of the research team. It will be kept securely.

Other third party researchers may wish to access anonymised data from this study in the future (anonymised data does not include names, addresses or dates of birth, and it is not possible to identify individual participants from anonymised data). If this is the case, the Chief Investigator will ensure that the other researchers comply with legal, data protection and ethical guidelines.

If you join the study, the data collected for the study, together with any relevant medical records, may be looked at by authorised persons from Norwich Medical School, the Research and Development department of the Norfolk & Norwich hospital and the Regulatory Authorities to check that the study is being carried out correctly. They all have a duty of confidentiality to you as a research participant.

Informing your General Practitioner (GP)

We would inform your GP of your participation in this study by writing; we are happy for you to discuss the study with them, including showing them this information sheet. We would ask your GP for details from your medical notes regarding your asthma diagnosis and we would write to them with your test results.

What will happen to the results of this study?

Once all the data from study participants has been collected it will be analysed; the results will be shown to the medical team at the Norfolk and Norwich University hospital, and may be published in a medical journal. No identifiable individual data will be published at any time.

Who is organising this study?

The research study is designed and managed by Adam Peel, a PhD student at the Norwich Medical School (UEA). The study is funded by the Asthma UK Centre for Applied Research, sponsored by the University of East Anglia, and hosted by the Norfolk and Norwich University Hospital.

Who has reviewed this study?

This study has been reviewed by a research ethics committee to ensure that the project is delivered in accordance with all legal and ethical guidelines. This study received a favourable outcome from the Cambridge South Research Ethics Committee.

Contact details for further information

Thank you for considering participation in this study. If you have any further questions please ask. If you choose to participate please retain this information sheet for future reference; contact details for the respiratory research group are below:

Mr Adam Peel
Norwich Medical School
University of East Anglia
Norwich Research Park,
Norwich, NR4 7TJ

Email:
respiratory.research@nnuh.nhs.uk
Telephone: 01603 289876
(24 hour answerphone)

University of East Anglia
Norfolk and Norwich University Hospitals NHS
NHS Foundation Trust

Bronchial Challenge Tests in Asthma: Their Effect on Volatile Organic Compounds in Exhaled Breath

Asthma Bronchial Challenge – The ABC Study

PREPARING FOR YOUR STUDY VISIT

Withholding medications

- Preventer inhaler (corticosteroids): 12 hours
  e.g. beclometasone
  budenoside
  fluticasone

- Reliever inhaler (short acting beta-agonists): 8 hours
  e.g. salbutamol
  terbutaline

- Long acting reliever inhaler (long acting beta-agonist): 24 hours
  e.g. salmeterol
  formoterol

- Combination inhaler (corticosteroids and long acting beta agonist): 24hrs
  e.g. fluticasone/salmeterol or budesonide/formoterol

- Inhaled NSAIDs (non-steroidal anti-inflammatory drugs): 8 hours
  e.g. sodium cromoglycate
  nedocromil sodium

- Ipatropium Bromide: 12 hours

- Theophylline: 24 hours

- Tiotropium bromide: 72 hours

- Antihistamines: 72 hours
  e.g. cetirizine
  fexofenadine
  loratadine

- Leukotriene recepetor antagonists: 4 days
  e.g. montelukast sodium

Other advice

On the day of the test:

- Avoid any caffeine e.g. tea, coffee, cola, chocolate
- Avoid vigorous exercise
- Please remember to bring your reliever inhaler (Ventolin/Salbutamol) and spacer (if you use one) to the study visit.

Note: if you experience any asthma symptoms please take your medications as usual and inform the study team. We would urge you to follow your personalised asthma plan and to prioritise your own care over study participation.

If you have any concerns over withholding your medication you may wish to arrange for a friend or family member to remain with you during this period.

Please contact us using the details below should you have any questions or if it has not been possible to withhold medications.

Mr Adam Peel
Norwich Medical School
University of East Anglia
Norwich Research Park
Norwich, NR4 7TJ

Email:
respiratory.research@nnuh.nhs.uk

Telephone: 01603 289876
(and 24 hour answerphone)

ASTHMA CONTROL QUESTIONNAIRE© Page 1 of 2

Please answer questions 1 - 6.

Circle the number of the response that best describes how you have been during the past week.

1. On average, during the past week, how often were you **woken by your asthma** during the night?
   - 0 Never
   - 1 Hardly ever
   - 2 A few times
   - 3 Several times
   - 4 Many times
   - 5 A great many times
   - 6 Unable to sleep because of asthma

2. On average, during the past week, how **bad were your asthma symptoms when you woke up** in the morning?
   - 0 No symptoms
   - 1 Very mild symptoms
   - 2 Mild symptoms
   - 3 Moderate symptoms
   - 4 Quite severe symptoms
   - 5 Severe symptoms
   - 6 Very severe symptoms

3. In general, during the past week, how **limited were you in your activities** because of your asthma?
   - 0 Not limited at all
   - 1 Very slightly limited
   - 2 Slightly limited
   - 3 Moderately limited
   - 4 Very limited
   - 5 Extremely limited
   - 6 Totally limited

