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Epithelial Permeability in Asthma

by

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PhD Thesis

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Table of Contents

List of Figures

List of Tables

Declaration of Authorship

I, Patrick Dennison declare that the thesis entitled “Epithelial permeability in asthma” and the work presented in it are my own work.

I confirm that;

1 This work was done wholly or mainly while in candidature for a research degree at this University;
2 Where any part of this thesis has been previously submitted for a degree or any other qualification at this University or other institution, this has been clearly stated;
3 Where I have consulted the published work of others, this is always attributed;
4 Where I have quoted the works of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5 I have acknowledged all main sources of help;
6 Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others, and what I have contributed myself.
7 None of this work has been published before submission

Signed: ……..

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All the work presented in this thesis was performed by myself except in the following instances:

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- Emily Swindle gave instruction/guidance on lab work

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List of Abbreviations

A.D.	Anno Domini
ARDS	Adult Respiratory Distress Syndrome
ALI	Acute Lung Injury
ATS	American Thoracic Society
BAL	Bronchoalveolar lavage
B.C.	Before Christ
BHR	Bronchial hyper-reactivity/responsiveness
BTS	British Thoracic Society
CCL	CC chemokine ligand
CT	Computerised tomography (scan)
CXCL	CXC chemokine ligand
DTPA	Diethylenetriaminepentaacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
FEV_1	Forced expiratory volume in 1 second
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FVC	Forced vital capacity
GH	Growth hormone
GINA	Global Initiative for Asthma
G-CSF	Granulocyte colony-stimulating factor
GMA	Glycol methalacrylate acrylic

GM-CSF	Granulocyte macrophage colony-stimulating factor
hCG	Human chorionic gonadotrophin
ICS	Inhaled corticosteroids
IL-1/3/4/etc	Interleukin-1/3/4/etc
KGF	Keratinocyte growth factor
KGFR	Keratinocyte growth factor receptor
NO	Nitric oxide
PAMP	Pathogen-associated molecular factor
SMART	Symbicort maintenance and reliever therapy
SOP	Standard operating procedure
SPECT	Single Photon emission computed tomography (scan)
TGF	Transforming growth factor
TLR	Toll-like receptor
UC	Ulcerative colitis

Section A: Abstract and Introductions

Abstract

Our knowledge and understanding of asthma have evolved over time, leading to new and improved treatments for this disease. Despite existing treatments however, there remains to date a significant proportion of asthmatics who remain poorly controlled, with unmet needs. Most existing treatments are based on the Th2-driven inflammation model of asthma, however there is increasing recognition of the importance of the epithelium in asthma pathogenesis. It has been proposed that the asthmatic epithelium is chronically damaged and unable to repair, with increased permeability as a result. Existing treatments do not address the epithelial damage directly, however there are now available recombinant growth factors that have been shown to have beneficial effects on epithelial healing. Our hypothesis was that modification of the epithelium, in effect boosting its repair using recombinant human keratinocyte growth factor (rhKGF), would lead to improvement in clinical parameters.

This was explored in several fashions. Firstly a randomised, double-blind, placebo-controlled clinical trial was performed using 20 poorly controlled, moderate asthmatics, with the active treatment group receiving parenteral rhKGF. Assessments before and after drug administration included objective, clinically relevant, measures of asthma such as airway hyperresponsiveness (AHR) measurements, spirometric measures, exhaled nitric oxide measurements and peak flow recording. Subjective, patient-centred assessments were also made using questionnaires to assess asthma control and quality of life, and bronchoscopy was performed to obtain samples to measure biological effects of the drug. KGF treatment resulted in a significantly greater improvement in the primary outcome of mannitol AHR, together with greater improvements in quality of life in the active treatment group compared to placebo. Other features (such as methacholine AHR, asthma control questionnaire scores, spirometric values, exhaled nitric oxide and peak flow variability) did not differ significantly between the groups, although this may be due to a greater than expected placebo response. Biological outcomes also did not differ significantly between the groups, although this may have been due to the sampling time-point used.

Concurrently to the clinical trial above, *in vitro* experiments were performed on cell cultures of epithelial cells from asthmatic and healthy donors, to verify and further explore the effects of KGF on an asthmatic epithelium. Specifically mechanical wounds were inflicted on the cultures, with assessment of the repair process using wound imaging, measurement of trans-epithelial electrical resistance (TER) and permeability to FITC-labelled dextran, in the presence and absence of KGF. As a subset of these experiments, some cultures were exposed to mechanical compression using air pressure, as a mimic for bronchoconstriction, to see if KGF was effective in these circumstances. Results confirm a biological effect for KGF on wound repair in the asthmatic epithelium, which can also partially overcome the deleterious effect of compression on wound healing. An intrinsic difference in wound healing between asthmatic and healthy cohorts, as previously reported, was not apparent.

Lastly the potential of nuclear medicine imaging, to assess epithelial permeability, was explored, for its potential use in future studies of asthma treatments addressing the epithelium directly. Unfortunately this was halted after a pilot study suggested potential methodological flaws – the results and conclusions from this pilot study are presented here, with suggestions for future studies in this area.

General Introduction

This thesis evaluates the involvement of altered epithelial permeability in asthma and its regulation, with separate thematic components focusing on clinical intervention, *in vitro* analysis of epithelial cell behaviour and diagnostic development. The first of these involved a clinical intervention trial with recombinant keratinocyte growth factor [KGF], to assess the clinical and biological impact of this growth factor in asthma. This was paralleled by *in vitro* studies to assess the impact of KGF on wound repair responses in primary epithelial cell culture models in asthma, whilst the final component related to developmental studies to enable *in vivo* measures of epithelial permeability with radiolabelled aerosol inhalation.

By means of introduction to the general themes in the thesis, asthma will be defined, including describing the epidemiology of the disease, the scale of the problem and a discussion of the current treatments and how they impact on the disease process. This will highlight current unmet needs in asthma and lead me to a more in depth and focussed discussion on the respiratory epithelium and its dys-regulation in asthma, explaining the rationale behind the programme of work within this thesis.

Following this general introduction, there will follow the separate introductions to the three components of the thesis outlined above.

Asthma – historical definitions.

Asthma has had many and varying definitions throughout the years, from the simple to the complex, with variations occurring as our knowledge of the disease has progressed. The term 'asthma' is said to be derived from the Greek term 'aazein'[1] (ααζειν), meaning to exhale with open mouth (to pant), and the term 'asthmati' was first used in Homer's Iliad in the description of a warrior at the end of a furious battle[1]. It was likely therefore to initially be used as a description for noisy/laboured breathing; it's first use as a medical term is thought to date from Hippocratic writings[1](c.460-360 B.C.), although it is unclear whether initial use of the term referred to a separate disease process, or a symptomatic description of severe breathing difficulties. A better clinical description, and recognition of asthma as a distinct clinical entity rather than a symptom, was made by the Greek physician, Aretaeus[2]. His definition/description remains much broader than our use of the term today, although it recognised and described the paroxysmal nature of the disease, the association with exercise, and it's origin in the lungs.

Galen (c130-200 A.D.) recognised asthma as a chronic disease[1], described additional clinical features such as increased breathing rate and increased chest expansion, and also began to develop our pathological understanding of the disease, attributing it to "obstruction of the trachea and bronchioles by 'thick secretions'".

These classical descriptions of asthma were refined and altered somewhat by various scholars during medieval ages[3], such as the recognition of asthma triggered by pollen exposure as described by the Islamic physician Abu Bakr Muhammad ibn Zakariya'al-Razi (865 – c.925)[4], although the term was still being confused with other pulmonary disorders at this time.

Further insights into the pathology and treatment of asthma, with an accompanying refinement of its definition, occurred during the Renaissance period. This was often accompanied by a distancing from the classical/Hippocratic notions of causative processes (which had failed to provide effective treatments). An extensive overview of asthma, including details on its causes, diagnoses, pathology and treatment was published by Sir John Floyer in 1698[5], and, whilst still relying on Gallenic ideas about the condition, separated out asthma more clearly from other pulmonary disorders.

In the 19th century, increased understanding of asthma was in part driven by improved diagnostic techniques such as the invention of the stethoscope and the use of auscultation by Laennec in 1819[6], and the invention of the spirometer by John Hutchinson in 1846[7]. Discoveries were made into the pathological findings in asthma, such as the identification of the possible link with eosinophils and the role of inflammation[8] and a link to other allergic conditions was forged. A separation of asthma into that stimulated by external stimuli e.g. animal dander, so-called 'extrinsic asthma', and that stimulated by internal mechanisms e.g. emotional stress, or 'intrinsic asthma', was put forward by Henry Salter in 1864[9].

In the 20th century, our understanding of asthma has further increased, with a parallel increase in our treatment options (see section 2.4). The concept of asthma as an inflammatory disorder has now been firmly established, together with an increased understanding of some of the molecular mechanisms behind some types of asthma and allergic diseases such as the role of IgE[10]. With increased understanding however, has come increased recognition of the heterogeneity of disease included under the term 'asthma'.

Asthma – current definition

It is perhaps not surprising then that a current definition from the Global Initiative for Asthma (GINA) defines asthma using an integration of clinical, physiological and pathological characteristics[11], describing it as:

"…. a heterogeneous disease, usually characterized 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 expiratory airflow limitation.

Further complexity can be introduced by the inclusion of separate definitions for 'severe asthma', 'refractory asthma', 'controlled' versus 'uncontrolled' asthma, 'acute' and 'chronic' asthma, and many more based on pathological features, clinical course or treatment responsiveness. This complexity reflects the aforementioned increasing recognition of the heterogeneity of asthma, with multiple phenotypes included under the term[12]. Whilst we are already seeing the beginning of treatment tailored to different phenotypes e.g. with anti-IgE therapy and anti-IL-5 therapy (see section 2.4.2), it is likely that in the future this will be the case more often.

Asthma - epidemiology

In 2004, GINA estimated that approximately 300 million people worldwide had asthma[13] with particularly high prevalence in developed countries such as the UK which has a reported prevalence rate of >15%[13, 14]. Indeed, there are no signs of decline in asthma prevalence, which is felt to be stable or continuing to increase in many parts of the world[13, 15].

Given the relatively high prevalence of the disease, it is perhaps unsurprising that the economic burden due to asthma is significant[16] with both direct costs related to medication, hospital admission, and physician contact, together with indirect costs related to lost school/work days and decreased productivity as a result of the disease. As discussed in section 2.5 below, these costs are skewed towards a relative minority of patients with poor control and/or more severe disease[17]. This would suggest a need to improve control in these patients, either through new strategies using existing treatments, or via the development of new treatments.

Asthma – current treatments

This section will outline how our evolving understanding of the pathophysiology of asthma has led to refinement of treatments and the development of new treatments for asthma. This will then lead to a discussion in the next section on the emerging role of the epithelium in asthma, and how this too may represent a path towards new ways of treating the disease.

Allergen avoidance

Lifestyle modifications for the treatment of asthma have been advocated since its early description, and indeed were a predominant management option for many early practitioners. Initial advice throughout the years has often included an element of the benefits of exercise, healthy diet, etc., which whilst possibly beneficial are not specific to asthma. However the avoidance of potential allergens which might precipitate bronchoconstriction in an asthmatic was recognised at least as far back as the 16th century, when the Italian physician Gerolamo Cardano advised John Hamilton, Archbishop of St Andrews and an asthmatic, to get rid of his feather bedding which apparently resulted in an improvement of symptoms[18]. Whilst initially based on history taking, the advent of skin-prick testing and testing for specific IgE allowed clearer identification of allergens for patients. Common allergens might include house dust mite, pets (cats, dogs), pollens (of different types of grass, tree and other plant pollens) or fungal spores[18]. There is also a recognised subcategory of asthma, 'occupational asthma' wherein the irritant/allergen is contained within the workplace, and where complete avoidance rather than reduction of exposure, is thought to be paramount.

Unfortunately there is a relative paucity of clinical evidence on the benefits of allergen avoidance, with the majority of studies, poorly controlled and employing inadequate interventions[18]. A Cochrane meta-analysis on dust mite avoidance studies did not find any significant clinical benefit and this is reflected in British Thoracic Society (BTS) guidelines which conclude that 'measures to decrease house dust mites have been shown to reduce numbers of house dust mites, but have not been shown to have an effect on asthma severity'[19]. Critics of this interpretation argue that interventions need to be multifaceted with several interventional measures, and targeted to particular phenotypes[20]. In addition however, where pet sensitisation is a provoking factor, there has yet be a clinical trial validating the benefits of reduced exposure, and getting rid of a family pet is often not a viable option. Finally, certain allergens e.g. outdoor allergens such as pollen/fungal spores may be impossible to avoid completely.

Taking the existing evidence therefore, there is an argument that whilst the detriments of allergen exposure to certain individual's asthma is well recognised, interventions to reduce exposure may be complex and/or clinically unsuccessful. This then leads to the question as to whether symptoms can be reduced despite exposure, in part through existing treatments (such as anti-inflammatory treatment, see section 2.4.2) but possibly also through modification of the epithelial barrier to such allergens (see section 2.6).

Pharmaceutical treatment

Clinically effective pharmaceutical treatments for asthma have really only emerged in the last 100 years or so, addressing different areas of pathophysiology. Most guidelines recommend a 'stepped' treatment plan, increasing the level of treatment if symptoms remain poorly controlled (and stepping down treatment if asthma is well controlled), illustrated by current BTS guidelines for management of asthma in adults (see Figure 1: Stepped treatment for asthma, taken from British guideline on the management of asthma, BTS[21])

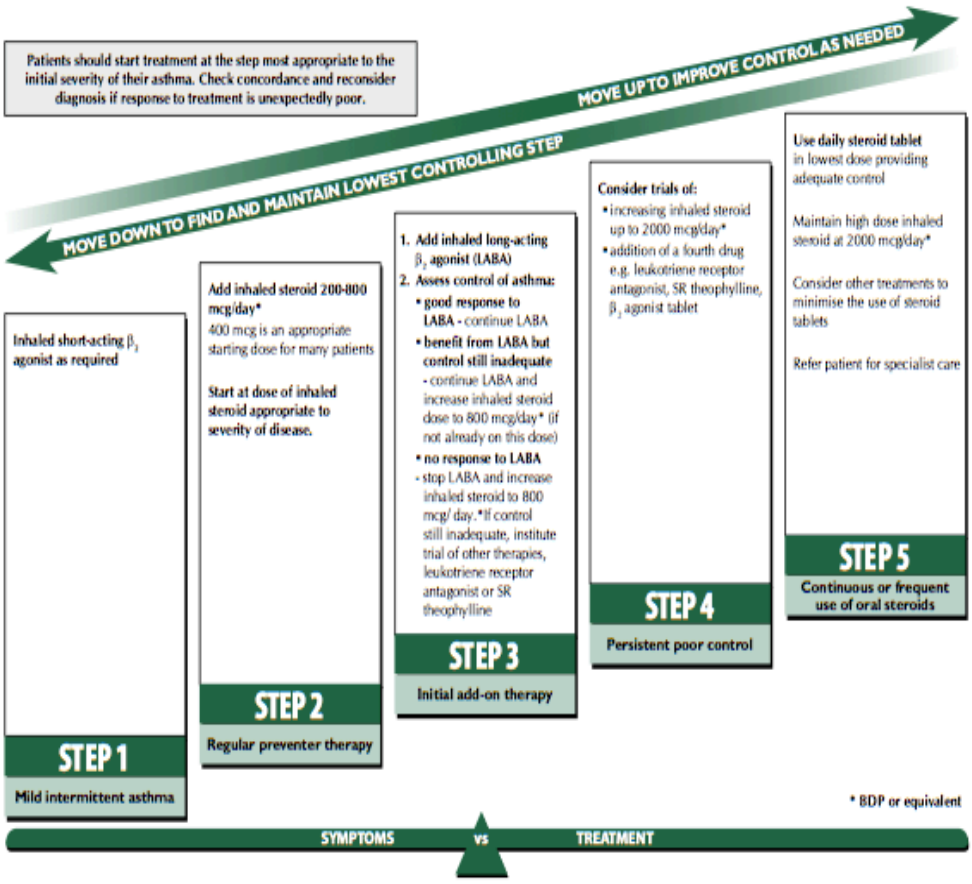


Figure 1: Stepped treatment for asthma, taken from British guideline on the management of asthma, BTS[21]

These treatments can be broadly divided into two areas, bronchodilator treatments and anti-inflammatory treatments, and have evolved as our knowledge of asthma has evolved. From the recognition of bronchoconstriction has come the development of effective bronchodilator therapy, whilst the understanding of the inflammatory nature of the condition has accompanied the use of inhaled corticosteroids, one of the mainstays of preventative therapy in asthma. Increased understanding of mediators involved in the inflammatory cascade had led to targeted developments such anti-leukotrienes and, most recently of all, monoclonal antibodies against specific targets e.g. anti-IgE treatments, anti-IL-5 treatments, etc.

Asthma – the unmet need

Unfortunately despite the available treatments for asthma, it is clear that there is an 'unmet need' in the asthmatic population. This is reflected in the so-called 'difficult asthma' population, a term which is used to cover a variety of situations, but including[22]:

- Patients who remain symptomatic despite maximal inhaled medication
- Patients with severe, life-threatening attacks, or frequent hospitalisation
- Patients who require high medication doses to maintain control

Estimates of the prevalence of difficult asthma vary, from 5%[22, 23] to 10%[24], and whilst this is a not insignificant proportion in itself, of further concern is the disproportionate burden caused by this subgroup of asthma, in terms of healthcare economics, utilisation of healthcare facilities and increased indirect costs such as increased absenteeism from work due to disease[25, 26].

Whilst this difficult asthma group may benefit from measures to address under-treatment, improve adherence, clarification of diagnosis and recognition and treatment of concurrent morbidities[22], there are likely to remain subjects who would benefit from increased therapeutic options.

Unfortunately despite our increasing understanding of the immunopathogenesis of asthma, there has been a relative paucity of new, clinically effective, pharmaceutical treatments for the disease. This is despite identification of potential targets for therapies through greater understanding of the widely accepted model for asthma, that is, as a Th2 cell driven inflammatory condition. Treatments against targets identified from this model, such as Il-4, IL-13 and IL-5 have sometimes been disappointing[27], and whilst these and other monoclonal antibody treatments in development[28] likely will have a role to play, they are likely to be effective only in subsets of patients.

This then leads to questions as to whether the Th2 model is wrong, or more likely incorrect for a proportion of patients, and/or whether there are missing features of asthma that are also important in mediating the expression of the disease. Emerging recognition of the role of the epithelium in asthma, as discussed in the next section, may well prove to be one of these missing features, and could provide new targets and new therapeutics for asthma. This may prove useful, again perhaps for subsets of patients, or indeed across the spectrum of asthma.

Asthma – the emerging role of the epithelium

Relatively recently attention has turned to the role of the respiratory epithelium in asthma, both in its important role in the immune response, and also with inherent defects which may be key to the clinical expression of asthma in an individual. To understand these theories, the following section will discuss the normal respiratory epithelium, together with the changes observed in asthma and how these changes might reflect a defective repair mechanism.

Normal epithelial structure in conducting airways.

The respiratory airway can be divided functionally into two parts – a conducting system bringing the air in, and a respiratory portion taking part in gas exchange. The conducting airways are divided by convention into the upper respiratory tract (extending from the external nares to the larynx) and the lower respiratory tract (extending from the larynx to the visceral pleura)[29]. This system of airways branches after the trachea to form the bronchi supplying the left and right lung, with subsequent dichotomous and asymmetrical branching to supply all areas of the lung. The trachea is supported by incomplete crescentic rings of cartilage (bridged dorsally by connective tissue and bands of smooth muscle), whilst the bronchi have irregular but frequent areas of cartilage. Distal to the last cartilage plate, the airways are defined as bronchioles, and the last of these whose function is purely conduction of air are called the terminal bronchioles. Distal to these are the respiratory bronchioles, which have their epithelium interrupted by alveolar ducts/sacs, and which take part in gas exchange.

The conducting airways are lined by a surface epithelium, which in the anterior nares is a stratified, keratinising, squamous cell type. Much of the upper respiratory tract has a pseudostratified, columnar, ciliate cell type epithelium with occasional mucus secreting cells, although in the larynx and pharynx there are areas of stratified squamous epithelium. In the lower respiratory tract there is a pseudostratified, ciliated columnar epithelium. Whilst there is a basal cell layer beneath the columnar ciliated cells, it is thought that all cells rest on the basement membrane (although not all reach the airway lumen), hence the term ‘pseudostratified’. In addition to the ciliated cells and basal cells, there are mucous cells, serous cells, Clara cells, neuroendocrine cells, indeterminate/transitional cells and migratory/inflammatory cells[29]. The proportions of these cells change dependant on the level of the airways. Beneath these lie the basement membrane acting as an extracellular scaffold, made up of type IV collagen, proteoglycans, laminin, entactin, nidogen and fibronectin. It consists of several layers, the lamina rara, lamina densa and lamina reticularis.

Beneath this lies the supporting lamina propria containing fibroelastic tissue and mucosa-associated lymphoid tissue; the epithelium and lamina propria together make up the airways 'mucosa'. Beneath this lies the submucosa, containing glandular tissue, smooth muscle, cartilage and lymphoid tissue (see Figure 2 Bronchial biopsy in GMA resin, x20 objective, stained for ZO-1 tight junction and haematoxylin staining, illustrating pseudostratified epithelial structure.).

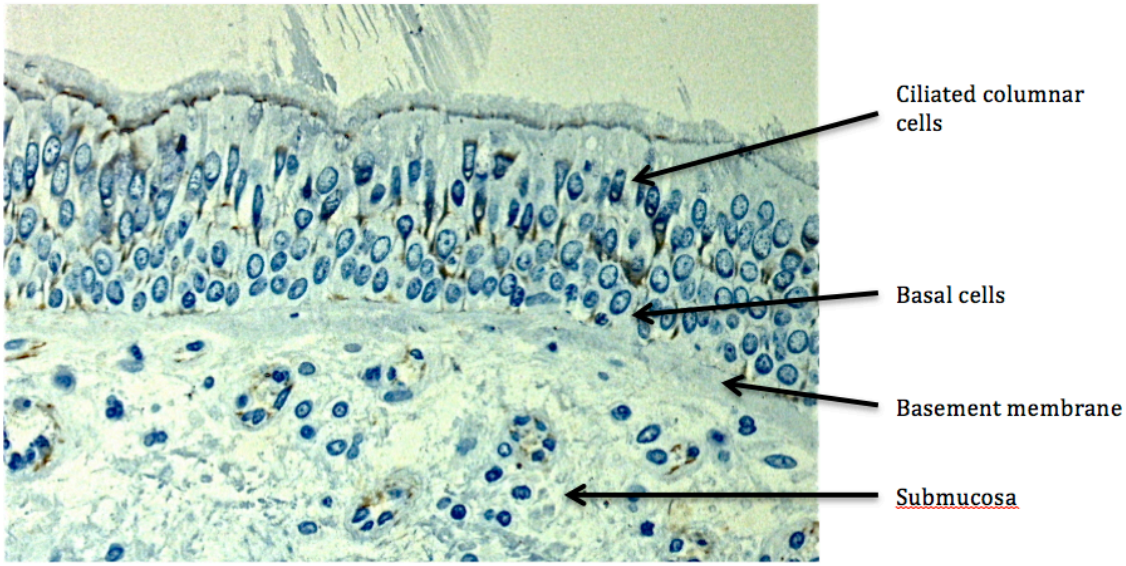


Figure 2 Bronchial biopsy in GMA resin, x20 objective, stained for ZO-1 tight junction and haematoxylin staining, illustrating pseudostratified epithelial structure.

Epithelial cells form contacts with other cells and structures via one of three types of junctional proteins[30, 31], see figure 3 below:

a) Adhering junctions e.g. zonula adherens (also known as intermediate junctions), macula adherents, hemidesmosomes, these join cells to the basal lamina and link to the cellular cytoskeleton via actin filaments
b) Tight junctions, which are located at the apicolateral borders of the cells and selectively regulate paracellular permeability to water, ions, macromolecules and cells. They maintain cell polarity and also divide the apical domain of the plasma membrane from the basolateral domain[32].
c) Gap ('nexus') junctions, which allow intracellular communication e.g. in coordinating ciliary activity.

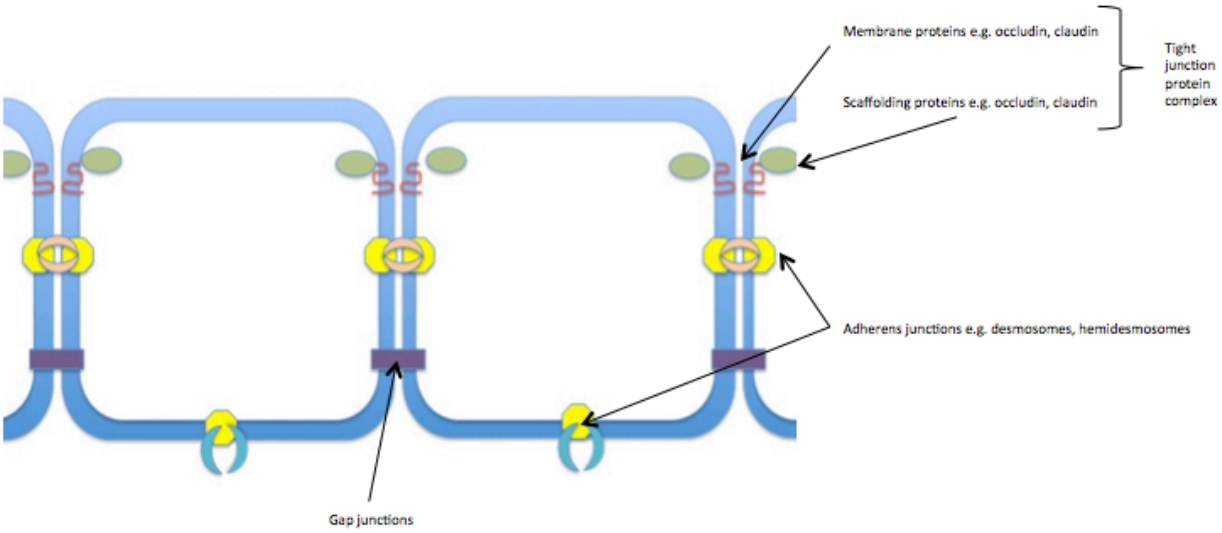


Figure 3: Cell junction molecules

The tight junctions are particular important therefore in the barrier function of the epithelium, and can be divided into two groups[33]:

- Membrane proteins e.g. occludin, claudins, tricellulin and junctional adhesion molecules (JAMs). These mediate cell-to-cell contacts, contain either one (JAMs) or four transmembrane domains (occludin, claudin, tricellulin) and also contain domains to bind to the scaffolding proteins (below)[34].
- Scaffolding proteins e.g. zona occludens (ZO) – 1/2/3, cingulin, MAGI-1 (membrane-associated guanylate kinase with inverted orientation-1), MUPP1 (multi-PDZ domain

protein) and others. These mediate signals from the surface to cytoskeletal actin filaments and activate signalling cascades within cells.

Disruptions of these tight junction proteins are seen in many diseases[32], and the changes in asthma are discussed below in section 2.6.3. In addition to a physical barrier, the epithelium maintains a chemical barrier – the proximal airways generate mucus and via mucociliary clearance can trap and clear particulates/pathogens, whilst surfactant lipoproteins, produced in the distal airways, make microbes less 'sticky' and aids bacterial and viral killing [35].

The role of the epithelium in the immune response.

In addition to the role of the epithelium as a physical/chemical barrier, we are increasingly recognising the role of epithelial cells in the immune response[35]. A full description of the role of the epithelium is beyond the remit of this thesis but in brief, we know that epithelial cells can produce a range of inflammatory cytokines in response to environmental stimuli or other pro-inflammatory cytokines, such as IL-6[36, 37], IL-11[38] and others. Epithelial cells can also affect survival of inflammatory cells via growth factor production such as granulocyte colony-stimulating factor (G-CSF)[39] or GM-CSF[40]. Epithelial cells can recruit inflammatory cells via production of chemokines such as CXCL8[36] or CCL11[41] together with leukocyte adhesion molecules such as ICAM-1 (intercellular adhesion molecule-1)[42]. Epithelial cells can produce a range of lipid mediators e.g. prostaglandins, and peptide mediators e.g. endothelins, to cause bronchoconstriction, inflammation and remodelling[35]. In addition the epithelium recognises pathogens via Toll-like receptors (TLRs), which bind to specific pathogen-associated molecular patterns (PAMPs), leading to the initiation of immune responses via some of the mechanisms mentioned above.

The epithelium in asthma

The suspicion that the bronchial epithelium was damaged in asthma first arose at the end of the 19th century, with the discovery of ciliated cells in the sputum of asthmatic patients by Curschmann in 1883, and the observation of detached bronchial mucosa in a post-mortem of a patient who died with status asthmaticus by Ellis in 1908. Further post-mortem studies supported this theory[43, 44], and with the advent of bronchoscopy, several studies showed increased numbers of epithelial cells in bronchoalveolar lavage (BAL)[45-47] and desquamation visible in endobronchial biopsies on light microscopy[48, 49] and using electron microscopy[50]. However there has been debate as to whether these findings represent artefactual damage from tissue sampling/processing[51], and in keeping with this,

there are studies showing no difference in epithelial cells in BAL[52-55] or sputum[56, 57], studies showing no difference in desquamation on bronchial biopsies[58-60], and post-mortem studies which have not seen increased desquamation[61]. It is worth noting though that several of these studies did show a difference e.g. higher numbers of epithelial cells in asthmatic sputum, higher degree of epithelial desquamation in asthmatic biopsies, although this was not statistically significant, often due to small numbers.

Alongside the debate over the above 'gross' morphological changes indicating epithelial damage/desquamation, there is however increasing focus on cellular markers indicating epithelial damage/injury in the asthmatic patient (with or without desquamation). Measuring growth factor/cytokine expression in this way is advantageous in that they should not be affected by any artefactual damage from tissue sampling/processing. In this way, Puddicombe et al[62] measured epidermal growth factor receptor (EGFR) expression in asthma. Epidermal growth factor (EGF) acts on the EGFR and has been shown to stimulate proliferation, migration and spreading of epithelial cells[63], it is also implicated in goblet cell production of mucin[64]. EGFR activation has been implicated in the epithelial response to injury via a number or different mechanisms, such as by chemical damage[65], tobacco toxins[66-68], mechanical stress[69], house dust mite[70] and mechanical wounding[71]. EGFR expression is increased in asthma, in proportion to disease severity[62] in this, other adult studies[64, 72, 73] and a paediatric population of asthmatics[74].

Further evidence of microcellular damage can be seen through analysis of the tight junctions, which as described in section 2.6.1 are responsible in part for the barrier function of the epithelium. Immunohistochemical staining has shown disruption of TJs in bronchial biopsies from asthmatics[75, 76], whilst epithelial cell cultures from asthmatic donors also display disrupted tight junctions and worsened transepithelial electrical resistance (TEER, a surrogate marker for barrier function). Electron microscopy analysis of bronchial biopsies has also shown disruption of junctional complexes[50, 58]. Many factors that have a clinical effect in asthma e.g. house dust mite[70], cigarette smoke[68], Th2 cytokines[77], can also disrupt tight junctions.

'Chronic wound' theory of asthmatic epithelium

Following on from this, it has been proposed that the epithelial repair mechanisms in asthma are insufficient, that is, there is a 'chronic wound' scenario with persisting damage/incomplete repair[78-80] of the epithelium. Interestingly, the epithelial defect in asthma may be systemic, as studies have suggested increased intestinal permeability in

asthmatics[81, 82], and mutations in the skin barrier gene filaggrin are associated with increased incidence of asthma (in association with eczema)[83] .

Support for this theory comes from

a) the observed persistent damage seen above (despite subjects receiving treatment for their asthma)
b) Cellular markers indicating increased proliferation levels (see below)
c) Impaired wound repair responses, to external stimuli, in cell cultures from asthmatic donors (see below)

With regards proliferative markers, the major markers analysed have been PCNA and Ki67. PCNA, a subunit of DNA polymerase delta, has a maximal expression in G1 and S phases, with decreased expression in G2 and M phases[84]. Ki67, a 345Kd protein, is present in all phases of the cell cycle, but is maximal during mitosis, and due to its short half-life (1-2 hours, versus 20 hours for PCNA[85]), will rarely be detectable in G_0[86]. Thus PCNA may overestimate proliferation as it is involved in DNA repair, and can be present in early G_0, but may show greater sensitivity in low frequency proliferation states due to its longer half-life. The two markers have shown correlation in studies looking at airway smooth muscle proliferation[87]. *Demoly et al* did *not* find a difference in PCNA expression in asthmatics versus healthy controls[88] although the study involved a mixture of patients with differing severities of asthma/differing treatments. Conversely, *Benayoun et al did* find increased expression of Ki67[89], but only in steroid-naïve/mild or ICS-treated/moderate asthmatics, and not in more severe asthmatics (on oral corticosteroids). Other studies have seen increased expression of Ki67 in severe asthma[74, 90] or mixed severity of asthmatics[91], sometimes in association with increased apoptosis markers[74]. Increased expression of cell cycle inhibitors[74, 92] has also been seen, in one study correlating with severity of asthma[89]. It is possible that the differing, sometimes conflicting, findings represent differing mechanisms of impairment of epithelial repair (see below) i.e. due to reduced proliferation in some cases, or increased apoptosis in others. Or the findings may relate to differing populations of asthmatics in each study, with differing definitions of mild/moderate/severe asthma, and thus on differing levels of treatment.

The 'chronic wound' theory is also supported by *in vitro* evidence, using cells cultured from asthmatic donors, which show impaired wound repair processes in response to mechanical wounds in adult[93] and paediatric populations[94, 95]. Asthmatic donor cell cultures have been shown to have increased susceptibility to damage by oxidants[96], cigarette smoke[97]

and ozone[98]. Indeed, as well as possible *intrinsic* differences in the asthmatic epithelium suggested by the above studies, other *in vitro* studies suggest that *extrinsic* factors relevant to asthma, can also impair wound healing, such as mechanical compression (as happens with bronchoconstriction)[99], viral infection[100] and corticosteroid treatment[101].

'Chronic wound' theory – potential mechanisms

The mechanisms behind the impaired repair response in asthma have still to be elucidated, Holgate et al[78] has speculated they may involve "primary genetic, secondary genetic (e.g. somatic mutations) or epigenetic (e.g. altered miRNA, histone acetylation/deacetylation, or DNA CpG methylation) changes to epithelial cell control pathways".

There has been some focus in this area on the Transforming Growth Factor Beta (TGF-β) and in particular TGF-β1. Amongst other effects e.g. chemotaxis of neutrophils[102], extracellular matrix (ECM) protein synthesis[103], and immmunoregulation[104], TGF-beta is implicated in wound healing with effects on cell migration and proliferation, and epithelial-mesenchymal transitition (EMT) (see below).

However the picture is not entirely clear – whilst TGF-beta expression has been seen to be increased in biopsies from asthmatics, particularly in severe asthma[105, 106], others have not seen this association[107, 108], or have shown increased expression of some but not all TGF-β isoforms in asthma[109].

Similarly whilst in some cell lines and primary cell cultures, TGF-β1 can increase cellular migration and boost wound repair times[110, 111], and promote epithelial-to-mesenchymal transition[112-114], other have seen an inhibition of epithelial wound repair[115, 116]. It has been suggested[63] that these contradictory studies may represent differences in cell origin (e.g. primary/cell line, airway/alveolar/nasal, etc.), or differences in concentration dependent responses, but the true picture remains unclear.

Additionally, and of relevance to the clinical study (section 2.8) KGF may have an antagonistic/inhibitory effect to TGF-β; the effects of KGF on alveolar epithelial cell proliferation were antagonised by TGF-β1[117], exogenous TGF-β1 may suppress KGF mRNA expression[118], and exogenous KGF decreased TGF-β in BAL fluid when given as a protective agent prior to intra-tracheal bleomycin in rats[119].

Whilst the true picture may require distinguishing cause and effect, TGF-β/TGF-β1 may well be implicated in some way in the defective wound repair process.

Consequences of a damaged epithelium in asthma

The consequences of a 'chronic wound' epithelium in asthma can be anticipated when one considers the main roles of the epithelium, as discussed above, namely it's role as a barrier (mechanical, chemical and immunological), and it's interaction with the immune system.

Firstly, the increased permeability seen with a damaged epithelium has been postulated to promote allergic sensitisation, this then causing the release of Th2 cytokines which can damage the epithelium and perpetuate the problem[120]. Evidence for this can be seen in a mouse study[121], wherein targeted knockdown of claudin-18, a tight junction protein, increased epithelial permeability and also led to higher serum IgE levels and increased sensitisation to allergen challenge.

Furthermore, the damaged epithelium may be self-perpetuating the problem, by 'driving' the immune response. Studies have shown the epithelium to be capable of secreting a number of inflammatory molecules in response to stimuli, which then promote Th2 inflammation, and this process appears to be enhanced in asthmatic cells. Cytokines include Il-33[122], thymic stromal lymphopoietin[123], and IL-25[124], and genome wide association studies have identified genetic differences in these molecules as associated with a small increased risk of development of asthma[120].

Therapeutic modification of the epithelium

Whilst we may not yet fully understand the full mechanisms, or the consequences, of the 'chronic wound' asthmatic epithelium, recognition of it's presence still allows the possibility of deriving therapies to directly target the epithelium, which may then lead to clinical improvement in a subject's asthma. By improving the damaged epithelium, we may see reduced effect of external stimulants such as allergen (due to improved barrier function) and also potentially reduced inflammation (due to reduced epithelial stimulation of the inflammatory response).

Whilst there are no existing human studies in this area in asthma or indeed any respiratory disease (see section 2.7.2), the concept of therapeutic modification of the epithelium has already been explored in non-respiratory diseases involving damaged epithelium, as below:

Modification of the epithelium in non-respiratory pathology

The two main non-respiratory areas where modification of the epithelium is currently being investigated include gastroenterological disease and dermatological disease. In both these

areas, animal evidence/models have generally been supportive (not summarised here), and therefore I shall focus on those agents/trials that have been performed in humans.

The inflammatory bowel diseases, ulcerative colitis (UC) and Crohn's disease, are also known for impaired epithelial repair, and interest in modifying the epithelium using growth factors is also present in this field[125]. Examples that have been trialled in humans include growth hormone (GH), epidermal growth factor (EGF), keratinocyte growth factor – 2 (KGF-2), GMCSF, G-CSF, trefoil factor and glucagon-like peptide. All of these have mechanisms which involve epithelial growth via migration/proliferation/decreased apoptosis, although with many, such as GH, the growth factor also has a multitude of other effects which may be contributing to any clinical benefit.

A randomised controlled trial (RCT) of hGH in Crohn's disease in adults showed significantly decreased disease activity scores in the treatment arm[126], whilst a RCT of EGF administered as an enema showed improved subjective and objective disease activity scores, with higher rates of remission in the treatment group[127]. Beneficial clinical effects were also seen in studies of glucagon-like peptide[128], GM-CSF[129, 130] and G-CSF[131]. However other studies have not shown a clinically significant effect, for example there has been a large RCT using FGF-10 (also known as keratinocyte growth factor – 2, KGF-2) in ulcerative colitis, where no increased effect at inducing remission was seen at a variety of doses[132]. This was despite reasonable data suggesting benefit in animal models of intestinal injury using FGF-10[133], and the authors speculated on possible reasons including incorrect dose, lack of importance of this mechanism (mucosal restitution) in UC, lack of effect of this agent on the epithelium in these circumstances/this patient group, incorrect time-points/end-points or simply due to insufficient power in the study. Whilst more research is recommended in this area[134] to establish which (if any) of these agents offer most clinical benefit, a tentative proof-of-principle has been established.

There has also been interest in this area from a dermatological perspective, looking at modifying/improving scar formation. Topical EGF applied to skin-graft donor sites enhanced the rate of human wound healing[135] in one study, although it did not affect wound healing of partial thickness wounds in a separate study[136]. Whilst animal studies continue in this field, of late there have been no further published human clinical trials, possibly due to a concern of malignancy.

Modification of the epithelium in respiratory pathology.

There are currently no published human clinical trials looking at targeted modification of the epithelium for clinical benefit in respiratory disease (some would argue the effects of current treatment is through this mechanism though). There have been calls for research[137] into such role in acute respiratory distress syndrome (ARDS) and acute lung injury (ALI) - conditions where an inflammatory cytokine cascade, precipitated by any one of a number of initial insults, causes damage to the epithelium and endothelium of the lung[138]. Indeed, a RCT for the use of keratinocyte growth factor, KGF, in patients with acute lung injury, is currently registered as recruiting patients to test this hypothesis (International Standard Randomised Controlled Trial number (ISRCTN) no. 95690673).

In asthma, some studies have suggested inhaled corticosteroids may improve epithelial repair processes, for example with inhaled budesonide[139, 140]. Some *in vitro* evidence however is suggestive of a deleterious effect of corticosteroids in epithelial repair, with induced apoptosis and prolonged mechanical wound repair in cultured cells[101, 141, 142]. Regardless, the studies discussed in section 2.6.3 (showing increased EGF expression/disruption of tight junctions in asthmatics) were in part performed in patients with moderate-severe asthma, that is, *already* on standard asthma treatment. This suggests that, for a proportion of patients at least, current treatment is at best either ineffective/only partially effective in enhancing epithelial repair.

One possible treatment option utilising this approach would be to use a drug already in licensed clinical use (although not specifically in asthma), namely recombinant human keratinocyte growth factor (rhKGF). The properties of KGF and the rationale for a potential benefit in asthma are explored in the following section.

Keratinocyte growth factor – overview

Keratinocyte growth factor (KGF), also known as fibroblast growth factor 7 (FGF-7), is a member of the fibroblast growth factor (FGF) family. Fibroblast growth factors are monomeric proteins, the first of which was discovered in 1974 and shown to be a mitogen for cultured fibroblasts[143]. They are involved in a large range of biological processes, affecting proliferation, differentiation, migration and apoptosis[144]. 18 mammalian fibroblast growth factors have been identified, grouped into 6 subfamilies[144], with 4 additional FGF homologous factors with similar structure to the other FGFs but which do not activate FGF receptors (FGFRs). Classically there are thought to be four FGF receptors, FGFR1-4[145], although recently a fifth has been postulated with slightly different

structure[146]. The FGFRs are trans-membrane protein tyrosine kinases, with two to three immunoglobulin-like domains and a highly acidic region in the extracellular domain. FGFs bind with high affinity to heparan sulphate proteoglycans (increasing their activity) before binding to the FGFRs, which dimerize and trans-autophosphorylate specific tyrosine residues in their cytoplasmic domain, thereupon acting via a number of cellular signalling pathways[145].

KGF was first isolated by from a human embryonic lung fibroblast line in 1989[147] using ultrafiltration and chromatography. It is a monomer with a molecular weight of 26-28kD, and was so named due to an apparent specificity in stimulating epithelial cells and in particular keratinocytes. Importantly, it demonstrated no detectable effects on fibroblasts or endothelial cells in this study, which set it apart from other growth factors known to possess epithelial cell mitogenic activity such as EGF, TGF-α and most of the other FGFs[147].

In addition to lung fibroblasts, KGF is expressed by other mesenchymal cells, including fibroblasts from other organs, microvascular endothelial cells[148], smooth muscle cells[149] and activated γδ T cells of the skin and intestine[150]. It is thought to be predominantly acting in a paracrine manner, i.e. not being expressed in the epithelium, although expression has been seen in ovarian[151], uterine[152] and lens[153] epithelial cells. KGF is induced by the inflammatory cytokines IL-1 and IL-6, platelet derived growth factor BB (PDGF BB) and transforming growth factor α[154, 155]. Interestingly, growth is suppressed with glucocorticoid treatment[156, 157].

The relative specificity of KGF for the epithelium is further suggested by studies looking at the expression of its receptor. KGF binds exclusively to the KGF-receptor (KGFR, s splice variant of the FGFR2 receptor known as FGFR2-IIIb). Miki et al[158], looked at exon-specific primers for the KGFR in mammary epithelial cells, fibroblasts, endothelial cells, melanocytes, monocytes and the tumour cell lines A1623 (anaplastic tumour groin node cells), OM431 (eye melanoma cells), A172 (glioblastoma cells) and Jurkat (lymphoma cells), and showed that expression was confined to the epithelial cells. A study[159] using in situ hybridisation in 14-16 day mouse embryos showed predominantly epithelial expression (but with some, largely weaker, expression in skeleton-muscular tissue i.e. perichondrium, cartilage of developing bones, developing skeletal muscle and visceral smooth muscle). This study also looked at KGF (but not KGFR) mRNA expression in human adult lung, liver, heart, stomach, spleen, kidney and peripheral blood lymphocytes, and found expression in stomach, heart, lung and kidney but not the others. Given the presence of epithelial cells in the stomach,

lung and kidney (but not the heart/spleen/liver/peripheral blood), this again is further evidence for a largely (although possibly not exclusive) expression of KGFR on epithelial cells.