4. In general, during the past week, how much **shortness of breath** did you experience because of your asthma?
   - 0 None
   - 1 A very little
   - 2 A little
   - 3 A moderate amount
   - 4 Quite a lot
   - 5 A great deal
   - 6 A very great deal

5. In general, during the past week, how much time did you **wheeze?**

0 Never
1 Hardly any of the time
2 A little of the time
3 A moderate amount of the time
4 A lot of the time
5 Most of the time
6 All the time

6. On average, during the past week, how many **puffs/inhalations of short-acting bronchodilator** (eg. Ventolin/Bricanyl) have you used each day?
(If you are not sure how to answer this question, please ask for help)

0 None
1 1 - 2 puffs/inhalations most days
2 3 - 4 puffs/inhalations most days
3 5 - 8 puffs/inhalations most days
4 9 - 12 puffs/inhalations most days
5 13 - 16 puffs/inhalations most days
6 More than 16 puffs/inhalations most days

Participant identification number ☐☐

British Thoracic Society – Asthma Diagnostic Guidelines

Diagnostic criteria	Yes Reported / documented in notes	No Absence of symptom is reported / documented	Unknown / unreported Presence or absence of symptom is not documented
Symptoms of wheeze, cough, breathlessness and chest tightness that vary over time	☐	☐	☐
History of recurrent episodes / attacks	☐	☐	☐
Episodes / attacks corroborated by variable peak flow when symptomatic and when asymptomatic.	☐	☐	☐
Response to trial of therapy noted	☐	☐	☐
If so, please state which test(s)	☐		
Recorded observation of wheeze (heard by a healthcare professional).	☐	☐	☐
Personal / family history of other atopic conditions	☐	☐	☐

Signs / symptoms to suggest alternative diagnoses present?	☐	☐	☐
Spirometry demonstrating airway obstruction	☐	☐	☐

Appendix 4 - ABBA study: patient information sheets and questionnaires

University of East Anglia

Norfolk and Norwich University Hospitals NHS

NHS Foundation Trust

Biomarkers in the management of acute asthma: a feasibility study

The ABBA Study: Asthma Breath Biomarker Assessment

Patient Information Leaflet

We would like to invite you to take part in a research study looking at markers of inflammation in the breath of patients with asthma.

Before you decide whether you would like to take part it is important for you to know why this study is being done and what it would involve for you if you do decide to take part. Please take time to read this information and to discuss it with others if you wish. Please talk to us if anything is unclear or if you would like more information.

What is the purpose of this study?

Existing ways of assessing asthma - such as peak flow meters - tell us about lung function and how well people are breathing but they don't actually tell us how inflamed their airways are. Measuring inflammation might give us useful information about how severe an attack is (or how severe it might become) and what treatments might be most effective. We also lack quick and easy tests to tell us whether bacteria are involved in triggering someone's asthma attack. A reliable bedside test for this might allow doctors to target their use of antibiotics more selectively.

Every time a person exhales, they breathe out water vapour and various gases. These contain many different substances that come from the lining of the airways. We hope that by capturing and examining these during an asthma attack we can discover which ones provide the most reliable and useful information about airway inflammation and infection.

We will be using three different devices - all of which have been used in studies before – to collect these substances for analysis. In the future these tests may have a place in the standard monitoring of patients with asthma so we also want to discover what patients think about the collection devices.

Why have I been chosen?

We have asked you to participate in this trial because you have been diagnosed with an asthma attack. We are approaching people attending the Norfolk and Norwich Hospital for asthma and we aim to recruit 100 patients to the study.

This will allow us to see if there is a relationship between the substances being measured in the breath and asthma attacks.

Do I have to take part?

No. The decision to take part is entirely up to you. If you decide that you would like to take part in the study you remain free to withdraw at any time. You do not need to give a reason if you decide to withdraw. If you decide to withdraw from the study or that you do not wish to take part, it will not affect the standard of care that you receive.

What will happen to me if I take part?

You will be asked to use the following three devices

a) The RTube – this requires you to breath normally into a chilled plastic tube for about 10 minutes. This causes moisture in the breath to collect on the sides of the tube; we can then sample this liquid.
b) The Niox Mino – this requires you to breathe into a machine in controlled manner for 6 to 10 seconds. This records the amount of nitric oxide (gas) that you exhale.
c) The ReCIVA Breath Analyser - this requires you to wear a face mask and breathe normally for less than 10 minutes. The machine captures the gases you breathe out, which can then be analysed. If you are stable and comfortable but still in hospital after 8 hours, we may ask you if you'd be willing to provide more breath samples (four) using this machine.

After using each device you will be asked to fill out a brief questionnaire telling us what you think of it. We will also ask you to fill out a questionnaire about your asthma control and perform a peak expiratory flow test; we will ask your permission to take a nasal swab and to provide us with a spit sample. The whole process should take approximately 60-80 minutes.

We will also ask to take a blood sample. At this visit it will be one tube of 5ml or less. At any subsequent visit it would be two tubes totalling 10ml or less.