Role of KGF

This section will discuss the postulated roles for KGF, looking at its cellular effects in the lung, potential roles in tissue development, its expression in injury and disease and finally looking at the use of exogenous KGF, largely as a cytoprotective agent.

Role of KGF – cellular effects in the lung

As said above, although KGF possesses similar mitogenic properties to other members of the fibroblastic growth factor family, its effects are said to be specific to the epithelium. Many of the effects of KGF appear to cause a protective effect, enhancing repair of injuries via multiple effects. Concentrating on the cellular effects in the lung, KGF administration has been shown to have the following effects in a mixture of cell lines/animal studies/primary bronchial cell cultures:

- Alveolar type II cell proliferation and increased migration/motility[160-162]
- Bronchial epithelial cell proliferation[163] and increased migration[164]
- Increased surfactant protein expression[161, 165-170]
- Increased fluid clearance from the alveolar space[171, 172]
- Enhanced DNA repair[173, 174]
- Decreased apoptosis of alveolar epithelial cells[175]

Role of KGF - development

A role for KGF in early development was suggested by studies looking staining in embryonic/developing tissues. Disruption of lung development can be caused by overexpression of KGF[176, 177], exogenous KGF [178], or by blocking KGF[179]. The disruption seems to be mainly related to branching morphogenesis, although there are suggestions that KGF is also likely to play a role in surfactant synthesis in the developing lung[180].

KGF has also been implicated in the development of the pancreas[181-183], bladder[184, 185], gastrointestinal tract[186-188], prostate[189], seminal vesicles[190], skin and hair[183, 188, 191-193], kidneys[183, 194] and cornea[195].

Finch and Rubin[196] however, point out that studies on temporal expression of KGF and FGF10 show earlier expression for FGF10 in the lung[197, 198] and other organs[199]. They argue that this suggests KGF either contributes less, or at a later stage, to organ development than say FGF10. This explanation, or indeed a differing role in different organ

system developments, would explain why knockout mice are viable (albeit with effects on several organ systems)[191].

Role of KGF in disease/injury

The role of KGF in disease and injury has been studied in the skin, lung, bowel and, to a lesser extent, bladder and kidney.

In the skin, KGF expression is up-regulated in response to injury in both mouse[200] and human cells[201]. Transgenic mice lacking a functional KGFR demonstrated impaired skin wound healing[193], whereas KGF expression and wound healing are reduced in diabetic mice[202] and mice treated with systemic steroids[157]. In psoriasis, a disease characterised by epidermal hyperplasia, KGF and KGFR were seen to have increased expression compared to normal skin[203, 204], leading the authors to suggest they may in some way be responsible for the pathology[203].

Similarly in the bladder and kidney, increased KGF has been observed after injury (using surgical wounding[205] or chemical injury[206] respectively). Whilst there have not been wounding models in the gastrointestinal tract, studies on inflammatory bowel disease[207-209] (where epithelial disease is frequently seen) showed increased levels of KGF expression in patients with the disease, and a correlation of the level of KGF expression with the degree of inflammation. Furthermore, KGF expression is also increased in intestinal damage seen in coeliac disease and noncoeliac enteropathy[210].

In the lung, increased KGF expression in response to injury has been seen in various animal models, including rabbits exposed to hyperoxia[211], rats exposed to α-naphthylthiourea[212] or bleomycin[213]. Studies looking at the expression of KGF in human disease have largely focussed on acute respiratory distress syndrome (ARDS) and acute lung injury (ALI). An initial study, looking at alveolar oedema fluid of acute lung injury patients, compared to a control group with hydrostatic oedema, found no difference in KGF levels [214]. However a further study, examining bronchoalveolar lavage (BAL) fluid in ARDS patients alone, found increased levels of KGF compared to controls, and showed a correlation of levels of KGF with prognosis[215]. This slight discrepancy may represent the differing categories in each study – ARDS is defined by more significant hypoxaemia on clinical result[138] and is likely to represent more significant lung injury , and also possibly the different time points used (the Stern study[215] collected samples on average at day 3, rather than < 48hrs for the Verghese study[214], and it has been suggested that expression develops at approximately day 4 in the ARDS process[118]).

In asthma, KGF expression has not been well studied. As part of an MD performed at this institution looking at epithelial repair in allergic airways disease[216], Dr R. Limbrey looked at immunohistochemical expression of KGF in both asthma and allergic rhinitis, together with ELISA analysis of BAL and nasal lavage fluid for KGF levels. Levels of each were compared to healthy controls, and levels were also measured in each compartment (nose and lung) following allergen challenge.

KGF was not detected in either BAL or lavage fluid of any of the these specimens despite the detection in ARDS patients as described above, and Dr Limbrey postulated that this may be due to a less severe epithelial insult with asthma, with levels below the detection limit of the assay, and that with concentration of the fluids it might have been possible to detect KGF[216]. Immunohistochemical analysis of nasal biopsies showed no difference in KGF expression between healthy patients and those with either perennial or seasonal allergic rhinitis, although it is not specified whether the rhinitic patients had active disease at the time of sampling, or what treatment (if any) they were taking at the time of biopsy. There was a trend for increased staining in the biopsies following allergen challenge when compared to a saline control, $p = 0.096$, with a pattern of both increased % staining and also possibly a more diffuse staining in the epithelial layer[216]. Immunohistochemical staining of bronchial biopsies in healthy controls, mild asthmatics and severe asthmatics showed a statistically significant increase in the mild group ($p = 0.037$, compared to healthy controls) but no significant difference in the severe group as compared to the healthy controls. It was postulated that this baseline difference, of lack of a difference, might be due to either the effect of steroids, which as said above have been shown in the skin to dampen down KGF expression[157] (as all the severe cohort of patients were noted to be on high dose inhaled corticosteroids and 50% were on oral corticosteroids). The other explanation put forward by Dr Limbrey was that this result might represent a failure of epithelial repair in severe asthma, and explain the persistence of disease in some subjects[216]. An statistically significant increase in KGF immunostaining post-allergen challenge in the biopsies was not seen ($p = 0.17$), although the time point of 6 hours may have been too early to detect this[216].

Role of exogenous KGF in cytoprotection

In view of the cellular effects of KGF on proliferation, cell migration, etc., many studies have explored the possibility of a cytoprotective effect of exogenous KGF on epithelial cells, to a variety of insults. In largely rodent models, KGF has been shown to protect against hyperoxia-induced damage [217, 218], chemical damage using acid instillation[219],

chemotherapy[220-222] or the rodenticide alpha-naphthylthiourea[223]. In addition, KGF pre-treatment has been seen to be protective in mechanical injury such as ventilation-associated damage[224-226], again in rodent models. In human cells, either using cell lines or cultures of primary bronchial epithelial cells, similar protective effects have been seen, with acid damage[174, 227, 228] or mechanical wounds[164].

Perhaps most relevant to asthma however, was a study by Tillie-Leblond et al[229] looking at the effect of KGF in a rat model of chronic asthma. The investigators used 44 rats, which were sensitised to ovalbumin (OVA) using intra-peritoneal injections of OVA, before being exposed to 6 nebulisations of OVA. Prior to the last nebulisation, animals received an intravenous injection of either KGF (1mg/kg, given over 3 days) or saline placebo. Outcomes looked at included epithelial permeability, assessed using radiolabelled tracers injected into the bloodstream and instilled into the lung (in ventilated rats), inflammatory cell counts in BAL, epithelial integrity and expression of tight junction proteins. They found that OVA challenge in sensitised animals caused increased permeability (indicated by increased leakage of the tracers), peribronchial inflammation and inflammatory cell influx into the BAL (composed of eosinophils, mononuclear cells, lymphocytes and granulocytes), increased epithelial damage and decreased levels of the tight junction proteins ZO-1 and β-catenin. Some animals had lavage/histopathological analysis performed 7 days later than the standard analysis (performed 2 days after last ovalbumin challenge, 3 days after KGF/saline pre-treatment), and these did not display a BAL inflammatory cell infiltrate, and any inflammatory cell infiltrate in the tissues was not significantly different from control, i.e. suggesting that the degree of inflammation had resolved by then.

KGF pre-treatment reduced the increased radiotracer permeability, reduced the increased lymphocyte and neutrophil counts in the BAL (but did not affect peribronchial inflammation, and did not affect the OVA-induced recruitment of eosinophils into the BAL), improved epithelial integrity and increased tight junction protein expression.

Clinical uses of recombinant human KGF

Recombinant human KGF for clinical use in humans was developed by the biopharmaceutical company Amgen, before the rights were later sold to the Swedish pharmaceutical company Biovitrium in 2008. It is produced using recombinant DNA technology in E. Coli bacteria[230, 231], and differs from endogenous human KGF in that the first 23 N-terminal amino acids have been deleted to improve protein stability[232]. It has the drug name Palifermin, and trade name Kepivance, and is currently licensed by both the

US Food and Drug Administration (FDA) and European Marketing Authority (EMA) for its effects on the gastrointestinal epithelium, namely the oral mucosa; it is indicated to decrease the incidence and duration of severe oral mucositis in patients with haematological malignancies receiving myelotoxic therapy requiring haematopoietic stem cell support. It has been shown to be efficacious (in reducing incidence/duration of severe mucositis) in this patient group in a large, phase III double blind, placebo-controlled, randomised trial[233], as well as other smaller studies[234, 235]. It has also been suggested to be efficacious for this use in non-haematological malignancies such as colorectal cancer[236] and head and neck cancers[237] although it is not licensed in these areas at the moment. These existing trials do not comment on any effects on respiratory conditions in participants in the trial.

It is supplied as a sterile, white, preservative-free lyophilised powder which must be reconstituted with sterile water[232], and given at a dose of 60mcg/kg/day, administered as an intravenous bolus, for 3 consecutive days before and 3 consecutive days after myelotoxic chemotherapy.

As Palifermin has undergone extensive human testing already for the above indication, its adverse effects are reasonably well known, and are based on RCT data and a pharmacokinetic study [232]. The most common serious adverse event (SAE) reported was skin rash, occurring in less than 1% of patients. Common non-severe adverse reactions to Palifermin often relate to its effect on the epithelium, and include:

- Dermatological reactions (rash, erythema, oedema, pruritus)
- Oral reactions (Oral dysesthesia, tongue discolouration, tongue thickening, taste alteration)
- Arthralgia
- Altered sensation (hyperesthesia, hypoesthesia, paraesthesia).

These reactions occurred at a median onset time of 6 days following the drug dosing, with a median duration of 5 days. Their frequencies vary from 10% (arthralgia) to 62% (rash) (NB with placebo rates of 5-50% respectively for these adverse effects).

For the purposes of our study (see below), we wished to use KGF in a ‘collapsed dose’ dosing regimen to minimise visits/disruption for patients – in this regimen KGF is given on one day at a dose of 180mcg/kg, followed by a second dose 11 days later also at a dose of 180mcg/kg (essentially condensing 3 days treatment into one day). This regimen has been used previously in other studies[238, 239] and has been reported to be well tolerated.

Summary

As discussed above, there is a reasonable body of evidence to suggest a disrupted epithelium in asthma, with incomplete repair/a chronic wound effect. KGF has been shown to have cytoprotective effects and to enhance wound repair in animal and human cells, of various epithelia. This then leads to the question as to whether KGF (or similar molecules) might represent a novel therapeutic area in asthma treatment.

Introduction – Clinical trial (Safety and Efficacy of parenteral KGF in moderate asthma subjects)

As discussed above, there is a growing recognition that the respiratory epithelium is damaged in asthma and unable to fully repair, leading to a 'chronic wound' phenotype. What is not known is whether treatments can be successful in enhancing the repair process, repairing and strengthening the epithelium, and whether this may then have clinical benefit in asthma.

The aims of the clinical study were to perform a randomised, double-blind placebo controlled trial assessing the safety and clinical efficacy of a course of rhKGF in moderate, poorly controlled asthmatics. Moderate asthmatics were defined as those taking steps 2-4 of BTS guidelines (see section 2.4.2) i.e. they were all on inhaled corticosteroids, plus or minus other maintenance therapy, but excluding those patients on oral steroids. Poor control was defined as a requirement for reliever inhaler (salbutamol or terbutaline) more than 3 times a week. This patient group (rather than say, mild or severe asthmatics) were chosen for a number of reasons:

a) Severe asthmatics are often a heterogeneous population[240-242], often with multiple co-morbidities. In a small pilot study, this may prove problematic in assessing either safety or efficacy of a novel treatment. In addition, the study was relatively intensive, requiring 2 bronchoscopies, which may not have been possible for some severe asthmatics.
b) Poor control despite existing treatment suggested an unmet need in these patients (although standard treatment would be to step up treatment as per guidelines in section 2.4.2), i.e. we could then explore whether KGF improved their symptoms (as opposed to a well controlled asthmatic, with minimal symptoms/minimal room for improvement).

As no previous studies had been done in this area, and therefore there were no indications of what possible size of clinical improvement would be expected, it was impossible to perform a power calculation to work out the number of patients required. The original intention had been to study 40 asthmatics (20 active and 20 placebo) but with the change in ownership of the Palifermin licence from Amgen to Biovitrium, the implied promise of free supply of drug and placebo from Amgen disappeared, and as Biovitrium were not interested in asthma as a target this figure had to be revised. The commercial cost of buying the drug (approximately £7,000 per patient) necessitated a significant reduction in the trial size and a

figure of 20 asthmatics was pragmatically determined, appreciating from experience of the principal investigator (Dr P Howarth) from previous trials, that with 10 in the =active treatment group, and 10 in the placebo group the study was likely to be significantly underpowered. However, as this was a proof of principle study the choice of 10 patients on active and placebo was a valid selection to inform as to whether a clinically relevant improvement signal could be identified, that would help determine whether it would be appropriate to progress to a larger clinical intervention study and to provide preliminary data for a power calculation for such a study.

Study outcomes

Study outcomes chosen were as follows:

- PRIMARY OUTCOME:
    - Change in PD_{15} Mannitol
- SECONDARY OUTCOMES (CLINICAL):
    - Change in PC_{20} methacholine.
    - Change in asthma symptoms and short acting β_2-agonist usage, assessed using Asthma control Questionnaire (ACQ).
    - Change in quality of life assessment, using Asthma Quality of Life Questionnaire (AQLQ).
    - Change in Exhaled NO (eNO).
    - Change in PEFR variability, assessed using diary monitoring.
    - Change in pre-bronchodilator FEV_1.
    - Safety readouts (clinical adverse event reporting)
- SECONDARY OUTCOMES (BIOLOGICAL)
    - Bronchial Biopsy analysis for mucosal/epithelial inflammation (assessed using cell differential), epithelial integrity/damage (assessed by immunohistochemical (IHC) staining for EGFR and tight junction proteins ZO-1 and Occludin), epithelial proliferation (assessed via IHC staining for Ki67), and markers of remodelling (assessed by IHC staining for basement membrane thickness).
    - Broncheo-alveolar lavage (BAL) analysis for inflammatory cell differential and surfactant levels (by ELISA analysis).
- EXPLORATORY OUTCOMES (BIOLOGICAL):
    - Bronchial biopsy analysis for Inflammatory cytokines (Il-8, GM-CSF, TNF-α, and TGF-β) and junctional proteins (p-120 and e-cadherin).
    - BAL ELISA analysis for the following markers: IL-8, Eosinophilic cationic protein (ECP), tryptase, IL-10, IL-13, histamine, Alpha-2 macroglobulin, sICAM-1, TIMP, MMP-9 and TARC.

Rationale behind study outcome choices.

To understand the rationale behind the choice of study outcome, it is necessary to explore the background behind each variable, as follows:

Primary clinical outcome (Change in PD15 Mannitol):

Mannitol can be used to assess the degree of bronchial hyper-reactivity (BHR, also referred to as bronchial hyper-responsiveness, or airway hyper-responsiveness) in asthma. BHR refers to an enhanced (compared to normal subjects) bronchoconstrictor response to a non-specific airway-narrowing stimulus[243]. BHR is thought to be due to two components:

- A 'fixed' or 'persistent' component due to structural alterations to the asthmatic airway (such as thickening of the sub-basement membrane, sub-epithelial fibrosis, airway smooth muscle (ASM) hypertrophy and hyperplasia, blood vessel proliferation and dilation and mucous gland hyperplasia[244]).
- A variable component relating to the degree of inflammation[245].

Whilst this model is somewhat over simplistic, for example in that long-standing inflammation is thought to contribute to the remodelling process, the distinction between the components is useful in understanding the different means of assessing BHR (see below).

BHR is seen in almost all patients with asthma[245], often in proportion to the severity of the disease[246], and can increase following allergen challenge[247, 248]. It can be assessed using 'direct' (see next section) or 'indirect' bronchoprovocation testing; direct challenges stimulate the airway smooth muscle directly, whereas indirect challenges cause the release of endogenous mediators which then cause the smooth muscle to contract[249]. Mannitol, along with other indirect challenges such as hypertonic saline, exercise or eucapnic hyperventilation, work by drying the airways/causing an osmotic gradient (in the case of mannitol, through deposition of the powder on the airway surfaces) – this leads to shifts in fluid from the airway surface liquid, epithelium and submucosa, and degranulation of inflammatory cells causing bronchoconstriction. The relevant inflammatory cells and mediators are not fully established, although mast cells, histamine and leukotrienes have all been implicated; in vitro studies have shown changes in osmolality to cause mast cell degranulation[250], whilst mannitol has been shown to cause release of histamine, PGD_2 and LTE_4[251]. Furthermore, studies have shown mast cell stabilisers[252, 253], antihistamines[254] and leukotriene receptor antagonists[253, 254] to all decrease AHR in asthmatics. Eosinophilic inflammation may be less important, as suggested by studies

looking at eosinophilic bronchitis wherein AHR to mannitol is not present[255]. Importantly though, as compared to the direct challenge bronchoprovocation tests, indirect tests such as mannitol are said to require currently *active* asthma[256], that is, a combination of:

- Asthma
- Airway smooth muscle which is responsive to inflammatory mediators
- Sufficient numbers of inflammatory cells
- Sufficient amounts of inflammatory mediators

This closer reflection of airway inflammation likely explains why, in trials of asthma treatments using both direct and indirect measures, larger improvements/changes in the indirect measures are often seen[257-261].

In addition to the physiological advantages as described above, practical features of the test including good repeatability, good tolerance and speed of completion[262], made this attractive as the primary outcome.

Secondary outcomes - clinical

To correlate with the primary outcome of PD_{15} mannitol, it was decided to perform PC_{20} methacholine testing in addition. Methacholine, and other direct bronchoprovocation methods (such as histamine) rely on the direct stimulation of airway smooth muscle receptors[263], and are thought to reflect more the 'fixed' component of BHR related to airway remodelling. This would explain the presence of direct AHR in non-asthmatic airway obstructive diseases such as COPD[264], and would also explain why, whilst 'direct' AHR does respond to asthmatic treatments such as ICS[265], as said above the changes may be less marked than those in 'indirect' measures of AHR.

The use of asthma control composite scores such as the ACQ is recommended both in guidelines for the management of asthma in a clinical setting[19] and, along with quality of life scores such as the AQLQ, diary monitoring and indeed measurement of BHR, is recommended in an ATS/ERS statement as an outcome measure for asthma control for clinical trials[266]. The ACQ and AQLQ are both well validated[267, 268], have defined levels for significant clinical improvement[269, 270], and are relatively quick and simple to administer.

Exhaled nitric oxide is also recommended, albeit with caveats, as a biomarker for use in asthma clinical trials in the ATS/ERS statement[266]. It is felt to correlate well with

eosinophilic airway inflammation[271], and shows changes in response to other asthma treatments such as inhaled corticosteroids[272]. As it was expected that the poorly controlled asthmatics in this trial may have a degree of eosinophilic inflammation, it was felt that the ease of repeatability of eNO would provide useful data to corroborate any changes seen in the biological specimens.

The data from pre-bronchodilator FEV_1 was readily available through the bronchoprovocation testing (for which FEV_1 was measured with the appropriate medications withheld), and it is recommended in the ATS/ERS guidelines[266] – in conjunction with reference values, % predicted values can be calculated and comparisons of treatment effects made between studies, and whilst it correlates poorly in some studies with outcomes such as quality of life[273] [274], it may be of use in predicting risk of future exacerbations[275] and was felt to be an important secondary outcome to assess.

PEFR variability can be measured using a variety of different equations, some of which are shown as examples below:

- Amplitude percentage mean = ((Highest PEF – Lowest PEF)/Mean PEF) x 100
- Amplitude percentage highest = ((Highest PEF – Lowest PEF)/Highest PEF) x 100
- Amplitude percentage lowest = ((Highest PEF – Lowest PEF)/Lowest PEF) x 100
- Maximum/minimum % = (Highest PEF/Lowest PEF) x 100

In addition, the above equations can be applied to calculate diurnal variability over a single day, then averaging the values over a period, or applied over the entire period. For example, PEFR variability could be calculated (using one of the above equations) for each day over a 2-week diary period, then averaged to give the average PEFR variability for this time, or the highest/lowest/mean PEF could be calculated for the period *as a whole*, and then used in the above equations. We elected to use the amplitude percentage mean equation for the period as a whole (see section 3.1.12), for ease of calculation and as it was hoped this would maximise level of variability and therefore more easily detect changes in the variability over the course of the trial, as might possibly improve with improvement in the epithelial barrier.

Adverse events were clearly important to monitor, as although these have been well determined in previous trials, these were in different patient groups, and therefore the possible adverse effects on an asthmatic population were not known.

Secondary outcomes - biological.

The biological outcomes chosen were selected to give a number of measures of potential effect of the active study drug:

Epithelial integrity

As it lies at the heart of the hypothesis behind this study, measuring epithelial integrity / permeability / damage was clearly important, although, as discussed in the introductory chapter, merely measuring the ratio of morphologically intact to damaged epithelium risked including changes due to artefact i.e. damage from bronchoscopic sampling. We elected to stain the GMA-embedded biopsies for EGFR, which as previously discussed is elevated in a number of in vitro epithelial injury models[65-67, 70], and has been shown to be increased in asthma in proportion to asthma severity[62, 64]. In a similar fashion, to examine microcellular damage i.e. increased permeability despite maintenance of a cellular barrier, we stained for the tight junctional proteins ZO-1 and Occludin, which are disrupted in asthma[75, 76], and KGF treatment has been shown to have protective effects on these proteins in one in vitro bronchial epithelial injury model[227], although this was not seen in a separate study[276]. These markers were only examined in intact, well-orientated epithelium (see methods). This avoids analysis of tangentially cut epithelium (which results in an excess of basal cells and loss of overlying columnar cells) but may result in underanalysis of physiologically (rather than artefactually) damaged epithelium.

An alternative method, which was considered and rejected, was simply to measure the total epithelium for each biopsy and then measure the intact epithelium, expressed as a percentage of the total. This was felt to be unfeasible for a number of reasons:

- Impact of the sampling method as detailed above, in that biopsies/epithelium might be damaged during the bronchoscopy.

- Impact of processing of the sample, for example a biopsy might display epithelium with only basal cells present initially, but later on might have fully intact epithelium, or not, with no way of determining ahead of time. It would then be not clear which of these was more reflective of the sample alone (without taking multiple samples at multiple depths, with associated waste of resources).

Epithelial inflammation

Whilst not expected to have a direct effect on epithelial inflammation, it might be expected that by increasing the barrier function/epithelial integrity and thus decreasing exposure to

external allergens, a secondary effect of KGF may be on epithelial inflammation. The degree of inflammation was assessed in two 'compartments', namely the epithelium itself, and in broncheo-alveolar lavage (BAL).

With regards the epithelium, GMA-embedded bronchial biopsies were analysed for inflammatory changes by looking at the presence of eosinophils, mast cells and neutrophils using immunohistochemistry. In mild asthma (patients on short-acting beta-agonist only), compared to healthy controls, there is a reasonable body of evidence showing that biopsies have increased numbers of eosinophils[277-282]. This does seem to reduce with asthma treatment (as opposed to BAL eosinophilia – see below), such as inhaled corticosteroids[283, 284] or leukotriene receptor antagonists[283]. In severe asthma however, despite treatment, some patients have persistent eosinophilia in biopsies[285] (leading to a phenotypic sub classification of 'eosinophilic' and 'noneosinophilic' severe asthma). The picture with mast cells is less clear; some studies show no differences in mild asthma[279, 285], whilst others do find differences[286]. Still other studies (in mild asthma) have found differences only in epithelial, not submucosal, mast cells[280, 281]. Increased numbers of (submucosal) mast cells have been reported in severe eosinophilic, but not noneosinophilic asthma[285]. As with eosinophils, there is some evidence that existing asthma treatments reduce mast cells, such as leukotriene receptor antagonists[287].

Prespecified secondary outcomes for BAL analysis concentrated simply on inflammatory cell differential (further analysis occurred for other direct and indirect markers of inflammation via ELISA analysis – see section below on 'exploratory outcomes''). BAL in healthy non-smokers contains predominantly macrophages, followed by lymphocytes and neutrophils, with comparatively small numbers of eosinophils and mast cells[288]. In asthmatics, BAL is said to contain increased numbers of eosinophils[46, 289-292], mast cells[293] and, as discussed previously in the introduction, possibly epithelial cells (dependent on studies)[45-48, 290]. BAL eosinophil counts have been found to increase (in asthmatics) in response to inflammation, either allergen induced[294, 295] or related to diurnal variability in nocturnal asthma[296]. Eosinophils and mast cells in BAL have also shown some correlation with physiological markers of asthma severity[297]. Interestingly, whilst levels of eosinophils have been seen to be decreased after initiation of certain asthma treatments such as an inhibitor of leukotriene formation[298], other treatments such as inhaled corticosteroids did not seem to have an effect on cell counts[290, 291].

Epithelial proliferation

As discussed in the introduction, Ki67 has been found to be increased compared to controls in mild-moderate asthma[89, 91] and severe asthma[74, 90], and may be reflective of the underlying impaired repair process in the epithelium. It was important to study this, both from a viewpoint that if the drug was effective, it might manifest in reduced levels of Ki67 expression due to a overall decreased drive for proliferation/repair, but also as a safety endpoint – whilst a temporary bronchial epithelial cell proliferative effect was expected from previous studies[163], it was necessary to check this had resolved (due to mitogenic concerns if there was on-going enhanced proliferation).

Epithelial remodelling

Whilst there are several histological changes in the remodelled asthmatic airway (as discussed in the introduction), it was decided to focus on reticular basement membrane (RBM) thickening. This is consistently seen in asthma of varying severities[49, 299-301], can be seen relatively early on in the disease[302] and seems to increase after allergen challenge [248, 303] i.e. may respond quickly as an outcome. The degree of RBM thickening seems to correlate to a certain extent with several physiological variables[304-306]. More recent work suggests that bronchoconstriction alone, without inflammation, can cause this remodelling[307]. The effect of existing asthma treatment in preventing remodelling and specifically RBM thickening is unclear, with variable evidence for ICS, and limited data for combination ICS/LABA inhaled treatment or newer treatments[308]. It was theorised that, by improving barrier function and therein reducing AHR/episodes of bronchoconstriction, or indirectly via effects on inflammation, KGF treatment might affect remodelling, although it was accepted that this was a relatively short-term study compared to others looking at other asthma treatments effects on RBM thickness[309].

Surfactant effect

The potential effect of KGF on pulmonary surfactant was specifically looked at in detail, due to

a) Increasing recognition of a role of surfactant in asthma
b) A direct effect for KGF on surfactant levels

Surfactant is a complex mixture of phopholipids, neutral lipids and proteins found within the lung. The lipid fraction makes up for ~90% of the mass, with the most abundant component of this being phosphatidylcholine (PC). The protein fraction accounts for ~10% of the mass, and is comprised of serum proteins and the surfactant-specific proteins SP-A, SP-B, SP-C and

SP-D. Surfactant proteins are secreted by alveolar type II cells and Clara cells[310]. Surfactant has a role in reducing surface tension at the air-liquid interface, but also an immunomodulatory role, with an ability to bind to and enhance clearance of allergens and complex interactions with immune cells in the lungs[310, 311].

Baseline levels of *certain* surfactant proteins may be increased in asthma - results are conflicting dependent on which SP is studied, and which area of the lung is sampled (e.g. alveolar surfactant from BAL versus airway surfactant from sputum)[312-314]. Allergen challenge seems to increase surfactant production in asthmatics, but not healthy controls, of the surfactant proteins B, C and D (but not A)[315]. It is postulated that surfactants may provide a negative feedback circuit attempting to reduce damage from inflammation[310]. Their ability to do so may be limited by enhanced degradation in the presence of pathogens relevant to asthma such as house dust mite[316]. Trials of administration of natural/artificial surfactant preperations have had mixed results[310], possibly due to the nonspecificity of the drugs i.e. containing mixtures of various amounts of lipids and surfactant proteins.

In vitro and *in vivo* animal experiments appear to demonstrate KGF enhancing surfactant production, with in particular increased secretion of phosphatidylcholine[167, 170], SP-A[161, 165, 166, 168], SP-B[161, 166, 168, 170, 317] and SP-D[161, 165, 169]. Thus it was decided to see whether, should KGF demonstrate a protective effect, it might be in some way mediated through manipulation of surfactant expression.

Exploratory outcomes

The initial range of biological markers chosen for the exploratory outcomes below were chosen on the basis of investigator experience, known links with asthma, and likely links with KGF/mucosal permeability. A broad range was chosen, essentially covering aspects of epithelial inflammation, remodelling and permeability. In addition some of these markers had only been studied in mild asthma, or severe asthma i.e. not in a 'moderate' group of asthmatics, or had only limited studies as a whole. The baseline data (prior to treatment) in the group as a whole might then have proven useful, independently of the study as a whole.

The markers included the immunohistochemical measurement in GMA-embedded biopsies of TGF-β, IL-8, TNF-α, GM-CSF, e-cadherin and p120, together with BAL ELISA analysis for Il-8, ECP, Tryptase, IL-10, IL-13, Histamine, TARC, α2-macroglobulin, sICAM-1, TIMP, and MMP-9.

In addition however, in measuring some cytokines in BAL using the ELISA method e.g. Th2 cytokines, matrix metalloproteinases, 'multiplex' ELISA analysis plates were used, which allowed simultaneous measurement of more markers than planned. This resulted in a large pool of exploratory outcomes, which could be criticised on the basis of the likelihood of finding a statistically significant result simply by chance, i.e. if not employing Bonferroni correction. This was accepted however, on the basis that the pulmonary effects in vivo of the drug had never been explored previously, and that by generating a large number of hypotheses, a number of variables could be thrown up that would then be suitable for exploration in future studies. A summary of the initial markers looked at, with brief descriptions of some of the evidence to date of involvement in asthma/relationship to KGF treatment (focussing on the areas studied i.e. BAL fluid and immunohistochemistry of biopsies), is included below (Table 1: Exploratory biomarkers for clinical trial and brief evidence of relevance in asthma/KGF).

Table 1: Exploratory biomarkers for clinical trial and brief evidence of relevance in asthma/KGF

Biomarker	Analysed in:	Evidence in asthma/KGF
TGF- β	GMA embedded biopsies	Increased expression in biopsies from asthmatics, secreted by eosinophils[105, 318] Increased levels in BAL in asthmatics vs healthy, further increase after allergen challenge[106] *In vivo* studies suggesting antagonistic effect of KGF on TGF- β[117-119]
IL-8	GMA embedded biopsies BAL ELISA	Increased levels in BAL from asthmatics vs serum levels[319] Reduced at baseline in biopsies from asthmatics compared to healthy controls, but with Increased expression after exposure to ozone [320] Increased expression in biopsies from severe vs moderate asthmatics[321]
TNF-α	GMA embedded biopsies	Reduced in biopsies from mild asthmatics vs healthy controls at baseline in one study[320], increased in another[281] Increased levels in BAL/biopsies in severe asthmatics vs healthy[322] Appears to disrupt barrier function in cell cultures[323]
GM-CSF	GMA embedded biopsies BAL ELISA	No difference at baseline, but higher expression in biopsies from asthmatics vs healthy controls after ozone exposure[320] KGF stimulated increases in GM-CSF levels in epithelial tissue in mouse model, leading to enhanced bacterial clearance in an infection model[324]
e-cadherin	GMA embedded biopsies	Reduced expression in cell cultures from asthmatic samples[91, 323], or in cell models in relation to allergen exposure[70] or TNF-α[325] Reduced baseline expression in biopsies from asthmatic vs healthy controls[91, 325].
P120	GMA biopsies	Reduced baseline expression in biopsies from asthmatic vs healthy controls[325]
ECP	BAL ELISA	Increased ECP levels in BAL in mild asthmatics after allergen exposure[326], increased in acute exacerbations vs bronchiolitis control group[327] Increased levels in asthmatics vs healthy controls at baseline[291, 328, 329], reduced after treatment with inhaled corticosteroids in one study[291] but not another[330]
Tryptase	BAL ELISA	No difference in asthma of varying severity vs healthy controls in one study[331] but increased in others[329], especially after challenge[332] May be a predictor of treatment failure[333], and decreased after treatment with inhaled corticosteroids[330]
Il-10	BAL ELISA	No increase in levels in BAL of sensitive asthmatics to allergen challenge versus non-responders[334]. Decreased levels in BAL from asthmatics vs controls[335]
Il-13	BAL ELISA	No increase in levels in BAL in asthmatics after allergen challenge in one study [334] but increased in another[336]. No difference at baseline in asthma in some studies[336, 337], undetectable in BAL in one study[292]
Histamine	BAL ELISA	Increased levels in BAL of asthmatics after allergen challenge in some studies[295, 332] but not others[334] Increased at baseline in mild asthmatics versus healthy controls[46]
TARC	BAL ELISA	Increased levels in BAL of asthmatics to allergen challenge vs non-responders[334]
α2-macroglobulin	BAL ELISA	Increased levels in BAL in asthmatics versus healthy controls[338, 339]
sICAM-1	BAL ELISA	Increased levels in BAL in asthmatics versus healthy controls[340, 341], with further increase after allergen challenge[341, 342]
TIMP	BAL ELISA	Increased levels in BAL in asthmatics versus healthy controls[343-345]
MMP-9	BAL ELISA	Increased levels in BAL in asthmatics versus healthy controls[343-346]

Aims of this research

The aims of this research were three-fold:

a) A clinical trial in asthma of a growth factor, KGF, which is known to affect epithelial repair at other mucosal sites. This part of the research aimed to establish/refute proof-of-principle of this therapeutic target in asthma (see Section B: Clinical Study - Safety and Efficacy of parenteral KGF in moderate asthma subjects).
b) In vitro experiments to complement the above research. Using bronchial epithelial cells in culture, it was hoped to further establish and explore in more detail the effect of KGF on the asthmatic epithelium (see Section C: *In vitro* studies of the effect of KGF on epithelial permeability).
c) An exploration of alternative means of assessing epithelial permeability in asthma, through non-invasive means. This aimed to use nuclear imaging in a novel fashion to demonstrate the increased permeability in asthma (see Section D: Assessment of epithelial permeability in asthma through nuclear imaging).

Introduction - *In vitro* studies (Assessment of the effect of KGF on epithelial permeability)

To parallel the clinical study of the effect of KGF on moderate asthmatics, it was decided to further explore the molecular effects using *in vitro* studies, specifically using mechanical wounds, in epithelial cell cultures in air-liquid interface, from both healthy and asthmatic donors, as explained below.

Substrate - Air-liquid interface cultures of epithelial cells

Many in vitro experiments studying bronchial epithelial cell biology are performed either on immortalised cell lines, or on primary bronchial epithelial cells grown as a submerged monolayer. Cell lines have practical advantages, such as cost, life span, decreased variability between experiments/donors and decreased incidence of cellular contaminants. However one can argue that the technique of air-liquid interface culture offers a more physiological model, a closer mimic to in vivo conditions and therefore more robust results.

The technique of ALI culture establishment was established and refined firstly in animal cells[347-349] and then developed in human epithelial cells [350]. Whereas monolayers are poorly differentiated, ALI cultures differentiate to the form a pseudostratified, polarised layer, which includes ciliated and goblet cells[350], and this in turn Is likely to have a bearing on their barrier properties. The transcriptional profile of ALI primary bronchial epithelial cultures more closely matches in vivo cells than submerged cell cultures or cell lines[351], and perhaps therefore unsurprisingly ALI cultures may respond differently to stimuli such as IL-13[352]. Thus the ALI culture technique was chosen for the in vitro experiments.

Mechanism for inducing epithelial permeability - IL-13 / house dust mite

Initial plans were to examine the possible protective/restorative effect of rhKGF against inflammatory cytokines present in asthma such as interleukin 13 (IL-13), together with possibly looking at more physiological stimuli such as house dust mite allergen. It was hoped to establish a reliable model in an epithelial cell line, before proceeding to primary bronchial epithelial cells in an air-liquid interface (ALI) culture. The 16HBE cell line is an immortalised epithelial cell line derived by SV-40 large T-antigen transformation of human bronchial epithelium[353] and has the advantage of retaining certain epithelial morphology and functions; monolayer cultures form an epithelial barrier with tight junctions and generation of transepithelial electrical resistance (TER). IL-13 was chosen as a cytokine bearing relevance in asthma – it is secreted by Th2 cells, mast cells and basophils[354], and in animal models and cell cultures seems to cause airway hyper-responsiveness[355],

eosinophilia[356] and increased mucus generation[357]. Furthermore, in a different cell line, IL-13 was shown to increase epithelial permeability (as measured by TER) and decrease expression of the tight junctions ZO-1 and occludin[77]. Dust mite also had been shown to disrupt tight junctions[358] and the two had been shown to work synergistically on the expression of inflammatory cytokines in primary epithelial cell cultures[359].

Initial experiments in this area were performed using a cell line, 16HBE cells. Unfortunately inconsistent results were obtained (data not shown), with poor potency of IL-13 in disrupting the barrier (requiring high, prolonged concentrations) and a failure of KGF treatment to differ from control transwells in a variety of conditions (given apically or basally, pre-treated or concurrently treated). It was unclear whether the latter related to the potency of the KGF, as a previous study looking at mechanical wounds in 16HBE cells had failed to see an effect of KGF[62], which the investigators postulated may reflect a lack of expression of the KGFR (although this seems unlikely given a study showing a protective effect of KGF in 16HBE cells exposed to H_2O_2[227]). It was postulated whether the intrinsic proliferation rate / recovery rate of the 16HBE cells was too high to allow a distinction between treated and untreated cells, however this also does not fit with existing studies which have shown 16HBE cells in culture to have a doubling rate somewhat between primary bronchial epithelial cells from asthmatic and healthy donors[360].

Given the disappointing results however, it was decided to modify the experiments, namely to proceed to a more definitive injury to the cell layer, namely a mechanical wound model, and to use primary bronchial epithelial cells in ALI culture instead of cell line monolayers. Initial experiments were then expanded, looking at the additional effect of mechanical compression, as this is used as a mimic for bronchoconstriction. Donor cells were taken from both healthy and asthmatic subjects, to see if there was a difference in the response rate to KGF. The rationale behind these choices is now discussed.

Mechanism for inducing epithelial permeability - Mechanical wounds

As discussed in the main thesis Introduction, in vitro studies seem to demonstrate impaired wound repair in asthmatic cells, with delayed healing seen in cells taken from either adult[93] or paediatric donors[94, 95]. In addition, as already said, extrinsic factors relevant to asthma can impair wound healing, such as mechanical compression (as happens with bronchoconstriction)[99, 361], viral infection[100], corticosteroid treatment[101] and even β2-agonists[362]. Various factors have been put forward to explain the possible intrinsic differences accounting for this, including:

- A failure/abnormality of the plasmin activation system, responsible for extracellular matrix deposition in addition to effects on epithelial migration, repair and proliferation[95].
- Dyssynchronous and slow mitosis with increased release of inflammatory and fibrogenic cytokines[93].
- Impairment of the remodelling and degradation of surplus extracellular matrix (ECM) components (laid down by fibroblasts as a provisional matrix/temporary seal for a wound)[363].

Again as already discussed, KGF has been shown in studies to boost mechanical wound healing in epithelial cells in both *in vitro* studies (using normal healthy bronchial epithelial cells and cell lines)[164] and in vivo studies (using rat tracheal epithelial cells)[364]. There are currently no published studies however exploring whether KGF is similarly effective in *asthmatic* epithelial cells.

Various techniques have been used to induce mechanical wounds on epithelial cell cultures, with techniques including:

a) Linear wounds or 'scrape wounds' using a metal spatula[99] or pipette tip[93, 361]

b) Cross wounds using pipette tips[100]

c) Circular wounds using bespoke wounding devices [95, 365]

Preliminary experiments performed showed most consistency in wound appearance using a 200µl pipette tip to produce linear scrape wounds across the full diameter of the transwell containing the epithelial ALI culture.

In the above experiments, one of the most common ways to assess wound repair is via imaging the wound using microscopy[93, 95, 99, 101, 361, 362] and then using software to analyse the images, either to calculate the total wound area[93, 101], or by looking at wound width[95, 99, 361], and expressing these at each time point as a % of baseline. Preliminary experiments (see results section XX) performed for these experiments suggested similar results with either method, but as there seemed to be occasional experiments where different parts of a wound healed at different rates, we elected to image the wound as a whole and measure overall area.

Transepithelial electrical resistance (TEER) is often used as an indirect measure of epithelial permeability/barrier function of the epithelium, particularly when looking at tight junction protein expression[76, 366]. TEER is a measure of permeability to the flux of ions across a barrier, and is commonly measured using a voltohmmeter with chopstick electrodes, one electrode sitting in apical medium and one sitting in basal medium. Decreased resistance suggests increased permeability, and disruption to the epithelium with mechanical wounds has a marked effect on TER (see results below). Whilst it was expected that TER would be relatively insensitive in measuring the wound repair whilst full thickness defects were still present, it was hoped that in measuring TER we would get an impression of return of functional integrity to the epithelium i.e. not just macroscopic closure but the formation of a functioning barrier.

Similarly to TER, assessment of epithelial layers using fluorescently labelled dextran molecules is mainly used in the assessment of tight junction integrity and paracellular permeability and has detected subtle differences between asthmatic and healthy donor cell cultures[76]. As for TER, whilst it might be expected that whilst a macroscopic defect was still present, FITC dextran permeability assay may be overwhelmed, it was hoped to measure the return of functional barrier integrity in the different conditions/at the different time points.

Mechanical Compression

Mechanical strain has also been shown to impair wound healing[99, 164, 361]. The mechanism of mechanical strain in these *in vitro* studies varies however. Some studies use a cyclic strain model, using vacuum pressure to rhythmically deform a silicone rubber substrate upon which the cells have been cultured[99]. This results in elongation of the cells rather than compression (although cells in the centre of the wells do undergo minor compression), and it can be argued that this applies less well to the in vivo experience of

cells undergoing bronchoconstriction (but is more relevant to ventilator-associated lung damage). A more relevant model for this is obtained by using compression – using bungs to provide air-tight seals for the cell cultures in transwells together with an air pump, humidified air (with 5% CO_2) can be supplied to the cells under pressure e.g. 30cm H_2O[361, 367, 368].

Mechanical strain appears to have differing effects on cultured cells taken from asthmatic donors with differing responses of fibroblasts[369] and also epithelial cells[370]. Indeed, the mechanical compression of epithelial cells as occurs with bronchoconstriction, has been proposed as a contributor to the airway remodelling which occurs in asthma[307].

Aims

Whilst KGF has been shown to have a protective effect against various insults in healthy epithelial cell cultures, the effect on cultures from asthmatic donors had not been established. As discussed previously, it is known that asthmatic epithelial cells remain intrinsically different from healthy cells even when maintained *in vitro* in the same environment, with differing responses to injury [360]. In addition many of the in vitro experiments have been performed on monolayer cultures, which again as discussed above may differ from ALI cultures in their responses.

Therefore it is unclear whether KGF would have the same, decreased, or enhanced cytoprotective effects on a pseudostratified epithelium from an asthmatic donor. The aims of the experiments therefore were as follows:

- Measurement and comparison of epithelial permeability in primary bronchial epithelial cell cultures grown in air-liquid interface cultures (in Transwells) from healthy and asthmatic volunteers. Permeability to be assessed through a combination of measurements of TER, FITC dextran permeability and time-lapse imaging as detailed below.
- Measurement and comparison of the effect on epithelial permeability, in the above patient groups, using scrape wounding +/- mechanical compression
- Measurement and comparison of the potential protective/reparative effect of rhKGF on cultures exposed to the above stimulants/situations.

Introduction – Imaging study (Assessment of epithelial permeability in asthma through nuclear imaging)

Assessment of epithelial permeability

The potential importance of the epithelium in asthma has been discussed above, together with the likelihood of a chronically damaged 'wound' phenotype with increased permeability. It is clearly important to be able to measure bronchial epithelial permeability, both for asthma and other lung diseases, and currently this is achieved by one, or more, of several methods:

a) Directly via bronchial biopsies which can then be stained immunohistochemically for markers of damage/permeability. Advantages of this method include a direct link with the factor of interest (c.f. indirect markers of permeability, below) and the ability to look at multiple molecules of interest at once. This technique is the most extensively used, and has been used in the evaluation of several types of asthma treatment. Disadvantages however that the procedure is invasive, relatively expensive, limited to specialist centres with relevant equipment/expertise, and has limited repeatability during a study due to practical/ethical reasons. This makes it potentially unsuitable as a biomarker of disease/treatment effect for larger studies.

b) Indirectly via cultures of cells taken from subjects with/without disease, or with differing severity of disease, assessing permeability via various techniques e.g. immunohistochemistry, transepithelial electrical resistance, FITC dextran permeability, etc. (see section 5). Advantages include the ability to assess the epithelium by several different methods, however the collection of cells still requires an invasive procedure with the disadvantages outlined above, and in addition the removal of cells from their standard environment, and subsequent culture in e.g. standard media (rather than the inflammatory milieu they may develop in normally) may have profound effects on the cells responses and thus the data obtained.

c) Indirectly via assessment of epithelial permeability in other more easily accessibly/measurable epithelia. For example, intestinal permeability has been assessed using a 'dual sugar technique' whereby differences are seen in the uptake of mono- and di-saccharides in both intestinal disease e.g. Crohn's [371] but also in asthma[82]. Disadvantages of this technique are that it is far from extensively used and thus has a very limited evidence base, and it is unclear (and perhaps unlikely) that treatment targeted towards one epithelia e.g. the bronchi in asthma, will not

have an effect on the intestinal epithelia (unless the treatment is systemic) and thus this technique will not be useful as a measure of treatment success.

d) Directly via radiological means – as will be discussed below. Advantages include non-invasive real time in vivo measurement with the potential for repeated measurements (although limited to a certain extent due to radioactive exposure). Disadvantages include conflicting evidence base as will be discussed below, and less evidence looking at modifiability with treatment.

In parallel with the clinical study of keratinocyte growth factor, it was decided to explore alternative means of assessing permeability as described above, and in particular to see if improvements could be made in the radiological techniques currently used. This would have the advantage as a potential biomarker/assessment of treatment response both to 'standard' asthma treatments but also clearly to those future treatments targeting epithelial permeability e.g. larger scale trials of KGF or similar molecules.

Current attempts to look at epithelial permeability through radiological means have mainly focussed on nuclear medical imaging, via assessment of the clearance of a radio-labelled compound such as 99^{m} technetium diethylenetriaminepentaacetate[372] (99mTc-DTPA, molecular weight 492 daltons, approximate molecular radius 0.57nm). 99mTc-DTPA is a hydrophilic compound that can be aerosolised and inhaled to deposit on the pulmonary epithelium. It is then either cleared from the lung through mucociliary clearance or through transcellular absorption between the epithelial cells to reach the pulmonary vasculature (after traversing the mucous layer (airways) or surfactant layer (alveoli)). The disappearance of 99mTc-DTPA from the lung can be measured using a gamma camera and a clearance rate can be established. This can also be correlated with the imaging of larger molecules such as technetium labelled human serum albumin (99mTc-HSA, molecular weight 66,000 daltons, commercially available as nanocolloidal particles with a molecular radius of less than 80nm) or technetium sulphur colloid (Tc-SC, insoluble particles, mean radius 300nm). These are not absorbed and therefore establish the rate of mucociliary clearance (although many studies have not performed this measurement in asthma as mentioned later). The rate of transcellular clearance is thought to reflect the 'permeability' of the epithelium. Through targeted deposition of the aerosol to differing areas of the lung, and selective imaging, one can attempt to distinguish between bronchial and alveolar permeability. Urine can also be sampled for radioactivity to indirectly assess permeability[373].

Radiological assessment of epithelial permeability in non-asthmatic conditions

Studies have already been performed in this fashion to look at other respiratory conditions, with results indicating increased permeability, such as in acute respiratory distress syndrome (ARDS)[374, 375] and various forms of interstitial lung disease[376-378].

Cigarette smoking has been particularly well studied with numerous studies showing increased permeability[379-382], increasing acutely with smoking[383] and partially resolving with abstention[384], although studies have often focussed on alveolar epithelial permeability rather than bronchial permeability. Interestingly, cigarette smoke has been shown to disrupt tight junctions in both histological[385] and cellular studies[386].

Radiological assessment of epithelial permeability in asthma

In asthma, where we are particularly interested in bronchial airway (rather than alveolar) epithelial permeability, and despite the work also suggesting impaired permeability histologically (see section 2.5.4), results from imaging studies have been less clear cut. Earlier studies have shown no difference in permeability in chronic stable asthma[380, 387-389], or an increase in permeability in acute but not chronic asthma[390]. Similarly, studies looking at the effectiveness of steroid inhalers have showed an improvement in ventilation but no change in permeability following treatment[391]. Potential criticisms of these studies include:

- Small study sizes
- Failure to correct for mucociliary clearance (which is known to be slowed in asthma[392]). Indeed, in a single study when mucociliary clearance was accounted for, permeability was shown to be increased in stable asthmatics[393].
- When mucociliary clearance is measured, this takes place on a separate day, raising the possibility of altered deposition of the two isotopes(either due to a change in breathing techniques, or altered permeation through the lungs due to differing levels of baseline bronchoconstriction), which could then affect the data.

There is a need for further clarification in this area therefore, to separate out permeability effects from mucociliary effects, to more closely separate out bronchial airway permeability from alveolar permeability, whilst avoiding the criticisms levelled at previous studies as detailed above. This can be accomplished through:

- Targeted deposition of particles into the airways alone using recently developed nebulisation techniques [394] (see section 5.3.4)

- Analytical techniques recently developed allowing 3D reconstruction of planar images to accurately dissect out bronchial airways from alveoli[395].
- Simultaneous assessment of both mucociliary clearance and epithelial permeability, by simultaneously administering both particles, with different radioisotope labels for each (namely technetium and indium, see section 5.3.3).

Aims

Primary objectives

- Measurement of In111-DTPA clearance in normal subjects, asthmatics and smokers and comparison for statistically significant difference.

Secondary objectives

- Measurement of mucociliary clearance using technetium labelled albumin as a micro-colloid, 99mTc-Nanocoll, in normal subjects, asthmatics and smokers and comparison for statistically significant difference
- Lung function and volume measurements (FEV1, FVC, FEF25-75, reversibility with salbutamol, FRC and TLC) together with provocation testing (PC20 methacholine) and exhaled nitric oxide (eNO) will be recorded and correlated with degree of permeability if possible.
- History of atopy will be recorded and correlated with permeability.

Section B: Materials and Methods

Methods - Clinical Study (Safety and Efficacy of parenteral KGF in moderate asthma subjects)

Subject recruitment – general

Subjects for departmental clinical studies are identified by several means:

A) A departmental database of volunteers who have expressed an interest in taking part in asthma research.
B) Patients attending out-patient asthma clinics at Southampton General Hospital
C) Local advertising within the hospital and local general practise clinics (in the form of leaflets/posters).

Eligible subjects are contacted by telephone or email and information sheets sent to them. After a minimum of 48 hours, subjects are re-contacted and if they are happy to take part, a screening visit is organised where full written consent is obtained. All protocols and study and related documents were approved by the Nottingham Research Ethics committee (ref no. 09/H0408/85).

Subject recruitment –inclusion criteria

Inclusion criteria for the study were as follows

- Age 18 - 60 years, either gender*
- Confirmed diagnosis of asthma for > 1 year as defined by BTS guidelines, requiring treatment with high dose inhaled corticosteroids +/- long acting beta2 agonists, with persisting symptoms requiring use of short-acting beta agonist therapy >3x/week*.
- Never-smoker, or ex-smoker having stopped > 1year ago with pack year history <10 pack years*.
- FEV1 >40%
- Subject must understand the procedures of the study and agree to participation in the study by providing written informed consent
- Subject considered fit enough to undergo lung function testing including provocation tests, and bronchoscopy.
- Subject must not be participating in another clinical trial or have done so within the last 12 weeks.

*Initially the study only included subjects who were 18-50 years old (rather than 18-60 years), on combination treatment with long-acting beta-agonists and inhaled corticosteroids

(rather than inhaled corticosteroids alone), and who had never smoked (rather than ex-smokers with <10pack year history). The above modifications were made partway through the study to improve recruitment chances and as it was felt that the potential biological/physiological effects of the drug would apply equally well in the larger group, and that the study remained safe and feasible in this larger group.

Subject recruitment –exclusion criteria

Exclusion criteria for the study were as follows

- Patients requiring regular maintenance oral steroids for their asthma, or those who are adhering to Symbicort 'SMART' single inhaler regime.
- Pregnancy (where pregnancy is defined as the state of a female after conception and until the termination of gestation, confirmed by a positive hCG laboratory test >5mIU/ml), an intention to become pregnant or breast-feeding (lactating.
- Subjects with active lung disease other than asthma
- Significant medical (cardiopulmonary, neurological, renal, endocrine, gastrointestinal, psychiatric, hepatic or haematological) co-morbidity, which in the view of the investigator could impact on the interpretation of results or participation in the trial, or which is uncontrolled with standard treatment.
- Current participation in another clinical trial or previous participation within the last 12 weeks.
- Alcohol or active drug abuse.
- On-going allergen desensitisation therapy
- Regular use of sedatives, hypnotics, tranquilisers
- Cancer or previous history of cancer
- Inability to understand directions for dosing and study assessment.
- Inability to be contacted in case of emergency.

Following recruitment, subjects were given a study number e.g. KGF01, KGF02, etc. Assuming patients completed pre-drug assessments (visits 2-4) successfully, they were then randomised by Southampton General Hospital pharmacy (keeping the investigators blinded), using a permuted block randomisation method (with blocks of 4) to ensure 10 patients randomised to each group. 37 patients were screened, and 21 patients were randomised (due to withdrawal of one patient after only one dose of the drug (see Results discussion below) to ensure 10 patients in each group having a complete course of the active treatment. 2 patients suffered exacerbations after the second bronchoscopy, requiring

treatments with systemic steroids, and as this would affect their subsequent data for visits 12 and 13, they were voluntarily withdrawn at this stage i.e. their data for visits 12 and 13 is 'missing', however data up to this point was included in the analysis

Subjects could be withdrawn from the study for the following reasons:

- Serious adverse event.
- Withdrawal of informed consent.
- Occurrence of an exclusion criterion that was clinically relevant and affected the subject's safety.
- Pregnancy.
- Lack of subject compliance.
- Occurrence of AE's where discontinuation of treatment was desired or considered necessary.
- Protocol violation.
- If investigator concluded it would be in the patient's best interests.
- The patient could voluntarily withdraw at any time, without giving a reason.

Study schedule – overview

The study consisted of 13 visits to Southampton General hospital over 6 - 7 weeks, as follows. At all visits adverse events were recorded, and throughout the study participants completed a diary card detailing symptoms and peak expiratory flow rate (PEFR). The study drug/placebo was given on day 0 and day 11. Details of which procedures were performed at which visit are shown in diagrammatic form below (see Figure 4: Diagrammatic representation of study visits and procedures) with a breakdown as follows.

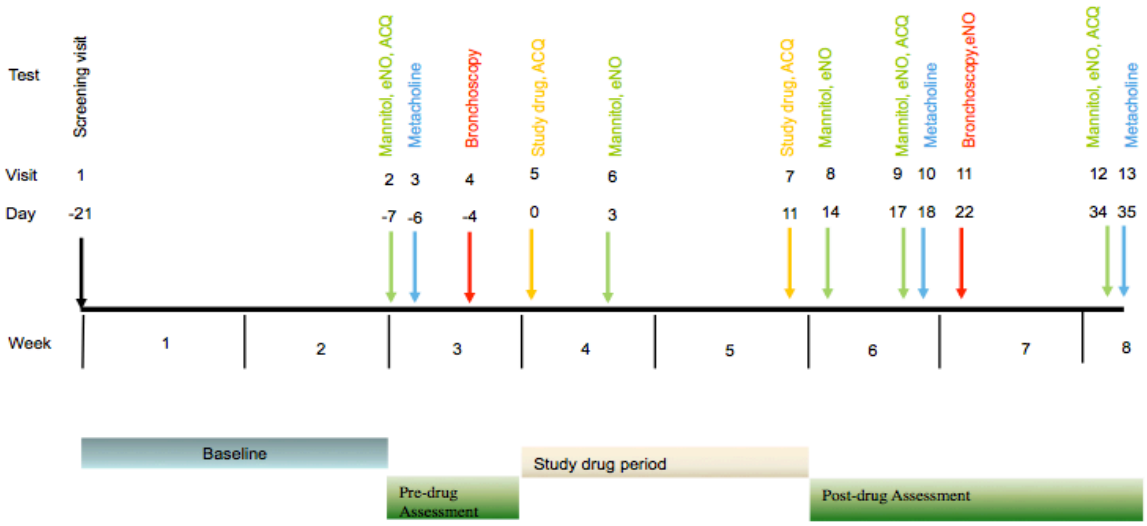


Figure 4: Diagrammatic representation of study visits and procedures

NB For increased participant flexibility, bronchoprovocation visits (visits 2, 3, 6, 8, 9, 10, 12 and 13) could be shifted one day later/earlier, although bronchoscopy/Study drug visits were fixed to comply with the planned dosing regime and to allow comparison of the biological samples between subjects. The screening visit needed to be at least 2 weeks before visit 2, but could be up to 4 weeks before.

Study schedule – breakdown

Screening visit (Day -21).

- Informed consent taken, inclusion/exclusion criteria checked, full medical history and examination performed.
- Vital signs (pulse rate, blood pressure, temperature, height, weight) taken
- Spirometry (FEV1, FVC, PEFR) performed
- Diary card and peak flow meter given
- Skin prick testing to common aeroallergens performed
- Exhaled nitric oxide (eNO) measured.

- Urine pregnancy test taken (if female and of childbearing age)
- Asthma quality of life questionnaire (AQLQ) and asthma control questionnaire (ACQ) completed

Visit 2 (Provocation visit) (Day -7 +/- 1 day)

- Mannitol bronchoprovocation test performed
- eNO measured (before provocation)
- ACQ administered.

Visit 3 (Provocation visit) (Day – 6 +/- 1 day)

- Methacholine bronchoprovocation test performed

Visit 4 (Bronchoscopy visit A) (Day - 4)

- Bronchoscopy performed

Visit 5 (Study drug/Placebo administration visit 1) (Day 0)

- Administration of either study drug or placebo
- ACQ administered.

Visit 6 (Provocation visit) (Day 3 +/- 1 day)

- eNO measured (before provocation)
- Mannitol test performed

Visit 7 (Study drug/Placebo administration visit 2) (Day 11)

- Administration of either study drug or placebo
- ACQ administered.

Visit 8 (Provocation visit) (Day 14 +/- 1 day)

- eNO measured (before provocation)
- Mannitol test performed

Visit 9 (Provocation visit) (Day 17 +/- 1 day)

- eNO measured (before provocation)

Mannitol test performed Visit 10 (Provocation visit) (Day 18 +/- 1 day)

- Methacholine test performed

Visit 11 (Bronchoscopy visit B) (Day 21)

- Bronchoscopy performed

Visit 12 (Follow-up provocation visit) (Day 35 +/- 1 day)

- eNO measured (before provocation)
- Mannitol test performed
- ACQ and AQLQ administered

Visit 13 (Follow-up provocation visit) (Day 36 +/- 1day)

- Methacholine test performed
- ACQ administered

Study outcome methods

Spirometry/reversibility

Spirometry was measured using a Vitalograph spirometer, recording FEV_1 (forced expiratory volume in 1 second) and FVC (forced vital capacity) from the best of 3 efforts, with % predicted for gender and height calculated using prediction tables based on equations from Hankinson et al[396]. There is no consensus as to the drug, dose or mode of administration of bronchodilators to assess reversibility[397] and various techniques have been used. In addition, there is no clear consensus as to what amount of change constitutes 'reversibility'[398]. An ATS/ERS task force[397] recommends expressing change as a per cent of predicted values, and states that increments of <8% (or <150mls) may be due to measurement variability, quoting from studies which have assessed fluctuations in a healthy population[399]. Differing cut-offs have been used over time, with the task force suggesting an increase in FEV_1 > 12% and >200mls, as constituting a 'significant' or 'positive' bronchodilator response. However it is known that longstanding asthma can be associated with non-reversible airways obstruction and decreased reversibility[400], and any cut-off is somewhat arbitrary. For the purposes of this trial, it was decided to use a cut-off of 11%, measured after inhalation of 2.5mg salbutamol via an air driven jet nebuliser (Respironics Sidestream (#4445) and mouthpiece (#1601)), with repeat FEV_1 measurement after 15 minutes. % Reversibility was then derived as follows:

$$\% \text{ Reversibility} = \frac{FEV1\ postbronchodilator - FEV1 prebronchodilator}{FEV1\ prebronchodilator} \times 100\%$$

Prior to reversibility, subjects were required to withhold certain medications as per departmental standard operating procedure (SOP) based in part on ATS/ERS recommendations[401](see below - Table 2: Types and durations of medications to withhold for reversibility test)

Time to withhold	Medication
6 h	SHORT ACTING BETA2 AGONIST INHALERS e.g. salbutamol,terbutaline
12h	LONG-ACTING BETA2 AGONIST INHALERS e.g. salmeterol, formoterol
12hrs	INHALED CORTICOSTEROIDS + LONG-ACTING BETA2 AGONIST INHALERS e.g. fluticasone/salmeterol (Seretide); budesonide/formoterol (Symbicort),
12h	THEOPHYLLINE TABLETS e.g. Nuelin SA, Slo-phyllin, Uniphyllin continus
72h	ANTIHISTAMINE TABLETSe.g. cetirizine, fexofenadine and loratadine.

Table 2: Types and durations of medications to withhold for reversibility test

If reversibility was <11%, on the discretion of the investigator, a second dose of 2.5mg nebulised salbutamol was administered to see if the peak response to bronchodilator had been achieved. In addition, again on the discretion of the investigator, if a patient did not achieve 11% reversibility patients could be brought back on an additional, separate visit to repeat the test (given the inherent variability in asthma).

Asthma control assessment.

Asthma control was assessed using the Asthma Control questionnaire (ACQ), developed by Juniper et al[267]. This validated, self-administered, questionnaire consists of 7 questions, looking at 5 major symptoms indicative of asthma control, together with a measure of FEV1 % predicted (pre-administration of bronchodilator) and amount of rescue bronchodilator use. It is scored based on a subject's experience of the week prior to the administration of the questionnaire, and each question has a 7-point answer scale (0 = no impairment, 6 = maximum impairment). The total of the seven questions is then averaged, to give a score between 0 and 6, with a lower score reflecting greater control. In general, the cut-off score of 1 can identify control of symptoms in asthma i.e. patients with scores below 1 are 'well'-controlled and those with scores above 1 are 'inadequately' controlled. However there is a grey area and therefore cut-offs of below 0.75 and above 1.5 have also been suggested[402] as giving a greater confidence in saying a patient's asthma is controlled. A change in the ACQ

(for example due to a change in treatment) of 0.5 is considered clinically relevant[270, 403]. The ACQ has been used as an outcome measure in multiple asthma trials.

Asthma quality of life assessment.

The effect of the patient's asthma on their quality of life was assessed using the standardised Asthma Quality of Life Questionnaire (AQLQ (S)), another standardised, validated questionnaire developed by the Juniper group[268]. It is also self-administered and consists of 32 questions in 4 domains – symptoms (12 questions), activity limitation (9 questions), emotional function (5 questions) and environmental stimuli (4 questions) (with 2 additional questions on overall limitation status). Questions are answered on a 7-point scale (7 = not impaired at all, 1 = severely impaired) on the basis of how the respondent has been over the previous **two** weeks (c.f. ACQ, which is based on the previous one week alone). The questionnaire gives an overall mean score (mean of all 32 responses) and also mean scores for each domain, with a lower score reflecting greater impairment. As with the ACQ, a change of 0.5 in the AQLQ score is considered clinically significant[269], and has also been used as an outcome measure in a number of asthma trials.

Permission to use the ACQ and AQLQ was obtained from the Juniper group.

Skin prick testing

Skin prick testing was performed using stock solutions of allergen extract to the following (all supplied by ALK Abello, UK):

Histamine dihydrochloride (positive control, 1 mg/ml)

Glycerol-saline (negative control)

Dermatophagoides pteronyssinus	Dermatophagoides farinae
Mixed grasses	Birch tree pollen
Rapeseed	Dog fur
Cat dander	Horse
Aspergillus fumigatus	Alternaria tenius

These were applied intra-dermally by pricking the skin of the volar aspect of the forearm with a sterile lancet through a bead of allergen extract. After 15 minutes, if a wheal formed at the site of the skin prick, its mean diameter was recorded (maximum wheal diameter plus

wheal diameter perpendicular to that in mm.). A positive result was indicated by a wheal of 3x3mm or more than the negative control. A subject is said to be 'atopic' if they display specific IgE sensitivity[404]; skin prick tests are accepted as a marker of specific IgE sensitivity[405].

Diary card monitoring

Subjects were supplied with a peak flow meter (Miniwright, Clement Clarke International Limited, Harlow, UK) to measure their peak expiratory flow rate (PEFR) at home. The best of three blows in the morning and evening, before taking any bronchodilators, was recorded in a diary card (see Section F: Appendices), along with daily recording of details of severity of chest symptoms and of short acting bronchodilator therapy. Diary card monitoring occurred continuously from screening visit to the end of the trial. Various equations have been proposed to calculate PEFR variability, for the purposes of this study this was calculated at intervals using the following equation for 'amplitude percent highest/maximum', as follows:

% PEFR variability = Highest PEFR (during period) – lowest PEFR (during period) X 100%

Highest PEFR

$$\frac{Highest\ PEFR\ during\ the\ period - lowest\ PEFR\ during\ the\ period}{Highest\ PEFR\ during\ the\ period} \times 100\%$$

This has been shown to correlate well with bronchial hyper-responsiveness and asthma symptoms [406, 407].

Mannitol

Indirect challenge to assess bronchial hyper-responsiveness was performed using spray-dried mannitol powder in gelatin capsule form, inhaled from a dry powder device (Osmohale^{TM}). Subjects were required to withhold the following medications for differing periods of time as detailed in manufacturer guidelines (see Table 3: Types and duration of medications to withhold for mannitol challenge).

As per manufacturer guidelines, the FEV_1 was measured before and 60 seconds after delivery of each dose (0, 5, 10, 20, 40, 80, 160,160,160 mg). The test did not proceed if the baseline FEV_1 was less than 50% predicted due to safety concerns. A positive response was achieved in 2 ways:

$\geq$15% fall in FEV_1 from baseline (using the *highest* post 0mg FEV_1 as comparator)

≥10% incremental fall in FEV_1 between consecutive Osmohale™ doses

The test continued until a positive response had been obtained as above or the maximal cumulative dose of 635 mg had been administered. The cumulative dose required to produce a 15% drop in FEV_1 was then calculated by linear interpolation using the following equation:

$$PD_{15} = \boldsymbol{Antilog}\left(\frac{((\mathbf{log}(\boldsymbol{C2})-\mathbf{log}(\boldsymbol{C1}))(\mathbf{15}-\mathbf{R1})}{(\mathbf{R2}-\mathbf{R1})} + \mathbf{log}(\boldsymbol{C1})\right)$$

where

R1 = % fall in FEV_1 prior to 15% fall R2 = % fall in FEV_1 ≥ 15% fall

C1 = cumulative dose of Osmohale prior to dose provoking 15% fall

C2 = cumulative dose of Osmohale to cause ≥ 15 % fall.

If a patient did not drop their FEV_1 by 15% by the end of the test i.e. no positive response, an arbitrary dose of 795mg was recorded (i.e. 160mg more than the last 635mg –this is discussed in the results section).

A response-dose-ratio (RDR) was also calculated, using the following equation:

$$RDR = \frac{\%\ \text{fall in FEV1 at the last dose}}{Total\ cumulative\ dose\ mannitol\ (mg\ administered)}$$

Following completion of the challenge with a positive result a bronchodilator was administered and the patient was monitored for 15 minutes to ensure their FEV_1 had returned to within 5% of pre-challenge level

Time to withhold	Medication
6-8 hours	INHALED NON-STEROIDAL ANTI-INFLAMATORY AGENTS e.g. sodium cromoglycate (Cromogen Easi-Breathe, Intal), nedocromil sodium (Tilade)
8 h	SHORT ACTING BETA-2 AGONISTS e.g. salbutamol (Ventolin), terbutaline (Bricanyl)
12h	INHALED CORTICOSTEROIDS e.g. beclomethasone dipropionate (Qvar, Clenil modulate), budesonide (pulmicort), fluticasone propionate (flixotide), ciclesonide (Alvesco)
12h	IPRATROPIUM BROMIDE e.g. Atrovent
24h	LONG-ACTING BETA-2 AGONISTS e.g. salmeterol (Serevent), formoterol (Oxis, Foradil)
24h	INHALED CORTICOSTEROIDS PLUS LONG-ACTING BETA-2 AGONISTS e.g. fluticasone and salmeterol (Seretide), budesonide and formoterol (Symbicort), beclometasone diproprionate and formeterol (Fostair)
24h	THEOPHYLLINE e.g. Nuelin SA, Slo-phyllin, Uniphyllin continus
72h	TIOTROPIUM BROMIDE e.g. Spiriva
72h	ANTIHISTAMINES e.g. cetirizine, fexofenadine and loratadine and others
4 days	LEUKOTRIENE- RECEPTOR ANTAGONISTS e.g. montelukast sodium (singulair), zafirlukast (accolade)

Table 3: Types and duration of medications to withhold for mannitol challenge

Methacholine

Direct challenge testing of airway hyper-responsiveness was assessed using methacholine provocation, using a modification of the method of Chai et al [408], as recommended by ATS guidelines[409], in a 5 breath dosimeter technique. As with mannitol, subjects had to withhold certain medications as per departmental SOP/guidelines; this was simplified by the fact that methacholine challenge was always performed the day after mannitol (see Table 4: Types and duration of medication to withhold for methacholine challenge).

Firstly, a baseline FEV_1 was obtained. The test did not proceed if the baseline FEV1 was less than 50% (due to safety concerns), and if the FEV1 was <70% but >50% then clinician advice was sought as to whether it was safe to proceed. The patient then inhaled 5 breaths (from functional residual capacity to total lung capacity) of physiological saline and a post-saline FEV1 was measured at 1 minute and 3 minutes. If the FEV1 post saline dropped by more than 10% then the test did not proceed (again due to safety concerns) and the patients PC_{20} (see below) was assigned a nominal value of '0'.

Assuming the saline did not result in >10% drop, the patient then inhaled 5 breaths of incremental doubling dilutions of methacholine from 0.03 mg/ml – 16 mg/ml administered by an Inspiron nebulizer (Bard Ltd, Sunderland, UK) using a Spira Elektra 2 Dosimeter, with an output of 0.33ml/min. FEV_1 measurements were made at one and three minutes after inhalation of each solution. The challenge was stopped when a fall in FEV_1 of > 20% below the post saline baseline was achieved or the 16mg/ml concentration was reached. If the patient failed to drop by 20% by the end of the test, again an arbitrary value of 32mg was assigned for their PC_{20}.

The non-cumulative concentration of methacholine required to produce a 20% fall in FEV_1 (PC_{20}) was calculated by linear interpolation between the last two points of the log dose response curve administered, using the following equation:

PC_{20} methacholine = antilog (logC1 + ((logC2-C1)*(20-R1)/(R2-R1)))
where
C1 = second-to-last methacholine concentration (concentration preceding C2).
C2 = final concentration of methacholine (concentration resulting in a 20% or greater fall in FEV1)
R1 = % fall in FEV1 after C1
R2 = % fall in FEV1 after C2.

Time to withhold	Medication
6-8 h	SHORT ACTING BETA-2 AGONISTS e.g. salbutamol (Ventolin), terbutaline (Bricanyl)
24hrs	LEUKOTRIENE- RECEPTOR ANTAGONISTS e.g. montelukast sodium (singulair), zafirlukast (accolade)
48h (NB This will have been stopped for the mannitol test for 24 hrs, so need only be held off until the second test)	LONG-ACTING BETA-2 AGONISTS e.g. salmeterol (Serevent), formoterol (Oxis, Foradil)
48hrs	INHALED CORTICOSTEROIDS PLUS LONG-ACTING BETA-2 AGONISTS e.g. fluticasone and salmeterol (Seretide), budesonide and formoterol (Symbicort),beclometasone diproprionate and formeterol (Fostair)
48h (NB This will have been stopped for the mannitol test for 24 hrs, so need only be held off until the second test)	THEOPHYLLINE e.g. Nuelin SA, Slo-phyllin, Uniphyllin continus
1 week	TIOTROPIUM BROMIDE e.g. Spiriva

Table 4: Types and duration of medication to withhold for methacholine challenge

Nitric Oxide

Fractional exhaled nitric oxide (F_eNO) was measured by chemiluminescence analyzer (Niox Flex, Aerocrine, Stockholm, Sweden), following guidelines on standardized techniques published by the ATS[410]. F_eNO was measured at a flow rate of 50ml/s. When the Niox Flex was not available for use, measurements were performed either using alternative analysers e.g. Niox Mino (Aerocrine, Stockholm Sweden) or NOBreath (Bedfont, UK). These machines also use a flow rate of 50ml/s and have been shown to have comparable results to the Niox Flex[411, 412].

Study drug/Placebo administration

Keratinocyte growth factor (KGF) was given intravenously, in a 'collapsed dose' regime of 180 mcg per kg on day 0 and day 11 as has been used previously in other trials[238]. Drug/placebo was administered by a clinician who remained blinded as to the treatment arm. Baseline vital signs were recorded, and the subject was cannulated according to local hospital policy. Study drug/placebo was administered as an intravenous bolus over 1-2 minutes and then baseline observations were repeated at 15-minute intervals for a total of 45 minutes.

Bronchoscopy

Bronchoscopy was conducted according to BTS guidelines[413] and local standard operating procedure. Participants were fasted for 4-6 hours before bronchoscopy. Participants were pre-medicated with nebulized salbutamol (2.5 mg) as a bronchodilator, and at the discretion of the clinician, sometimes also intravenous atropine (0.6 mg), to reduce airway secretions. Mild sedation was achieved where required using intravenous midazolam (up to 5mg) and/or intravenous alfentanyl (up to 500mcg), titrated to effect.

Local anesthesia was achieved with lignocaine spray and gel applied to the nasal passages, soft palate and pharynx. A flexible bronchoscope was then passed either through the nose or mouth into the lungs with additional lignocaine spray being applied to prevent/reduce coughing.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed with pre-warmed (37^{o}C) 0.9% saline, with the bronchoscope wedged within an upper sub-segmental bronchus, and the lavage recovered by gentle suction into a fluid trap. Six aliquots of 20ml were used for lavage, with variable return (approx. 30-40mls). The BAL samples were then sieved (using a 100µm nylon filter (BD Falcon cell strainer, Marathon Lab. Supplies. London, UK) and then centrifuged at 1300G for 10 minutes at 4°C. The supernatant was separated and aliquoted prior to storage at -80^{o} C until further analysis. The cell pellet was re-suspended in PBS and cytocentrifuge slides (Thermo Shandon Ltd, Runcorn, UK) were prepared. A total cell count was achieved using a Neubauer hemocytometer and the trypan blue exclusion method. Differential cell counts were performed manually on cytocentrifuge slides stained with rapid Romanowsky stain (Raymond Lamb Ltd, Eastbourne, UK).

Bronchial brushes

Epithelial cells were obtained for tissue culture and also mRNA analysis. This was performed using a sheathed cytology brush (Olympus BC-202D, Keymed, UK), which was rubbed gently against the epithelium of a second or third generation bronchus four to five times, removed from the bronchoscope and then agitated in PBS to remove the cells. Four such brushings were obtained from the initial bronchoscopy (two brushings for culture, two for mRNA) and two from the second bronchoscopy (for mRNA alone, cells not taken for culture).

Bronchial biopsy

Bronchial biopsies were performed from the large airway carinae (3rd and 4th airway divisions) using disposable (single use) forceps ((Bard, Ref 100503, size: 1.8mm) (Keymed

(Medical & Industrial Equipment) Ltd., OLYMPUS Group Company, Southend-on-Sea, UK)). Up to eight biopsies were taken. Samples were then further processed for GMA analysis, mRNA extraction, whole mount staining and electron microscopy analysis (see separate sections below).

Bronchial biopsy immunohistochemistry analysis

Endobronchial biopsies were processed into GMA resin for immunohistochemical staining, according to the protocol described by Britten et al[414]:

Embedding:

The biopsies were fixed in ice-cold acetone (Fisher Scientific Loughborough, UK) containing protease inhibitors (2mM phenyl methyl sulphonyl fluoride (Sigma, Poole, UK) and 20mM iodoacetamide (Sigma, Poole, UK)) and kept overnight at –20°C. The following day the samples were transferred into acetone (as above) at room temperature for 15 minutes and then into methyl benzoate (Fisher Scientific Loughborough, UK) for a further 15 minutes. The tissue was then immersed in GMA monomer plus 5% methyl benzoate (solution A, Polysciences Inc., Warrington, USA) at 4°C for 3 x 2 hour periods at 4°C, with the GMA solution replaced between each incubation. The tissue was finally embedded in GMA resin (prepared by mixing GMA solutions A + B and benzoyl peroxide) in flat-bottomed capsules (Taab, Aldermaston, UK) and left to polymerize overnight at 4°C. The resin blocks were stored in airtight containers containing silica gel at -20°C.

Cutting:

Samples were roughly trimmed, then cut into 2μm sections using a microtome (Ultracut, Leica, Milton Keynes, UK) and floated onto ammonia water (1:500) to be picked up onto 0.01% poly-L-lysine (Sigma-Aldrich, Poole, UK) coated glass slides (Knittel Glaser, Baunschweig, Germany). Two non-contiguous sections were placed on each slide. Toluidine blue staining was performed to assess the best sections for immunostaining. Once cut, sections were either stained that day or stored wrapped in aluminium foil at -20°C for up to two weeks in order to preserve tissue antigenicity.

Staining:

Biopsy sections on PLL coated slides were initially incubated with 0.1% sodium azide (Fisher Scientific, Loughborough, UK) and 0.3% hydrogen peroxide (Sigma-Aldrich, Poole, UK) in ROW for 30 minutes to inhibit endogenous peroxidases. The slides were then washed with TBS for 3 x 5 minutes, prior to the addition of blocking medium (DMEM with 20% FBS and

BSA) for 30 minutes. Slides were then drained and primary antibodies applied at appropriate dilutions (as determined by titration) under coverslips overnight at room temperature.

Slides were then washed with TBS for 3 x 5 minutes, drained and biotinylated second stage antibodies applied at appropriate dilutions for 2 hours at room temperature. Slides were washed again with TBS for 3 x 5 minutes, drained, and streptavidin biotin-peroxidase complexes (stABC-HRP complex, Dako, Stockport, UK) applied for 2 hours at room temperature. Following TBS wash (3 x 5 minutes) either AEC (AEC substrate pack, Launch diagnostics, Longfield, UK) or DAB (liquid DAB substrate pack, Launch diagnostics, Longfield, UK) substrates were applied for 20 or 10 minutes respectively at room temperature as required.

Slides were rinsed in TBS and then running water for 5 minutes, prior to counterstaining with Mayer's haematoxylin (90 seconds) and a further running water rinse. Finally sections were sealed with aqueous mounting medium (AbD Serotec, Kidlington, UK) incubated at 80°C for 30 minutes and allowed to cool prior to coverslipping using Pertex (Surgipath, Peterborough, UK).

Primary antibodies used in immunohistochemistry, their source, chromogen and working dilution are shown in the table below (Table 5: Primary antibodies used in immunohistochemistry, their source, chromogen and working dilution**).** All antibody dilutions were established by titration, and absence of nonspecific staining established by isotype controls.

Table 5: Primary antibodies used in immunohistochemistry, their source, chromogen and working dilution

Antigen	Supplier, Clone/catalogue number	Type	Chromogen	Dilution
Eosinophilic cationic protein (EG2)	Diagnostics development, #mAb593	Mouse mono	AEC	1:2000
Mast cell tryptase (AA1)	Abcam, #Ab2378	Mouse mono	AEC	1:1000
Neutrophil Elastase	Dako #NP57	Mouse mono	AEC	1:1000
Epidermal growth factor receptor (EGFR)	Brooke lab, Floss	Sheep	DAB	1:400
Transforming growth factor beta-2 (TGF-β2)	Abcam, #Ab36495	Mouse mono	DAB	1:100
Collagen III	Chemicon #0603024755	Mouse mono	DAB	1:1500
GM-CSF	R&D Systems, #MAB215	Mouse mono	DAB	1:160
Interleukin 8 (IL-8)	eBiosciences, #BMS136	Mouse mono	DAB	1:300
TNF-alpha	Cell Tech 52B B3	Mouse mono	DAB	1:150
E-cadherin	Invitrogen #13-700	Mouse mono	DAB	1:600
P120	BD Biosciences #610133	Mouse mono	DAB	1:800
Zona occludens 1 (ZO-1)	Life technologies #33-9100	Mouse mono	DAB	1:50
Occludin	Life technologies #33-1500	Mouse mono	DAB	1:150
Ki67	Dako, #M7240	Mouse mono	DAB	1:200

Image analysis

Image analysis was performed by a blinded observer. A single observer performed analysis for all staining except IL-8, TNF-alpha and GM-CSF, which was all analysed by a second, blinded single observer, to remove intra-observer variation.

Image analysis occurred on a Zeiss KS400 light microscope with Zeiss computer software (Image Associates, Bicester, UK), using bespoke computer programmes for analysis of area/% staining as follows:

Biopsy characteristics

Biopsy sections at the beginning and halfway through the sections cut for staining runs were stained with toluene blue to highlight biopsy architecture. These example sections were used to calculate representative submucosal areas for the biopsy, using the image analysis software to outline the submucosa, excluding areas of damage/cartilage, muscle, blood vessel and glands. The software then measured the area in mm^2 (Figure 5: Highlighting (in green) valid submucosa in a given bronchial biopsy section using the image analysis software.).

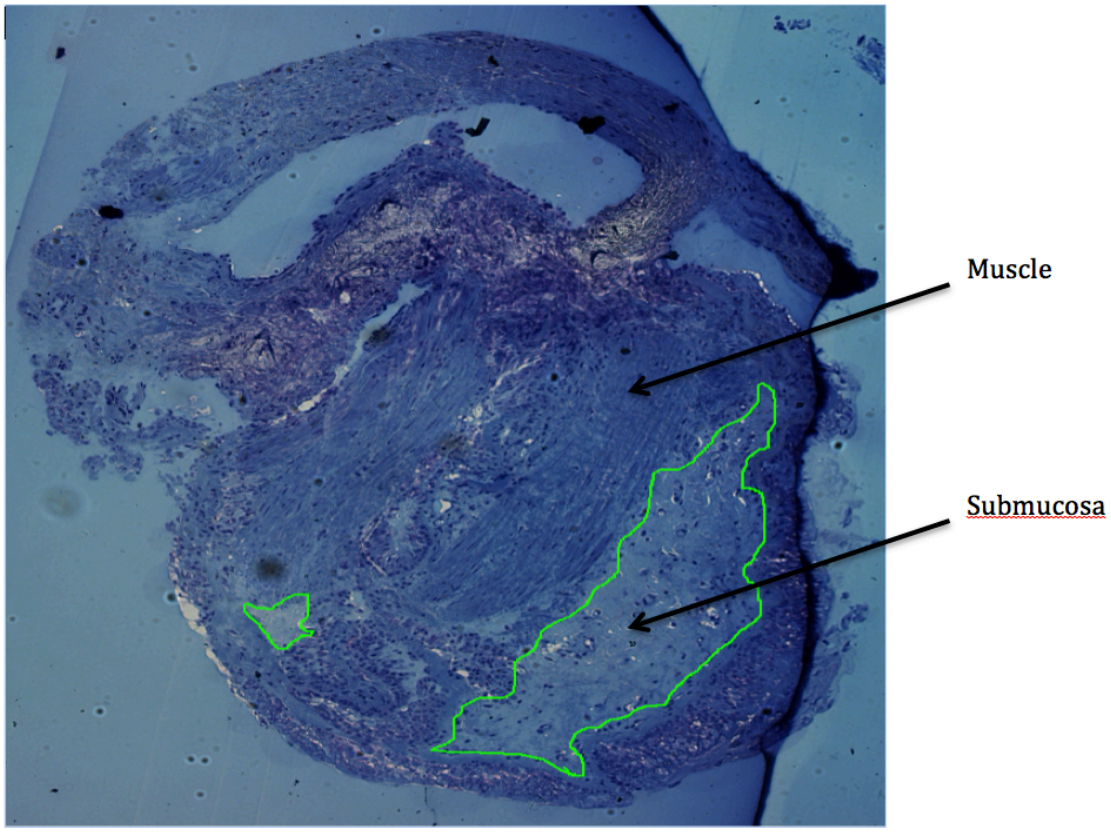


Figure 5: Highlighting (in green) valid submucosa in a given bronchial biopsy section using the image analysis software. X25 magnification, areas of muscle and submucosa arrowed.

In addition, the length of complete, non-tangential epithelium could be measured using this software, as shown below (Figure 6: Highlighting (in green) length of valid epithelium in a bronchial biopsy using the image analysis software. x25 magnification in above image, x100 close-up below.). Tangential epithelium (signified by an excess of basal cells with no overlying columnar cells), was not analysed, nor was damaged epithelium (signified by either no epithelial cells, or a single layer of basal cells with no overlying columnar cells), also shown below.

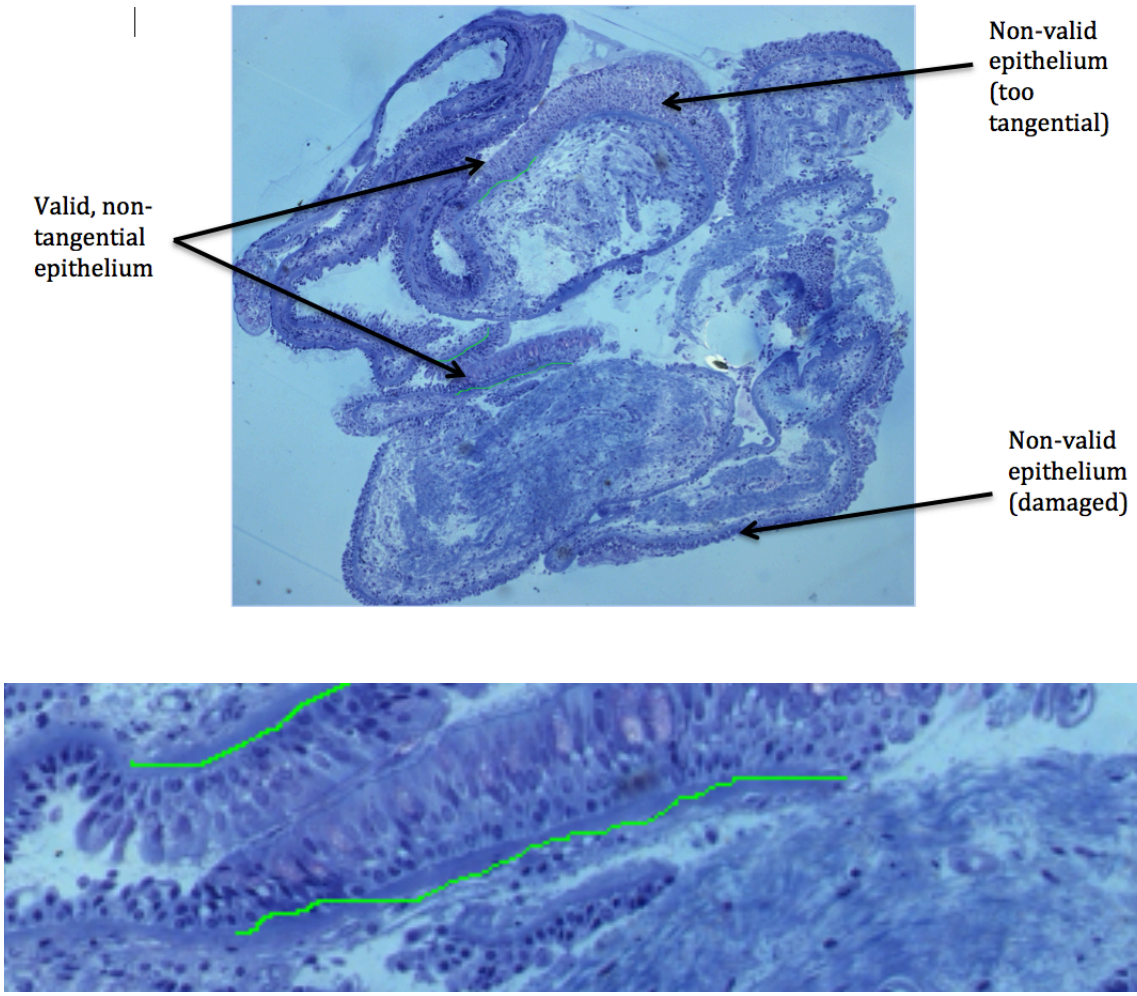


Figure 6: Highlighting (in green) length of valid epithelium in a bronchial biopsy using the image analysis software. x25 magnification in above image, x100 close-up below. Areas of non-valid epithelium marked with arrows.