We will ask your permission to use details from your medical notes; this will include details of your medical history, height and weight, and results of any tests that you might have had done as part of your normal hospital care. After you have performed the tests and completed the questionnaires there will be nothing further at this visit (although we will continue to monitor your hospital records in order to record your treatment and discharge from hospital).

With your permission we would repeat these tests:

1. At your follow-up outpatient appointment at the asthma clinic (in approximately 6 weeks).
2. If your asthma is not fully controlled at the time of your follow-up appointment we may invite you to attend at a future date when your asthma is stable and controlled.
3. In the event of you experiencing another asthma attack or deterioration of symptoms before the study end date (December 2018). If this were to happen we would ask you to contact the research team – if convenient - and attend the hospital for assessment.

We would also contact you every three months to enquire about any asthma attacks you might have had.

What do I have to do?

If you agree to take part in this trial we will ask you to:

1. Perform the breathing tests.
2. After each test provide us with feedback on it (by filling out a brief questionnaire).
3. Complete an asthma control questionnaire and provide any other samples.
4. Return for the follow-up assessments.
5. Contact us and attend the hospital for assessment if you experience another asthma attack or deterioration in your symptoms.

What is the investigation being tested?

Collecting exhaled breath condensate and exhaled breath gas is a way of measuring substances which tell us about conditions within the lung. We would like to know whether these tests can predict things such as the frequency or severity of attacks or response to treatment. We would also like to know what patients think of these tests and of using them during an attack.

What happens to my samples?

The exhaled breath and other samples will be used exclusively for research and will be treated as a gift. Breath gas samples will be analysed by the University of Manchester. All samples will be stored anonymously and labelled with a unique code i.e. not your name or personal details. Please see section *Will my taking part in the study be kept confidential?* below for more details.

If any of the samples remain unused at the end of this research project these will be stored, in a linked-anonymised form (as described in *Will my taking part in the study be kept confidential?* below), by the Norfolk and Norwich University Hospital NHS Foundation trust so that they might be used in future ethically

approved research projects including by third parties. Samples will be stored in this way for up to 10 years before being destroyed.

What are the possible disadvantages of taking part?

In addition to participating in the tests now, we would be asking you to undertake tests at your routine follow-up appointment. This would add approximately 1 hour to the length of your follow-up appointment and involve a blood test. We might also ask you to come in for another assessment when your asthma is stable and controlled. We would reimburse your transport costs for this visit but it is an additional appointment which you would not normally have. Finally, we would be asking you to contact us - and attend for an assessment - in the event of experiencing another asthma attack or worsening of your symptoms. Again, we would reimburse your travel costs but this is an appointment and a blood test which you would not normally have as part of 'usual care'.

What are the potential benefits of taking part?

The results from this study may help us to develop better ways of assessing and monitoring asthma, and better ways of directing treatment. Your views on how acceptable the tests are are also important. In the future these tests may have a place in the standard monitoring of patients with asthma and it is important to know whether patients would be happy to perform the tests and under what circumstances.

What happens when the research study stops?

Once you have performed the follow-up assessment/s you will have completed the study – unless you experience another asthma attack within the remaining study period. Once the data has been collected from all participants the results will be compiled. We will send you a copy of these results so that you know what the outcome of the study was. We will invite a sample of patients to participate in a focus group where they can discuss the study and provide us with feedback.

What if there is a problem?

The contact details for the research team are shown at the end of this sheet. If you have any concerns please contact the research team. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints mechanisms (or Private Institution). Contact details can be obtained from the Patient Advice and Liaison Service (PALS) 01603 289036.

What will happen if I don't want to carry on in the study?

You have the right to withdraw your consent at any time. If you wish to withdraw from the study you do not have to give a reason for this. Withdrawing from this study will not affect your present or future treatment in any way.

Once data has been entered onto the database it is not be possible to remove it; this means that although you are free to withdraw from the study at any point, once your results have been included in our database we cannot remove this data.

Will my taking part in this study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled with the strictest confidence. All information which is collected about you during the research will be held securely in accordance with the Data Protection Act. It will be kept for 10 years after which it will be disposed of securely. All of your data (your questionnaire information, samples and clinical details) will be labelled by a code and will not have your name or any other details about you on it. We refer to this as linked anonymised data as although your sample is anonymous it can be linked to you by a code. The code will only be known by your hospital doctors and members of the research team. It will be kept securely.

Other third party researchers may wish to access anonymised data from this study in the future (anonymised data does not include names, addresses or dates of birth, and it is not possible to identify individual participants from anonymised data). If this is the case, the Chief Investigator will ensure that the other researchers comply with legal, data protection and ethical guidelines.

If you join the study, the data collected for the study, together with any relevant medical records, may be looked at by authorised persons from Norwich Medical School, the Research and Development department of Norfolk & Norwich Hospital and the Regulatory Authorities to check that the study is being carried out correctly. They all will have a duty of confidentiality to you as a research participant.

Informing your General Practitioner (GP)

We would inform your GP of your participation in this study by writing; we are happy for you to discuss the study with them, including showing them this information sheet.

What will happen to the results of this study?

Once all the data from study participants has been collected it will be analysed; the results will be shown to the medical team at the Norfolk and Norwich University hospital, and may be published in a medical journal. No identifiable individual data will be published at any time.

Who is organising this study?

The research study is being organised by the respiratory research group, which is based at the Norfolk and Norwich University hospital and Norwich Medical School (part of the University of East Anglia). The study is also being supported by the Asthma UK Centre for Applied Research.

Who has reviewed this study?

This study has been reviewed by a research ethics committee to ensure that the project is delivered in accordance with all legal and ethical guidelines. This study received a favourable outcome from the London-Fulham Research Ethics Committee.

Contact details for further information

Thank you for considering participation in this study. If you have any further questions please ask. If you choose to participate please retain this information sheet for future reference; contact details for the respiratory research group are below:

Mr Adam Peel
Norwich Medical School
University of East Anglia
Norwich Research Park
Norwich, NR4 7TJ

Email:
respiratory.research@nnuh.nhs.uk

Telephone: 01603 289876

University of East Anglia Norfolk and Norwich University Hospitals NHS NHS Foundation Trust

Biomarkers in the management of acute asthma: a feasibility study

The ABBA Study: Asthma Breath Biomarker Assessment

Patient Information Leaflet

We would like to invite you to take part in a research study looking at markers of inflammation in the breath of patients with asthma.

Before you decide whether you would like to take part it is important for you to know why this study is being done and what it would involve for you if you do decide to take part. Please take time to read this information and to discuss it with others if you wish. Please talk to us if anything is unclear or if you would like more information.

What is the purpose of this study?

Existing ways of assessing asthma - such as peak flow meters - tell us about lung function and how well people are breathing but they don't actually tell us how inflamed their airways are. Measuring inflammation might give us useful information about how severe an attack is (or how severe it might become) and what treatments might be most effective. We also lack quick and easy tests to tell us whether bacteria are involved in triggering someone's asthma attack. A reliable bedside test for this might allow doctors to target their use of antibiotics more selectively.

Every time a person exhales, they breathe out water vapour and various gases. These contain many different substances that come from the lining of the airways. We hope that by capturing and examining these during an asthma attack we can discover which ones provide the most reliable and useful information about airway inflammation and infection.

We will be using three different devices - all of which have been used in studies before – to collect these substances for analysis. In the future these tests may have a place in the standard monitoring of patients with asthma so we also want to discover what patients think about the collection devices.

Why have I been chosen?

We have asked you to participate in this trial because you have been diagnosed with asthma and have experienced an attack within the last 12 months. We are approaching people who attend the Norfolk and Norwich Hospital for asthma and we aim to recruit 100 patients to the study. This will allow us to see if there is a relationship between the substances being measured in the breath and asthma attacks.

Do I have to take part?

No. The decision to take part is entirely up to you. If you decide that you would like to take part in the study you remain free to withdraw from the trial at any time. You do not need to give a reason if you decide to withdraw. If you decide to withdraw from the study or that you do not wish to take part, it will not affect the standard of care that you receive.

What will happen to me if I take part?

We will arrange an appointment at the Norfolk & Norwich Hospital in order to discuss the study, answer any questions you might have, and seek your consent for participation. Alternatively, if you have an outpatient appointment scheduled at the respiratory clinic we will arrange to see you before/after your appointment. If you participate we will ask permission to use details from your medical notes; this will include details of your medical history, height and weight, and results of any tests that you might have had done as part of your normal hospital care.

There would be nothing further at this appointment. We would, however, ask you to contact the research team and attend the hospital for an assessment if you experienced a worsening of symptoms or asthma attack any time between now and December 31st 2018 (the study end date). During this assessment you would be asked to use the following three devices:

- The RTube – this requires you to breath normally into a chilled plastic tube for about 10 minutes. This causes the moisture in the breath to collect on the sides of the tube; we can then sample this liquid.
- The Niox Mino – this requires you to breathe into a machine in a controlled manner for 6 to 10 seconds. This records the amount of nitric oxide (gas) that you exhale.
- The ReCIVA Breath Analyser - this requires you to wear a face mask and breathe normally for less than 10 minutes. The machine captures the gases you breathe out which can then be analysed.

After using each device you will be asked to fill out a brief questionnaire telling us what you think of it. We would also ask you to fill out a questionnaire about your asthma control, perform a peak expiratory flow test and have a blood test

(two tubes totalling 10ml or less). In addition we would ask permission to take a nasal swab and to provide us with a spit sample.

The whole process should take 60 – 80 minutes in total. With your permission we would repeat these tests during a follow-up appointment at a time when your asthma was stable and controlled.

We would also contact you every three months to enquire about any asthma attacks you might have had.

What do I have to do?