Cell count

Once the area of submucosa and length of epithelium was calculated, the number of nucleated cells stained with AEC chromogen in said submucosa/epithelium could be counted manually using a light microscope (at x40 magnification) to give a value for cells per length epithelium (cells/mm) or per area of submucosa (cells/mm^2) as appropriate (see example image - Figure 7: Example of bronchial biopsy stained for mast cells (AA1)).

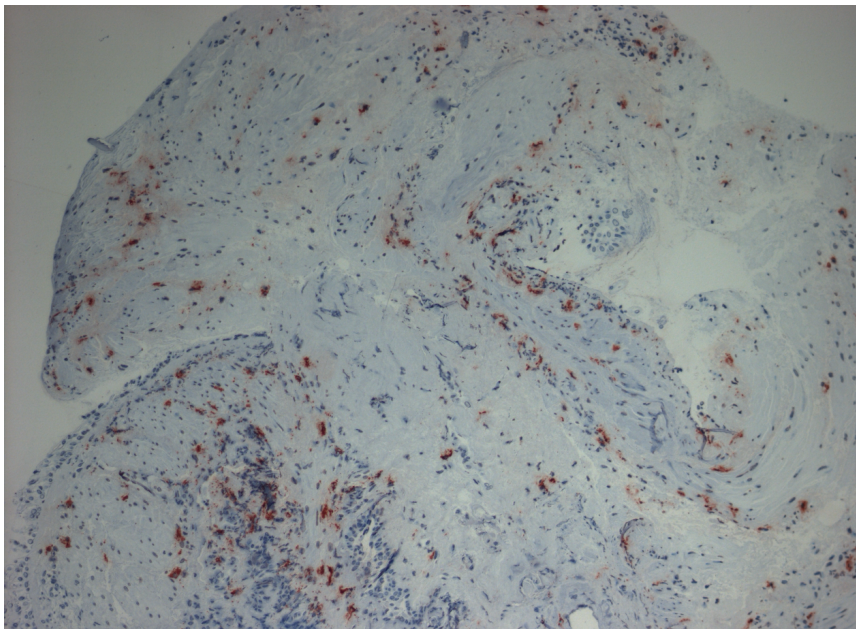


Figure 7: Example of bronchial biopsy stained for mast cells (AA1), x40 magnification, AEC immunohistochemistry using Abcam antibody, 1:1000 dilution

Two biopsy sections were analysed for each variable, either in different biopsies (each patient had 2 bronchial biopsies embedded in GMA), or if only one biopsy was analysable, in the same biopsy with sections separated by at least 20μm. Mean values were then recorded using these 2 measurements.

Diffuse epithelial staining

Using the area of intact/valid epithelium as described above, sections were analysed for % staining using the imaging software and based on red, blue and green colour balance (see figure 8 below). As with cell counts, 2 sections were analysed and the mean average value recorded.

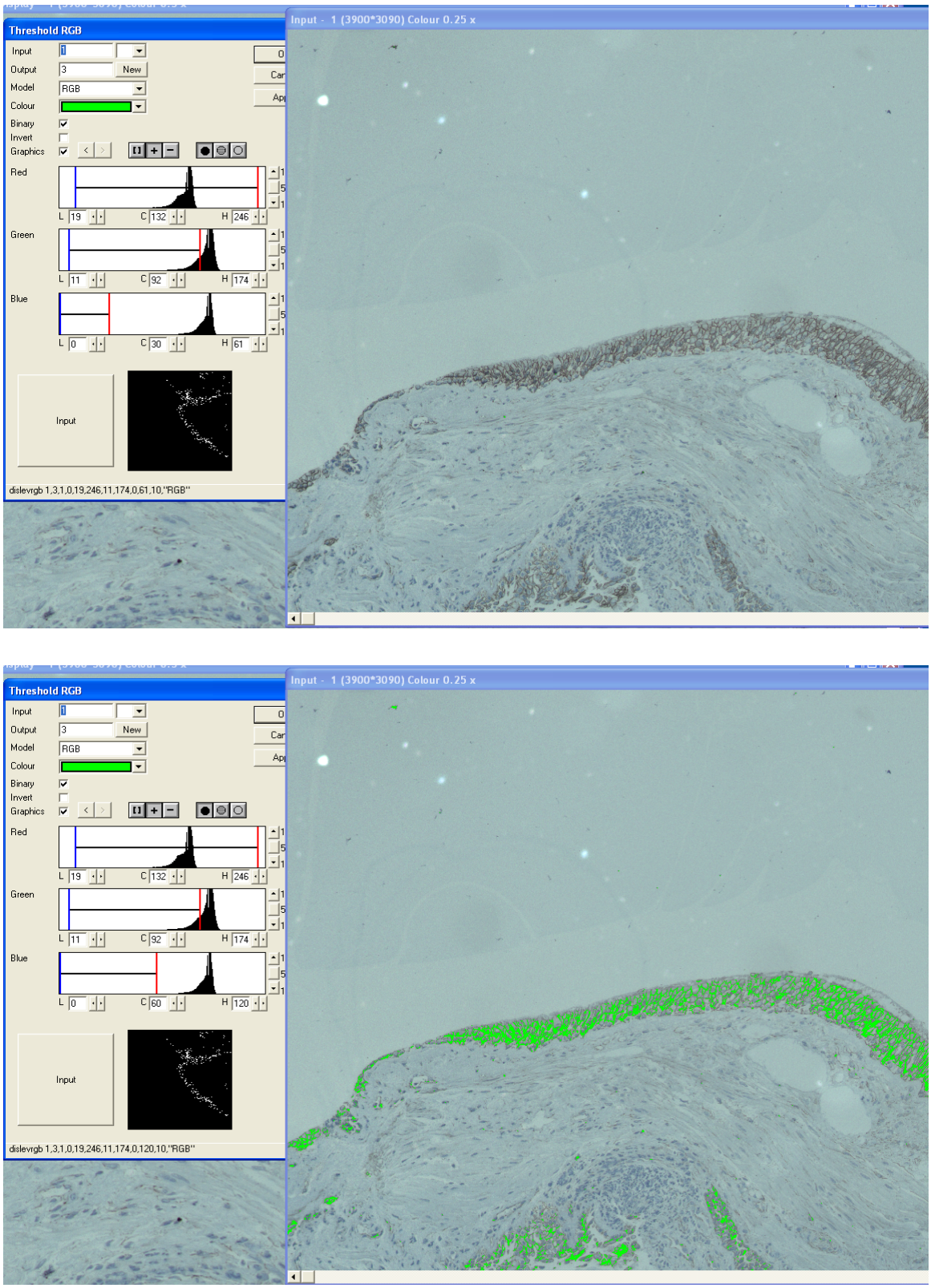


Figure 8: Example of RBG balance matching procedure to determine % epithelial staining. Above is original staining, below is RBG balance adjusted to match said staining as closely as possible.

Tight junctional analysis

The pattern of tight junctional staining precluded use of the above techniques for quantifying the degree of staining – instead semi-quantitave scoring was employed (by a blinded individual) using the following guide to scoring (as previously used[76]):

- 3 = strong and regular punctuate staining of TJs along epithelial surface
- 2 = irregular staining along epithelial surface, but clear TJs evident
- 1 = patchy staining, only a few TJs evident
- 0 = no staining evident

Examples of the above staining patterns are displayed below (see Figure 9: Examples of TJ staining: A = score 3, B = score 2, C = score 1, D = score 0). Only well orientated, intact epithelium was analysed. Occasionally a biopsy would display different staining in different epithelial areas – multiple scores were then assigned, with an average score given for that biopsy. An average of at least 2 sections was taken as the final value for that subject.

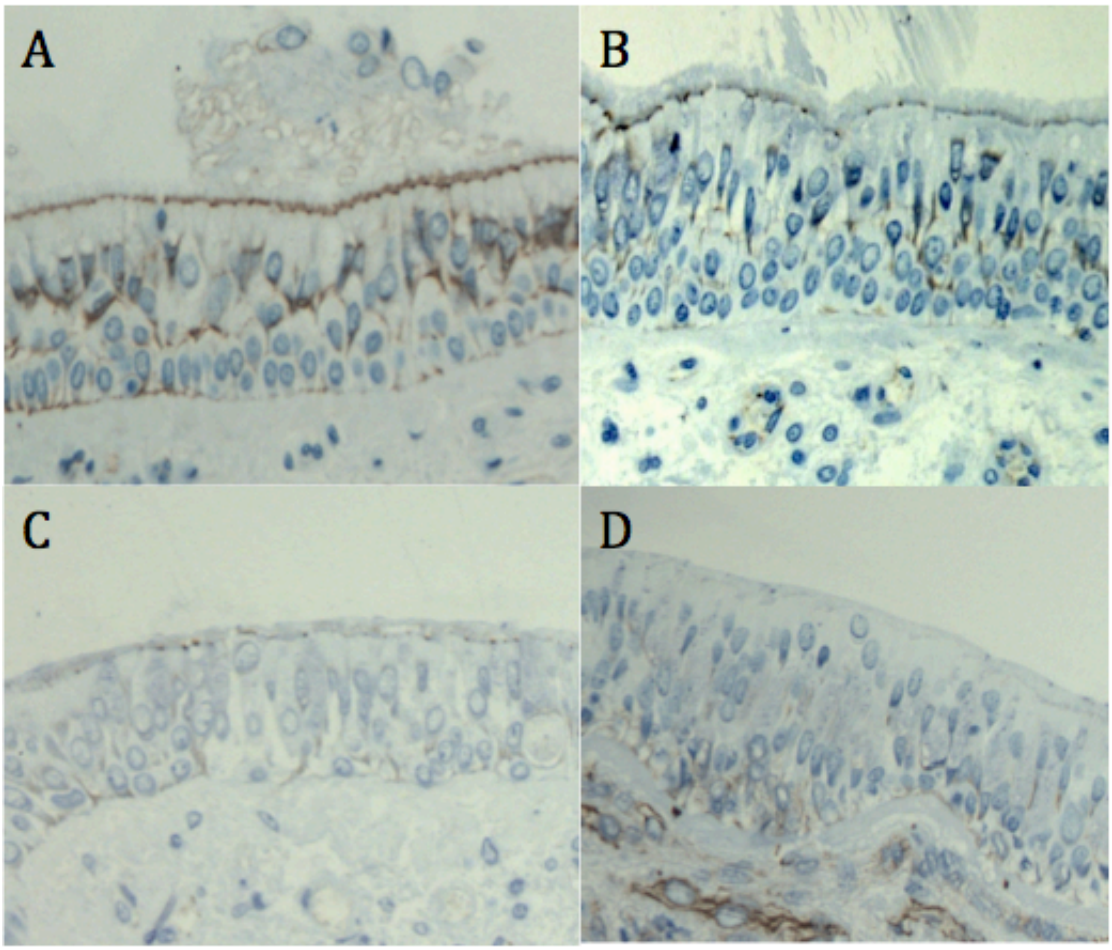


Figure 9: Examples of TJ staining: A = score 3, B = score 2, C = score 1, D = score 0. X100 magnification, DAB staining, using mouse monoclonal antibodies from Life Technologies at dilutions as specified above.

BAL enzyme-linked immunosorbent assay (ELISA)

Most ELISA's were performed using commercially available kits, processed according to manufacturer instructions (details in table below). Tryptase and alpha-2-macroglobulin ELISA's were developed in house according to the method below.

Target	Manufacturer	Catalogue No.
Histamine	Neogen corporation, Lansing, USA	409010
YKL-40	Quidel Corporation	8020
MPO	Hycult Biotech, Cambridge Bioscience, Cambridge, UK	HK324
Osteopontin	R+D System, Oxford, UK	DY1433
Groalpha	R+D System, Oxford, UK	DY275
Eotaxin	R+D System, Oxford, UK	DY320
sICAM-1/CD54	R+D System, Oxford, UK	DCD540
ECP	Caltag	RG-7618-D
TIMP-1	R+D System, Oxford, UK	DY970
MMP9	R+D System, Oxford, UK	DY911
IL-10	R+D System, Oxford, UK	DY217B
CCL-17/TARC	R+D System, Oxford, UK	DY364
RANTES	R+D System, Oxford, UK	DY278
ENA-78	R+D System, Oxford, UK	DY254
IL-8	R+D System, Oxford, UK	DY208

Table 6: Manufacturer and catalogue number for ELISA assays

Tryptase 'in house' ELISA method

- Coat ELISA plate (Greiner bIo-One 96 well Half-area plate, Catalog no 675001) with 50µl of 1:2000 ammonium sulphate-precipitated antiserum (EAR, see below):tryptase (made up with 50mM sodium carbonate, pH 9.6), cover and incubate overnight at 4^{o}C.
- Wash plates with 200µl of PBS-T (Phosphate buffered saline/ 0.05% Tween20) twice (2 min per wash).
- Block non-specific protein binding sites with 150 µl of 2% BSA (Bovine serum fraction V albumin) dissolved in PBS-T. Cover the plate and incubate at room temperature for at least 60 min. Wash the plate twice (as above).
- Apply 50 µl of the samples or standards (tryptase purified from human skin, applied at doubling dilutions from 100ng/ml down to 0.78ng/ml). Incubate at room temperature for 90min. Wash the plate five times (as above)
- Add 50 µl of the detecting antibody: 0.2µg/ml biotinylated AA5, diluted in PBS with 0.1% BSA and incubate for 90 min at room temperature. Wash the plate twice (as above).
- Add 50 µl of 1/10000 dilution of extravidin peroxidise buffered aqueous solution (Sigma-Aldrich, E2886, made up with PBS/0.1%BSA) and incubate at room temperature for 30 min. Wash the plate twice (as above).
- Develop with 50ul of Tetramethlybenzidine (TMB) substrate (see below).
- Stop the reaction 5 minutes later with 50 µl 2M sulphuric acid.
- Read absorbances at 490nm, compare with standard curve, adjust for any dilution of sample. Lower limit of detection 0.5ng/mL.

Ammonium sulphate-precipitated antiserum: Precipitate polyclonal rabbit immunoglobulin overnight with 45% saturated ammonium sulphate at 4^{o}C, before pelleting in a centrifuge (29000G for 1 hr), dialysing against PBS and freezing for later use.

TMB substrate: Make up sodium acetate buffer using 1.5g of sodium acetate trihydrate in 100 ml of distilled water (ph 8.1to 8.3), adjusted to pH to 5.5-6.0 with glacial acetic acid. Make up TMB solution using 6mg/ml TMB in DMSO. Add 200µl of above TMB solution to 12mls of sodium acetate buffer, mix well and add 1.2µl of 30% H_2O_2 before mixing well again.

Alpha-2-macroglobulin 'in-house' ELISA method

- Coat microtitre plates (Fisher-Scientific, Leicester, UK) with sheep anti-alpha2macroglobulin (The Binding Site Ltd, Birmingham, UK), using 100 μl per well at a dilution of 1:500 in 50mM sodium carbonate (pH9.6), incubating overnight at 4^{o}C.
- Wash plates with 200μl of PBS-T (Phosphate buffered saline/ 0.05% Tween20) three times (2 min per wash).
- Make up standards using alpha2macroglobulin (Sigma Aldrich), diluted in PBS/1% Tween20 (2-100ng/ml), and apply either standards/duplicates of BAL to wells for 90 minutes at 37^{o}C.
- Wash plates with 200μl of PBS-T (Phosphate buffered saline/ 0.05% Tween20) three times (2 min per wash).
- Add 100 ml per well of 1:1000 peroxidase-conjugated sheep anti-alpha2macroglobulin (The Binding Site Ltd, Birmingham, UK) for 90 minutes at 37^{o}C.
- Wash plates with 200μl of PBS-T (Phosphate buffered saline/ 0.05% Tween20) three times (2 min per wash).
- Develop with 100 ml per well of 0.4mg/ml o-Phenylenediamine (Sigma Aldrich) dissolved in phosphate buffer (pH7) containing 0.01% H_2O_2.
- Stop the reaction 10 minutes later with 50 μl 2M sulphuric acid.
- Read absorbances at 490nm, compare with standard curve, adjust for any dilution of sample. Lower limit of detection 2ng/mL.

Multiple ELISA Assay plates

In addition, although not planned in the original data analysis, we obtained access to several commercial multiple analysis assays, which overlapped with some of the ELISA measurements above (offering a chance to verify the above results) and also offering an enhanced coverage of common inflammatory cytokines. These are detailed in the table below; prior to analysis in the Luminex 30-plex and MSD 7-plex plates, BAL samples were concentrated using centrifugal concentrators (Vivaspin 6, FDP-875-102J).

Multiple analysis ELISA plate type	Supplier/Catalogue number	Analytes
Human TH1/TH2 7-Plex	Meso Scale Discovery K15011A-4	IFN-ϒ, Il-10, IL-12, IL-13, IL-2, IL-4, Il-5
Luminex Cytokine Human 30-plex panel	Invitrogen LHC6003	Growth factors (EGF, FGF-basic, HGF, VEGF) Chemokines (Eotaxin, IP-10, MCP-1, MIG, MIP-1α, MIP-1β, RANTES) Cytokines (G-CSF, GM-CSF, IFN- α, IFN-ϒ, Il-1β, IL-1RA, IL-2. IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, TNF- α)
Fluorokine MAP Multiplex Human MMP Panel	R+D, LMP-000B	MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, MMP-13.

Table 7: Manufacturers and catalogue numbers for multiple analysis ELISA plates, with analytes measured specified.

BAL Surfactant protein analysis using mass spectometry

Samples (500 µl BAL) had volumes adjusted to 800µl with 0.9%NaCl. An internal standard (1nmole of DMPC) was added and then 2ml of methanol were added followed by 100µl 800µl of dichloromethane. The samples were centrifuged at 3000rpm, 4-10˚C for 10 minutes. The supernatant was carefully drawn off to a new drying tube. To the supernatant 1ml distilled H_2O and 1ml dichloromethane were added and centrifugation repeated. The lower (dichloromethane) layer was then carefully aspirated, placed in a new drying tube and dried in a concentrator at 37˚C under continuous low flow of N_2 gas. Once dry a further 500µl dichloromethane was added, samples were transferred to brown-glass mass spectrometry bottles and the drying process was repeated. Once drying was completed the bottles were capped, labelled and stored at -20˚C prior to analysis by electrospray ionisation tandem mass spectrometry (ESI MS/MS).

Mass spectrometry scans of each lipid extraction were performed in positive and negative ionisation and then for precursors of specific lipid classes, namely, m/z 184 (PC), m/z 241 (PI), m/z 153 (PG, PA and PI).

Statistical analysis/Data entry

IBM SPSS statistics version 19 was used for statistical analysis of the clinical study. Clinical data was entered into an SPSS database by 2 separate people (double data entry) and the databases compared. Any discrepancy resulted in referral back to the original study documents to ensure no data entry mistakes occurred. In addition, upper and lower limits were set for the variables to flag up possible errors in entering data.

Once the final database of clinical data was accepted i.e. after it had undergone data 'cleaning' as detailed above to eliminate entry errors, it was locked to prevent alterations once unblinding was underway. Between-group analysis was performed, as planned in the initial data analysis plan for the study. This was performed using paired t test for normally distributed data, or Kruskal Wallis testing for nonparametric data. In addition, for data where there was imbalance in the baseline values between the groups, linear regression was performed to account for this.

Methods - *In vitro* studies (Assessment of the effect of KGF on epithelial permeability)

Subject recruitment

As for the clinical trial, subjects were identified by a departmental database of volunteers who had expressed an interest in taking part in asthma research, and also patients attending outpatient asthma clinics at Southampton General Hospital.

Eligible subjects were contacted by telephone or email, and information sheets sent to them. After a minimum of 48 hours, subjects were re-contacted and if they were happy to take part, a screening visit was organised where full written consent was obtained.

Samples for cell culture were obtained both from the clinical trial "Safety and Efficacy of parenteral KGF in moderate asthmatic subjects" (REC no. 09/H0408/85) and from a separate trial "Pathophysiology of airway diseases such as asthma and COPD" (REC no 05/Q1702/165).

Inclusion/Exclusion criteria

Inclusion/Exclusion criteria for the KGF clinical study are detailed in Section B 1.1.1-3. For the study "Pathophysiology of airway diseases such as asthma and COPD", criteria were slightly different (NB COPD criteria not detailed as no COPD samples were used):

Inclusion criteria - general:

- Age 18-75 years.
- For female patients: menopausal >2yr or with childbearing potential but taking efficient contraception and having a negative pregnancy test.

Inclusion criteria – asthmatic cohort:

- Confirmed diagnosis of asthma (>1yr).
- Regular, current anti-asthma medication.
- PC_{20} to methacholine of <8mg/ml.
- For chronic severe disease: requirement of high dose inhaled corticosteroids in combination with long acting $beta_2$ agonists with persisting symptoms and at least 2 courses of oral steroids.
- Baseline FEV_1 >40% of predicted.

Inclusion criteria – healthy cohort

- No diagnosis of respiratory disease.

- No significant response to methacholine administered at 16mg/ml.
- Non-smoker, ex-smoker who quit >1yr prior to study and smoking history <10 py.

Exclusion criteria – general:

- Diagnosis or documented history of broncho-pulmonary aspergillosis or uncontrolled infections.
- Any clinically significant cardiopulmonary abnormalities not related to asthma.
- Past or present tuberculosis, systemic lupus erythematosus or multiple sclerosis.
- Any clinically significant neurological, renal, endocrine, gastrointestinal, hepatic or haematological abnormalities uncontrolled with standard treatment.
- History of psychiatric, medical or surgical disorders which may interfere with study.
- Alcohol and recreational drug abuse.
- Diagnosis of immunodeficiency requiring treatment.
- Treatment with immunomodulators (theophylline or leukotriene receptor antagonists will be allowed).
- Ongoing allergen desensitisation therapy.
- Regular use of sedatives, hypnotics, tranquillisers.
- Positive hepatitis viral antigens or antibodies.
- Blood donation within 3 months either end of study.
- Live immunisation <4 weeks prior to study.
- Oxygen therapy.
- Inability to understand directions for dosing and study assessment.
- Inability to be contacted in case of emergency.
- Participation in another study at the same time or within a prior 3-month period.

Skin prick testing

For the study "Pathophysiology of airway diseases such as asthma and COPD", skin prick testing was performed as detailed in Section B (1.1.6).

Nitric Oxide

For the clinical trial "Safety and Efficacy of parenteral KGF in moderate asthmatic subjects", measurement of F_eNO is detailed in Section B (1.1.6). For the study "Pathophysiology of airway diseases such as asthma and COPD" F_eNO was measured using the Logan LR2500 gas analyser (Logan Research Ltd, UK), set at a flow rate of 100ml/s.

Reversibility

For the study "Pathophysiology of airway diseases such as asthma and COPD", reversibility was performed as detailed in Section B, (1.1.6).

Methacholine challenge

For the study "Pathophysiology of airway diseases such as asthma and COPD", methacholine challenge was performed as detailed in section B, (1.1.6) for the asthmatic cohort. For healthy controls, an accelerated dosing regime was used (as allowed by ATS guidelines[409]) wherein following saline inhalation, doses of 1mg/ml, 4mg/ml and then 16mg/ml are used.

Bronchoscopy

For the study "Pathophysiology of airway diseases such as asthma and COPD", bronchoscopy was performed as detailed in section B (1.1.6) although numbers of brushings/biopsies/amount of BAL varied according to general laboratory requirements (as samples were used by several investigators on different studies). For my *in vitro* studies, bronchial brushings were used in cell culture experiments, as detailed below.

Establishing/Maintaining an ALI culture

Cells were obtained at bronchoscopy as previously described, and then taken to air-liquid interface in a modification of the method of Gray et al[415] . (NB Method described below is for 2 brushings, amounts differed if >2 brushings obtained)

Cells in suspension in PBS had an equal amount of RPMI1640 medium without L-Glutamine (Life Technologies, Cat No: 31870-025), containing 20% foetal bovine serum (FBS) (heat inactivated) (Life Technologies, Cat No: 10108-165) and penicillin / streptomycin (5000IU/ml Penicillin, 5000ug/ml Streptomycin) (Life Technologies, Cat No: 15070-063), added to them. They were then centrifuged at 1000rpm, 5min at room temperature to form a pellet. The supernatant was discarded and the cell pellet re-suspended in 0.5ml Bronchial epithelial cell basal media (BEBM) (Lonza, Cat No: CC-3171) containing growth factors (BEGM SingleQuot Kit Suppl. & Growth Factors ((Lonza, Cat No: CC-4175)). Cells were counted and then seeded in collagen pre-coated T25cm2 tissue culture flasks (Fisher Scientific, Loughborough, UK), together with an additional 3.5ml of BEGM. Cells were then incubated at 37^{o}C in a CO2 incubator, with BEGM replaced at 24 hours and thereafter every 2 days until the cells reached 70-80% confluence. The cells in the T25 flasks were then trypsinised ((Trypsin, 0.5% (10x) with EDTA 4Na (Life Technologies, Cat No: 15400)) and reseeded into collagen pre-coated T75 flasks (Fisher Scientific, Loughborough, UK). Once the cells had reached 70% confluence in the T75 flasks, they were again trypsinised, spun down (as above) and the cells

counted. Cells were then diluted to a concentration of 0.35 x 10^6 cells/ml (350,000/ml) in BEGM and 200µl of the cell suspension applied to the apical compartment of a collagen pre-coated transwell in a 24 well plate (Corning Life Sciences, Amsterdam, Netherlands) (giving a concentration of 0.7 x 10^5 cells/ml). 0.5ml of BEGM was added to the basal compartment of each transwell (i.e. underneath). HBSS (1ml) was added to all unused wells in the plate. Cells were then incubated at 37^{o}C in a CO2 incubator.

The cells were inspected daily, and once they had formed a confluent monolayer, the BEGM was removed from the apical compartment. Simultaneously the BEGM was removed from the basal compartment and replaced with 300µl 1xALI medium. The date of this procedure was taken as ALI day 0. The cells were then maintained in ALI culture with daily changes of medium and weekly measurement of transepithelial resistance. Cells were used only after ALI day 21 to allow a pseudo-stratified layer with ciliated cells to develop.

Epithelial wounding and KGF application

After a minimum of 21 days in ALI culture as detailed above, wounds were made using a 200µl pipette tip to 'scrape' the epithelium linearly from one side of the transwell to the other. The wounds were made directly after measurement of the TEER on the day of the experiment, before HBSS had been removed. After wounding, HBSS was aspirated and 2 further 'washes' with HBSS were made to remove any cell debris, before re-measuring TEER.

Cells were not starved, but basal media was replaced with either standard media, or media supplemented with either 5mg/ml or 50mg/ml KGF (R+D Systems (Catalogue number 251-KG), reconstituted in PBS) immediately following the wounding

TEER measurement

Trans-epithelial electrical resistance (TEER) measurement occurred pre-wound, post-wound, 24 hours post wound/KGF application, or 48 hours post wound/KGF application. TEER was measured using an Epithelial Voltohmmeter (EVOM) (World precision instruments) with chopstick electrodes. Electrodes were sterilised by spraying with Industrial Methylated Spirits (IMS) (containing 65-75% ethanol and 1-10% methanol) and then leaving immersed in IMS prior to use. 100µl of pre-warmed Hanks Balanced Salt Solution (HBSS) w/o Ca2+ or Mg2+ (Life Technologies Cat No: 14170-138) was added to the apical compartment, and after 15 minutes, chopsticks were removed from IMS (which was allowed to evaporate and the electrodes rinsed in fresh HBSS) and used to measure TEER, moving from left to right as quickly as possible so that temperature of the cells remained as close to 37^{o}C as possible. HBSS was then aspirated, and cells returned to incubator for further culture/experiments.

Wound image analysis

Wounded cell cultures were placed inside a temperature and humidity controlled microscope housing and multiple images were taken of the entirety of the wound using the x5 objective of Leica DMI6000B microscope. Imaging was repeated at 4 hourly intervals for the first 24 hours. Images were combined to form a composite image of the entire wound using Cell^P imaging software (Olympus, UK) (see figure 7 below), and then imported into Image J (Image J is a public domain, Java-based image processing program developed at the National Institutes of Health, USA). This programme allowed manual tracing of the wound image (see figure 8), and used this to calculate total wound area at baseline and at the different time points to give a % original wound.

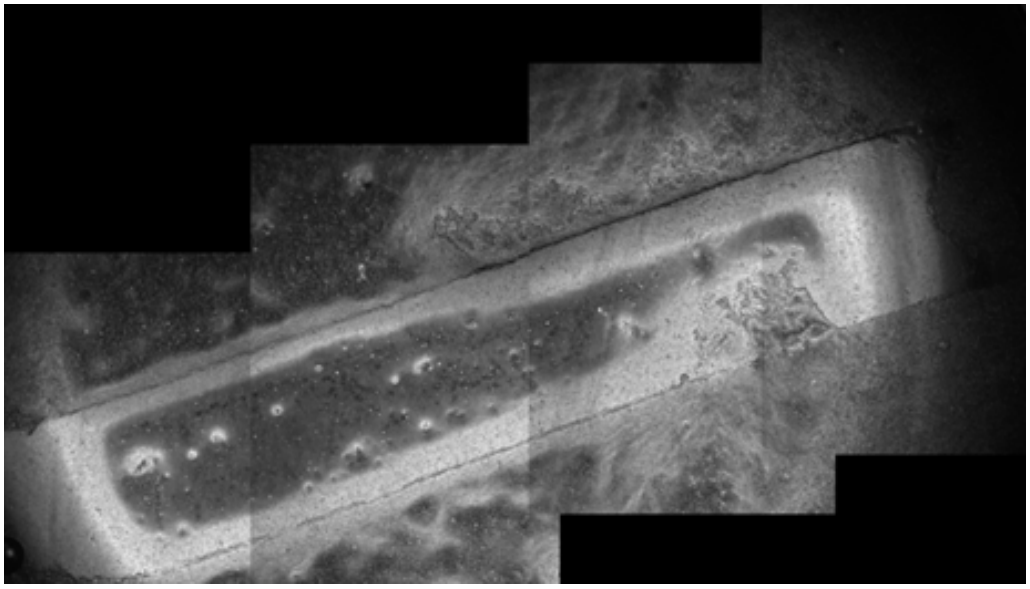


Figure 10: Composite imaging of scrape wound on ALI culture of epithelial cells. Images recombined using Cell^P imaging software, before being exported into Image J software (below)

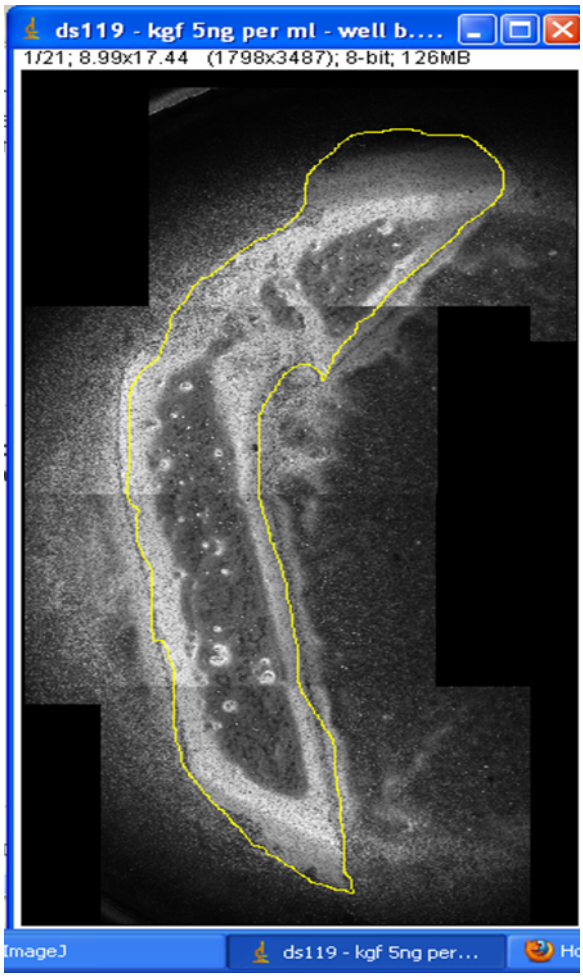


Figure 11: Tracing wound area using Image J software to delineate wound edges (in yellow)

FITC Dextran permeability

As well as TEER, permeability was assessed using a FITC Dextran flux assay in some, but not all cultures, at either 24- or 48-hour time-points (these cultures could not subsequently be used for any further measurements, as the application of the FITC dextran apically may have altered their phenotype/properties).

At the requisite time point, 50µl of Phosphate-buffered saline (PBS) containing 2mg/ml of FITC-dextran 4 kDa (Sigma) was applied to the apical surface of cultures and incubated for 24 hours at 37^{o}C (50µl was chosen as the lowest amount of fluid which still coated the entire apical surface, in an attempt to minimise the effect of re-submerging the ALI culture). After 24 hours, 100µl of media was removed from the basolateral compartment (aspirating up and down several times before removing to ensure media was mixed and representative). Samples from each experiment condition were then placed on a Nunc black 96-well microplate, together with 2 sets of standard curve samples (generated starting at 1 mg/ml and halving concentrations to a minimum of 0.000195 mg/ml). Immunofluorescence was measured using a Fluoroskan Ascent FL2.5 reader (Thermo Fisher, Loughborough, United Kingdom).

Compression

In later experiments, some cultures underwent compression with 30cm H_2O pressure for the first 4 hours. A bespoke compression apparatus was developed and optimised by C. Grainge, who kindly gave permission for its use. A schematic diagram is shown in figure 9. Essentially, cylinders of compressed 5%CO_2 in air were obtained from BOC Special Gases (BOC Ltd, UK) and stepped down from cylinder pressure to approximately 1 bar pressure using a 2-stage regulator (BOC Ltd, UK). Gas was then piped to a low-pressure regulator (Regulus 3, Tescom, Germany), which enabled precise control of pressure from 0 - 45 cm of water (cmH_2O). Gas was then piped via a T connector to Magnahelic pressure gauge (Dwyer Instruments, Wycome, UK) and downstream to a three-way tap (BD Connecta, Becton, Helsingborg, Sweden) allowing pressure venting as needed (figures 13-4). All tubing from this point was Sterile luer lock tubing (Starstedt, Numbrecht, Germany) heated to 37°C. Gas was then piped via a humidification chamber (Leica, Germany) to a manifold of sequential 3 way taps. From this manifold, gas was piped to 17G stainless steel needles (Scientific laboratory supplies, UK) passed through 7mm solid rubber bungs (Fischer Scientific, UK), and cut off flush with the end of the rubber bungs (Figure 15). The rubber bungs were then inserted into transwells to establish a pressure tight seal, and pressurised to 30 cmH_2O. Non-pressurised transwells had the same bungs as shown in Figure 15 inserted, but no

tubing/pressure was applied. The system was maintained in a custom manufactured Perspex box at 37°C and humidified 5%CO_2 piped at atmospheric pressure to the non-pressurised cells. The apparatus was calibrated using the column of water prior to use, with the Magnahelic pressure gauge being calibrated to provide an accurate pressure read out. Cells were then compressed, or subjected to 'sham' compression with bungs inserted into the transwells and atmospheric pressure 5% CO_2 for a 4 hour time period. Imaging of wounds occurred before and after compression.

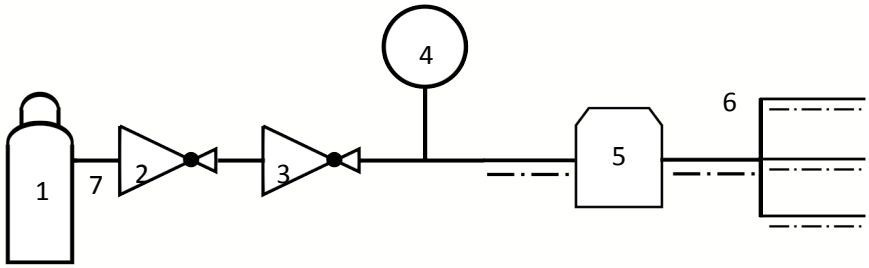


Figure 12: Schematic diagram of cell pressurisation system.

Components: 1. Cylinder of 5% CO_2 in air, 2. Step down regulator, 3. Regulus 3 low-pressure regulator, 4. Magnahelic pressure gauge, 5. Humidification chamber, 6. Manifold. Dashed lines indicated heated tubing.

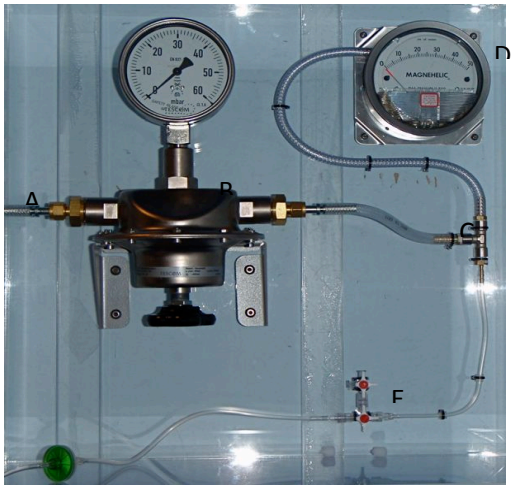


Figure 13: Custom manufactured compression apparatus.

A. Inlet of filtered 5% CO2 in air at approximately 1bar pressure. B. Low pressure regulator (Regulus 3, Tescom, Germany) enabling precise control of pressure from 0 - 45 cm of water (cmH_2O). C. 'T' connector. D. Magnahelic pressure gauge (Dwyer Instruments, Wycome, UK). E. Three way tap (BD Connecta, Becton, Helsingborg, Sweden) allowing pressure venting as needed.

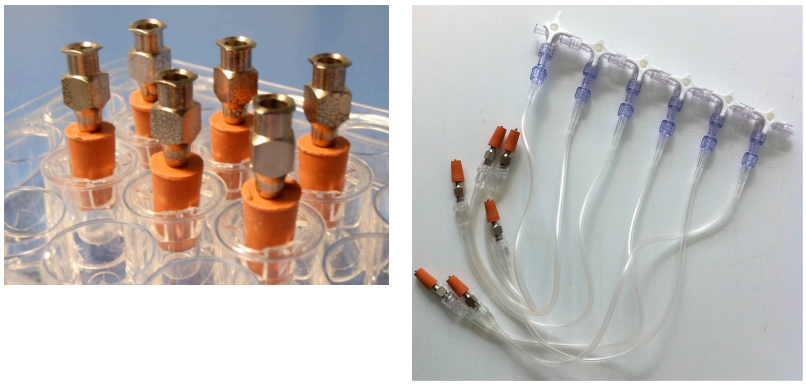


Figure 14: The pressure manifold and bungs used for the compression apparatus. Bungs shown wedged in the transwells (left) and were then connected via tubing and 3 way taps (right) to compression apparatus (figure 14).

Statistical analysis/Data entry

IBM SPSS statistics version 19 was used for statistical analysis of the in vitro study results. Non-parametric data was analysed using Kruskal-Wallis testing for across group comparisons and Mann-Whitney U testing for between group comparisons. Normally distributed data was analysed using paired t testing for across group comparisons, and using two-sample t test/one-way ANOVA testing for between groups (dependant on number of groups). P values of <0.05 were taken as significant.

Methods - Imaging study (Assessment of epithelial permeability in asthma through nuclear imaging)

Study design/schedule

This was a single centre study occurring at University Hospital Southampton NHS Foundation trust. The study consisted of a screening visit, followed by two imaging visits.

Screening visit

A screening visit occurred to assess eligibility and stability. At the visit, written consent was taken, a medical history was taken and physical examination was performed. Lung function testing including spirometry, reversibility, methacholine provocation testing and exhaled NO measurement also occurred. Skin prick testing to common allergens was performed. A blood sample to establish urea and creatinine was taken to establish normal renal function. In all women of childbearing age, a urine pregnancy test was taken to exclude pregnancy. At the screening visit the patient was given a study number, for example BRUPERM001, BRUPERM002, etc. Full methods for the above procedures are given in section 2.3.

Imaging visit A

The patient then returned between 14 and 28 days later for the imaging visits. Radiopharmaceuticals were prepared on the day of visit A and nebulised via AKITA inhalational device as detailed in section 2.3.8. Imaging occurred with the patient in the supine position, for 1-2 hours, as detailed in section 2.3.9.

A cumulative urine sample was collected at 2 hours, and the subject was given a container to collect their urine over the subsequent 24-hour period.

Subjects were asked to refrain from caffeine containing drinks, strenuous exercise or excessive alcohol for 24 hours preceding and following this visit (to minimise effects on mucociliary clearance). Spirometry and oximetry was undertaken before and after imaging.

Imaging visit B

The subject then returned 24 hours later for one further planar image of the thorax together with a transmission scan. They also supplied the 24-hour cumulative urine sample.

Subject recruitment

The initial plan for the study was to have 4 cohorts of subjects, with 10 subjects in each group:

a) Healthy non-smokers (to act as a negative control)
b) Healthy smokers (to act as a positive control)
c) Non-smoking, controlled asthmatics (as test group)
d) Non-smoking, non-controlled asthmatics (as test group).

However it was decided, as the technique was relatively unproven, to perform an initial pilot study, using 3 healthy non-smokers and 3 non-smoking, controlled asthmatics, to check the methods involved. Following analysis of this initial data (see below), the planned technique was found to be flawed and the study was not continued to the remaining subjects.

Inclusion/Exclusion criteria for each cohort are detailed in section 2.3.2.

10 subjects were screened (3 healthy controls and 7 asthmatics, with 7 asthmatics failing screening due to insufficient bronchial reversibility/bronchial hyper-responsiveness).

Preliminary work

Whilst the technetium and indium emit gamma rays at differing wavelengths (140kev for the technetium, and 173 and 247 keV for the indium respectively), it is known that higher energy gamma rays can contribute to lower energy windows, due to 'down-scatter' of photons. This has been recognised previously in studies using mixtures of isotopes [416], and one way to correct for this is to estimate the amount of scatter using an additional window immediately above the imaging window [417]. Experiments were therefore performed using a phantom (a device chosen to mimic the conditions of the body parts in the actual study) by J Fleming, using a 210keV window, which allowed the calculation of a correction factor for this down-scatter.

Targeting airways

It was important to target the initial deposition of the radiopharmaceuticals as best possible to the airways, the site of pathology in asthma rather than, say, the alveoli. The site of inhaled drug deposition within the lung is broadly affected by 3 areas[418]:

a) Respiratory tract morphology/anatomy.
b) Aerosol characteristics e.g. size of particle, shape, electrical charge, etc.
c) Ventilatory parameters.

Whilst it was not possible to directly control/modify the first factor above, the remaining 2 could be modified for the study to optimise deposition in the airways. With regards aerosol characteristics, whilst the need to specifically use Technetium/Indium/DTPA/Nanocoll (to

allow simultaneous imaging and assessment of different clearance techniques) constrained the option of modifying aerosol features somewhat, the type of nebulizer (and thus particle size) was controllable. Particles > 6µm tend to deposit in the oropharyngeal regions, 2-6µm within the central airways, and <2µm within more distal regions such as the alveoli[419].It is also important to note though that smaller particles <1µm have less impaction and may therefore be more likely to be expired/not retained in the lungs[420]. The AKITA device was connected to an LC®Plus jet nebulizer (Pari Respiratory Equipment) which generates particles with a mass median diameter (MMD) of 3.8µm, which was felt to be optimal for this study.

With regards ventilatory techniques, the majority of previous studies in this area have not used targeting at all, simply employing tidal breathing whilst administering the radiopharmaceutical [380, 389, 390, 421, 422], and relying on particle size. Tidal breathing however preferentially deposits particles in the larger airways (thus potentially missing a major site of pathology in asthma, the small airways). Others have used dosimeters with controlled breathing patterns, wherein a device is set to deliver the dose after an initial inspirational volume [387, 393, 423], although this does not take account of differing amounts of dead space and conducting airways volumes between subjects.

This study used an AKITA device (manufactured by Activaero®, Germany, pictured below with jet nebuliser attached, figure 44) to allow individualised, targeted administration of the radiopharmaceuticals. This device is designed to use ‘smart cards’ with predetermined targets for inhalation flow rate, inhaled volume and breath holding. The patient actuates the device with a slight inspiratory effort, and thereafter the device takes over control of the inhalation, ventilating the lung with a small positive pressure at a constant flow rate, whilst also giving the patient a display feedback on the time remaining for each breath. The device also controls the timing of the pulsing of the aerosol during the inspiration phase, which can be modified to try to optimise deposition in the conducting airways rather than, say the alveoli or dead space airways.