If you agree to take part in this trial we will ask you to:

1. Contact us and attend the hospital for assessment if you experience an asthma attack or deterioration in your symptoms
2. Perform the breathing tests as part of this assessment
3. After each test provide us with feedback on it (by filling out a brief questionnaire)
4. Complete an asthma control questionnaire and provide any other samples (including a blood sample).
5. Return for a follow-up assessment.

What is the investigation being tested?

Collecting exhaled breath condensate and exhaled breath gas is a way of measuring substances which tell us about conditions within the lung. We would like to know whether these tests can predict things such as the frequency or severity of attacks or response to treatment. We would also like to know what patients think of these tests and of using them during an attack.

What happens to my samples?

The exhaled breath and other samples will be used exclusively for research and will be treated as a gift. Breath gas samples will be analysed by the University of Manchester. They will be stored anonymously and labelled with a unique code i.e. not your name or personal details. Please see section *Will my taking part in the study be kept confidential?* below for more details. If any of the samples remain unused at the end of this research project these will be stored, in a linked-anonymised form (as described in *Will my taking part in the study be kept confidential?* below), by the Norfolk and Norwich University Hospital NHS Foundation trust so that they might be used in future ethically approved research projects including by third parties. Samples will be stored in this way for up to 10 years before being destroyed.

What are the possible disadvantages of taking part?

Enrolment in the study will add time to your outpatient appointment (<30 minutes). We will ask you to contact us - and attend for an assessment - in the event of experiencing an asthma attack or worsening of your symptoms. This assessment would last approximately 1 hour and involve a blood test as well as the breathing tests. We would also ask you to come in for a follow-up assessment when your asthma was stable and controlled. We would reimburse your transport costs for these visits but they are additional appointments which you would not normally have as part of 'usual care'.

What are the potential benefits of taking part?

The results from this study may help us to develop better ways of assessing and monitoring asthma, and better ways of directing treatment. Your views on how acceptable the tests are are also important. In the future these tests may have a place in the standard monitoring of patients with asthma and it is important to know whether patients would be happy to perform the tests and under what circumstances.

What happens when the research study stops?

Once you have performed the follow-up assessment you will have completed the study – unless you experience another asthma attack within the remaining study period. Once the data has been collected from all participants the results will be compiled. We will send you a copy of these results so that you know what the outcome of the study was. We will invite a sample of patients to participate in a focus group where they can discuss the study and provide us with feedback.

What if there is a problem?

The contact details for the research team are shown at the end of this sheet. If you have any concerns please contact the research team. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints mechanisms (or Private Institution). Contact details can be obtained from the Patient Advice and Liaison Service (PALS) 01603 289036.

What will happen if I don't want to carry on in the study?

You have the right to withdraw your consent at any time. If you wish to withdraw from the study you do not have to give a reason for this. Withdrawing from this study will not affect your present or future treatment in any way. Once data has been entered onto the database it is not be possible to remove it; this means that although you are free to withdraw from the study at any point, once your results have been included in our database we cannot remove this data.

Will my taking part in this study be kept confidential?

Yes. We will follow ethical and legal practice and all information about you will be handled with the strictest confidence. All information which is collected about you during the research will be held securely in accordance with the Data Protection Act. It will be kept for 10 years after which it will be disposed of securely. All of your data (your questionnaire information, samples and clinical details) will be labelled by a code and will not have your name or any other details about you on it. We refer to this as linked anonymised data as although your sample is anonymous it can be linked to you by a code. The code will only be known by your hospital doctors and members of the research team. It will be kept securely.

Other third party researchers may wish to access anonymised data from this study in the future (anonymised data does not include names, addresses or dates of birth, and it is not possible to identify individual participants from anonymised data). If this is the case, the Chief Investigator will ensure that the other researchers comply with legal, data protection and ethical guidelines.

If you join the study, the data collected for the study, together with any relevant medical records, may be looked at by authorised persons from Norwich Medical School, the Research and Development department of the Norfolk & Norwich hospital and the Regulatory Authorities to check that the study is being carried out correctly. They all will have a duty of confidentiality to you as a research participant.

Informing your General Practitioner (GP)

We would inform your GP of your participation in this study by writing; we are happy for you to discuss the study with them, including showing them this information sheet.

What will happen to the results of this study?

Once all the data from study participants has been collected it will be analysed; the results will be shown to the medical team at the Norfolk and Norwich University hospital, and may be published in a medical journal. No identifiable individual data will be published at any time.

Who is organising this study?

The research study is being organised by the respiratory research group, which is based at the Norfolk and Norwich University hospital and Norwich Medical School (part of the University of East Anglia). The study is also being supported by the Asthma UK Centre for Applied Research.

Who has reviewed this study?

This study has been reviewed by a research ethics committee to ensure that the project is delivered in accordance with all legal and ethical guidelines. This study received a favourable outcome from the London-Fulham Research Ethics Committee.

Contact details for further information

Thank you for considering participation in this study. If you have any further questions please ask. If you choose to participate please retain this information sheet for future reference; contact details for the respiratory research group are below:

Mr Adam Peel
Norwich Medical School
University of East Anglia
Norwich Research Park
Norwich, NR4 7TJ

Email:
respiratory.research@nnuh.nhs.uk

Telephone: 01603 289876

Biomarkers in the management of acute asthma: a feasibility study.

The ABBA Study: Asthma Breath Biomarkers Assessment

	Yes	No	Don't know
During the period in which you were enrolled on the study, would you say that you had experienced an asthma attack?	☐	☐	☐

If yes, how many? ☐

Thinking about the most severe of these asthma attacks did you:

(please tick as many boxes as appropriate)

- Manage it on your own / without medical assistance ☐
- Contact primary care (GP or walk-in centre) ☐
- Contact secondary care (e.g. attend A&E) ☐
- Receive a course of oral steroid tablets (e.g. prednisolone) ☐
- Receive a course of antibiotics ☐
- Not applicable (did not experience an asthma attack) ☐

During the period in which you were enrolled on the study, did you experience any of the following?

	Yes	No	Don't know
- Decreased peak flow (>20% decline) for 2 days or more	☐	☐	☐
- Decreased peak flow of > 30% at any time?	☐	☐	☐
- Waking at night and needing a reliever inhaler (> 2 consecutive nights)	☐	☐	☐
- Increased reliever inhaler (4 puffs a day more than usual)	☐	☐	☐
- Increased use of steroid / combination inhaler	☐	☐	☐
- Starting oral steroid tablets	☐	☐	☐
- Accessing acute medical care (e.g. emergency GP appointment or hospital attendance)	☐	☐	☐

	Yes	No	Don't know
Did you feel that your asthma was back to 'baseline' or back to your usual level of control at any point during the study?	☐	☐	☐
Did you feel that your asthma was <u>fully</u> controlled *at any point* during the study?	☐	☐	☐
During the course of the study was your diagnosis of asthma changed to something different?	☐	☐	☐
Did you receive any new medical diagnoses during the study?	☐	☐	☐

If yes, what?

During the course of the study were your asthma medications changed?	☐	☐	☐
During the course of the study did you start an injectable 'biologic' drug such as Omalizumab or Mepolizumab?	☐	☐	☐
During the course of the study did you start a long-term maintenance course of steroids?	☐	☐	☐

Invitation to focus group

We would like to invite you to participate in a focus group (group discussion) about the ABBA study. This would be a meeting of 6 – 8 participants from the study. The purpose is to gather the opinions of study participants, both on the breathing devices which were used and the study methods more generally.

The group will meet at the University of East Anglia and the meeting will last no longer than one hour. Refreshments will be provided and travel costs reimbursed. The discussion will be recorded (audio, not video); any contributions made to the discussion will be anonymised; and names will not be used when converting the audio recording to a typed manuscript. One or more member of the respiratory research team will be present to chair the meeting.

This is a valuable stage in the research process, allowing us to gather the thoughts and opinions of participants in a more in-depth fashion than a questionnaire would allow.

If you would like to participate, please tick this box ☐

If you would like further information, please could you let us know by calling us on 01603 289876 (24 hour answering machine) or by email at respiratory.research@nnuh.nhs.uk.

Focus Group Questions

Open / Generic

- How did you find participation in the study? What did you think of the study?
- Was there anything you particularly liked or disliked about the study? Or the way in which it was run?
- Did you have any concerns about the study? Was there anything you questioned during your participation (why am I doing this)?
- What do you think the strengths or weaknesses of the study were?

Recruitment strategy

- Why did you agree to participate?
- Was there anything that made you think twice about taking part?
- Can you think of any reasons why you might have said no to participation in the study? Why do think people might not want to take part?
- How might we recruit more patients?
- Why do you think people might have dropped out? Did you consider dropping out at any point, and if so why?

Ethics

- How did you feel about being approached about the research while you were in hospital?
- What did you think of the information you were given about the study (when deciding whether to participate)? Did you feel like you had sufficient information on which did base a decision about participation?
- Did you feel like you were given sufficient time to consider your participation?

Measurements in the acute exacerbation & hospital settings

- How did you feel about being asked to contact the team / come in for assessment during an exacerbation?
- How easy or difficult was it to contact the team? to book an assessment? to come to the hospital and attend for assessment?
- What do you think of the assessments we were asking you to perform?
- Do you think they were appropriate given the symptoms you were experiencing? How did you feel about performing the assessments while experiencing an exacerbation?

Device specific questions – devices present as reminder / prompt

- What did you think of using the Niox Vero? Did you have any difficulty using the Niox Vero?
- What did you think of using the RTube? Did you have any difficulty using the RTube?

- What did you think of the VOC breath analyser? Did you have any difficulty using the VOC breath analyser?
- Was any one of them more user-friendly / easier / more comfortable to use than the others?

Questionnaire assessment

- What did you think of the AAQ(R)? Did you think it adequately captured how you felt about the acceptability of devices? Is there anything you would want to add? Anything you felt but couldn't express in the questionnaire?

Measurements in the outpatient setting

- As above
- For those who attended a stage 3 assessment - How did you feel about coming in for the follow-up assessment visit?
- Can you imagine doing any of the tests at home?
- How would you feel about doing any of these tests at your GP practice?