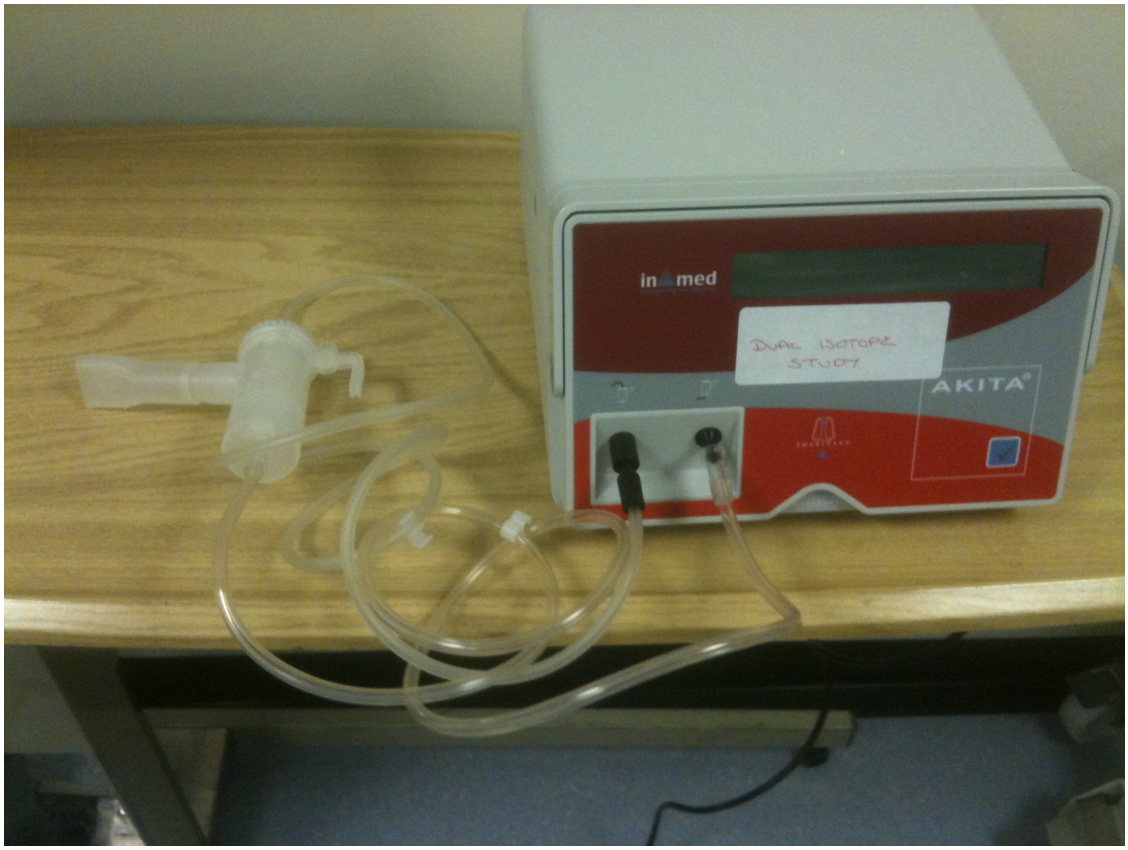


Figure 15: AKITA device (Activaero) with attached jet nebuliser

After discussion with Activaero with regards optimising deposition in the airways, the following parameters were suggested:

- A degree of individualisation i.e. rather than individual smartcards for each patient in the study, the use of 1 of 7 smart cards based on the patient's height. As dead space volume and conducting airways volume seem to be proportional to height [424, 425], equations to approximate these from height were derived (personal communication between J Fleming (Honorary Professor in Nuclear Medicine Physics) and Activaero). Thus for each smartcard the device required a different inhalational volume, with a different sized aerosol bolus, delivered at a different time-point in the inhalation (Table 8: Smart Card parameters for AKITA device to allow targeted airway deposition for different body heights).
- A slower than normal inhalational flow rate set at 100ml/sec, as this has been found to increase airways deposition [426, 427], with a fast, forced exhalation after each breath.

Group No.	Body Height [m]	Vd calculated [L]	Bolus Depth [ml]	Bolus Width [ml]	Inhalation Volume [ml]
1	1.17	53	80	56	160
2	1.385	80	120	84	240
3	1.555	107	160	112	320
4	1.695	133	200	140	400
5	1.825	160	240	168	480
6	1.945	187	280	196	560
7	2.05	213	320	224	640

Table 8: Smart Card parameters for AKITA device to allow targeted airway deposition for different body heights using different inhalational boluses delivered at different time-points in the inhalation. Smart card was chosen dependant on subject's height.

Image analysis

Raw data was given to Michael Bennett (Biomedical Research Unit (BRU) title, Southampton General Hospital, who wrote a bespoke programme using Matlab computing language to perform the analysis. Initial analysis was done on the planar images. Essentially, the transmission image was used to generate a lung mask/define lung contours from where to measure the emitted radiation (figure 45). Attenuation, that is the loss of detection of an emitted photon due to either absorption/scatter out of the detector field of view, was calculated using CT data (figure 46). This was then used, together with the lung masks, on planar images at each time point for the Tc99m and In111 (figure 47) to determine counts. These were plotted in clearance curves (figure 48) and best-fit curves were plotted using the following equation (to give a rate constant 'b').

$y(x) = a*\exp(-b*x)$

From these results, clearance rates could be determined, as reflected by the % count clearance (compared to the original count) for either technetium or indium, at various time points – as the majority of clearance occurs in the first 2 hours, we decided to concentrate on 1 and 2 hour time points. Furthermore, by subtracting the rate constants for the best-fit curves for technetium from indium, a figure to represent epithelial permeability could be calculated.

Typical images received are shown below (images from subject PERM001, a healthy control):

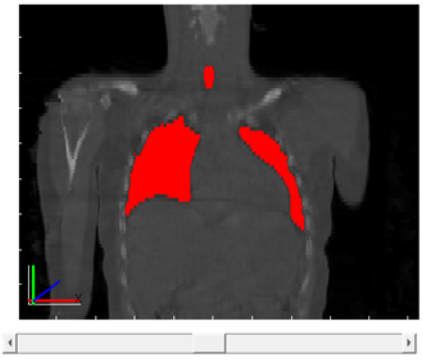


Figure 16: Example of lung mask generated to determine regions of interest

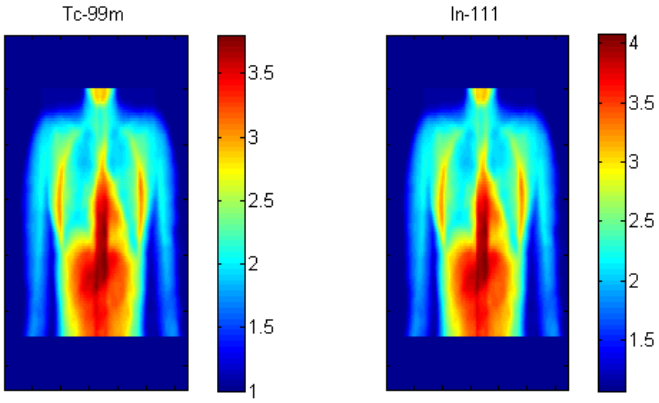
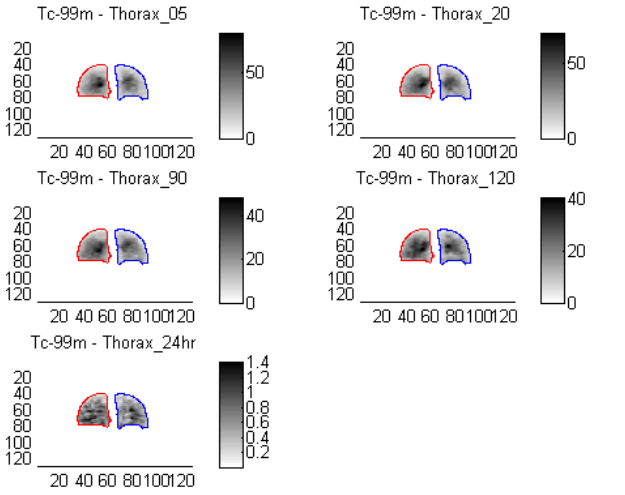


Figure 17: Example of calculation of attenuation for each radioisotope



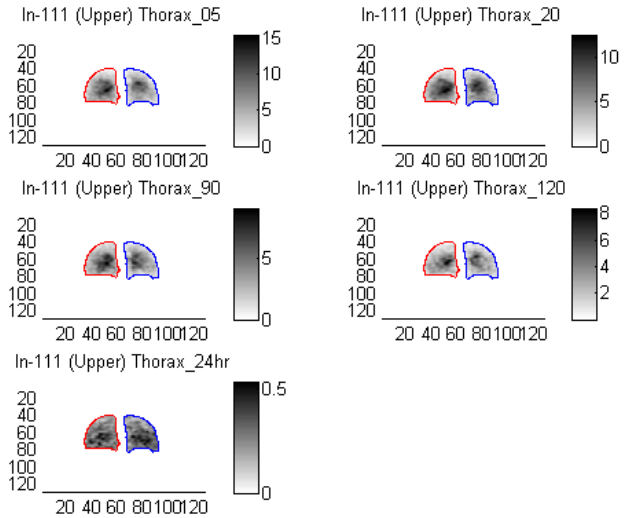


Figure 18: Example of activity counts for each radioisotope

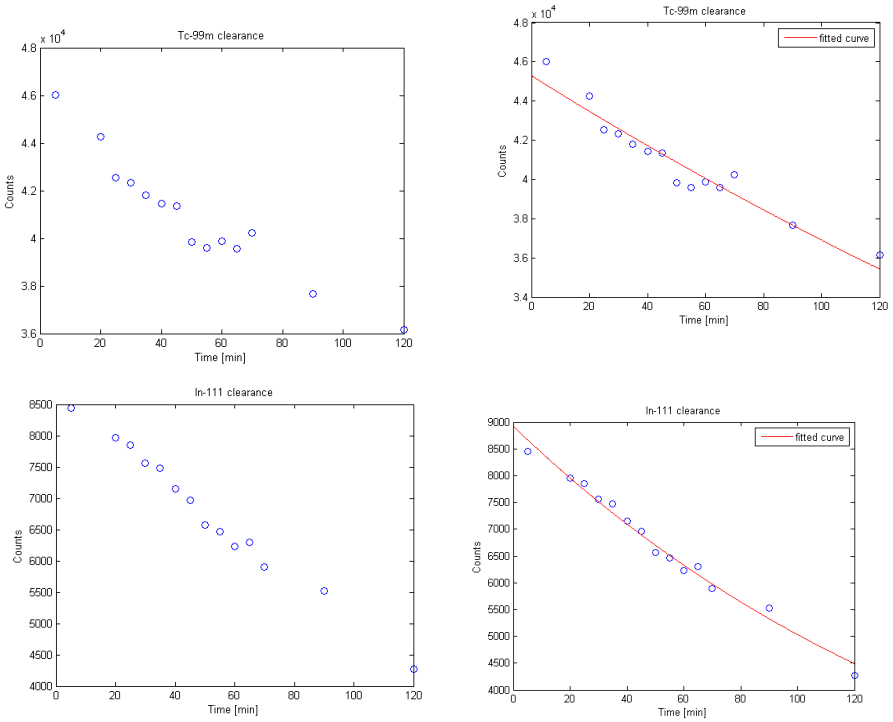


Figure 19: Examples of clearance data for each radioisotope, with best-fit curves fitted

Section C: Clinical Study - Results and Discussion

Safety and Efficacy of parenteral KGF in moderate asthma subjects – Clinical Results

Subject Recruitment/Withdrawal

As per the below diagram (Figure 20 CONSORT flow diagram for clinical study) 37 subjects were screened. 16 were excluded as they fail d to meet inclusion criteria, mainly due to insufficient FEV1% reversibility, and a failure to decrease FEV1 by 15% despite reaching the maximum dose of mannitol. One patient withdrew after receiving one dose of drug/placebo, therefore to achieve our target of 10 patients in each arm receiving the full dose of drug/placebo, their randomisation number was reassigned and an additional patient was recruited (making 21 in total). 2 patients were withdrawn after the second bronchoscopy (and before visits 12 and 13) due to asthma exacerbation requiring oral corticosteroids (as this would then affect testing in these visits) – both these patients turned out to be in the placebo arm once unblinding had taken place.

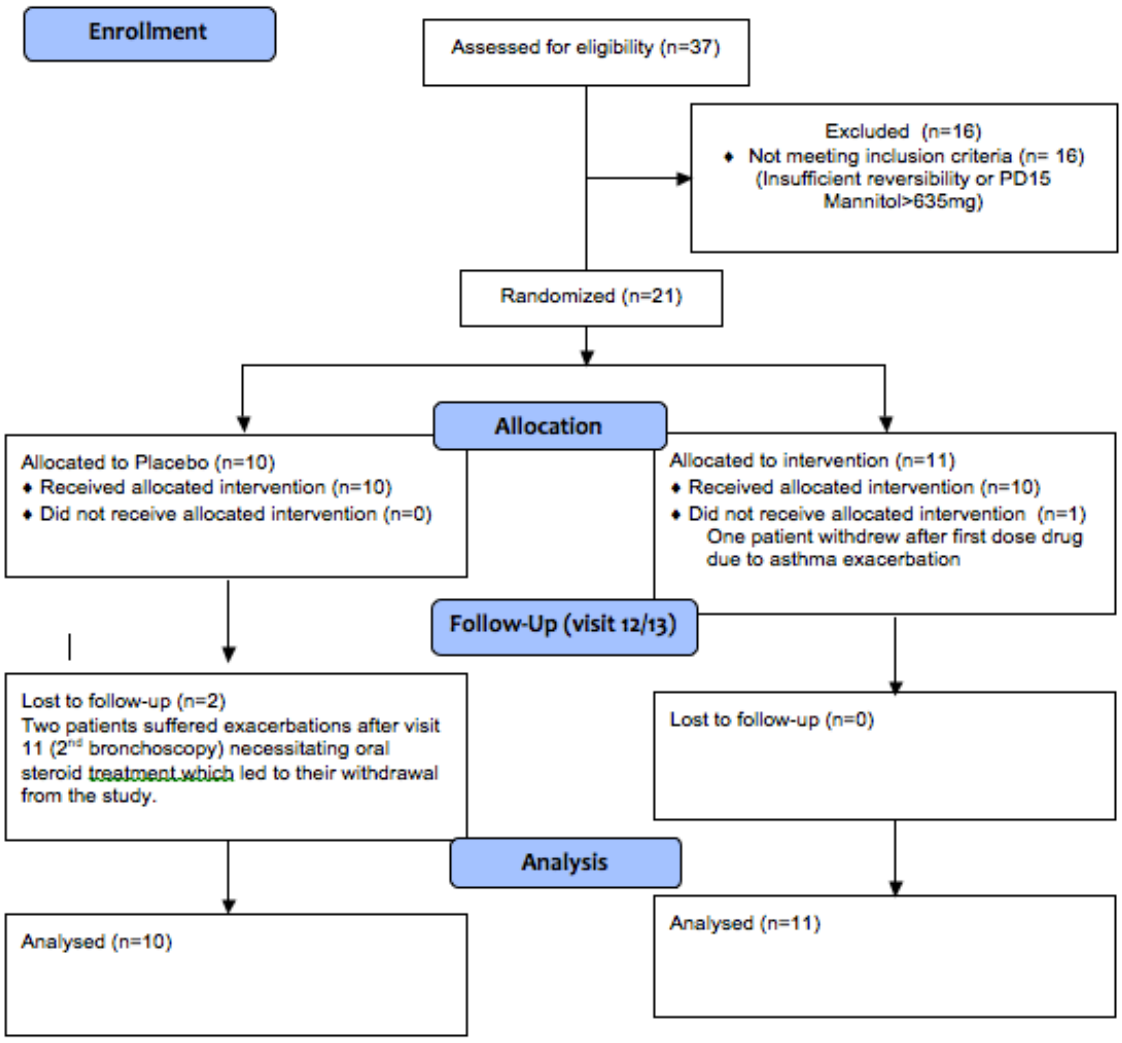


Figure 20 CONSORT flow diagram for clinical study

Demographic data/Baseline characteristics

Baseline characteristics for the group are detailed below (Table 9: Baseline demographic data).

	Overall N=21	Placebo N=10	KGF N=11	P value
Age in years, mean (SD)	39.1 (13.5)	41.1 (12.7)	37.3 (14.5)	0.5
Sex, n (%) male	6 (29)	2 (20)	4 (36)	0.4
FEV1 (% predicted), median (IQR)	86.6 (69.8, 98.0)	85.2 (69.8, 88.5)	93.3 (67.7, 102.6)	0.2
FVC1 (% predicted), median (IQR)	93.9 (88.9, 109.0)	92.3 (84.4, 95.0)	109.0 (88.9, 112.7)	0.07
PD_{15} mannitol, median (IQR)	52.0 (28.8, 228.9)	168.7 (41.5, 237.6)	36.7 (24.0, 173.8)	0.09
PC_{20} methacholine, median (IQR)	3.1 (0.4, 5.6)	4.5 (3.1,10.0)	1.2 (0 .1, 4.8)	0.1
ACQ score, mean (SD)	2.0 (0.6)	2.2 (0.6)	1.9 (0.6)	0.3
AQLQ score, median (IQR)	5.2 (4.2, 5.7)[a]	4.9 (3.9, 5.5)[b]	5.2 (4.2, 5.8)	0.6
Atopic, n (%)				
Yes	18 (86)	7 (70)	11 (100)	0.05
No	3 (14)	3 (30)	0 (0)	
eNO, median (IQR)	28.9 (16.0, 45.2)	17.3 (12.7, 37.3)	36.6 (26.9, 58.9)	0.04
PEFR variability, median (IQR)	19.6 (14.4, 27.3)	21.9 (19.2, 25.7)	16 (12.5, 28.8)	0.4

P values are for the comparison of the group treated with KGF versus the placebo group.

[1]2 Subjects did not react to methacholine challenge within the 0-16mg range, and 2 subjects experienced a >10% drop with saline precluding safe challenge – not included in the calculation of the mean/range above.

[a]n= 20, as [b] n = 9 in placebo group due to incorrect completion of AQLQ form

Table 9: Baseline demographic data for the KGF clinical study, for the group as a whole (overall), and shown for each treatment group

It can be seen that by chance, despite randomisation, the two groups were not perfectly matched, with higher levels of atopy and exhaled nitric oxide in the KGF treatment group. Otherwise, as might be expected given the inclusion criteria, both groups displayed elevated ACQ scores indicating uncontrolled asthma symptoms, slightly reduced quality of life questionnaire scores, and significant levels of BHR and PEFR variability, with no significant differences between the groups. Spirometry did not show marked obstruction in either group, again as might be expected in moderate asthma (cf. severe asthma)

Pearson's testing was used to test for correlations in the baseline demographic data (in the group as a whole), and there was a strong correlation between ACQ and AQLQ ($r = -0.662$, $p = 0.02$) as might be expected (with worsening asthma control, one might expect a greater deleterious impact on quality of life). There was a moderate correlation between PD_{15} Mannitol and PC_{20} Methacholine levels, reflecting the overlap in their measurement of bronchial hyperresponsiveness ($r = 0.484$, $p = 0.026$). There was also a moderate correlation between PEFR variability and FEV1% predicted ($r = -0.482$, $p = 0.031$), which may result from those with lower FEV1 values having greater degrees of variability in their obstruction to the airways.

Surprisingly, correlation was not seen between PD15 and ACQ ($r = -0.18$, $p = 0.43$), AQLQ ($r = -0.24$, $p = 0.33$) or eNO ($r = -0.22$, $p = 0.33$). Nor was it seen for PC20 and ACQ ($r = 0.04$, $p = 0.85$), AQLQ ($r = -0.45$, $p = 0.051$) or eNO ($r = -0.05$, $p = 0.83$). Some of these correlations have been seen in other, albeit larger, studies[428].

Clinical Results - Primary outcome – change in PD15 Mannitol

Changes in PD15 Mannitol – raw data

PD_{15} Mannitol was measured at several time-points over the course of the trial (visits 2, 6, 8, 9, 12), and the relationships of these to the study drug/timetable are shown in the figure below (Figure 20: Diagram showing measurement of Mannitol in relationship to study drug/study timetable).

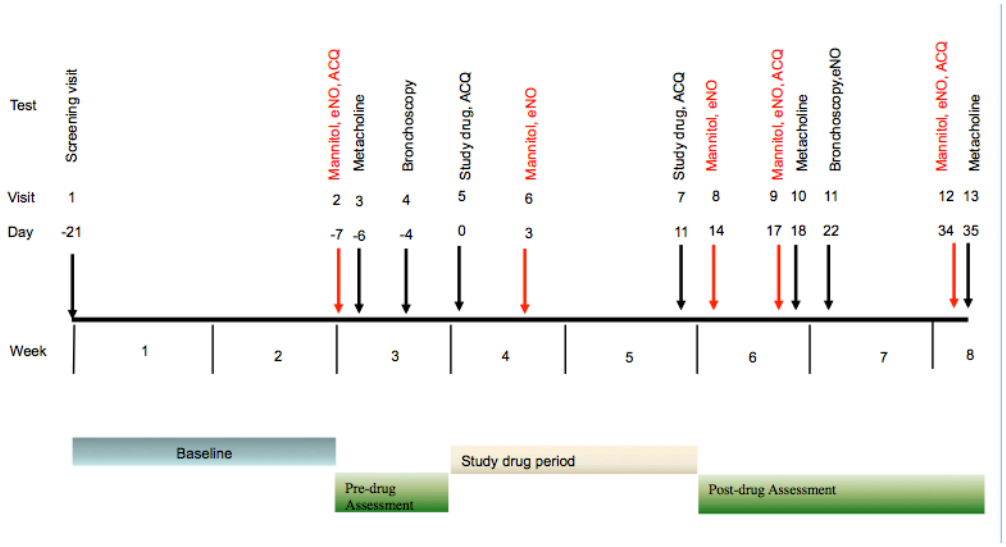


Figure 20: Diagram showing measurement of Mannitol in relationship to study drug/study timetable

Note that whilst n= 21 for the baseline n numbers, this number decreases to between 16 and 20 for subsequent visits, due to patient withdrawal/inability to perform test (it is unsafe to perform a Mannitol PD15 test if the baseline FEV_1 was less than 50%. Also, as previously discussed, 2 patients in the placebo arm had exacerbations and their data is missing for visit 13).

The change in PD15 (Δ PD15) from baseline for the two groups are shown in the table below (Table 10: Mannitol PD15 values, and changes from baseline (visit 2), for active and placebo group) with Mann-Whitney U testing used to compare KGF treatment versus placebo:

Visit	N	Placebo treatment		KGF treatment		P value	P value (adj)
		PD15	Δ PD15	PD15	Δ PD15		
Baseline (2)	21	168.7 (267.2)		36.7 (149.8)			
6	20	216 (249.2)	76.6 (131.5)	149.5 (228.1)	49.9 (173.4)	0.45	0.48
8	19	313 (349.1)	170.8 (248.9)	172.4 (520.3)	166.5 (328.4)	0.81	0.87
9	20	338.5 (350)	190.2 (337.2)	528.5 (552.2)	289.8 (481.1)	0.23	0.37
12	17	329.7 (511.2)	182.6 (253.6)	289.46 (489.5)	215.3 (228.2)	0.773	0.84
Values shown are medians with IQR in parentheses. N numbers shown for each visit. P value calculated using Mann-Whitney U test (unadjusted) and using linear regression adjusting for baseline inequality in PD_{15} at visit 2.							

Table 10: Mannitol PD15 values, and changes from baseline (visit 2), for active and placebo group, at visits 2, 6, 8, 9 and 12

It can be seen that (as also shown in the previous table) the two groups are mismatched at baseline, with the KGF group as a whole having a lower median PD_{15} mannitol level i.e. with more bronchial hyperresponsiveness. Whilst the two groups changes in PD_{15} mannitol do not differ from each other when compared statistically therefore, as a change *compared to baseline*, the KGF group increased by a larger extent (explored statistically when looking at the doubling dose change, see below). Another point this raises however is that the more severe group have a greater likelihood of improving spontaneously, in a 'regression to the mean' phenomenon. To counter this, one can perform linear regression, adjusting for the baseline inequality – whilst this does not make a difference to the above p values (which remain insignificant), it does to the figures explored below. It is important to note though, that performing linear regression assumes normality of the data, and with the small numbers in the study, this was not the case (see also discussion).

Changes in PD15 Mannitol – Response Dose Ratio (RDR)

There is another problem with analysing the above raw data change in PD15 as an outcome, mainly relating to those subjects who failed to drop within the dose range of Mannitol i.e. did not achieve a 15% drop in FEV_1, despite having the maximum cumulative dose of 635mg. As discussed in section B 1.4.6.6, these subjects were given an arbitrary value of 795mg for their PD15 (one 160mg dose more than 635mg).

Visit	Entire group			Placebo group			KGF group			P value	P value (adj)
	N	RDR (% fall/mg)	Δ RDR	N	RDR (% fall/mg)	Δ RDR	N	RDR (% fall/mg)	Δ RDR		
Baseline (2)	21	0.269 (0.46)		10	0.07 (0.30)		11	0.41 (0.63)			
6	20	0.069 (0.15)	-0.05 (0.38)	10	0.07 (0.06)	0 (0.16)	10	0.1 (0.29)	-0.28 (0.4)	0.03	0.87
8	19	0.053 (0.04)	-0.05 (0.4)	10	0.05 (0.04)	-0.04 (0.28)	9	0.06 (0.28)	-0.17 (0.47)	0.29	0.21
9	20	0.040 (0.05)	-0.07 (0.4)	10	0.05 (0.03)	-0.02 (0.16)	10	0.03 (0.14)	-0.37 (0.39)	0.02	0.78
12	17	0.050 (0.08)	-0.08 (0.37)	8	0.04 (0.06)	-0.04 (0.33)	9	0.05 (0.16)	-0.3 (0.46)	0.34	0.32

Δ RDR = change in RDR compared to baseline (visit 2) value. Values shown are medians with IQR in parentheses. P values are comparing Δ RDR for active (KGF) treatment group vs. placebo, using Mann-Whitney U test, or for adjusted P value, using linear regression, with baseline RDR as independent factor

However this then does not allow discrimination between two subjects (or indeed the same subject at different time points) who dropped by 14.5% at 635mg, and one who dropped by only 3%. Therefore many studies use the 'response-dose ratio' (RDR) (see section B 1.4.6.6 for calculation), which does not plateau out and can discriminate between the subjects in the example above.

Looking at the RDR for the group as a whole (

), we can see that over the course of the study there is a decrease in RDR (signifying a decrease in bronchial hyper-reactivity) i.e. an increasingly negative Δ RDR (change in RDR compared to baseline (visit 2)). This change in RDR (comparing RDR values to baseline for the *whole group*), is statistically significant using Wilcoxon signed ranks test for paired data, p = 0.004 (visit 6), 0.002 (Visit 8), 0.001 (Visit 9), and 0.003 (visit 12).

Table 11: Response dose ratios for mannitol at visits 2,6,8,9 and 12, and changes in RDR compared to baseline (visit 2)

Visit	Entire group			Placebo group			KGF group			P value	P value (adj)
	N	RDR (% fall/mg)	Δ RDR	N	RDR (% fall/mg)	Δ RDR	N	RDR (% fall/mg)	Δ RDR		
Baseline (2)	21	0.269 (0.46)		10	0.07 (0.30)		11	0.41 (0.63)			
6	20	0.069 (0.15)	-0.05 (0.38)	10	0.07 (0.06)	0 (0.16)	10	0.1 (0.29)	-0.28 (0.4)	0.03	0.87
8	19	0.053 (0.04)	-0.05 (0.4)	10	0.05 (0.04)	-0.04 (0.28)	9	0.06 (0.28)	-0.17 (0.47)	0.29	0.21
9	20	0.040 (0.05)	-0.07 (0.4)	10	0.05 (0.03)	-0.02 (0.16)	10	0.03 (0.14)	-0.37 (0.39)	0.02	0.78
12	17	0.050 (0.08)	-0.08 (0.37)	8	0.04 (0.06)	-0.04 (0.33)	9	0.05 (0.16)	-0.3 (0.46)	0.34	0.32

Δ RDR = change in RDR compared to baseline (visit 2) value. Values shown are medians with IQR in parentheses. P values are comparing Δ RDR for active (KGF) treatment group vs. placebo, using Mann-Whitney U test, or for adjusted P value, using linear regression, with baseline RDR as independent factor

Dividing the data into those on active (KGF) treatment and those on placebo (in the above table), we can see that the KGF group has greater decreases (equalling less hyperresponsiveness) in the RDR than the placebo group, at all time points, although this is only statistically significant at visit 6 and visit 9. Applying linear regression analysis to the data to account for baseline differences however results in non-significant p values.

Mannitol – doubling dose difference

A final way to look at the primary outcome data, via the doubling dose difference, addresses the issue that there will always be some variability within a subject without any change in treatment on repetition of the test, and therefore a clinically significant change is only indicated by more than one doubling of the initial PD_{15} mannitol dose[429]. Combining this with RDR values rather than PD_{15} values for the reasons outlined above, a doubling dose difference in RDR can be calculated using the following equation[430]:

Doubling dose difference (DDD (RDR) = (log (RDR visit 2) – log (RDR visit 6/8/9/12))/ log (2).

Applying this to the data gives the following results (see below, table 12), which show that whilst both groups, on average, had clinically significant improvements i.e. >1 doubling dose, the KGF group improved by almost 3 doubling doses at peak. This is clinically significant, as in the data above, at visit 6 and visit 9, although again this is unadjusted for baseline.

DDD for mannitol PD15 RDR				
Visit	N	Placebo	KGF	P value
6	20	0.47 (1.23)	1.68 (1.09)	0.03
8	18	1.42 (1.4)	2.13 (1.97)	0.4
9	20	1.11 (1.62)	2.89 (1.74)	0.03
12	17	1.57 (1.4)	2.29 (1.82)	0.38
Values shown are mean with standard deviation in brackets P value calculated using t test, comparing active vs placebo				

Table 12: Doubling dose difference in RDR at visits 6, 8, 9 and 12

Mannitol – correlation with other outcomes.

Correlation testing using Pearson's testing was performed on the group as a whole to determine whether changes in the primary outcome reflected other changes i.e. was there a correlation between changes in mannitol hyper-reactivity and secondary outcomes such as AQLQ, methacholine and ACQ. This was tested using changes at visit 9, when one might expect peak biological effect (and compared to changes in the other markers as close to visit 9 as data allowed). Moderate correlation was seen for changes in mannitol and AQLQ, although this only became statistically significant using RDR changes rather than PD15 changes:

- Correlation for change PD_{15} Mannitol at visit 9 with change AQLQ: r = 0.514, p = 0.06
- Correlation for change RDR Mannitol at visit 9 with change AQLQ: r = -0.536, p = 0.05

Changes in PD_{15} mannitol also showed moderate correlation with changes in ACQ score at visit 9, although strangely this was not the case for changes in RDR:

- Correlation for change PD_{15} Mannitol with change ACQ (visit 9): r = - 0.590, p = 0.006
- Correlation for change RDR Mannitol with change ACQ (visit 9): r = 0.01, p = 0.967

Surprisingly, no statistically significant correlation was seen between changes in mannitol and changes in methacholine, although, as shown in the next section and discussed later, the changes in PC_{20} methacholine were less marked:

- Correlation for change PD_{15} Mannitol at visit 9 with change PC_{20} methacholine at visit 10: r = 0.226, p = 0.368
- Correlation for change RDR Mannitol at visit 9 with change PC_{20} methacholine r = 0.034, p = 0.895

Clinical Results – Secondary Outcomes

Methacholine

We can apply the same analysis techniques to PC_{20} methacholine, although for this outcome we cannot calculate RDR (as cumulative dose is unknown, the test relies on looking for a drop in FEV_1 in relation to a set *concentration*, not *dose*). For subjects who failed to drop within the methacholine dosing range therefore, an arbitrary dose of 32mg was assigned. In addition, some subjects dropped their FEV_1 by 10% with the control, normal saline nebulisation – which precludes proceeding with the test, due to safety concerns. These patients were assigned an arbitrary dose of 0.015 mg (half the concentration of the lowest dose of methacholine).

PC_{20} methacholine was measured at visits 3, 10 and 13 respectively (Figure 21: Diagram showing timetable for measurement of methacholine in relationship to study drug/timetable).

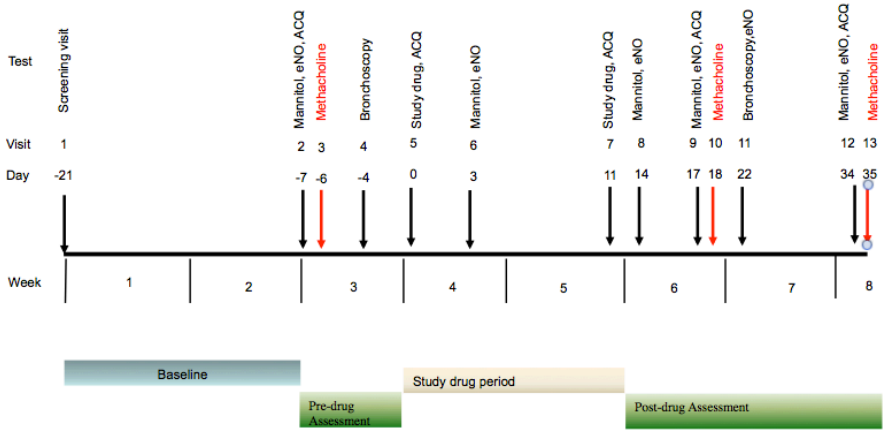


Figure 21: Diagram showing timetable for measurement of methacholine in relationship to study drug/timetable

As a group (Table 13: PC_{20} methacholine for entire group and separate placebo and KGF groups at visits 10 and 13.), there was an increase in PC_{20} (indicating decreased hyper-reactivity) at visit 10 and less so at visit 13, which is somewhat in keeping with the decrease in BHR shown by the Mannitol PD_{15} results, although the changes are less marked. When the data is divided into the active (KGF) and placebo treatment groups, we can see that the KGF group had a larger increase in their PC_{20} levels, although this was not statistically significant.

PC_{20} Methacholine										
Visit	Entire group			Placebo			KGF			P value
	N	PC_{20}	Δ PC_{20}	N	PC_{20}	Δ PC_{20}	N	PC_{20}	Δ PC_{20}	
3	21	3.09 (7.41)		10	4.46 (7.94)		11	1.21 (4.69)		
10	18	5.78 (30.8)	0.16 (7.32)	9	8 (19.67)	-0.04 (12.23)	9	1.52 (31.02)	0.31 (14.35)	0.45
13	16	4.48 (5.59)	0.2 (8.2)	8	4.28 (4.84)	-0.39 (5.02)	8	6.73 (24.17)	0.195 (7.6)	0.46
Values shown are medians with IQR in parentheses. P value calculated using Mann-whitney U test, comparing change PC_{20} in KGF group vs placebo, 2-tailed										

Table 13: PC_{20} methacholine for entire group and separate placebo and KGF groups at visits 10 and 13.

In the same fashion as methacholine, clinically significant changes in methacholine are signified by an improvement by more than one doubling dose, and therefore the doubling dose is usually computed when PC_{20} is calculated, using the following formula[431]:

(Log (PC_{20} at later visit) – Log (PC_{20} visit 3))/ log (2).

By performing this analysis, we can see that, as reflected in the 'raw' data above, there is a greater improvement in the KGF group, although this remains statistically insignificant (Table 14: Doubling dose change in PC_{20} methacholine)

Visit	Placebo		KGF		P value
	N	Doubling Dose	N	Doubling Dose	
10	9	0.05 (-1.59, 1.69)	9	1.52 (-1.65, 4.69)	0.36
13	8	0.18 (-1.47, 1.83)	8	1.62 (-1.73, 4.97)	0.38
Values shown are mean with 95% CI in brackets P value calculated using t test, equal variances not assumed					

Table 14: Doubling dose change in PC_{20} methacholine at visits 10 and 13

ACQ

ACQ was measured several times over the course of the study, at visits 5, 7, 9 and 12 (Figure 22: Diagram showing timetable for measurement of ACQ in relationship to study drug/study timetable). The baseline ACQ score for the group as a whole had a mean value of 2.04 +/- 0.13 (SE), indicating poor control.

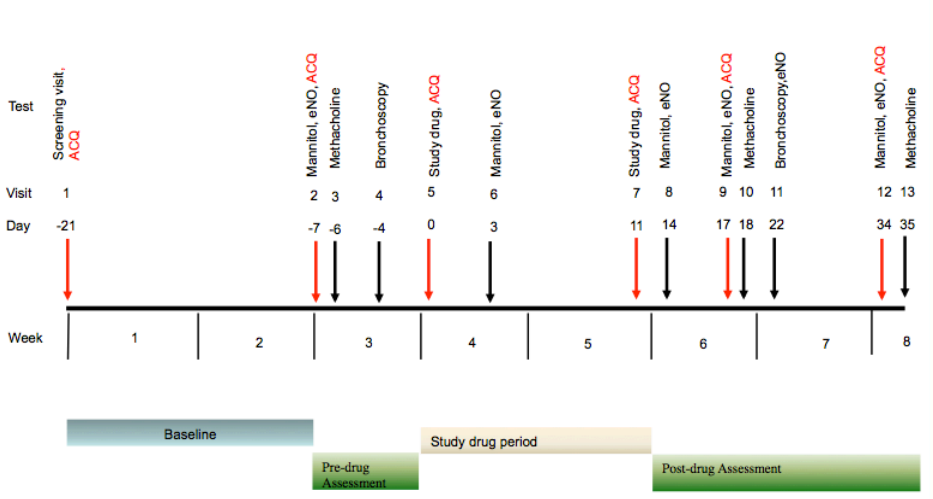


Figure 22: Diagram showing timetable for measurement of ACQ in relationship to study drug/study timetable

Visit 1 ACQ values were taken as baseline (although visits 2 and 5 were also performed before study drug/placebo administration). In keeping with the improvement in Mannitol over the time course of the study as a whole, there was a similar improvement in ACQ (where lower values indicate better control); at visit 12 ACQ had decreased in the group as a whole to a mean of 1.32 +/- 0.18 (SE), with a p value of 0.003 (Wilcoxon signed ranks test) (Figure 23: ACQ score for entire group at different visits.).

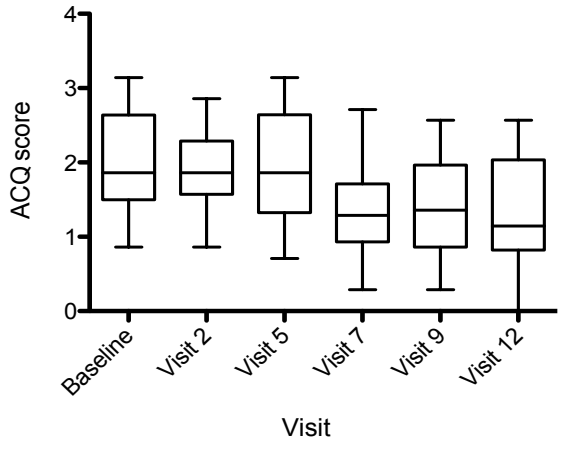


Figure 23: ACQ score for entire group at different visits.

Dividing this data, as before, into those subjects on active treatment (KGF) and those on placebo (Figure 24: ACQ data split into the two treatment groups and Table 15: ACQ data split between treatment groups) we can see as expected that neither group changes significantly between visits 1, 2 and 5, as these are all pre-treatment. Both groups seem to improve by visits 7/9/12, and whilst there is a suggestion from the raw data that the KGF group improves to a larger extent, this is not statistically significant. Taking the average of visits 1, 2 and 5 as a 'pre-drug' average, did not affect the results i.e. there was no significant difference in improvement between groups.

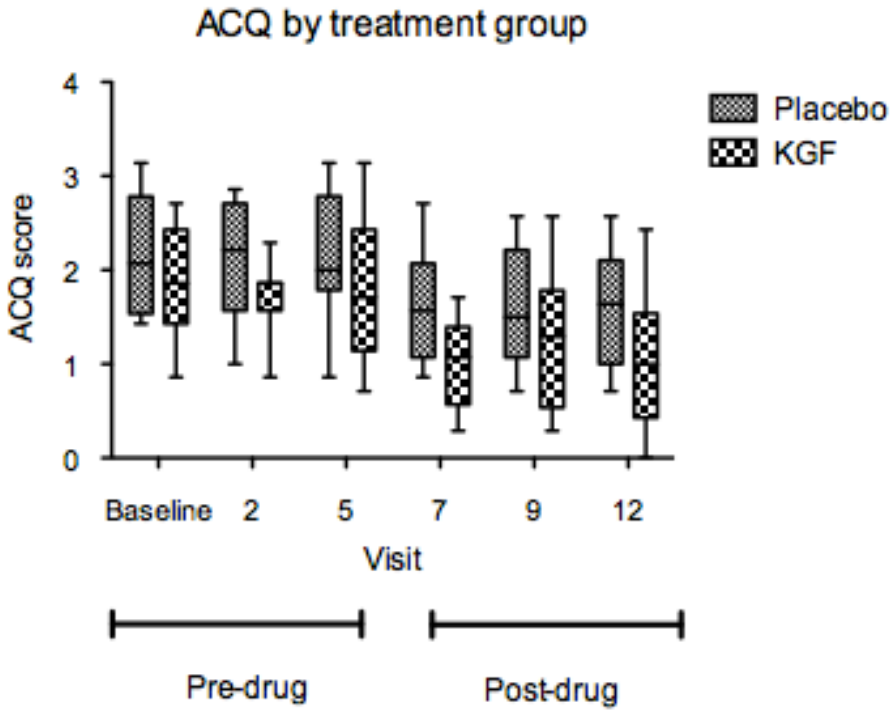


Figure 24: ACQ data split into the two treatment groups

ACQ							
Visit	N	Placebo		KGF		P value (t –test)	P value Adjusted for baseline
		ACQ Mean (SD)	Δ ACQ Mean (SD)	ACQ Mean (SD)	Δ ACQ Mean (SD)		
Pre-drug							
1	21	2.19 (0.64)	-	1.91 (0.57)	-	-	
2	21	2.14 (0.63)	-0.04 (0.66)	1.74 (0.43)	-0.17 (0.44)	0.6	0.21
5	20	2.16 (0.70)	-0.09 (0.77)	1.77 (0.77)	-0.14 (0.66)	0.9	0.55
Post-Drug							
7	19	1.54 (0.62)	-0.64 (0.95)	1.02 (0.46)	-0.92 (0.55)	0.68	0.08
9	20	1.60 (0.64)	-0.59 (1.01)	1.26 (0.73)	-0.66 (0.70)	0.88	0.36
12	18	1.59 (0.65)	-0.52 (0.90)	1.10 (0.78)	-0.81 (0.64)	0.56	0.25

Table 15: ACQ data split between treatment groups

AQLQ

AQLQ was only measured twice in the study, at the beginning (visit 1) and at the end (visit 12). Data was not normally distributed so median values (with IQR) are shown. In keeping with the ACQ/ other clinical data, over the time-course of the study the group improved as a whole with a mean increase (as opposed to ACQ where an increase signifies worsened control, increases in AQLQ signify better quality of life) of 0.52 (Table 16: AQLQ measurement). Full data sets were not achieved in 2 patients at baseline (failure to complete questionnaire fully) and in 6 patients at visit 12 (due to 3withdrawal due to exacerbation, 3 failures to complete questionnaire fully). (Data is shown in Table 16: AQLQ measurement and represented in graphical form in Figure 25: Boxplot graph showing AQLQ values at baseline and end of trial, divided into active and placebo treatment groups).

- The change in AQLQ was higher in the active treatment group despite the low numbers, with a statistical significance of p = 0.035 (unadjusted data).

AQLQ										
	Entire group			Placebo			KGF			P value
	N	AQLQ	Δ AQLQ	N	AQLQ	Δ AQLQ	N	AQLQ	Δ AQLQ	
Baseline (Visit 1) Total	19	5.22 (1.5)		11	5.13 (1.6)		8	5.22 (1.6)		
Visit 12 Total	14	6.22 (1.2)	0.52 (1.35)	7	5.53 (2)	0.19 (1.2)	7	6.53 (0.78)	1.03 (0.75)	0.04
Δ AQLQ = change in AQLQ compared to baseline (visit 1) value Values shown are medians with IQR in parentheses P values are comparing Δ AQLQ for active (KGF) treatment group vs. placebo, using Mann-Whitney U test										

Table 16: AQLQ measurement at beginning and end of trial, for entire group and separated into treatment arms, with mean change in AQLQ from visit 1 noted.

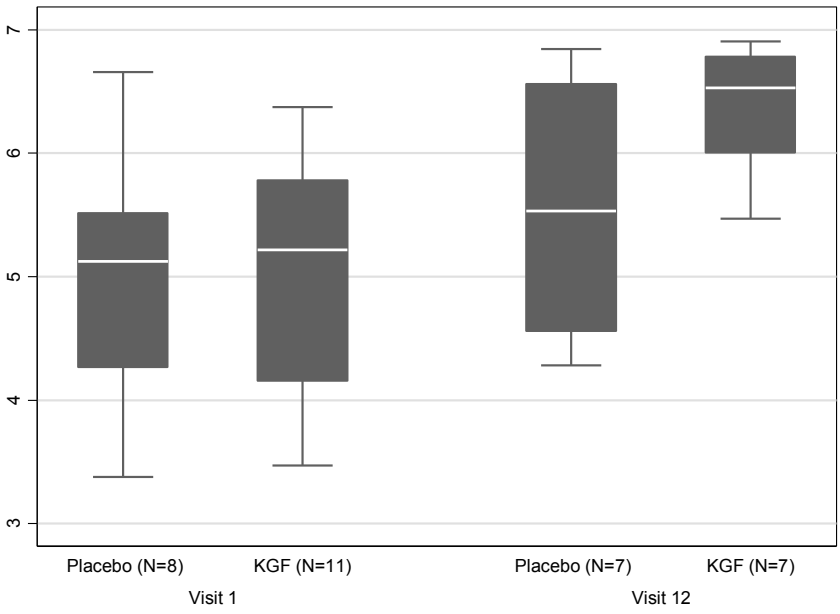


Figure 25: Boxplot graph showing AQLQ values at baseline and end of trial, divided into active and placebo treatment groups. N numbers shown, reduced due to incomplete completion of questionnaire or withdrawal.

Looking at the different subdomains assessed by AQLQ, the main differences between active treatment and placebo appear to be in the symptom score and activity limitation score, reflected in the p values:

- Symptom score p = 0.16
- Activity limitation p = 0.08
- Emotional function p = 0.7
- Environmental p = 0.75

Pre-bronchodilator FEV1/exhaled nitric oxide/PEFR variability

In contrast to the above data, no significant differences between the KGF and placebo groups were seen in the secondary outcomes of pre-bronchodilator FEV_1, exhaled nitric oxide or PEFR variability, nor was there any difference for the group as a whole over the course of the trial (Table 17: % predicted FEV_1 over course of trial, Table 18: Exhaled nitric oxide levels result, Table 19: PEFR variability results).

Note that for exhaled nitric oxide, visit 2 values are used as the baseline below as a full dataset was available (due to equipment failure not all subjects had a FeNO measurement for visit 1).

Visit	Entire group			Placebo		KGF		P value
	N	FEV_1 % predicted	Δ FEV_1 % pred.	FEV_1 % predicted	Δ FEV_1 % pred.	FEV_1 % predicted	Δ FEV_1 % pred.	
Baseline	21	86.6 (29.9)		85.22 (26)		93.3 (35)		
2	21	90.0 (32.0)	4.1 (11.5)	86 (37)	5.8 (11.3)	92.1 (29.4)	4.1 (12)	0.36
3	21	88.4 (36.6)	4.1 (13.9)	89.1 (36.3)	4.9 (11.7)	88.4 (38.1)	-2.8 (19.3)	0.12
6	20[1]	91.2 (35.5)	4.9 (13.4)	91.2 (28.4)	6.9 (8.3)	92.1 (42.5)	2.3 (13.8)	0.15
8	20[1]	86.0 (31.1)	6.5 (18.2)	86 (30.9)	9.4 (17.9)	89.3 (35)	-3.4 (18.5)	0.13
9	20[1]	92.2 (37.4)	5.9 (9.3)	89.1 (23.5)	6.2 (9.1)	96 (40.8)	3.1 (13,2)	0.36
10	20[1]	91.5 (36.9)	4.7 (14.1)	89.2 (29.7)	4.7 (9.5)	95.7 (45.5)	3.9 (21.9)	0.76
12	18[1]	91.3 (34.4)	10.7 (18.3)	86.7 (29.9)	11.8 (13.3)	93.6 (40.5)	4.8 (21.4)	0.37
13	17[1]	91.3 (35.8)	6.4 (18.2)	87.4 (25.2)	9.5 (15.7)	98 (41.9)	6.4 (17.2)	0.44

[1] = n reduced due to patient withdrawals

Δ FEV_1 = change in pre-bronchodilator FEV_1 compared to baseline (visit 1) value

Values shown are medians with IQR in parentheses

P values are comparing Δ FEV_1 for active (KGF) treatment group vs. placebo, using Mann Whitney U test

Table 17: % predicted FEV_1 over course of trial, for entire group and separated into treatment arms

Visit	Entire group			Placebo			KGF			P value
	N	eNO (ppb)	Δ eNO (ppb)	N	eNO (ppb)	Δ eNO (ppb)	N	eNO (ppb)	Δ eNO (ppb)	
1	19[1]	24.5 (31.6)		9	19 (19.3)		10	46.4 (30.9)		
2 (Baseline)	21	28.9 (34.7)		11	17.3 (25.2)		10	36.6 (32)		
6	20[1]	40.4 (43.2)	-4.5 (12.6)	10	18.4 (21.8)	3.5 (6.0)	10	55.7 (24.2)	7.3 (30.4)	0.65
8	19[1]	30.7 (50.5)	0.76 (13.7)	10	17.6 (15.7)	-0.9 (9.3)	9	61.8 (39.3)	0.8 (26.8)	1.00
9	20[1]	27.5 (32.9)	-0.12 (17.1)	10	22.3 (22.0)	4.6 (13.6)	10	38.9 (32.4)	-8.1 (31.2)	0.08
12	18[1]	30.7 (39.4)	-0.8 (27.1)	8	21.8 (25.8)	6.7 (21.5)	10	46.8 (42.1)	-3.6 (46.5)	0.53

Δ eNO = change in exhaled nitric oxide compared to visit 2 value

[1] N= reduced to patient withdrawal/equipment malfunction

Values shown are medians with IQR in parentheses

P values are comparing Δ eNO for active (KGF) treatment group vs. placebo, using Mann-Whiney U test

Table 18: Exhaled nitric oxide levels results over course of trial, for entire group and separated into treatment arms

Visit	Entire group			Placebo			KGF			P value
	N	PEFR Variability %	Δ PEFR Variability %	N	PEFR Variability %	Δ PEFR Variability %	N	PEFR Variability %	Δ PEFR Variability %	
2	20	19.6 (14.2)		9	21.9 (11.6)		11	21.2 (16.3)		
5	20	22.4 (16.5)	-1.35 (13.1)	9	22.7 (16.2)	0.5 (11.33)	11	19.2 (17.6)	-3.2 (21.26)	0.47
7	19	20.4 (12.9)	-4.4 (10.8)	9	21.7 (17.2)	-3.4 (12.5)	10	20.4 (14.8)	-4.6 (10.1)	0.68
12	16	20.1 (9.9)	0.64 (10.3)	6	23.2 (9)	-0.44 (18.1)	10	16.7 (10.7)	0.7 (7.7)	1.00

[1] n reduced due to incomplete diary/failure to return/withdrawal

Δ PEFR variability = change in PEFR compared to baseline (visit 2) value

Values shown are medians with IQR in parentheses

P values are comparing Δ PEFR for active (KGF) treatment group vs. placebo, using Mann-Whitney U test

Table 19: PEFR variability results over course of trial, for entire group and separated into treatment arms

Correlation

Before moving on to discuss the above results, it would be useful to look at the data and see if there are correlations between the clinical values i.e. do the patients who display most improvement in AHR also display improvement in ACQ, etc, as this might support the results being significant due to effect of the drug rather than chance. (NB correlation between clinical data and biological results are dealt with in the next chapter). Correlations were performed using Pearson's correlation coefficient.

Over the course of the trial, the change in AQLQ correlated strongly with the change in ACQ, whether the latter was measured at visit 7 ($r = -0.86$, $p<0.000$), visit 9 ($r = -0.811$, $p<0.000$) or visit 12 ($r = -0.919$, $p<0.000$). This correlation was maintained even if the group was analysed separately (active treatment and placebo) rather than as a whole, though with less significant p values (maintained <0.05 though). The change in PD15, measured as change in RDR, and looking at the group as a whole, correlated with AQLQ at visits 6 ($r = -0.536$, $p = 0.05$) and 9 ($r = -0.536$, $p = 0.05$) but not visits 8 (-0.377, $p = 0.18$) or visit 12 ($r = -0.41$, $p = 0.151$). RDR change (at visits 6, 8, 9 or 12) also failed to correlate with changes at ACQ at visits 7, 9 or 12. Changes in PC20 failed to correlate with changes in ACQ or AQLQ.

Discussion – Clinical results

This section will discuss the above changes, in clinical outcomes, or lack of, and their implications. (Further discussion of the trial as a whole, integrating clinical and biological outcomes, will occur at the end of this section).

Changes in AHR

The group as a whole improved clinically over the time course of the study, with statistically significant improvements in PD_{15} Mannitol showing decreased bronchial hyper-responsiveness, and significant improvements in subjective measures of asthma such as ACQ and AQLQ. The changes in mannitol reactivity showed some correlation with AQLQ scores, duplicating the findings of other asthma clinical trials which have shown a good correlation between mannitol AHR and QOL scores before/after various treatments[430, 432].

In the active treatment group, there were statistically significant greater changes in RDR and, in the more clinically relevant doubling dose difference, than the placebo arm. It is unfortunate that, due to the small size of this pilot study, baseline inequalities in the two groups arose by chance, and that correcting for this baseline inequality negated the statistical significance of the change. Whilst from a statistically rigorous point of view one can argue that this is necessary to assess changes due to regression to the mean[433], the two groups were not statistically significantly different in baseline PD_{15} levels, and overstating the importance of regression to the mean might result in a type II statistical error. In addition, the statistical method used for correction, linear regression, only applies to normally distributed data – even with logarithmic transformation the data still did not appear convincingly normally distributed, and thus correction for baseline is effectively not feasible. Regardless, it could be argued that in a small proof of concept study such as this, this initial data seems quite promising, but that it would need to be verified in a larger group.

What also seems to be apparent from the raw data though, is an apparent discrepancy between the mannitol and methacholine results i.e. that there is less improvement in hyperresponsiveness measured using the latter compared to the former, either for the group as a whole, or for the active treatment group. There are relatively few trials that have used both these *particular* measures as outcomes when looking at asthma treatment, the only one being a recent study looking at the effects of the addition of a combination steroid/long-acting beta agonist inhaler, versus increasing steroid dose alone, in smoking asthmatics[434]. Outcomes included both mannitol and methacholine testing, and improvements were seen in both groups (but comparatively more in the combination inhaler group) to a similar level in both mannitol and

methacholine outcomes. However as already explained in our rationale behind choice of outcomes, one would *expect* differences as the two measures are assessing different aspects of BHR (fixed versus variable components), and in other trials of (different) indirect and direct bronchoprovocation testing, the changes in the indirect tests appear to be greater. One might expect that in a short study such as this, any active treatment would be unlikely to have significant remodelling effects (and of course it may have no effects on this aspect of asthma at all, regardless of length of treatment), but that by modifying the degree of epithelial damage/permeability it might reduce inflammation and thus disproportionately improve indirect BHR.

There are other possibilities for the discrepancy between the two tests. It may reflect an unforeseen mechanism of action of KGF which affects one but not the other test, e.g. whether KGF in some way might affect the ability of methacholine to cross the epithelium, whilst not affecting the fluid shift required for mannitol to have an effect. The length of treatment may affect changes in the two measures i.e. it may be that changes in mannitol AHR occur with shorter durations of treatment whereas methacholine AHR only changes with more prolonged treatment. Lastly there is debate over the best methods for measuring AHR with methacholine, with increased sensitivity reported with the 'tidal breathing' method versus the '5 breath dosimeter' method[435, 436], and whilst ATS/ERS recommendations do not recommend withholding inhaled corticosteroids routinely for a methacholine challenge[437] (and therefore they were not withheld for this study), they were withheld for the mannitol test. Steroids have been shown to reduce AHR to methacholine when given for a prolonged period as a treatment[265, 438-441], and even a single dose of inhaled corticosteroid has been shown to affect direct AHR results[442, 443]. It may be therefore that the dose of inhaled corticosteroid taken by subjects on the morning of the methacholine challenge was sufficient to 'blunt' the improvement in methacholine reactivity.

Changes in AQLQ and ACQ

The improvements in ACQ and AQLQ in the group as a whole might be expected given the improvement in AHR, as said above previous studies have shown good correlation between subjective measures of asthma and AHR. AQLQ improves by a statistically significantly greater amount in the group on KGF compared to placebo, and this appears to be mainly due to effects on symptom score and activity limitation subdomains. This may be that whilst the physiological effects of the drug might fairly rapidly impact on these areas (through effects on BHR), the subdomains addressing the 'emotional' impact of an individuals asthma and how they might avoid 'environmental' stimuli would only change with longer treatment/more sustained improvement

of a subject's symptoms. It is surprising then, that the differences between the groups are not significant for ACQ, as the two overlap somewhat in the symptom areas they address. In case this reflected the lack of improvement between the two groups in spirometrical outcomes e.g. FEV_1 % predicted (see also discussion below), analysis was also performed on ACQ-6 scores (this simply averages the first six questions in ACQ, rather than 7, and thus does not include a score for FEV_1). However the changes between the groups remained statistically insignificant (data not shown). Looking at the data, we can see that a significant proportion of the placebo group improved by 0.5 points on the scale following the 'drug' (placebo), and it may be that the placebo effect is greater on subjective measures such as ACQ, compared to objective measures such as BHR; however this would then not explain the improvement in AQLQ, and in the literature, the placebo effect can be seen on objective measures such as FEV_1[444] and BHR[445] as well as subjective questionnaires[446].

Changes in other clinical outcomes

The remaining clinical outcomes - pre-bronchodilator FEV_1, exhaled nitric oxide and PEFR variability, are fairly unchanged throughout the trial, regardless of treatment group. FEV_1 is used as an outcome in a multitude of asthma trials, and in certain studies changes in FEV_1 have correlated well with changes in AHR and symptom scores, for example with ICS treatment[290]. Furthermore, baseline FEV_1 has been used in the prediction of future exacerbation rates[447-449]. However other studies have shown dissociation between FEV_1 and patient reported symptoms of asthma[450, 451], or in some cases the degree of inflammation[452]. From an understanding of the physiological effects of KGF, one might expect that the degree of baseline bronchodilation (and thus FEV_1), would not be directly affected (as it is not known to have bronchodilator properties, but would only indirectly affect this (via a reduction in stimulation of external stimuli, and possible reduction in inflammation). It may be therefore that the current trial is too short to see such an effect, or that the time points chosen were insufficient to correct this i.e. with more prolonged treatment and a longer follow-up, changes may become apparent (or it may become apparent that KGF may be completely independent of this measure).

Similar explanations would apply to exhaled nitric oxide (eNO). Exhaled nitric oxide is produced endogenously in cells by NO synthases and is used as a surrogate biomarker for airway inflammation, in particular eosinophilic inflammation[453]. eNO has been shown to correlate well with eosinophil presence in BAL [454] and sputum[455], and with mucosal eosinophils in bronchial biopsies in some studies[456] (but not others[457]). However the sensitivity and specificity in the above studies are relatively low, eNO can be affected by diet, age, gender and others, and the range of values for eNO in the healthy and asthmatic populations overlap[453]. In

general however, the upper level of normal for eNO is felt to be 25ppb at a flow rate of 50ml/sec (the flow rate used in this study)[453, 458], and significant changes are felt to be a change of ≥20% if baseline eNO ≥ 50ppb, and a change of 10ppb if baseline <50ppb. The baseline for the group as a whole was at the upper end of 'normal'/lower end of 'asthmatic' range at ~25ppb, which fits with the low proportion of BAL eosinophilia seen in sputum analysis (using a 'normal range' for BAL eosinophils as ≤ 2%[288], only 4 of the 20 subjects had an elevated BAL eosinophil count). The lack of any clinical change in the group as a whole, or in the two treatment groups may relate to this low baseline, or, as with FEV_1, due to KGF failing to have an effect on this biomarker, or incorrect length of treatment/choice of time points to see a change in this biomarker.

Given our original hypothesis regarding epithelial permeability and the effect of KGF, one might have expected to see an effect on PEFR variability, as this has been suggested by some as a surrogate marker of AHR[459], and has also been shown to correlate with patient's perception of asthma severity[460]. PEFR variability has been shown to have some correlation with AHR[461-463], in particular with diurnal variation of indirect AHR[464], although it has been argued that the correlation is overall quite poor[459], with a proportion of patients with AHR lacking variability, and a wide variation in degree of variability for a given level of AHR. However as seen in the results, PEFR variability remained reasonably constant in the group as a whole (and did not seem to differ greatly in active vs. placebo groups). This may be due to a more consistent effect of KGF (i.e. improving both morning and evening PEF measurements) or lack of effect of KGF on this outcome.

Future Work

There are suggestions of benefit in the results shown from KGF treatment, although the improvement was not as marked as might be hoped. It is important to learn from this pilot study to plan possible future studies in this area, and in part to 'streamline' future studies i.e. focus on those areas which might best be expected to confirm (or refute) a role for the drug/other similar drugs, whilst reducing unnecessary testing/visits.

Clearly one comment that would be relevant to all outcomes assessed would be whether the pilot study was adequately powered to detect changes in the variables measured. As discussed in the methods section, the cost of the drug precluded a larger sample size as had been originally planned, the biological effect of the drug was not at all known, and the inherent variability in these outcomes in this patient group was not clear. Analysis of the existing data set does provide estimates and measures of variability, which could be used in order to select suitable outcome measures and sample sizes for future trials (assuming a similar population was used).

With this in mind PD_{15}, and namely the DDD in RDR, does seem a valid primary outcome for future studies. The test is easy to perform, objective, carries clinical significance, and fits with a plausible biological effect of the drug i.e. a reduction in bronchial hyper-reactivity. Assuming a significance level of 0.05 and power of 80%, and based on data from the study (where standard deviation of DDD at visit 9 in the placebo and KGF groups was 1.62 and 1.74 respectively), a sample size of 92 subjects (46 in each group) would be required to detect a difference of one DDD. For a more optimistic effect of 2 doubling dose differences, 26 patients would be required. PC_{20} methacholine would not seem to add much to a future trial – if this pilot study can be believed, the effects are less marked on PC_{20}, there is not a strong body of evidence that one BHR test is more significant clinically than another, and the test was more involved/added to the visits for the patient.

ACQ and AQLQ should be included in future trials whilst subjective and perhaps more susceptible to placebo effect, they are directly relevant to the most meaningful goal for future asthma treatments, that is, a reduction in patient symptoms and improvement in quality of life. The questionnaires are (reasonably) simple and quick to complete, and have been well validated.

PEFR diary monitoring and exhaled nitric oxide measurement could perhaps be dropped from a future study (pre-bronchodilator FEV_1 will be measured regardless as part of the mannitol PD_{15} test) given no apparent response in this pilot study – whilst they did not add to the visit burden per se, these tests do carry a cost burden (eNO), and a small but potentially relevant time implication for the subject (both in visits e.g. performing the eNO test, and outside visits e.g. completing the PEFR diary).

Other impacts on future work e.g. in terms of biological outcomes/tests and more general implications will be discussed in the relevant chapters/general discussion at the end of this section.

Safety and Efficacy of parenteral KGF in moderate asthma subjects – Biological Results (pre-specified)

The next section will deal with those biological outcomes that were *pre-specified* as secondary outcomes for the clinical trial i.e. not those that were exploratory outcomes (which will be covered in the next chapter). Because the analysis of some of these outcomes was dependant on the specimens being both collected and analysable, n numbers are shown; for example, not all patients' biopsies displayed good quality tangential epithelium for tight junction analysis.

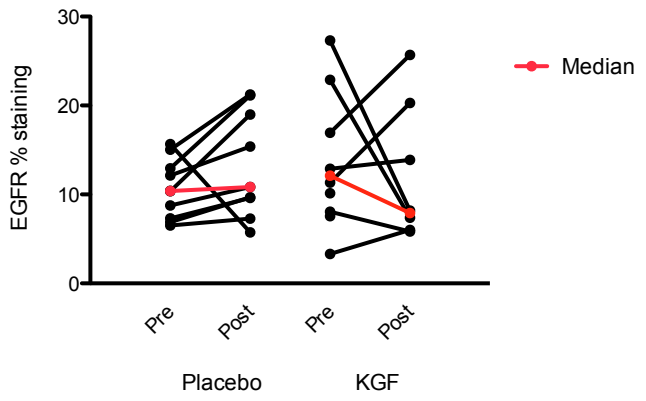
The results are presented in sections corresponding to those biological effects it was hoped to assess, namely

- Epithelial integrity
- Epithelial inflammation
- Epithelial proliferation
- Epithelial remodelling
- Surfactant effect

Examples of the GMA staining for the epithelial and mucosal cytokines/cells are shown in each section, following the tabulated results.

Epithelial integrity – EGFR

The table below (Table 20: EGFR staining in GMA-embedded bronchial biopsies in clinical trial) shows the results for EGFR staining in the GMA-embedded biopsies, in graph form with the figures below. The two groups had similar baseline levels of staining (p = 0.374, Mann-Whitney U test), and whilst there was a decrease in the mean and median values for the KGF treated group post-treatment, neither group had a statistically significant change compared to pre-drug values (p values of 0.11 and 0.87 respectively for placebo and KGF using Wilcoxon signed Ranks test (WSRT)). In addition, the change in EGFR was not significant *between* the groups (p = 0.491 using Mann-Whitney U testing). The level of % staining for EGFR (10-12%) is slightly lower than that previously reported in asthmatics, more akin to the upper limit of healthy patients/milder asthmatics[62, 64, 72], although not so far removed as to suggest that the technique/results were invalid.



	Placebo			KGF			P Value
	Pre-drug	Post-drug	Change	Pre-drug	Post-drug	Change	
EGFR % epithelial staining	10.39 (7.2, 13.5)	10.84 (8.4, 20.1)	2.8 (1.4, 7.2)	12.11 (8.6, 21.4)	7.9 (6.4, 18.7)	1.01 (-15.5, 8.7)	0.491
N	10	9	9	8	9	8	

Values shown are medians with IQR in parentheses

Change based on patients where pre-drug and post-drug suitable for analysis

p values are comparing changes in cell differential (Post-Pre) between the groups using Mann-Whitney U testing

Table 20: EGFR staining in GMA-embedded bronchial biopsies in clinical trial, graph showing individual patients, and medians before and after treatment (above), with table showing median values for groups as a whole and statistical testing (below).

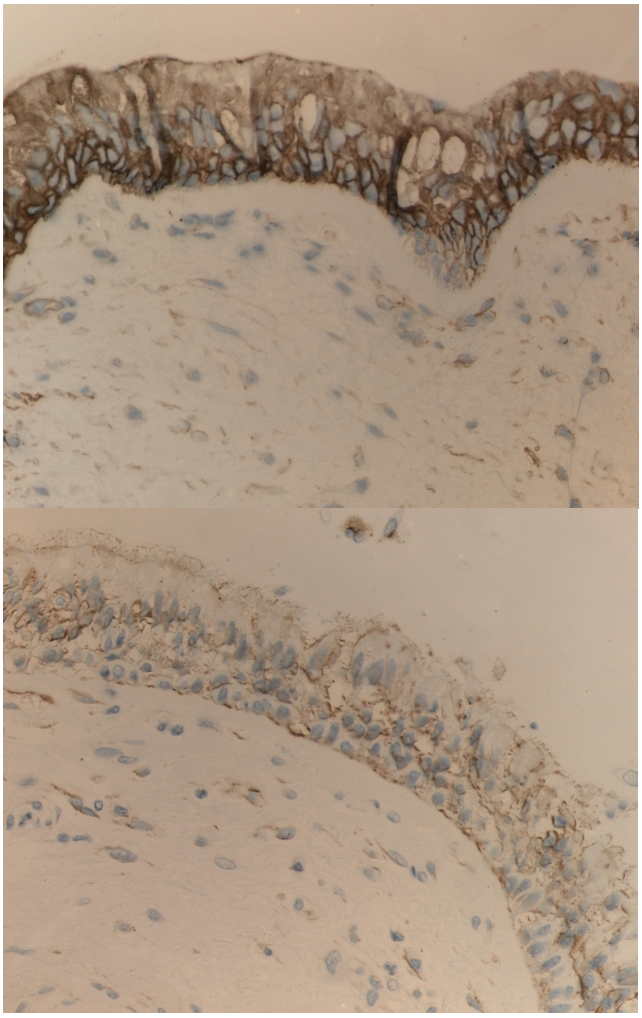


Figure 26: Examples of EGFR staining in bronchial epithelial biopsies from KGF clinic trial. Biopsies obtained, embedded in GMA and stained as described in earlier Methods section. More intense staining seen in biopsy above and less intense staining in image below (different subjects). Sections orientated with lumen at top and mucosa at bottom. X400 magnification

Epithelial integrity – Tight junctions

Looking at the tight junctional staining in bronchial biopsies (Table 21: Tight junction staining scores in GMA-embedded bronchial biopsies), we can see that overall both groups' scores improved at the second bronchoscopy, for ZO-1 staining. Comparing between the groups, a significant difference in the change in staining was not seen (p = 0.957, MWU test). It may be important to note however, that this improvement occurred in only 6/9 of the placebo group (66%) versus 7/7 in the KGF group (100%). Furthermore, comparing *within* the groups for pre- and post- treatment, a statistically significant difference (compared to baseline) was only seen for the KGF group (p = 0.03, Wilcoxon Signed Ranks test) and not for the placebo group (p = 0.396, WSRT).

There appeared to be less of an improvement in Occludin scores – only 3/9 (33%) improved in the placebo group versus 5/7 (56%) in the KGF treated group. Neither group significantly increased compared to baseline values (p = 0.89 for placebo and p = 0.15 for KGF group, WSRT, comparing post-treatment values to baseline), nor was there a significant difference between the groups comparing the change in Occludin staining (p = 0.31, MWU test).

	Placebo				KGF				P value between group
	Pre-drug	Post-drug	Change (Post-Pre)	P value within group	Pre-drug	Post-drug	Change (Post-Pre)	P value within group	
ZO-1 Score	2 (1, 2.63)	2.5 (1.63, 2.75)	0.5 (0, 1.5)	0.396	2.13 (1.63, 2.5)	2.5 (2.25, 2.75)	0.5 (0.25, 1)	0.026	0.957
Occlud Score	1.83 (1.5, 2.5)	2 (1.5, 2.5)	0 (0, 0.5)	0.893	2.25 (1.0, 2.5)	2.75 (1.71, 3.0)	0.5 (-0.16, 2)	0.149	0.31
Values shown are medians with IQR in parentheses Changes/Improvements in score based on patients where pre-drug and post-drug suitable for analysis p values using Mann-Whitney U testing on change in Zo-1/occludin score, active vs placebo									

Table 21: Tight junction staining scores in GMA-embedded bronchial biopsies. Median scores shown with change in median score and statistical testing within and between groups

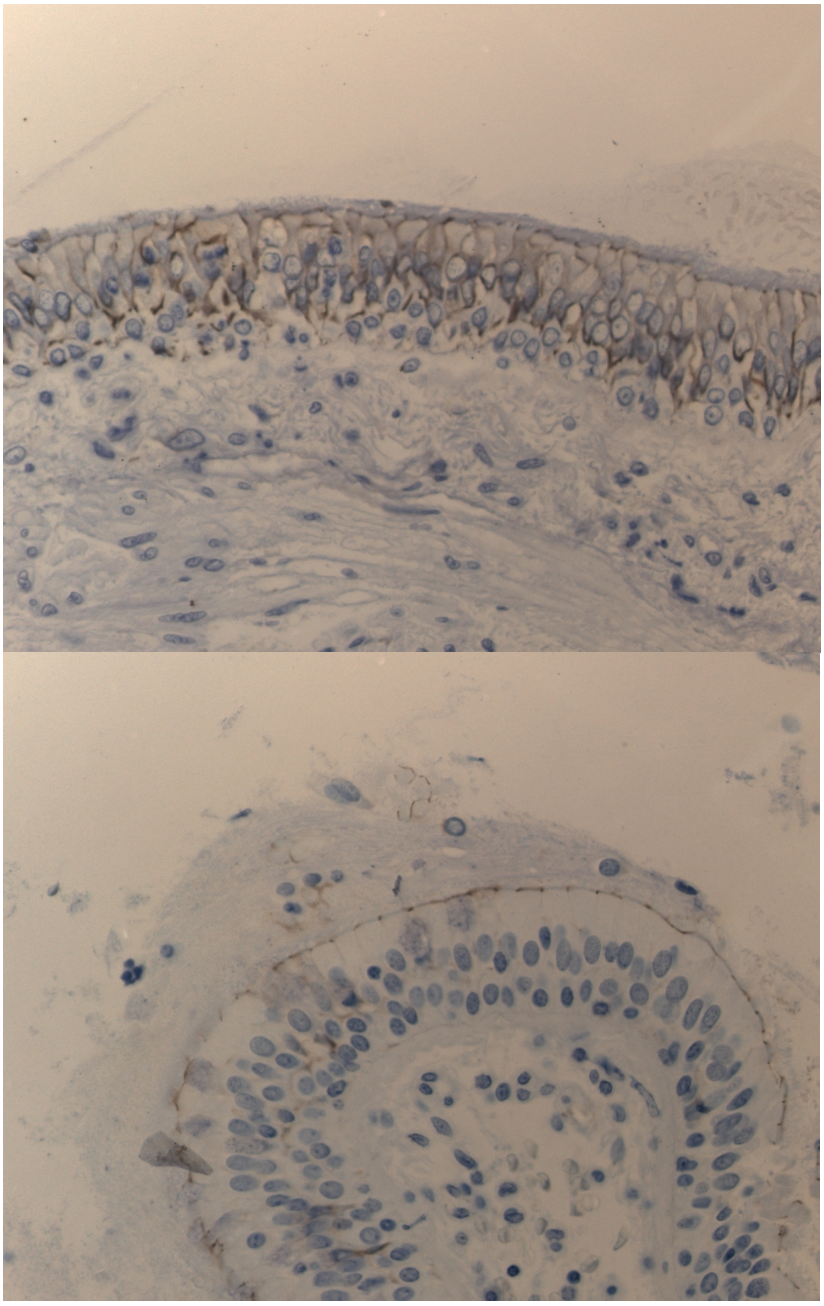


Figure 27: Example of Tight junction staining in bronchial epithelial biopsies in KGF clinical trial. Biopsies obtained, embedded in GMA and stained as described in earlier Methods section. Above image shows staining for occludin, below image shows staining of ZO-1. Further example images of ZO-1 staining seen in methods section, to demonstrate scoring system. Sections orientated with lumen at top and mucosa at bottom, in the lower image the biopsy has curled around on itself and has lots of mucus present on surface of epithelium. X400 magnification

Epithelial inflammation

Epithelial inflammation was assessed by looking at several compartments: the cellular differential in the BAL, the epithelium itself and submucosal area (in bronchial biopsies).

BAL cell differential

This is shown below (Table 22: Broncheoalveolar lavage cell differential in active and KGF groups pre and post treatment). Macrophages were predominant as expected, and the proportions were similar to those reported in other studies although with perhaps a higher percentage of epithelial cells seen (although there is a degree of variation between studies dependant on the population studied [465-470].

Cell Type	Placebo				KGF				P Value (between groups)
	Pre-drug % cells	Post-drug % cells	Change	P value (within group)	Pre-drug % cells	Post-drug % cells	Change	P value (within group)	
Epithelial	11.75 (4.6, 18.6)	4.4 (2.3, 17.8)	-2.85 (-4.9, 7.9)	0.799	8.8 (7.2, 20)	5.9 (2.2, 9.1)	-2.75 (0.18, 13.4)	0.037	0.5
Eosinophil	0.25 (0, 1.3)	0.38 (0.2, 1.7)	0.25 (-0.6, 0.6)	0.766	1.38 (1.1, 3.8)	2.25 (0.4, 3.1)	-0.25 (-0.9, 0.5)	0.553	0.45
Neutrophil	2.5 (1.1, 4.5)	1.9 (2.4, 3.5)	-0.15 (-1.2, 2.8)	0.54	1.88 (1.1, 8.4)	4.4 (0.7, 10.2)	-0.63 (-4.7, 2.1)	0.441	0.31
Macrophage	84.5 (75.6, 90.7)	91.3 (77.7, 93.1)	-1.6 (-7.6, 3.6)	0.61	81.7 (72.1, 89.1)	81.7 (73.5, 90.1)	-5.3 (-9.0, 2.1)	0.203	0.52
Lymphocyte	1.13 (0.6, 1.8)	1.25 (0.8, 2.6)	-0.13 (-0.6, 0.3)	0.324	1.4 (0.6, 2.8)	0.88 (0.3, 4.8)	-0.13 (-1.8, 0.9)	0.858	0.85

N = 20, Values shown are medians with IQR in parentheses

p values comparing changes in cell differential (Post-Pre) within the groups are calculated using Wilcoxon Signed Ranks testing, and between the groups using Mann-Whitney U testing.

Table 22: Broncheoalveolar lavage cell differential in active and KGF groups pre and post treatment.

There was no significant difference between the groups at baseline for any of the cells except eosinophils, which whilst low overall, were statistically higher at baseline in the KGF group ($p = 0.04$, Mann-Whitney U testing). The % epithelial cells in the BAL, following treatment, seemed to decrease in both groups, and whilst comparing *between* the groups, the change in epithelial cells (and all other cells) was not significantly different, when comparing *within* the groups there was a significant change, but only for the KGF group post-treatment (WSRT, $p = 0.037$),

Bronchial biopsies

Below are shown the changes in inflammatory cellular staining in the submucosal and epithelial compartments respectively (Table 23: GMA bronchial biopsy cell differential in two treatment arms, before and after treatment- submucosal compartment, and Table 24: GMA bronchial biopsy cell differential in two treatment arms, before and after treatment- epithelial compartment). There was relatively little staining of eosinophils or mast cells in the epithelial layer (for both groups). In the submucosa, the predominant cell type was neutrophils, then mast cells and eosinophils.

Comparing *within* the groups, in the placebo group surprisingly there was a significant change pre to post treatment in epithelial mast cells (WSRT, p = 0.037, this *within* group data not shown in table), given the low numbers involved this is of questionable significance. In addition, looking within the placebo group, epithelial eosinophils and any submucosal cells did *not* change significantly post-treatment. The KGF group also did not display any significant changes pre-post treatment for either submucosal or epithelial cells – whilst there did seem to be a reduction in mast cells and eosinophils in the submucosa post treatment this was not statistically significant. The difference in changes in cell counts *between* the groups was also not significant for any of the cells, in either compartment.

Cell Type	Placebo			KGF			P Value
	Pre-drug cell count (cells/mm^2)	Post-drug cell count (cells/mm^2)	Change	Pre-drug cell count (cells/mm^2)	Post-drug cell count (cells/mm^2)	Change	
Eosinophils	0.34 (0, 6.7)	0.77 (0, 4.5)	-0.68 (-5, 0.7)	7.81 (6.4, 10.5)	1.97 (1.5, 8.8)	-3.11 (-8.6, 0.6)	0.56
Mast cells	17.15 (8.1, 40)	26.83 (10.1, 29.1)	1.52 (-11.4, 6.9)	25.27 (10.6, 40.5)	13.19 (7.3, 27.9)	-4.17 (-13.1, 6.5)	0.773
Neutrophils	59.01 (27.5, 77.7)	25.07 (20.4, 77.3)	-23.63 (-54.5, 31.9)	41.85 (18.1, 63.2)	28.48 (19.1, 68)	-0.09 (-19.2, 10.5)	0.27
N	10	9	9	9/10	8/9	7-9	–
Values shown are medians with IQR in parentheses p values are comparing changes in cell differential (Post-Pre) between the groups using Mann-Whitney U testing p values for within group data not shown in this table							

Table 23: GMA bronchial biopsy cell differential in two treatment arms, before and after treatment- submucosal compartment

Cell Type	Placebo			KGF			P Value
	Pre-drug cell count (cells/mm^2)	Post-drug cell count (cells/mm^2)	Change	Pre-drug cell count (cells/mm^2)	Post-drug cell count (cells/mm^2)	Change	
Eosinophils	0 (0, 0.1)	0	0 (-0.3, 0)	0 (0, 0.8)	0	0 (-0.8, 0)	0.762
Mast cells	0.79 (0.4, 2.2)	0 (0, 0.97)	-0.55 (-2.0, -0.1)	1.57 (0.5, 3.2)	0 (0, 3.1)	-0.48 (-1.6, 0.8)	0.491
N	10	9	9	6	8	6	

Values shown are medians with IQR in parentheses

p values are comparing changes in cell differential (Post-Pre) between the groups using Mann-Whitney U testing

p values for within group data not shown in this table

Table 24: GMA bronchial biopsy cell differential in two treatment arms, before and after treatment- epithelial compartment

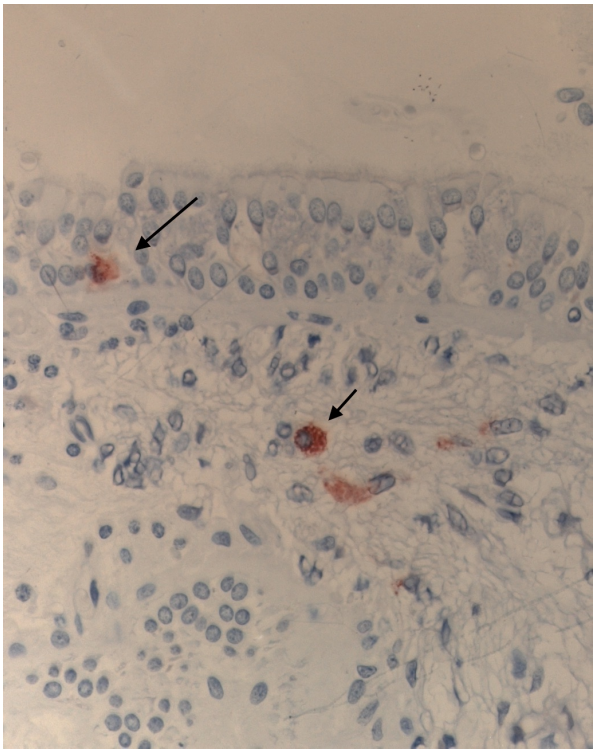


Figure 28: Example of AA1 (mast cell tryptase) staining in bronchial epithelial biopsies in KGF clinical study. Biopsies obtained, embedded in GMA and stained as described in earlier Methods section. Mast cells seen within epithelium (long arrow) and also in mucosa (short arrow). Sections orientated with lumen at top and mucosa at bottom. X400 magnification

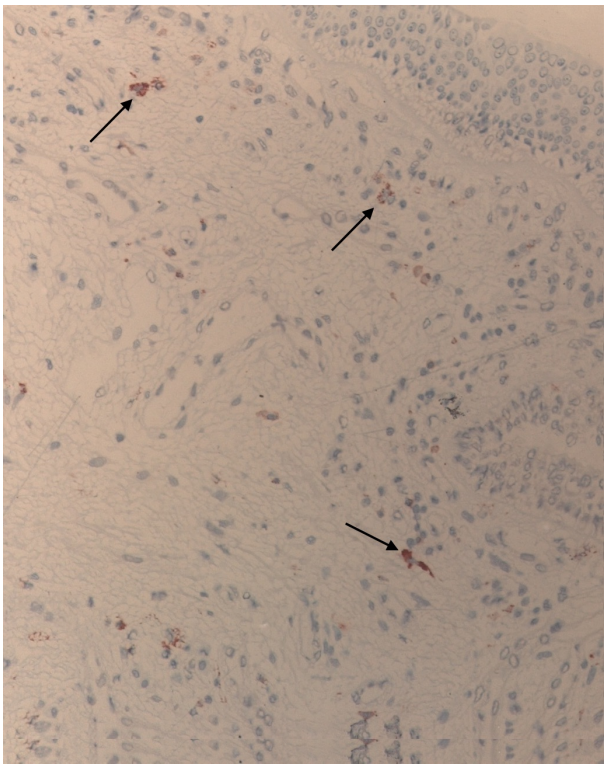


Figure 29 Example of EG2 staining for eosinophils in bronchial epithelial biopsies in KGF clinical study. Biopsies obtained, embedded in GMA and stained as described in earlier Methods section. Eosinophils seen in mucosa predominantly (marked with arrows), and rarely in epithelium, as reflected in results above. Sections orientated with lumen at top right, with mucosa below. X200 magnification

Epithelial proliferation

As previously stated, epithelial proliferation was assessed looking at Ki67 staining in the epithelium. Neither group changed significantly from baseline (WSRT, p = 0.68 for placebo group, p = 0.61 for KGF group, data not shown in table), nor was there a significant difference in the change *between* the groups (Table 25: GMA Bronchial biopsy - Epithelial proliferation).

Cell Type	Placebo			KGF			P Value
	Pre-drug	Post-drug	Change	Pre-drug	Post-drug	Change	
Ki67 Cells/mm^2	5.60 (0.6, 11.3)	6.60 (1.5, 8.2)	-2.04 (-5.9, 6.5)	5.25 (3.4, 12.2)	1.88 (1.3, 1.9)	-0.82 (-9.6, 5.3)	0.958
N	10	9	9	8	9	8	

Values shown are medians with IQR in parentheses

p values are comparing changes in cell differential (Post-Pre) between the groups using Mann-Whitney U testing

p values for within group data not shown in this table

Table 25: GMA Bronchial biopsy - Epithelial proliferation marker (Ki67), showing median values for two treatment groups, before and after treatment with median change, and statistical testing on change between groups.

Epithelial remodelling

Epithelial remodelling was assessed by studying collagen band thickness in the GMA-embedded biopsies (Table 26: GMA bronchial biopsies - epthelial remodelling). There was no significant change before/after treatment for either *within* group (placebo group (p = 0.86, WSRT), KGF group (p = 0.37)), or between the groups in the change observed ().

Cell Type	Placebo			KGF			P Value
	Pre-drug	Post-drug	Change	Pre-drug	Post-drug	Change	
Collagen band thickness (µm)	8.67 (7.5, 10.5)	9.08 (7.4, 11)	0.00 (-1.6, 2.6)	11.14 (8.6, 12.3)	12.03 (10.2, 15)	2.02 (-2.5, 4.5)	0.453
N	10	9	9	9	9	9	

Values shown are medians with IQR in parentheses

p values are comparing changes in cell differential (Post-Pre) between the groups using Mann-Whitney U testing

p values for within group data not shown in this table

Table 26: GMA bronchial biopsies - epthelial remodelling assessed using collagen band thickness in two treatment groups, median values shown before and after treatment with median change and statistical testing between the groups.

Surfactant effect

Surfactant was measured by both ELISA (Surfactant Protein D, see Table 27 below), and via mass spectrometry as part of a general lipid profile (see exploratory outcomes, next chapter) on BAL samples. Looking at the ELISA values, neither KGF nor placebo group had significant changes compared to baseline (p = 0.58 and p = 0.51 respectively, WSRT). There was no significant difference in the change between the groups (p = 0.6, MWU test, see table below).

Cell Type	Placebo			KGF			P Value
	Pre-drug	Post-drug	Change	Pre-drug	Post-drug	Change	
Surfactant protein D (ng/ml)	23.67 (7.6, 43.2)	37.89 (8.4, 42.6)	3.45 (-8.9, 15.0)	20.15 (8, 50.8)	13.35 (8.8, 74.8)	-3.22 (-7.9, 4.9)	0.60

Values shown are medians with IQR in parentheses

p values are comparing changes in cell differential (Post-Pre) between the groups using Mann-Whitney U testing

p values for within group data not shown in this table

Table 27: BAL – BAL Surfactant Protein D levels measured in two treatment arms using ELISA, before and after treatment, with median change and statistical analysis on between group difference.

Correlations

We explored correlations between the clinical and biological outcomes e.g. did those who improved clinically also improve in the biological markers studied. As might be expected given the disappointing biological results, overall there was poor/inconsistent correlation between biological values and clinical data, whether it was baseline results or changes in those values before and after treatment (data not shown). In addition, with the sheer number of variables being compared, apparent correlations where seen might have been expected to arise by chance.