Barriers to undertaking a larger study:

- What do you think might be the problems with doing a study like this on a larger scale?

Facilitators to undertaking a larger study:

- Is there anything you can think of that would make this study easier to run?
- Is there anything you can think of to make this study easier for patients to do?

Dissemination of findings:

- How would you like to find out the results of this study?
- What would be the best way of disseminating the findings of this study to the public?

Others:

- What do you think of personal asthma action plans? (If you have a personal management plan, do you use it? how useful do you find it? what triggers you to take your medication?
- What do you think of peak expiratory flow meters? (do you pay more attention to changes in symptoms or to peak flow? how regularly do you do them? do they tell you anything that you don't already know?)
- How would you feel about your treatment being guided by the results of a test such as this? (how would you feel about a personal asthma action plan that required you to use another device? Do you feel like you need more information on your lungs in order to manage your asthma more effectively? Would you like a second source of information / a second way of testing your asthma?)

- Would you recommend participating in a research study to friends or family? (Would you recommend participating in *this* research study to friends or family?)

Appendix 5 – Documents relating to the development of the AAQ-R questionnaire

Dear member of the AUKCAR Patient Advisory Group (PAG),

Thank you very much for taking the time to read this letter and for your help in designing this questionnaire.

Background:
I am a PhD student at the University of East Anglia (UEA) in the process of designing a study looking at markers of inflammation. By doing this we hope to aid the development of new methods to assess the severity of attacks and guide treatment. As part of this study we will be using two or more devices to identify and measure markers found in the exhaled breath of people with asthma. One of the devices has been used a lot in research but is not used in general practice, the other is in more widespread use. However, there is no information on what patients think of these devices, particularly what they think of using them when having an asthma attack. In addition to gathering data on the exhaled breath markers themselves we also want to find out how acceptable the devices are to patients. When deciding whether a device is useful in clinical practice it is crucial to understand whether patients think it is an acceptable test. This questionnaire has been designed as a way of assessing this; it would be given to study participants (aged 18+) after using the devices. The pictures below show the sort of devices we are talking about.

How you can help:
The questionnaire has been designed with input from respiratory doctors but we would really like your opinion to help determine whether the questionnaire is fit for purpose. We want to make sure if someone with asthma was asked to fill in the questionnaire after using a breathing device, that it would really capture whether they thought the device was acceptable.

Below is a draft copy of the questionnaire that will be used to gather study participants' views and also a feedback sheet. If you choose to help, please read the questionnaire (you do not need to fill this out) and then complete the feedback sheet, answering as many or as few of the questions as you wish. There may well be things that you consider important that we have not mentioned so please add any comments you might have.

Thank you for taking the time to get involved; any feedback would be greatly appreciated. The study is being organised by the UEA respiratory research group, which is based at the Norfolk and Norwich University Hospital and Norwich Medical School (part of the UEA). The results of this feedback exercise will be incorporated into the questionnaire design. If you have any further questions or if you would rather arrange to give your feedback by phone please contact me using the details below.

Yours faithfully

Adam Peel

Contact details

Mr Adam Peel
School of Medicine
University of East Anglia
Norwich Research Park
Norwich
NR4 7UY

Email: respiratory.research@nnuh.nhs.uk

Asthma UK Centre for Applied Research

Question	Response from PPI	Summary	Amendments made
If several similar breathing devices were available for monitoring asthma, what sort of things would you use to decide between them?	A) My primary concern regarding the test relates to the status of the patients asthma at the time of any test. Where the patient is experiencing difficulty breathing then any test will result in difficulties for the individual and may impact negatively on the results. B) Ease of use, ability to understand what information the devices give C) What size and shape they are and how large they are. And how easy they might be to use.	A) Ease of use Suitability for acute setting B) Ease of use Ability to understand the results C) Ease of use	None required – the AAQ captures ease of use and suitability for setting
What sort of concerns (if any) might you have about a new device for assessing asthma?	A) Impact on breathing! Need for continuous exhaling! Confidence in accuracy of results! Not being use as a test case! Length of time before receiving results! B) I would be concerned that doctors, etc, would rely completely on the device and not take patients' symptoms into account. But if it's clear to everyone that the device is one more tool to help manage patients' asthma I think it could be very helpful. C) That it would not take too much time to use. D) Most patients would find the 2nd device easier to use than the first one - may find it difficult when having a more severe attack - knowing asthmatics - relief is the only important word - so co-operation might just be limited during one	A) Impact on breathing; confidence in the results; speed of getting results B) Reliance on results rather than patients symptoms C) Time it takes	The AAQ captures concerns about potential impact on breathing in the question 'do you think it would make your symptoms worse'; it addresses confidence in the results. The AAQ does not capture the time taken to do test or the time to get results. Time taken to do the test / use the device is partly captured under ease of use.

Do the options for responding seem appropriate (e.g. very easy, easy, difficult, very difficult)? Do you think there should be, for example more or fewer options?	A) Where seeking guidance on scale, it may be better to ask the patient to score the issue on a scale lying between 1 and 10 rather than placing a mark on a line. Probably will result in higher accuracy and easier for patients to understand.	A) Dislike of VAS as a line without marker numbers	Changed blank VAS line to one with number.
	B) The options seem appropriate, but somehow on my copy they were rather mixed up. There were also long strings of numbers which may have had to do with images, but anyway were rather confusing.	B) Options seem appropriate	Investigated this but changing to 3-point Likert would give insufficient discrimination in the data. No change made.
	C) The options for responding are ok but I find them frustrating in that they do not allow people to express what they actually think or feel, so I think there should be boxes below quite a number of them to allow for further comment. That way you would receive more detailed information.	C) Free text boxes with each question	Other respondents disagree that it is too long. No change made.
	D) The questionnaire looks very good but too many different options in answering the questions - people would probably waver over making a decision when there are multiple answer questions - me included. (They) will lose interested and waver over the answers - result this is going to take too long - and he will end up with not getting a true picture because of the length - hope this makes sense to him - needs to make less options. The options seem appropriate, but somehow on my copy they were rather mixed up. There were also long strings of numbers which may have had to do with images, but anyway were rather confusing. The options for responding are ok but I find them frustrating in that they do not allow people to express what they actually think or feel, so I think there should be boxes below quite a number of them to allow for	D) Too many different options (5-point Likert too much) Questionnaire too long	The PPI group member who reported 'long strings of numbers' uses equipment to assist with reading due to sight impairment so the formatting may have been an issue. It depends on whether the questionnaire will be given electronically as to whether or not this would matters for the study.) Insufficient space for free type box after every question but added one to the end of the questionnaire; the researcher can encourage respondents to use

	further comment. That way you would receive more detailed information.		the questions as a prompt for comments.
Is the length of time it would take to complete the questionnaire acceptable?	A) Filling the form will take very little time but explanation will have to be very clear to assist an understanding of what is required. B) Questionnaire is rather long but it could be a necessity to get a complete picture but this needs to be a simple and easily understood - otherwise you will not get co-operation C) Yes, even if someone is helping the patient, such as reading out the questions for those with reading difficulties. It would still not take very long to do. D) Yes, it is brief and to the point.	A) Yes, won't take too long; will require some explanation B) Rather long, could be simplified C) Yes, won't take too long D) Yes	3 participants versus 1 on being too long therefore no changes made.
Are any of the questions difficult to understand, distressing, or pointless? If so which ones and how could we improve them?	A) A clearer explanation of the devices to be used, their benefits to asthma sufferers and resulting improvement to care may incentivise readers to complete the questionnaire. B) No C) Not that I'm aware of.	A) N/A – they will get a better explanation in the actually study B) No C) No	
Are we missing any key questions? If so what?	A) Those who complete the questionnaire may wish to complete it on-line. Is that the proposed route? If so the patient may wish to view the test results, will you facilitate access via a URL link? I don't think so.	A) Not currently planned.	
Do you have any other comments?			

Appendices

AAQ-R

Initials ☐☐☐ Study ID No ☐☐☐ Name of device ☐

Assessment of Acceptability Questionnaire

How easy/difficult did you find it to use the device?

Very easy	Easy	Neither easy nor difficult	Difficult	Very difficult
⬭	⬭	⬭	⬭	⬭

How comfortable/uncomfortable did you find the test?

Very comfortable	Comfortable	Neither comfortable nor uncomfortable	Uncomfortable	Very uncomfortable
⬭	⬭	⬭	⬭	⬭

How bothered/embarrassed were you by the test?

Very	Moderately	Slightly	Not at all
⬭	⬭	⬭	⬭

How willing would you be to have your asthma monitored using this device in the future?

Very happy	Somewhat happy	Neither happy nor unhappy	Unhappy	Very unhappy
⬭	⬭	⬭	⬭	⬭

This device would be appropriate for use in the following situations:
(please mark either yes or no for each situation)

	Yes, suitable	No, not suitable
• During a mild attack/exacerbation?	⬭	⬭
• During a moderate attack/exacerbation?	⬭	⬭
• During a severe attack/exacerbation?	⬭	⬭

This device would be appropriate for home use

Strongly agree	Somewhat agree	Neither agree nor disagree	Disagree	Strongly disagree
⬭	⬭	⬭	⬭	⬭

How likely do you think it is that using this device might make your symptoms worse?

Very likely	Somewhat likely	Neither likely nor unlikely	Unlikely	Very unlikely
⬭	⬭	⬭	⬭	⬭

How believable is it that this device could give useful information?

Very believable	Somewhat believable	Neither believable nor unbelievable	Somewhat difficult to believe	Unbelievable
⬭	⬭	⬭	⬭	⬭

Overall, how acceptable did you find the use of the assessment device?
(please circle a number)

Very acceptable

Neither acceptable nor

Not at all acceptable

We value your opinion and experience; please feel free to add other comments.

(these may be positive or negative)