Discussion – Biological Results

Overall, the biological results from the trial were disappointing, failing to show a clear effect of KGF in several different areas. Given the proven effect of KGF in vitro, and the apparent clinical effects in vivo, reasons for the lack of effect could be ascribed to one of several reasons:

a) Lack of an effect of KGF in vivo on those variables studied: Whilst this is possible, and may account for some of the lack of responses seen, it seems unlikely to be the sole cause. Many of the outcomes have a plethora of in vitro data to support a bronchial epithelial effect, in human and animal models. For example, KGF has been shown to increase Ki67 staining of pulmonary epithelial cells in animal models when applied endogenously[160, 163, 169, 471] or through viral transcription[169, 472], in cultured human bronchial epithelial cells[163], and in humans in other epithelial cell lines[238].

b) Incorrect time-point: the second bronchoscopy was performed 11 days after the second dose of the drug/placebo. Looking at the data on proliferative effect as mentioned above, the peak effect appeared to be at 48 hours, with a decreasing response seen at 72 hours or beyond[169, 238]. Some biological effects have been seen to persist beyond this time-point e.g. up to 90 hours[218] but it is possible that the time-point chosen was not optimal to see a difference.

c) Insufficient numbers: This pilot study was small and the limited numbers of patients (and also downstream i.e. analysable biopsies/completed results) would naturally limit the sensitivity to detect a difference. For example, there is a natural variability to inflammatory cells even in normal healthy subjects[473], and our population of asthmatics were somewhat heterogenous in their existing treatments and may have displayed similar heterogenousity in their inflammatory responses, affecting the sensitivity of the analysis. Indeed, a study looking at the repeatability of inflammatory cell counts in biopsies from asthmatics[474] by Sont et al, their findings suggest that our study might be too small to detect differences, particularly for between-group differences. This study suggested a study size of 13-48 subjects in this population is required to detect a doubling/halving in cell number.

d) Insufficient treatment length: Conversely to the above argument with regards performing bronchoscopy too late after treatment to see a biological effect, there is a separate argument that perhaps the overall study was too short to see an effect, particularly with remodelling. For example, when looking at the effects of inhaled corticosteroids on remodelling, shorter (6-8 week) studies have seen no change in RBM thickness[430, 475,

476] despite improved AHR[430]), whereas some longer term studies in this field have seen remodelling effects[476-478].

e) Specific concerns/Limitations relating to the outcome chosen/method of analysis (see below).

Limitations of methods of analysis

Biopsy derived outcomes

Immunohistochemistry analysis of GMA-embedded biopsies was chosen as the technique for a number of biological outcomes. GMA-embedding carried a number of benefits, including host site experience (with well-characterised antibodies for subsequent staining), an ability to cut thin sections (2μm) and thus maximise the biopsies, and good morphology presentation. Disadvantages are limited, and are mainly restricted to those in immunohistochemistry as a whole i.e. if using polyclonal antibodies, lack of specificity. Similarly whilst the count of inflammatory cells by an observer introduces a level of subjectivity/possibility for error, as opposed to automated cell counts, the latter is less well studied[479] and attempts were made to minimise bias by performing the analysis blinded, by a single operator. The limitations instead apply to the biopsies themselves, where there might be several concerns:

a) Were sufficient biopsies taken? Due to biopsies which were not of sufficient quality (e.g. containing cartilage only, no tangential epithelium, or limited amount of epithelium which 'ran out' with subsequent sectioning), not every outcome could be measured in every participant. Bronchoscopies were time limited due to patient tolerance and effects of sedation, and therefore only a limited number of specimens (including biopsies) could be taken/attempted. However additional biopsies were taken for proposed mRNA analysis and electron microscopy analysis (which has subsequently not occurred), and therefore it is arguable that if these had not been attempted, more/better quality GMA biopsies would have been possible.

b) Were the biopsies of sufficient quality? When assessing the submucosal compartment, samples were used if they had $\geq$ 0.4mm^2 area visible. The area of submucosa required for an 'acceptable' biopsy is not universally specified. Many studies in the field do not include information regarding surface area, or when specified, accept differing sizes e.g. 0.2mm^2[279] or 0.1mm^2[480]. A sample area of 0.3 to 0.5 mm^2 has been suggested[481] and there is some evidence to suggest similar cell counts for large and small biopsies[473]. Similarly there is even less guidance on the acceptable length of epithelium for measurement of epithelial outcomes,

although a length of 1mm for assessment of RBM thickness has been proposed[482] – we elected not to exclude lesser areas of epithelium to maximise biopsy inclusion.

c) Are the biopsies representative of the processes occurring in the lung? This is particularly of note as the biopsies are by necessity taken mainly from carinal areas, in relatively proximal airways, leading to concern over their validity in reflecting the lung as a whole[481, 483]. There are varying suggestions as to numbers of biopsies required to be representative, and it may depend on the variable studied. Proposals include 2[481], 3[484] or 4[483].

Bronchial biopsies remain a useful tool in the evaluation of biological effect of asthma treatments despite the above limitations, but it might be fair to say that whilst they have successfully shown changes in many other treatments, such as inhaled corticosteroids[283, 477, 478], the changes are not universally seen between all studies despite an acknowledged effect. It may be that with repeated, larger studies, with differing timepoints, a biological effect in vivo of KGF becomes apparent.

Bronchoalveolar lavage derived outcomes

Bronchoalveolar lavage as a method of assessment of biological effect also comes with limitations that have been recognised[481] but is felt to be complementary to endobronchial biopsy in that it might 'sample' more peripheral airways[485]. The converse argument to this however is that BAL samples of say, surfactant, may be influenced more by alveolar surfactant levels rather than airway surfarctant levels (which may be better reflected by measuring sputum surfactant) – in our study we would be most interested in changes in the airways (rather than alveoli) as the site of the disease.

Another major limitation which applies equally to our study would be the variable return in sample between patients. Whilst in a minority of cases this might have been due to differing amounts of saline instilled (not all patients were able to tolerate the full amount of saline instillation specified in the protocol), the majority simply reflected the natural variability in this technique – it is not sure whether this represents an element of the disease process or not. It is also unclear whether adjustments should or could be made for the variable return; in the majority of studies they are not.

As with the bronchial biopsies, whilst the limitations of BAL analysis do not detract from its usefulness as a tool, and BAL analysis has undoubtedly shown some changes after treatment effect using recognised treatments e.g. inhaled corticosteroids[290, 476], effects are variable.

Again, a larger study, with differing time-points, may be able to establish whether there is any effect of KGF on BAL markers.

Future work

The timing of the bronchoscopy was probably crucial to the successful demonstration of a biological effect of KGF, although it was hoped that such effects might persist to the time point chosen (and potentially the clinical benefits from the treatment did persist). Another reason for choosing this time point in the study for bronchoscopy, is that the effects of BHR testing on bronchial biopsies is not well established, although looking at the reverse i.e. effects of bronchoscopy on BHR, there is some evidence of deleterious effect short-term[486]. In this study the bronchoscopy was 48-72 hours after BHR testing (and subsequent BHR re-testing 7-12 days later.

A counter-argument would be that both groups would be equally affected by any influence of the BHR test on the bronchoscopy. When designing the schedule of any future studies, whilst priority must be given to evaluating the primary (likely clinical) outome chosen, to consolidate evidence for biological as well as clinical effect, an earlier time point for bronchoscopic sampling should be chosen. The markers chosen for this study are probably equally valid for future studies, although it may be that occludin staining could be omitted (as potentially a lesser effect seen) and/or measurement of basement membrane thickness (dependant on the length of follow-up of future studies and thus the likelihood of seeing remodelling changes).

In addition due to the time-limited nature of some of the bronchoscopies, one option would be to remove the BAL as a sample/outcome and instead concentrate on GMA biopsies alone – by increasing biopsy numbers one could increase likelihood of an analysable sample and reduce likelihood of sampling error.

Lastly, another question that arises is whether two bronchoscopies are needed. Whilst this is beneficial to look at before and after treatment changes, it does affect patient willingness to participate in a study (anecdotal evidence), has cost/manpower implications for a trial, may or may not be influenced by/influence other outcome testing (as described above), and does have a very low but not negligible general complication rate[487]. With larger numbers in a future trial, such that population heterogeneity in the biopsy results is less pronounced and there is less need to look at results before and after, possibly a single bronchoscopic visit/assessment may suffice.

Safety and Efficacy of parenteral KGF in moderate asthma subjects – Biological Results (exploratory)

GMA immunohistochemistry

In addition to the above pre-specified biological outcomes, other variables were explored in both GMA immunohistochemistry and ELISA analysis, casting a wide net to see if there were any hitherto unforeseen effects from the KGF treatment. Sections of epithelium from biopsies were stained immunohistochemically for TGF-beta2, IL-8, TNF-alpha, GM-CSF, E-cadherin and p120 as described in the Methods section, and then a % epithelial staining was calculated using imaging software as described in the Methods section, with results shown below (Table 28: GMA bronchial biopsy - Epithelial exploratory outcomes). There were no significant differences between the groups for any of the markers studied, nor did they change significantly within the groups.

	Placebo			KGF			P Value
	Pre-drug	Post-drug	Change	Pre-drug	Post-drug	Change	
TGF-beta2 % staining	1.56 (0, 4.2)	3.02 (1.4, 4.9)	0.58 (-0.6, 1.6)	2.27 (0.1, 3.7)	1.25 (0.7, 3.8)	-0.31 (-2.2, 1.4)	0.50
IL-8 % staining	28.5 (18.9, 30.5)	35.8 (29.4, 42.2)	7.22 (0.7, 12.8)	25.77 (15.8, 39.9)	28.53 (25.9, 37.8)	3.82 (-10, 11.7)	0.4
TNF-alpha % staining	19.06 (15.3, 25.8)	15.77 (12.0, 20.4)	-4.67 (-11.4, -0.7)	19.11 (17.2, 29.1)	18.94 (14.4, 30.0)	1.25 (-19.8, 11.3)	0.4
GM-CSF % staining	16.00 (13.4, 26.0)	17.13 (11.7, 21.1)	-1.93 (-5.5, -0.9)	19.53 (15.0, 25.0)	18.77 (10.9, 30.1)	2.05 (-5.7, 6.7)	0.2
E-cadherin % staining	14.62 (9.4, 21)	19.56 (12.3, 24.9)	6.39 (2.5, 9.6)	13.28 (5.9, 18.9	18.02 (11.2, 19.9)	0.84 (-4, 13.3)	0.7
p120 % staining	13.74 (6.8, 22.4)	15.87 (10.8, 23.6)	5.43 (3.4, 8.5)	9.85 (9.1, 11.1)	17.82 (13.1, 19.1)	7.95 (6.4, 10.1)	0.245
Values shown are medians with IQR in parentheses, n = 14-17 dependant on no. of acceptable biopsies p values are comparing changes in cell differential (Post-Pre) between the groups using Mann-Whitney U testing							

Table 28: GMA bronchial biopsy - Epithelial exploratory outcomes assessed using immunohistochemistry staining for analytes as specified in table, median values for each treatment group before and after and median change shown, with statistical analysis on between group.

ELISA

Results from the ELISA analysis are shown below, both from single analysis and multiplex plates (tables 29-31). Several markers were effectively not detected (and are not included in the tables), including many of the interleukins (apart from those detected only on the concentrated BAL used for the 30-plex plate), TARC, MMP-12, MMP-13, MIP1alpha/beta, RANTES, IFN-gamma, MMP-1 and MMP-3. The Human TH1/TH2 7-plex plate is not shown as it did not detect significant levels in any of the wells (this itself corresponded to the other plates i.e. low levels of IFN-gamma and many of the interleukins).

Where there was overlap between the plates i.e. the same cytokine analysed on different plates e.g. MMP-9, IL-8, whilst the absolute concentrations were different, the trend i.e. higher in one group than another, decreasing/increasing post-treatment, etc, agreed between the two plates.

The only markers where the change pre to post treatment was statistically significantly different ($p<0.05$) between the two groups, were YKL-40, IL-1Ra and MIG (see discussion for the limitations in interpreting these results). MMP-9 will also be discussed – whilst the reduction seen in the active treatment group was not statistically significant compared to control, it was consistent across two different ELISA plates.

	Placebo			KGF			P Value
	Pre-drug	Post-drug	Change	Pre-drug	Post-drug	Change	
MMP-2	261.9 (175.7, 735.1)	263.4 (215.6, 305.0)	63.1 (-552.1, 190.4)	383.9 (221.9, 802)	451.6 (287.2, 705.6)	-26.5 (-196.7, 26.1)	0.45
MMP-7	1750 (1516.2, 2756)	1567.1 (1361, 2060.4)	8.5 (-1063.3, 444.3)	1463.3 (740.7, 4162.3)	2655.1 (634.8, 4310.1)	-266 (-1372.3, 917.7)	0.94
MMP-8	282.6 (137.4, 852.2)	477.9 (136.8, 761.8)	53.8 (-564.7, 447)	649.3 (352.6, 1872.6)	507.5 (0, 1726.3)	-479.8 (-1882.3, -239.2)	0.10
MMP-9	1326.4 (694.8, 3466.1)	1633.4 (663.7, 3351.8)	264.5 (-1487.4, 2293.7)	3001.6 (1348.5, 11,234)	2276.6 (536.2, 3933.1)	-2194.9 (-7424.2, 281)	0.11
All values shown are pg/ml Values shown are medians with IQR in parentheses p values are comparing changes in cell differential (Post-Pre) between the groups using Mann-Whitney U testing MMP-1/3 data not shown as not detected							

Table 29: ELISA: Fluorokine MAP Multiplex Human MMP Panel Multiplex plate

	Placebo			KGF			P Value
	Pre-drug	Post-drug	Change	Pre-drug	Post-drug	Change	
YKL-40 ng/ml	11.72 (7.5, 20.0)	10.5 (7.9, 11.6)	-2.73 (-6.8, 1.1)	8.19 (6.7, 10.6)	10.19 (8.0, 12.6)	1.63 (-0.1, 5.6)	0.02
MPO ng/ml	3 (1.9, 4.2)	2.66 (2.4, 9.2)	0.76 (-0.5, 5.2)	5.53 (4.6, 9.0)	3.96 (2.5, 15.2)	-2.21 (-6.5, -0,7)	0.11
MMP-9 pg/ml	440.2 (257.8, 7548.5)	519 (198.1, 1017)	100.1 (-196, 642.6)	1182.5 (487.6, 2280)	772.2 (238.9, 1214)	-606.4 (-813.1, -34.7)	0.15
GM-CSF	3.12 (3.1, 3.3)	3.24 (3.1, 5.7)	0.01 (-0.03, 2.4)	3.12 (2.9, 3.4)	3.22 (3, 3.9)	0.24 (-0.1, 0.5)	0.54
Histamine ng/ml	0.36 (0.2, 0.9)	0.56 (0, 1.0)	0.17 (-0.2, 1.0)	0.94 (0.8, 1.4)	0.65 (0.4, 1.1)	-0.14 (-1.1, 0.26)	0.41
Tryptase ng/ml	0.39 (0.3, 0.5)	0.46 (0.4, 0.5)	0.08 (-0.0, 0.1)	0.52 (0.4, 1.0)	0.53 (0.4, 0.7)	-0.1 (-0.4, 0.2)	0.94
sICAM-1 ng/ml	30.19 (17.8, 36.0)	36.7 (16.8, 58.9)	9.3 (-3.4, 23.0)	26.18 (11.8, 36.3)	25.11 (14.5, 61.6)	2.54 (-2.6, 15.8)	0.65
ECP ng/ml	0 (0, 0.4)	0.5 (0, 0.9)	0.5 (0, 0.9)	4.3 (3, 5.5)	1.3 (0.2, 5.3)	-1.9 (-4.5, 1.6)	0.41
α2-macroglobulin ng/ml	11.9 (4.6, 46.0)	16.6 (15.2, 23.0)	0.1 (-29.7, 10.0)	33.7 (17.3, 69.4)	60.6 (12.7, 171)	10.5 (-8.2, 17)	0.23
Opn pg/ml	592.85 (500.7, 1669)	837.3 (412.3, 1350)	-68.8 (-350, 55.5)	1314 (1143, 2688)	1217.5 (738.2, 1912)	-389.2 (-811, 185)	0.26
TIMP pg/ml	2310 (2174, 4974)	2720 (2091, 4330)	-23.8 (-2186, 778)	5913 (2607, 10,220)	4613 (2067, 15686)	-2152.5 (-6294, 398)	0.50
GRO-alpha pg/ml	806.25 (505.7, 1740)	921.4 (734.5, 1557)	279.1 (-804.6, 607)	1114.5 (841.1, 1522)	968.1 (552.6, 1412)	-247.25 -603.8, -64)	0.53
Eotaxin pg/ml	8.72 (5.9, 22.7)	10.91 (0, 15.4)	-1.08 (-2.6, 0.11)	2.16 (0, 18.3)	3.58 (0, 10.0)	0 (-6.2, 0)	0.88
RANTES pg/ml	0.72 (0, 9.7)	2.45 (0, 6.9)	0.85 (0, 4.7)	3.58 (0, 18.8)	4.27 (0.6, 7.2)	-0.93 (-11.7, 3.62)	0.6
ENA-78 pg/ml	63.72 (23.2, 84.1)	39.99 (23.5, 58.7)	-10.41 (-38.6, 11.7)	38.47 (5.2, 51.5)	29.02 (0, 45.5)	-6.12 (-15.2, 0)	0.66
IL-8 pg/ml	8.39 (3.1, 20.6)	13.9 (10.2, 22.6)	7.08 (-2.9, 14.2)	16.61 (9.7, 37.2)	19.44 (8.2, 29.4)	-8.78 (-16.3, 8.2)	0.36
Values shown are medians with IQR in parentheses p values are comparing changes in cell differential (Post-Pre) between the groups using Mann-Whitney U testing							

Table 30: ELISA results - single marker analysis

	Placebo			KGF			P Value
	Pre-drug	Post-drug	Change	Pre-drug	Post-drug	Change	
VGF	4.29 (2.6, 5.5)	4.89 (3.3, 9.2)	0.92 (0.2, 2.8)	4.24 (3.6, 4.5)	4.44 (3.1, 5.6)	-0.1 (-2.4, 1.6)	0.40
EGF	0.28 (0.1, 0.4)	0.8 (0.4, 1.4)	0.33 (-0.4, 0.5)	0.64 (0.4. 1.4)	0.79 (0.3, 3.0)	-0.26 (-0.6, 0)	0.33
HGF	12.02 (8.3, 14.4)	20.7 (13.6, 40.1)	4.74 (-2.0, 34.4)	13.66 (9.7, 27.0)	16.82 (13.7, 40.9)	5.54 (-1.1, 11.6)	0.76
FGF-basic	0.3 (0.3, 0.4)	0.7 (0.3, 1.4)	0.15 (-0.1, 1.0)	0.3 (0.2, 0.6)	0.42 (0.2, 0.5)	0.0 (-0.1, 0.2)	0.29
IFN-alpha	2.77 (2.7, 2.8)	2.9 (2.7, 5.2)	0.13 (0, 1.7)	2.82 (2.7, 3.1)	2.9 (2.7, 3.6)	0.2 (-0.1, 0.5)	0.29
IL-6	2 (1.7, 2.3)	3.3 (2.6, 4.6)	0.79 (0.3, 2.5)	2.57 (1.7, 2.9)	2.21 (2.0, 2.7)	-0.39 (-0.7, 0.6)	0.10
IL-12	3.08 (2.8, 3.6)	3.15 (2.9, 5.1)	0.23 (0.1, 0.9)	3.11 (2.3, 6.3)	3.86 (2.0, 4.2)	0.69 (-0.6, 1.0)	0.69
IL-13	3.46 (3.4, 3.7)	3.59 (3.4, 6.2)	0.17 (-0.1, 1.7)	3.43 (3.3, 3.7)	3.42 (3.3, 4.3)	0.1 (-0.1, 0.7)	0.38
IL-15	0.77 (0.3, 1.0)	0.79 (0.5, 2.9)	0 (-0.3, 0.6)	1.23 (0.7, 2.9)	3.82 (1.9, 4.8)	1.22 (0, 1.7)	0.38
Eotaxin	0.59 (0.34, 0.98)	1.09 (0.75, 1.27)	-0.23 (-0.5, 0.8)	0.8 (0.8, 2.5)	0.91 (0, 2.3)	-0.77 (-0.8, 0.3)	0.69
MCP-1	8.21 (6.9, 24.8)	12.1 (9, 14.4)	4.01 (-14.4, 5.9)	13.34 (5.1, 19.2)	13.34 (9.6, 18.2)	-1.59 (-5.3, 4.9)	0.51
IL-5	0.31 (0.3, 0.4)	0.36 (0.3, 0.6)	0.04 (0, 0.3)	0.35 (0.3, 0.4)	0.3 (0.3, 0.5)	0.04 (-0.1, .1)	0.35
IFN-gamma	1.73 (1.6, 2.1)	1.95 (1.6, 2.9)	0.21 (0, 1)	1.51 (1.3, 2)	1.82 (1.5, 2.6)	0.41 (-0.1, 0.8)	0.6
TNF-alpha	0.37 (0.3, 0.5)	0.48 (0.4, 1)	0.08 (0, 0.8)	0.38 (0.4, 0.6)	0.4 (0.4, 0.6)	0.05 (-0.1, 0.2)	0.31
IL-1Ra	13.65 (11.6, 20.5)	25.7 (16.5, 83.3)	6.31 (1.5, 20)	32.9 (24.8, 270)	23.4 (12.4, 316)	-11.65 (-332, 0.52)	0.05
IL-7	5.45 (5.2, 6.3)	5.8 (4.6, 8.5)	-0.05 (-0.5, 2)	5.1 (4.6, 5.8)	5.2 (4.3, 6.8)	0.48 (-1.1, 1)	0.9
MIG	2.07 (0.4, 5.9)	1.89 (0.3, 5.4)	-0.36 (-1.2, 1.9)	7.64 (0.4, 93.3)	10.16 (2.9, 132.2)	5.61 (-0.2, 69.5)	0.03
IL-4	2.59 (2.6, 2.9)	2.67 (2.5, 4.8)	-0.7 (-0.1, 1.7)	2.56 (2.5, 2.8)	2.57 (2.3, 3.6)	0.08 (-0.3, 0.7)	0.69
IL-8	13 (10, 51.4)	24.51 (15, 69.2)	8.94 (-1.5, 21.9)	30.6 (19.8, 113.5)	38.7 (12.8, 86.8)	-15.1 (-93.7, 2.6)	0.09
IP-10	26 (14.1, 31.3)	30.4 (21.1, 59.8)	7.65 (-1.5, 22.8)	38.4(22.8, 190.4)	71.1 (30.9 148.9)	1.89 (-10.8, 20.2)	0.63
GM-CSF	3.12 (3, 3.3)	3.21 (3, 5.8)	0.01 (0, 2.4)	3.09 (2.9, 3.6)	3.22 (2.9, 4.3)	0.24 (-0.5, 0.7)	0.54

All values shown are pg/ml, Values shown are medians with IQR in parentheses

p values are comparing changes in cell differential (Post-Pre) between the groups using Mann-Whitney U testing

IL-10, MIP-1A/B not shown as minimal levels detected

Table 31: Multiple ELISA plate - Luminex Cytokine Human 30-plex panel

Phospholipid analysis by mass spectrometry

Mass spectrometry data showed the % composition of different phosphatidylcholines, phosphatidylinositols and negatively ionising glycerophospholipids in the BAL, together with the amount per extraction of the different phosphatidylcholine components (nmoles), in both groups before and after treatment (see table below - Table 32: Phospholipid analysis in BAL by mass spectrometry). T-testing was used to compare before and after treatment, and there were no significant changes in any of the components, in either group.

	Placebo			KGF		
% composition by component	Pre-drug	Post-drug	P value	Pre-drug	Post-drug	P value
Phosphatidylcholine						
LPC16:0	1.42	0.82		0.78	0.93	
PC14:0/16:0	8.82	9.25		8.86	8.73	
PC16:0a/16:0	2.16	2.22		2.26	2.16	
PC14:1/18:0	11.82	12.11	All NS	10.74	10.65	All NS
PC16:0/16:0	56.81	56.96		55.88	56.68	
PC18:2/16:0	4.71	4.54		5.06	5.27	
PC18:1/16:0	10.87	10.87		12.42	11.65	
PC18:0/16:0	1.6	1.55		1.81	1.76	
PC18:2/18:0	1.79	1.66		2.17	2.16	
Glycerophospholipids						
LPA16:0	28.28	16.91		19.02	20.14	
LPG18:1	8.08	6.17		6.53	6.82	
PA14:0/14:0	3.02	3.07		4.21	5.13	
PG16:0/18:2	5.57	5.68	All NS	5.1	6.84	All NS
PS16:0a/18:1	1.96	2.81		5.51	5.47	
PG16:0/18:1	21.59	22.73		19.74	19.82	
PS16:0a/18:0	3.09	2.24		3.11	5	
PG18:1/18:1	10.82	10.21		9.88	11.07	
PG18:0/18:1	8.78	9.27		9.36	8.42	
Phosphatidylinositol						
PI14:0/14:0	6.3	5.5		4.91	6.19	
PI18:1/12:0	8.71	7.63		9.07	9.55	
PI14:0/16:0	6.58	6.64		7.17	6.40	
PI18:1/16:0	12.69	13.52	All NS	12.25	11.91	All NS
PI18:0/16:0	5.4	6.03		6.24	6.93	
PI18:2/18:0	16.63	14.17		15.47	15.97	
PI18:1/18:0	14.66	14.8		12.97	13.54	
PI18:0/20:4	7.58	7.99		7.4	8.23	
Amount per extraction(nmol)						
LPC16:0	2.6	2.13			2.11	
PC14:0/16:0	11.48	10.5		8.9	9.37	
PC16:0a/16:0	2.89	2.56		2.29	2.34	
PC14:1/18:0	15.9	14.5		11.25	11.44	
PC16:0/16:0	73.92	66.84	All NS	57.66	61.92	All NS
PC18:2/16:0	6.03	5.17		5.29	5.82	
PC18:1/16:0	14.15	12.79		13.06	12.55	
PC18:0/16:0	2.03	1.81		1.87	1.86	
PC18:2/18:0	2.26	1.89		2.3	2.25	
Values shown are medians (IQR), p values comparing changes in (Post-Pre) within groups using t-testing, NS= non-significant						

Table 32: Phospholipid analysis in BAL by mass spectrometry

Discussion – Exploratory outcomes

As with the pre-specified biological outcomes, the exploratory results are largely disappointing, failing to show a clear effect from KGF treatment. Clearly the same general explanations can still apply here i.e. this may be due to lack of effect of KGF, incorrect time-point, insufficient numbers, insufficient treatment length or specific limitations from the method of analysis.

A discussion of every marker analysed, and interpretation of the lack of statistically significant findings in this trial, would not be helpful, as in part the above arguments regarding lack of power, etc. would repeatedly apply. There were some significant results, and whilst these may have occurred through chance they will now be discussed as to biological plausibility, together with important negative findings/areas in which we expected to see effect, but did not.

GMA immunohistochemistry – lack of effect on TGF-β

TGF-β2 staining was hoped to be of interest, as mentioned in the Introduction and Methods chapter of this section, increased expression of TGF-β1[105, 106, 318, 488] and TGF-β2[109, 488] has been reported in asthmatic biopsies, and *in vitro* studies had shown possible antagonistic effects of KGF and TGF-β1[111, 112, 117].

Levels of staining were relatively low however – one possible explanation for this is that whilst TGF-β can be secreted by almost all immune cells[489], a major source in the asthmatic epithelium/airway is eosinophils[105], and as seen in the previous chapter, the biopsies taken had relatively low levels of epithelial eosinophilia.

We also did not attempt to stain for the other TGF-β isoforms i.e. 1 and 3, which may or may not have shown differences.

ELISA - Statistically significant findings

Whilst the limitations of multiple analysis/lack of Bonferroni correction apply with the large number of molecules analysed by ELISA as stated above, potential effects of KGF treatment were seen with YKL-40, IL-1R and MIG, and these will be briefly discussed.

YKL-40 is one of the 'chitinase-like proteins', so called as they can bind to chitin (a biopolymer in the walls of fungi and helminths, together with insects and crustaceans) but lack the ability to degrade chitin seen with 'chitinases'. They may have a role in parasitic defence, and have been shown to protect against oxidant-induced injury and augment TH2 inflammation, although their full biological function is still being elucidated[490]. In asthmatics as compared to healthy controls, ykl-40 levels are raised in the serum[491, 492] and lungs[491, 493] and may correlate

with disease severity[491]. Interestingly, other studies have found correlation of serum ykl-40 levels with poor asthma control[493, 494] and atopy[494], which would fit in with our study population, and that levels *reduced* after treatment of asthma[493]. It is disappointing therefore, that whilst the change in ykl-40 BAL levels in the KGF group compared to the placebo group was statistically significant, median levels appeared to *increase* post-treatment in the KGF group, and decrease in the placebo group – the opposite of what we might expect if the treatment was leading to reduced inflammation (although without further clarity on the role of ykl-40 it is difficult to interpret this fully).

Interleukin 1 receptor antagonist (IL-1Ra) binds non-productively to the IL-1 receptor, IL-1R. IL-1R is the receptor for IL-1α and IL-1β, binding of which can cause a wide range of effects but with relevance to asthma, IL-1β may play a role in airway hyper-responsiveness of asthmatic smooth muscle[495]. IL-1β production is increased in the submucosa, epithelium and BAL from asthmatics[496, 497], can induce eotaxin which itself is an eosinophil chemoattractant[498], and promotes recruitment of eosinophils[499]. IL-R knockout mice had reduced eosinophilic inflammation in response to ovalbumin sensitisation[500].Polymorphisms in the IL-Ra gene have been associated with increased risk of asthma[501], and higher levels of IL-1Ra have been found in BAL fluid from asthmatics (vs healthy controls)[502]. Perhaps most relevant is a study by Shyamsundar et al[503], looking at the effect of KGF in attenuating a human model of ARDS – KGF increased BAL concentrations of Il-1Ra (NB in healthy controls). Again frustratingly, our results, whilst significant, did not quite show the expected increase in the KGF treatment group, rather a median *decrease* in IL-1Ra levels. Our detection levels of IL-1Ra were significantly lower than reported by others (3,000-5,000 pg/mln[503], and 2000 pg/ml[502] vs our study showing 13-32 pg/ml), and as has already been discussed, our bronchoscopy was performed at a significantly later time point when the biological effects may have resolved.

Monokine induced by interferon-gamma (MIG), also known as CXCL9, is a chemoattractant for activated T lymphocytes[504], signalling through the CXCR3 chemokine receptor. It can be expressed by bronchial epithelial cells[505], and in animal models can reduce eosinophil recruitment[506]. Some studies have found increased levels of MIG in BAL fluid from asthmatics compared to healthy controls[502, 507], or in mild but not severe asthma[508], whilst in a paediatric study, serum levels were lower which was suggested to reflect a diminished antagonizing effect of Th2 cytokine production[509]. As the exact function is still not clear, and studies have not looked at treatment effects on BAL MIG, it is difficult to know what to 'expect', but as with IL-1Ra, our detected levels in BAL were an order of magnitude below that reported by

other studies[502], and whilst there was an apparently statistically significant greater decrease in BAL levels of MIG after treatment in the KGF group, numbers were small and may not reflect a true effect.

Lastly, as part of the inflammatory remodelling in asthma, matrix metalloproteinases (MMPs) are thought to be continually degrading the extracellular matrix (ECM)[346]. MMP-9 levels are higher in BAL from asthma patients than healthy controls[343, 344, 346], but were not affected by ICS treatment[346]. The KGF treated group had a non-significant trend towards decreased MMP-9 levels post treatment, and whilst this may be evidence of a beneficial effect, again this is the opposite of that reported by Shyamsundar et al (in healthy controls)[503].

Phospholipids – lack of evidence of effect

As discussed in the methods and introduction part to this section, KGF has been shown to have effects both on surfactant proteins *in vitro*[161, 165, 166, 168] and *in vivo*[503], and on phosphatidylcholine in vitro[167, 170]. However lung surfactant phospholipid is actually a complex mixture of individual molecular species, with differing combinations of fatty acyl moieties on a glycerophosphate backbone[510]. Phosphatidylcholines are the principal component of this, and dipalmitoyl PC (PC16.0/16.0) appears to be critical for the surface tension lowering effect of surfactant[314, 510], with decreased levels of dipalmitoyl PC found in sputum but not BALF from asthmatics[511]. Sadly there was no significant changes in composition or concentration of any of the different phospholipid molecular species, again possibly reflecting incorrect timepoint.

Future work

It is difficult to know how much interpretation to read into the above results/lack of, given the comments regarding time-points etc. Whilst a future study may wish to reduce exploratory outcomes and/or focus perhaps on a more select group i.e. not attempting to detect interleukins, just looking at MMP-9, ykl-40, MIG, IL-1Ra, etc, it would depend on the population/intent/resources for said future work.

Safety and Efficacy of parenteral KGF in moderate asthma subjects – Adverse events

Adverse events

Adverse events were seen in both groups (Table 33: Adverse events by treatment group in clinical study) but were significantly higher in the KGF group, due to increased incidence of dermatological and gastroenterological events, as is already known with this drug from trials in other patient groups[512]. The median duration was 4 days for all adverse events (2 days for dermatological AE, and 7 days for gastroenterological AE). All adverse events were mild or moderate in severity. 3 asthma exacerbations occurred during the trial, one in the treatment arm (starting before drug administration), and 2 in the placebo arm (post-bronchoscopy). There was no statistically significant difference between the groups and whilst this exacerbation rate may seem high for a comparatively short trial, the patient population chosen (uncontrolled asthmatics, slightly elevated eNO levels) were at risk of this complication.

	Placebo	KGF	P value
Number of patients experiencing any AE with either dose	5/10	11/11	0.01
Dermatological AE with either dose	1/10	8/11	0.05
Gastroenterological AE with either dose	3/10	9/11	0.02
Respiratory AE (asthma exacerbation) during trial	2/10	1/11	0.486
Other AE with either dose	3/10	2/11	0.535

Table 33: Adverse events by treatment group in clinical study

Implications for future work

Whilst the adverse events listed above are in keeping with the effects of the drug, and did not lead to withdrawal of subjects, the high incidence is of concern, both for future studies and for the potential viability of this particular drug as a treatment option. KGF has been administered in nebulised form in animal studies[513], but not in any human trials, and whilst this might reduce dermatological effects, it may not affect/may potentiate gastroenterological effects. One option would be nebulisers which would activate only on inspiration and which could be modified to target the small airways (such as the AKITA device used in Section D of this thesis, or an I-neb®), together with vigorous rinsing to minimise oropharyngeal deposition/side-effects.

Clinical study - overall discussion/conclusions

In conclusion, this small pilot study provides important proof-of-concept evidence for the therapeutic role in asthma, of compounds that directly improve epithelial permeability. This opens up a new area of therapeutics in asthma, and may apply to other areas of respiratory disease.

The main effects seem to be on airway hyperresponsiveness and quality of life, although it is hoped that with larger, future studies, effects would also be seen on day-to-day asthma control. Biological effects were not seen in our studies, but are likely given other studies in the field, and with increased numbers/altered timing of sampling in future studies, should also be seen and would provide further proof of principle.

Adverse effects were common though, and may require alternate methods of delivery of drug. Lastly, only 21 patients (56%) were randomised following screening, and 2 of these patients withdrew during the study due to exacerbations, so the feasibility of recruiting larger numbers of patients and eligibility requirements should be considered for future studies – this might be achieved by 'streamlining' future studies i.e. cutting down on numbers/types of assessment to make them less arduous/more attractive to volunteers, and make it less likely that an exacerbation will occur during the trial.

Another possibility might be splitting future work into several smaller trials e.g. performing a small study in healthy volunteers, of nebulized KGF with bronchoscopy thereafter to establish biological effect and safety of administration, before proceeding to asthmatic patients (see later overall discussion at end of thesis).

Section D: *In vitro* Studies – Results and Discussion

Results

Patient demographics (all experiments)

Baseline characteristics for the two groups are shown below in table 34.The asthmatic group was significantly younger, and (in keeping with the obstructive nature of the disease) had statistically significantly lower FEV_1% predicted and FEV_1/FVC %). The asthmatics had a mixture of severity, classified below according to the level of treatment they were on (as per BTS guidelines, [19]). Not all cultures were used in all experiments, and therefore n numbers are quoted for each section below.

	Asthmatic cohort (n = 15)	Healthy cohort (n = 21)	P value
Sex (% Female)	66%	61.9%	
Age	42.5 (11.5)	29.1 (11.6)	0.002
FEV_1 % predicted	75.0 (26.2)	101.4 (15.7)	0.001
FEV_1/FVC %	68.1 (18.0)	82.9 (7.5)	0.003
BTS Stage	Stage 2: n = 1 Stage 3: n = 4 Stage 4: n= 6 Stage 5: n = 3	N/A	
Values shown are means with standard deviation in parentheses P values calculated using t-test, equal variances assumed			

Table 34: Baseline characteristics for two groups of subjects used in *in vitro* studies

Wound analysis data

Single image versus whole wound imaging

Wound image analysis was initially performed on single images taken at a transverse section of the wound, with wound area calculation as described previously. However it was later decided to move to imaging of the entire wound and reconstruction of the images, due to 2 reasons:

a) Different areas of the wounds were sometimes observed to move at differing speeds.

The compression experiment required initial imaging of the wound, removal of the cell plate to set up in the compression apparatus, and then subsequent reimaging after compression and subsequently. This meant that it could not be guaranteed that exactly the same section of the wound would be imaged (if imaging a section only) and thus it would be better to image the entire wound.

It is important to note though, that the initial data from the 'single image' wound analysis was similar to the data generated from the 'entire wound' – as shown below (Figure 30: Graphs comparing single image vs. whole wound imaging in estimating wound closure), illustrating the data generated by both single images and whole wound images for a collection of experiments in the same donor cultures (n= 5, all asthmatic).

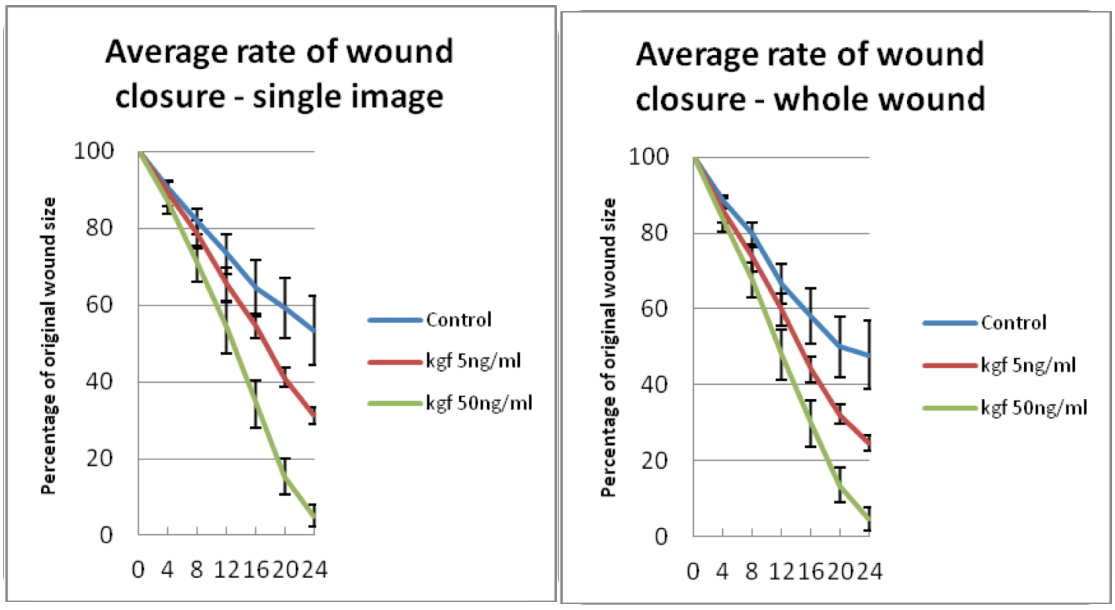


Figure 30: Graphs comparing single image vs. whole wound imaging in estimating wound closure. % wound (compared to original size wound) plotted against timecourse in hours. Statistical testing not shown. N= 5, all asthmatic

Whole wound analysis

Data is illustrated below in figure 28. Not all the cultures underwent time-lapse analysis due to equipment failure and also in initial experiments (as said above), single image analysis only was performed, and is not included in the following figures/statistical analysis. N= 12 for asthmatic cohort, n = 6 for healthy cohort.

Asthmatic vs. Healthy cohort, untreated cells

Firstly, comparing asthmatic cell cultures to healthy cell cultures in their ability to repair the wound (without additional KGF treatment), we saw similar rates of wound repair (Figure 31: Graph comparing healthy to asthmatic cell cultures in wound closure, assessed by time-lapse imaging) with no statistical difference between the groups at the 24-hour time point (p = 0.964).

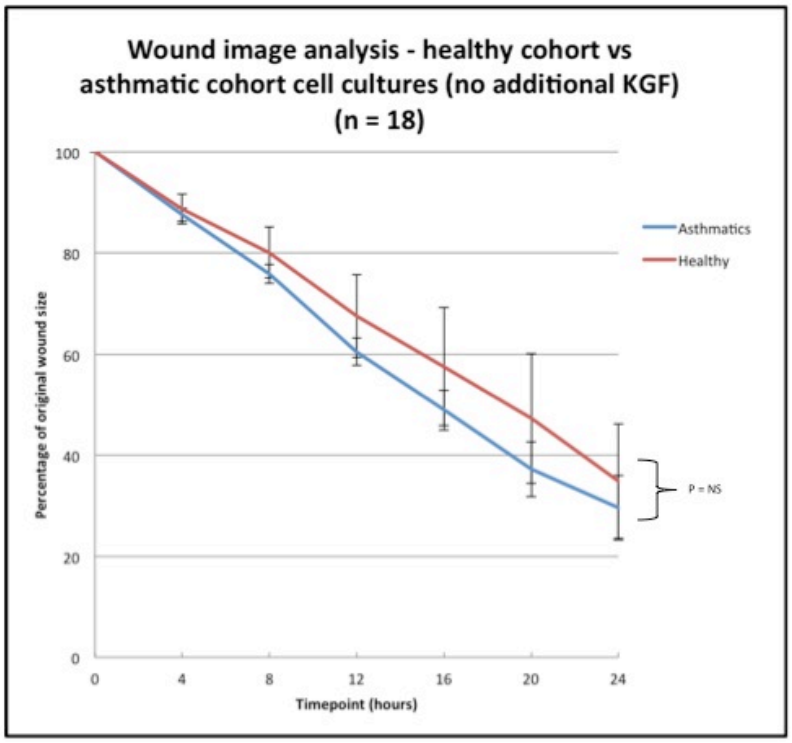


Figure 31: Graph comparing healthy to asthmatic cell cultures in wound closure, assessed by time-lapse imaging. % of original wound remaining plotted against timepoint (hours), separated into two groups, asthmatic and healthy. N= 18, 12 asthmatic and 6 healthy.

Effects of KGF

In the asthmatic cohort, KGF increased wound repair in a dose-dependent manner, this approached statistical significance at the 24-hour time point comparing control to 5ng/ml KGF ($p = 0.059$, Mann-Whitney U test), and was significant comparing control to 50ng/ml ($p = 0.000$). The difference between 5ng/ml and 50 ng/ml KGF was not significant ($p = 0.134$). (Figure 34).

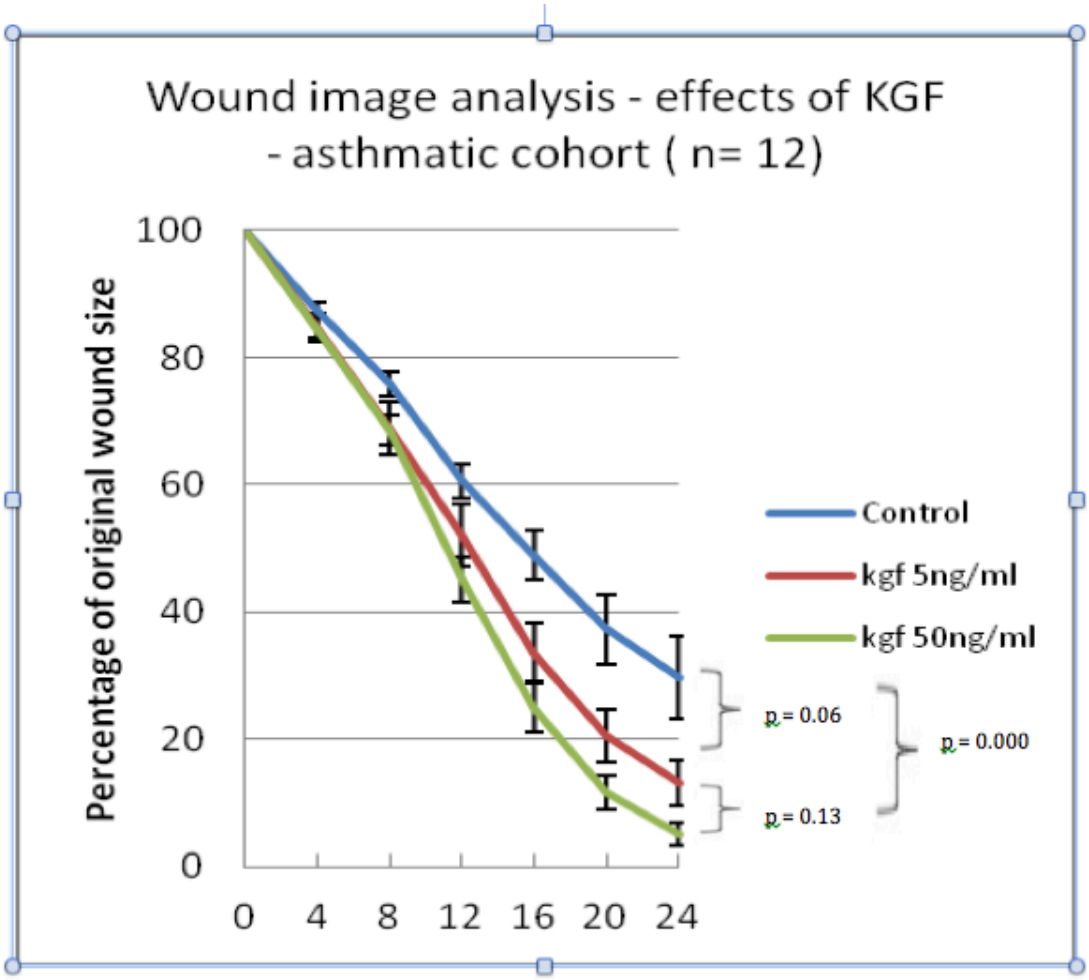


Figure 32: Graph showing effects of KGF on wound closure, asthmatic cohort. Percentage of original wound plotted against timepoint in hours. Statistical significances shown, comparing control to 5ng/ml KGF, control to 50ng/ml KGF, and comparing 5ng/ml to 50ng/ml KGF

In the healthy cohort, the improvement was less marked, with no clear difference between control and 5ng/ml KGF at 24 hours (p = 0.937, Mann-Whitney u test), and the difference between control and 50ng/ml KGF was also not significant (p = 0.180), nor was there significant difference between 5ng/ml and 50ng/ml KGF (p=0.18) although a large degree of variability was noted.

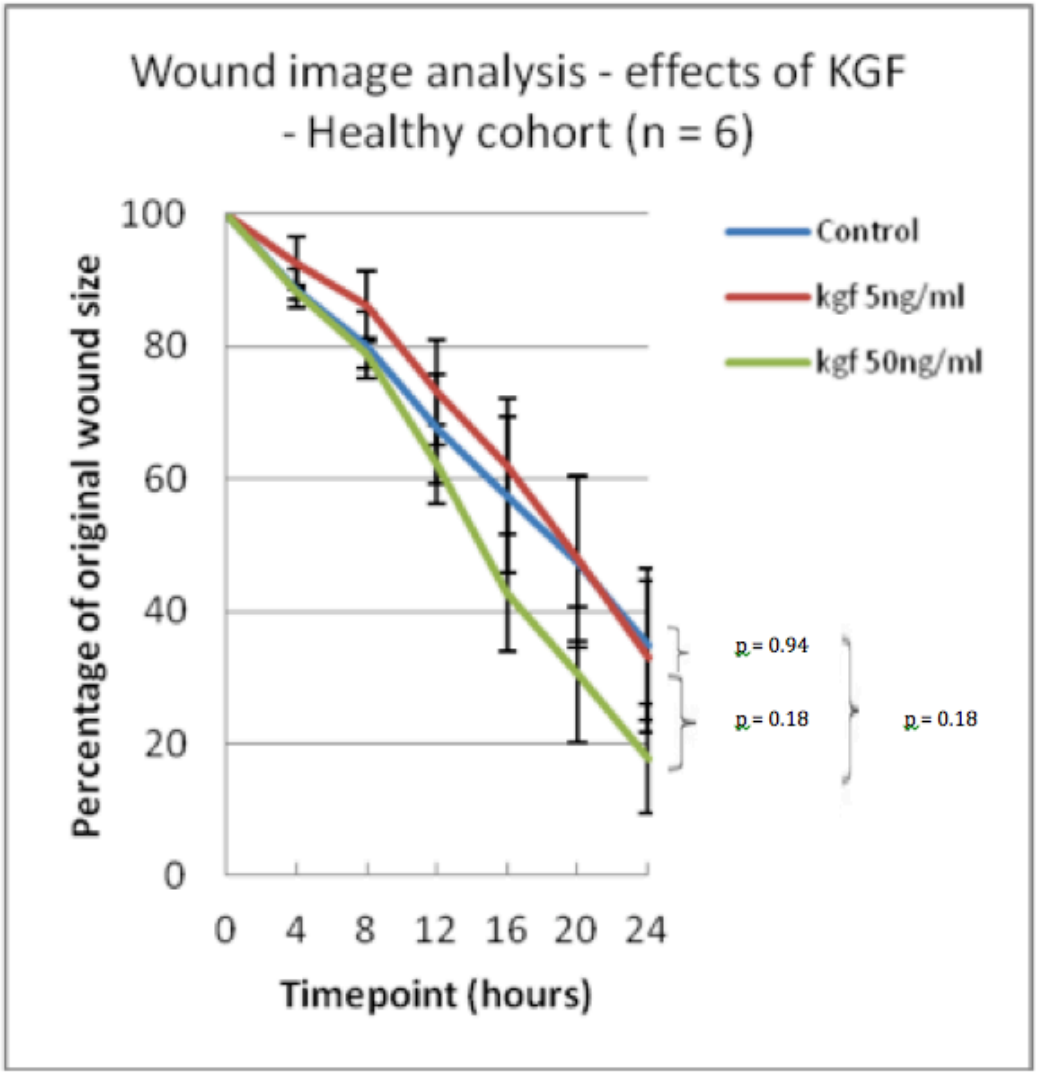


Figure 33: Graph showing effects of KGF on wound closure, healthy cohort. Percentage of original wound plotted against time-point in hours. Statistical significances shown, comparing control to 5ng/ml KGF, control to 50ng/ml KGF, and comparing 5 ng/ml to 50 ng/ml KGF

Effects of compression

Compression was only possible on 10 donor cell cultures (7 asthmatic and 3 normal). Dealing with the effects of compression alone initially, i.e. no KGF treatment, and comparing healthy versus asthmatic cultures, the results are shown below (Figure 31). Whilst in this smaller group the asthmatic cells actually seemed to do better i.e. recover faster, there was no significant difference with sham compression at the 24 hour time point between healthy and asthmatic cells ($p = 0.138$, MWU); this would be expected given the results with a larger group (not involved in compression) as shown above. Similarly, looking at the effects of 'real' compression, when comparing the healthy and asthmatic groups directly there remains no difference ($p = 0.138$, MWU). Whilst compression did seem to have a relatively greater effect on asthmatic cultures i.e. asthmatic cultures had a greater deleterious effect from compression than healthy, there was no significant difference in this 'change in wound healing with compression' between the groups ($p= 0.569$, MWU, data not shown in below figure).

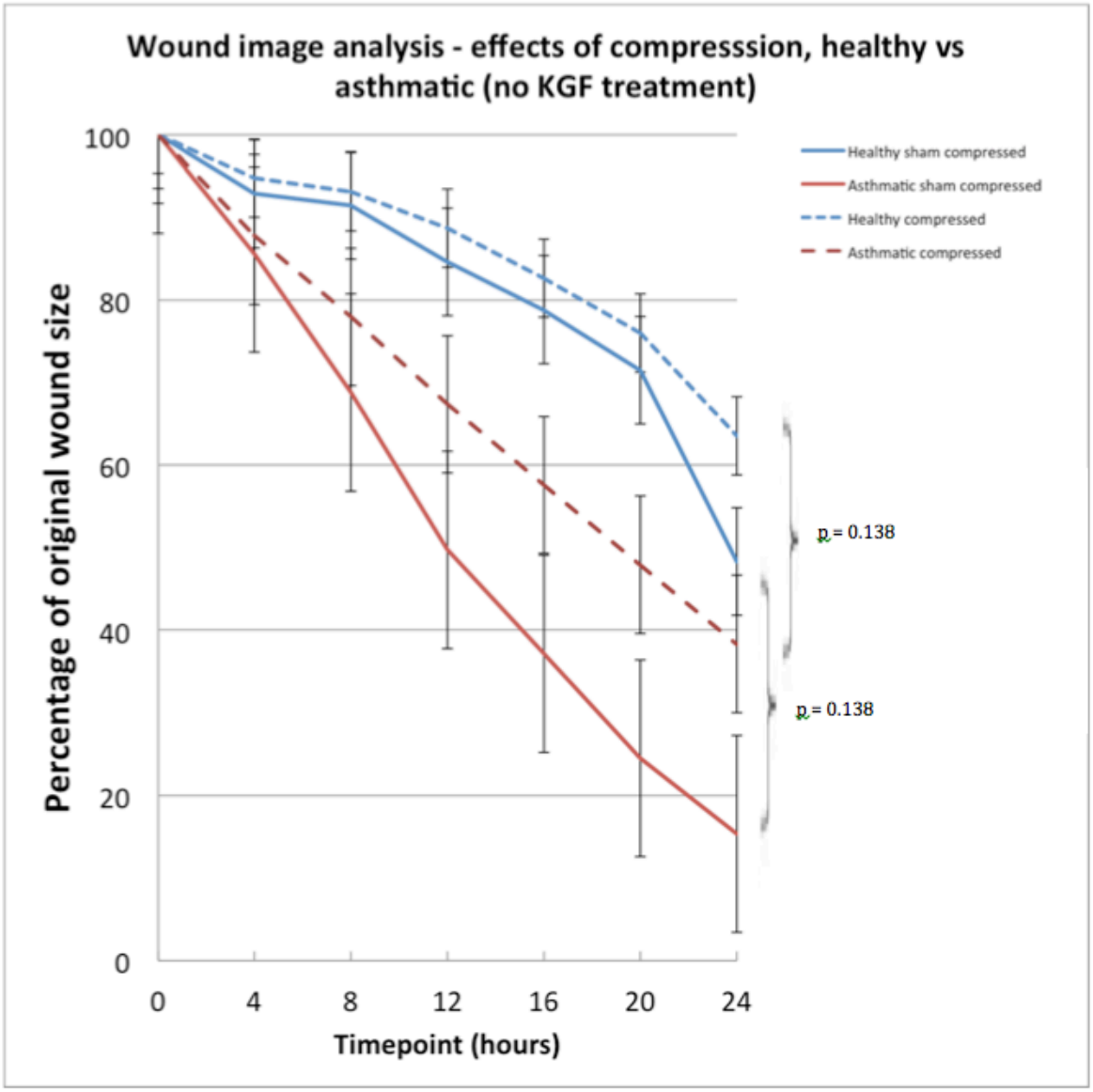


Figure 34: Wound image analysis comparing effects of compression on healthy vs. asthmatic donor cultures. Whilst the asthma cultures appeared to heal faster in this subgroup, there was no significance difference from healthy cultures, either when compressed or not (see p values above comparing healthy vs. asthmatic, for compressed and sham compressed).

Given the above, it was decided to pool the data when analysing the effects of KGF i.e. the data now reflects a mixture of asthmatic and healthy cultures (Figure 35: Graph showing the restorative effects of KGF on wound healing despite compression, pooled cell cultures from asthmatics and healthy donors).

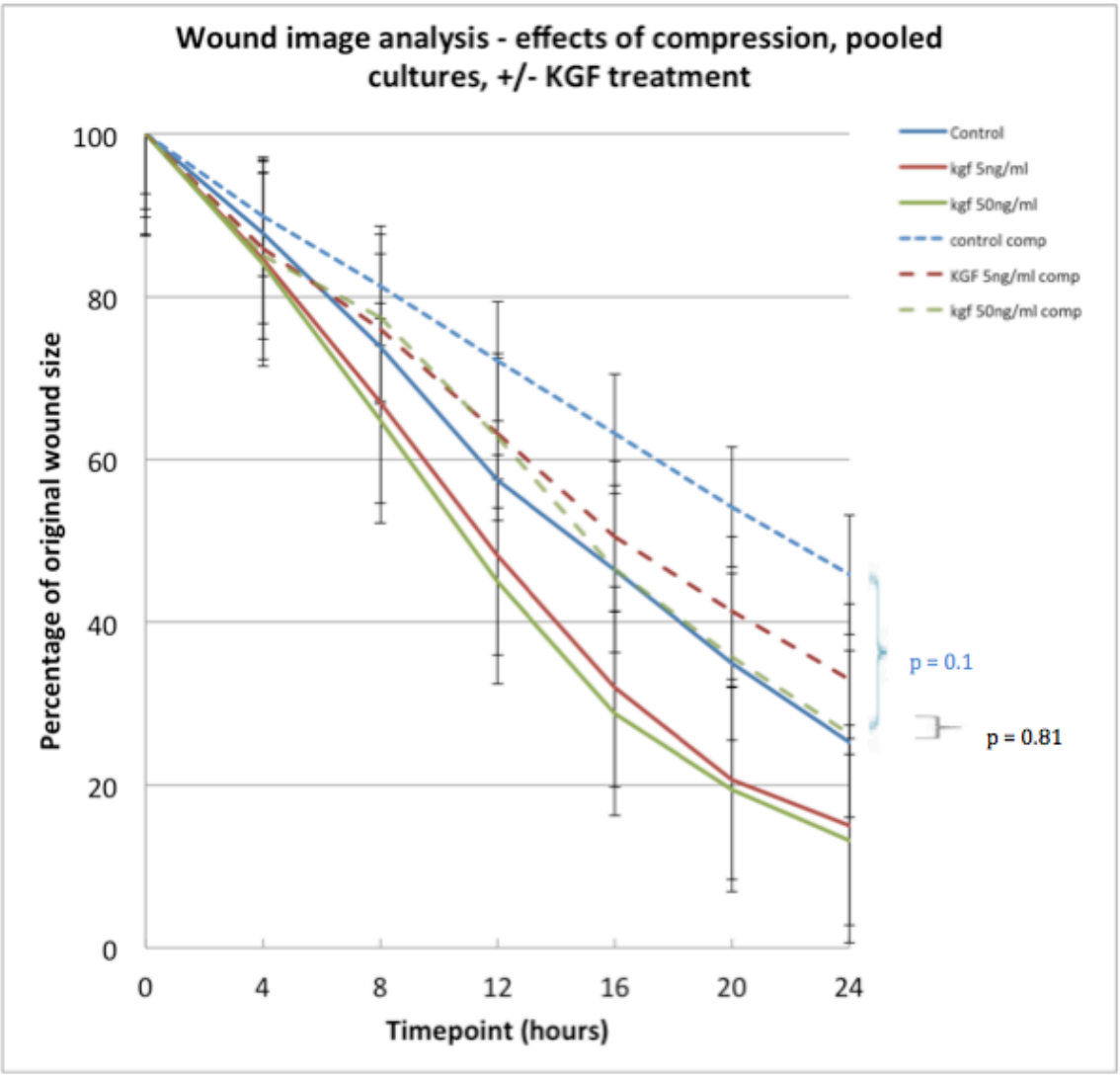


Figure 35: Graph showing the restorative effects of KGF on wound healing despite compression, pooled cell cultures from asthmatics and healthy donors. P values showing that compression approached significance for reducing wound healing vs. non compressed, p = 0.1, and that the 'compressed but KGF 50ng/ml treated group' was not statistically different from the 'non-compressed, non-KGF treated' control, p= 0.81

Looking at the non-KGF treated, control, cell cultures initially, whilst at the 24 hour time point those that were sham compressed had a mean wound area of 25.2 % (compared to baseline), the compressed cells had reduced repair, with a mean wound area of 45.9 %. This difference approached but did not reach statistical significance though (p = 0.1, Mann-Whitney U test).

As expected from the results in the non-compression experiments, treatment with KGF boosted repair – in the 'sham' compressed group, 50ng/ml KGF treatment decreased wound area to a mean of 13.18% vs. 25.2% in the control (non-KGF treated) group, this approached statistical significance (p= 0.06, MWU). 5ng/ml had a lesser effect; only decreasing wound area to a mean of 15.02%, with a similar significance compared to no KGF treatment (p=0.06, MWU).

KGF was almost completely effective in reducing the deleterious effects of compression - wound area in the compressed cultures treated with KGF 50 ng/ml was down to mean of 26.29 % , with no statistically significant difference between the 'compressed but KGF (50 ng/ml) treated' group and the 'control, sham compressed' group (p = 0.81, MWU).

TEER data

Baseline values

Transepithelial electrical resistance was measured for the all cell cultures, with n = 21 (healthy cohort) and n = 15 (asthmatic cohort). The healthy cohort had significantly higher baseline TEER readings - median values 1310 Ω for asthmatic cohort (IQR 525) and 1548 Ω for healthy cohort (IQR 681 Ω), p = 0.028, MWU (Figure 36: Baseline TEER readings (Ohms), asthmatic and healthy donor cell cultures).

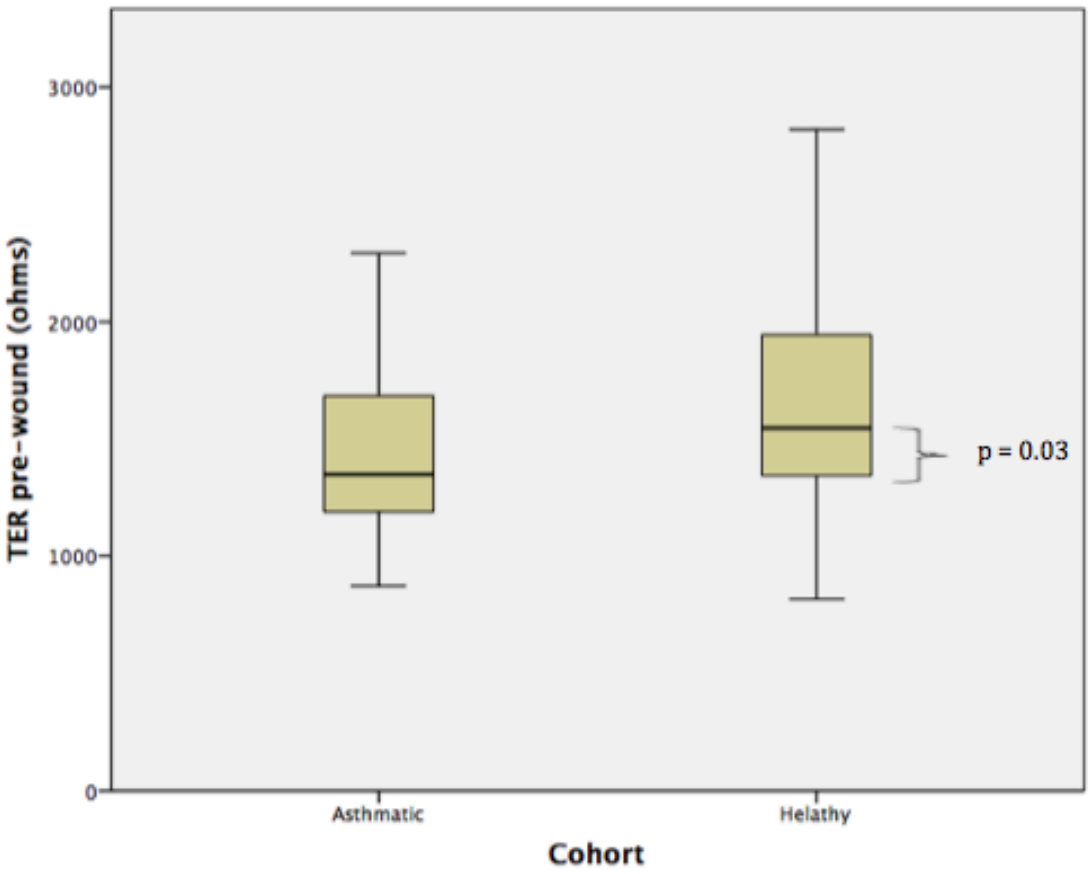


Figure 36: Baseline TEER readings (Ohms), asthmatic and healthy donor cell cultures. Healthy cultures had statistically significantly higher values than asthmatic cultures, p = 0.03, MWU.

There was no difference between the groups in the `post-wound' TEER values - median values 440 Ω for asthmatic group (IQR 66 Ω) and 460 Ω for healthy cohort (IQR 70 Ω), p = 0.112, MWU (data not shown in graph form).

24 hour time point values

At 24 hours, the TEER values for the untreated cells (i.e. no KGF) were statistically similar for both asthmatic and healthy cohorts - median 400 Ω (IQR 732 Ω) in asthmatics, median 428Ω (IQR 240 Ω) in healthy cohort, p = 0.7, MWU. Both groups had not significantly improved compared to post-wound values (p =0.733 for asthmatic cohort, p = 0.511 for healthy cohort, WSRT).

In the healthy cohort, treatment with either KGF 5 ng/ml or 50 ng/ml improved TEER values statistically significantly after 24 hours (when compared to post-wound values), to medians of 635 Ω (IQR 213) and 704 Ω (IQR 379) respectively, p = 0.000 and 0.000, WSRT. In the asthmatic cohort, treatment with KGF 5 ng/ml or 50ng/ml also increased TEER values (compared to post-wound values) to medians of 599 Ω (IQR 733 Ω) and 765 Ω (IQR 461 Ω) respectively, p values of 0.05 and 0.01, WSRT.

There was no significant difference *between* the cohorts however i.e. asthmatic vs healthy, in their TEER response to KGF at either 5 ng/ml or 50 ng/ml (p = 0.96 and 0.502 respectively, MWU).

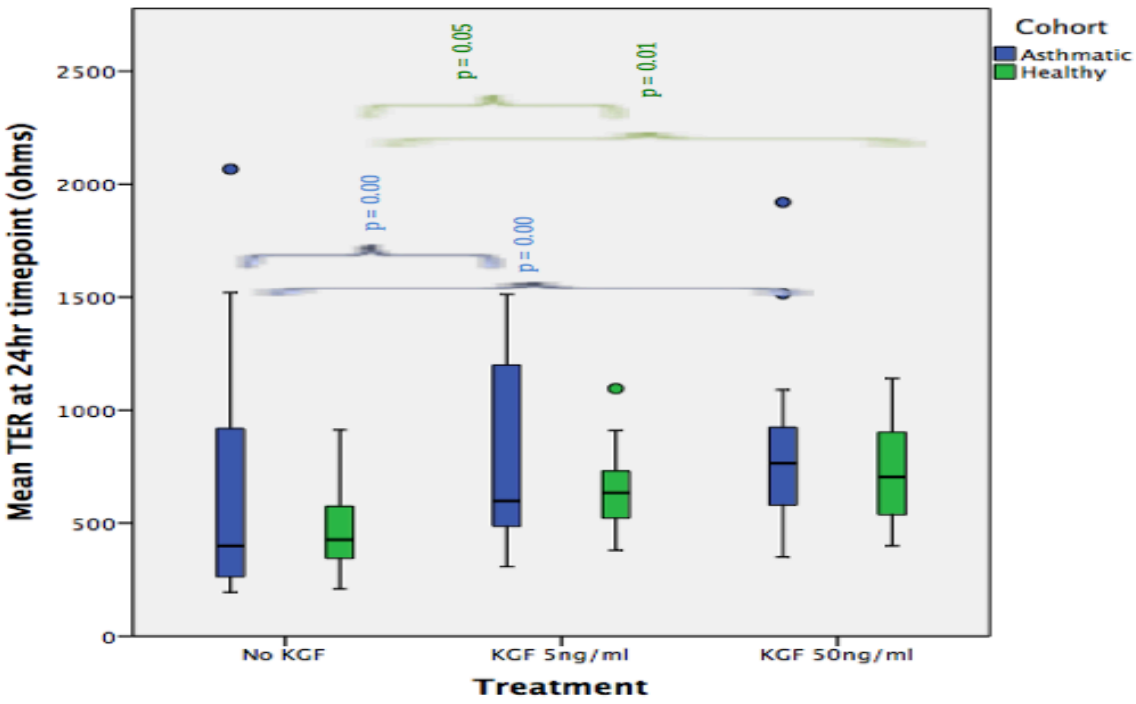


Figure 37: TER values at 24 hour time point, separated into cohorts, +/- KGF treatment. Significant differences were seen with either 5 ng/ml KGF or 50 ng/ml KGF, in either cohort, p values shown. There was no significant difference between the cohorts, p values not shown

48-hour time point values

Data is represented in graphical form in Figure 38: TER values at 48 hour time point, separated into cohorts, +/- KGF treatment. At the 48 hour time point, for the untreated cells, TER values had still not statistically significantly improved from post-wound values in either cohort, although absolute values had increased as might be expected (medians increased to 523 Ω (IQR 220 Ω) for healthy cohort, p = 0.21, and 405 Ω (IQR 724 Ω) for asthmatic cohort, p = 0.38, WSRT).

In the healthy cohort, as at 24 hours, at the 48hr time point treatment with either KGF 5ng/ml or 50ng/ml did significantly increase TER values compared to post-wound values. The untreated group had a median of 523 Ω (IQR 220 Ω) as stated above, but this increased to a median of 819 Ω (IQR 318 Ω) (p = 0.001, WSRT, compared to post-wound values) in the KGF 5ng/ml group. In the healthy, 50ng/ml KGF group at 48hrs, median TER was 830 Ω (IQR 490), (p = 0.000, WSRT, compared to post-wound values). Similarly in the asthmatic cohort, treatment with KGF 5ng/ml significantly increased TER compared to pre-wound values – whilst the untreated group as above had a median TER 405 Ω (IQR 724 Ω), in the 5mg/ml KGF group median TER was 640 Ω (IQR 705 Ω), (p = 0.02, WSRT, compared to post-wound values). Treatment with KGF 50ng/ml increased median TER to 935 Ω (IQR 480) with p = 0.003, WSRT, compared to post-wound values.

As at 24 hours, there was no significant difference *between* the asthmatic and healthy groups at this time point, either with KGF 5ng/ml treatment (p= 0.45, MWT) or KGF 50ng/ml treatment (p= 0.94. MWT).

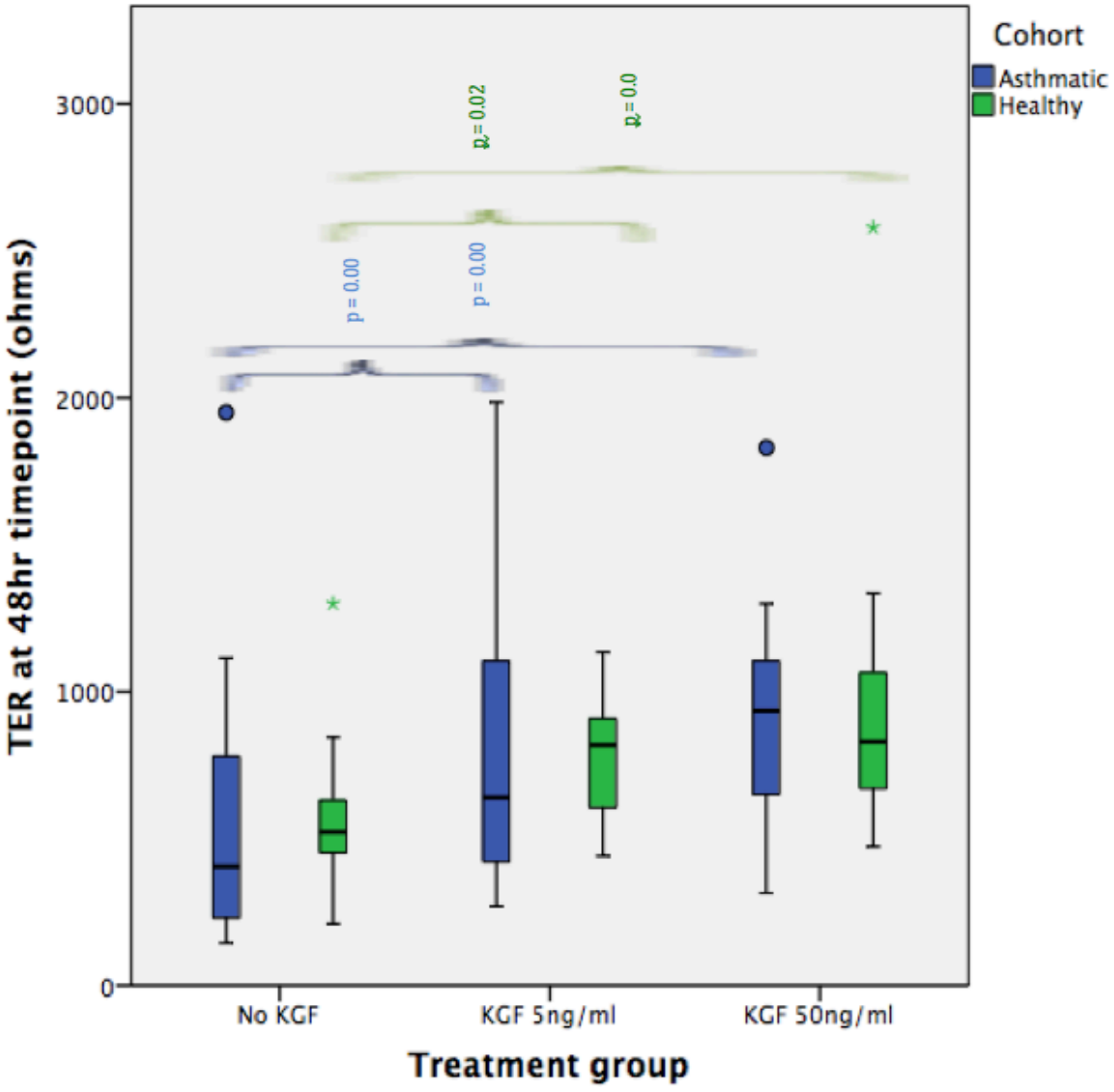


Figure 38: TER values at 48 hour time point, separated into cohorts, +/- KGF treatment. Treatment in either cohort, asthmatic or healthy, at either dose of KGF, 5ng/ml or 50ng/ml, significantly improved TEER values, p values shown. There was no significant difference between the groups however, p values not shown.

Effects of compression on TER

Compression/Sham compression was performed on n= 11 subjects, 6 from the healthy cohort and 5 from the asthmatic cohort. At baseline, TER pre-wound was similar for the compressed and sham-compressed group (medians of 1267 Ω (IQR 661 Ω) and 1313 Ω (IQR 855 Ω) respectively, p = 0.82, MWT). In this smaller group, there was not quite a significant baseline TER difference between the asthmatic and healthy cultures (as was seen in the above larger group), with medians of 1150 Ω (IQR 550 Ω) and 1490 Ω (IQR 753 Ω) respectively, p = 0.11, MWT.

TER post wound was similar between compressed and sham-compressed, p = 0.56,MWT but was by chance different between healthy and asthmatics, with median post-wound TER 425 Ω in the asthmatic cultures and 525 Ω in the healthy cultures, p = 0.02, MWT.

Effects of compression ALONE on TER, asthmatic vs. healthy.

Dealing with the effects of compression alone initially i.e. only looking at cultures which did not receive KGF treatment, in general in this small subgroup, TER recovered more slowly than the above experiments (where compression/sham-compression was not used (see data above)), and this may reflect non-optimal conditions e.g. temperature/humidity in the compression chamber. TER values are shown in the table below, and were in the main not significantly different from post wound values i.e. in either healthy or asthmatic cultures, compressed or compressed, the TER had not improved at 24 or 48 hour time-points (except for the asthmatic cultures, which were significantly worse at the 24 hour time-point compared to immediate post wound values).

Condition	TER value (Ω) 24hr time point	TER value (Ω) 48hr time point
Healthy, sham compressed	435 (155)	515 (80)
Healthy, compressed	428 (98)	475 (235)
Asthmatic, sham compressed	303 (134)	398 (281)
Asthmatic, compressed	295 (40)	505 (238)

Table 35: Non-KGF treated cultures, TER values at 24 and 48 time-points, compressed and sham compressed. There were no statistically significant differences between the groups at any time-point.

Effects of compression combined with KGF on TER, asthmatic vs healthy

Because of the smaller numbers, and the lack of a clear distinction between asthmatic and healthy cultures in the above results, it was decided to combine asthmatic and healthy results together when looking at the effects of compression on the KGF response. Data is shown below, and it can be seen that for the first 24 hours, compression appears to blunt the response to KGF treatment (Figure 39: TER values at 24 hour time point, mixed cohort, separated into compressed and sham compressed cells, +/- KGF treatment). This effect was seen in both asthmatic and healthy cultures to a similar extent (data not shown). This effect however was not statistically significant for KGF 5ng/ml i.e. comparing TER values between compressed and non-compressed, p value was 0.173, MWU test, and approached but did not reach significance for KGF 50ng/ml (p = 0.07, MWU test) either. At the 48 hour time point, the effects of compression were no longer apparent i.e. KGF seems to be just as effective whether compression took place or not.

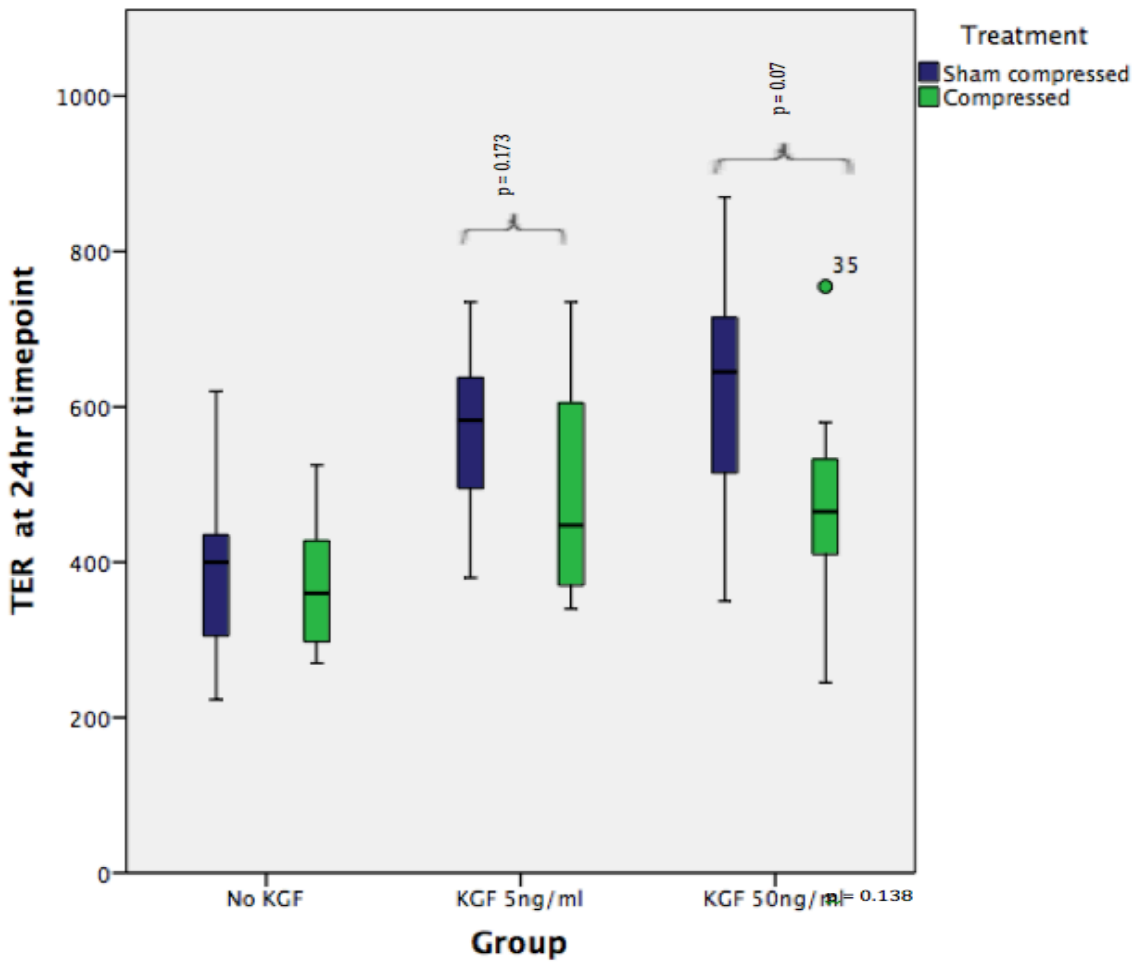


Figure 39: TER values at 24 hour time point, mixed cohort, separated into compressed and sham compressed cells, +/- KGF treatment. Compression blunts the response to KGF, however this did not reach statistical significance at 5ng/ml KGF and only approached significance at 50ng/ml KGF, p values shown.

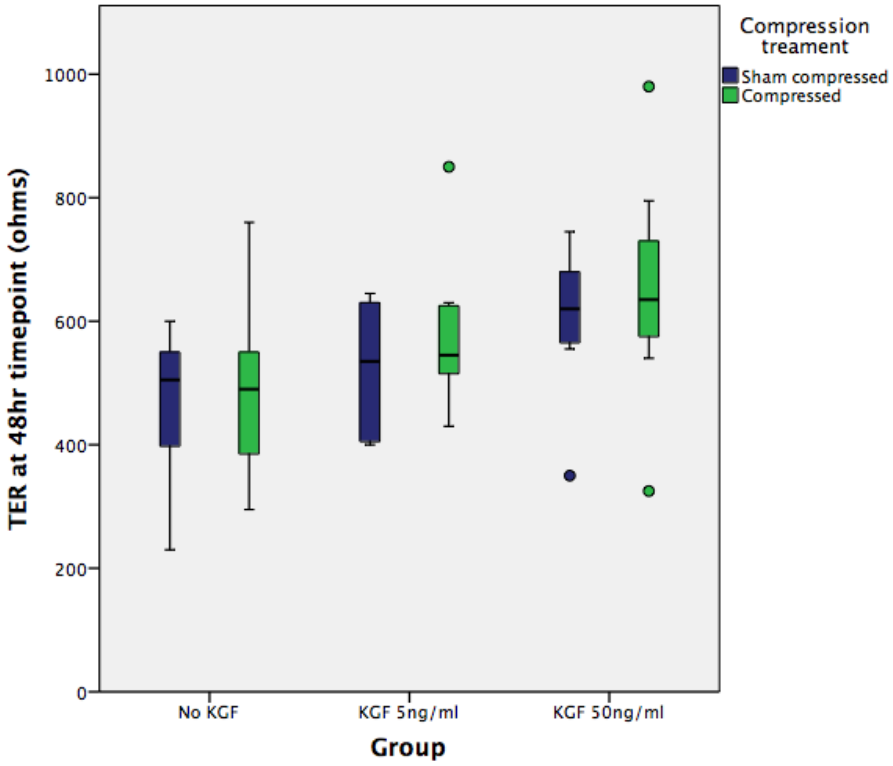


Figure 40: TER values at 48 hour time point, mixed cohort, separated into compressed and sham compressed cells, +/- KGF treatment. The effects of compression appear to have worn off, with no significant difference in the response to KGF (p values not shown)

1.1.1.1 *Assessment of permeability by FITC dextran*

Shown below are the results of the FITC dextran permeability assays, performed at the 24 (n = 14, 6 asthmatic, 8 healthy) and 48-hour time points (n=9, 3 healthy, 6 asthmatic). There was no significant difference between the asthmatic and healthy groups, at either 24 or 48 hours, at any KGF concentration (0, 5 or 50mg/ml), and therefore the results have been pooled. Furthermore, because of significant variability between donors, it was decided to normalise the values compared to control cells i.e. the graphs plot the FITC dextran concentration measured in the basal media (having permeated the epithelial barrier), as a *percentage of the values for control* (untreated) cells.

Variability was large, and whilst KGF treatment appeared to reduce the permeability to FITC (i.e. reduced concentration in the basal media), no statistical differences were seen at 24 hours. At 48 hours however, both groups were significantly better (less permeable) than baseline (with p = 0.05 for 50mg/ml vs. control cells, and 0.000 in 5mg/ml group vs. control).

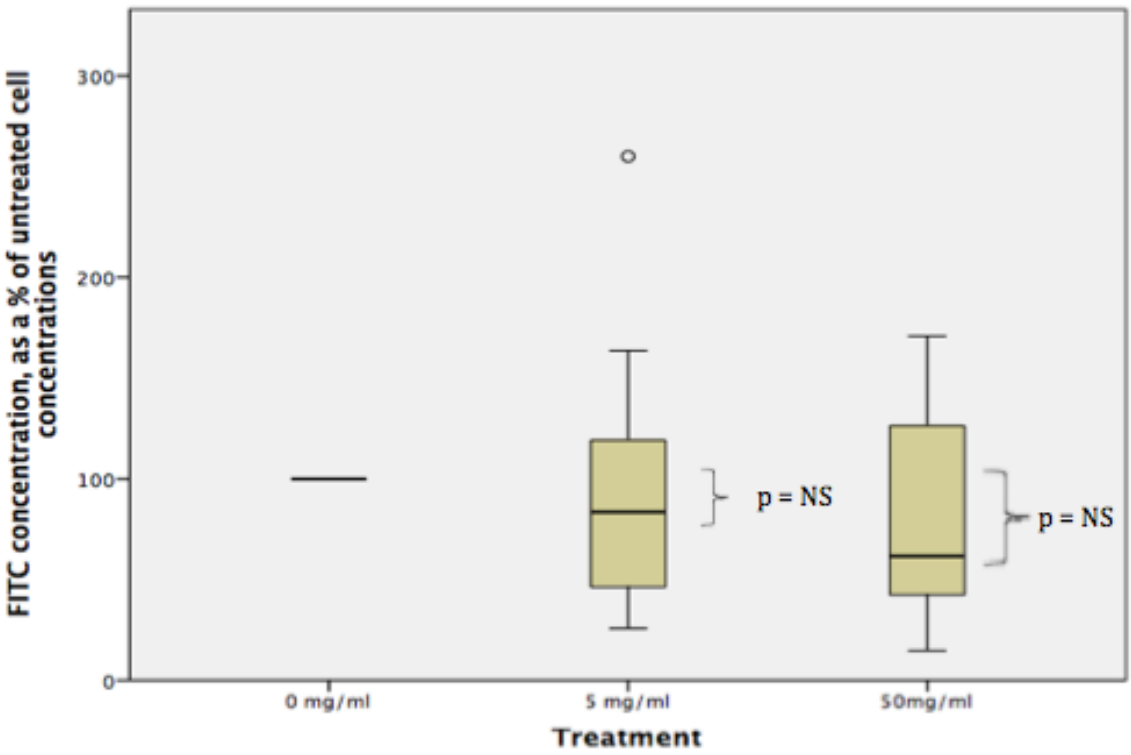


Figure 41: FITC dextran assay, mixed cohort, control versus KGF treatment at 5ng/ml or 50ng/ml, concentration measured at 24 hour time point. No significant difference seen with KGF at this time point, p values shown, although large variability

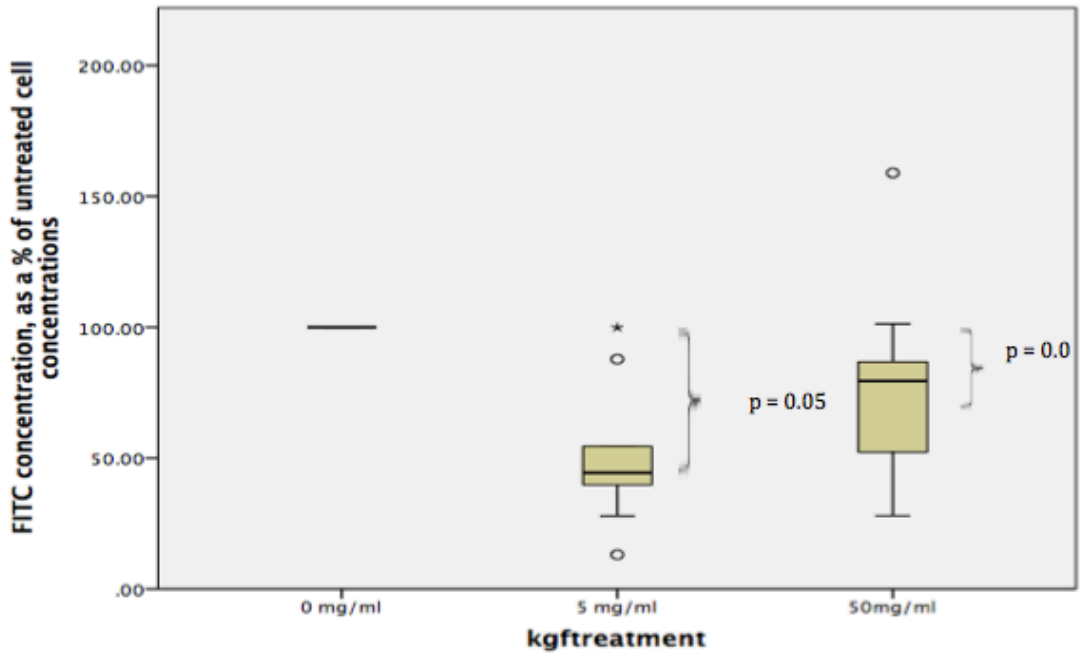


Figure 42: FITC dextran assay, mixed cohort, control versus KGF treatment at 5ng/ml or 50ng/ml, concentration measured at 48 hour time point. KGF at either dose significantly reduced permeability to FITC dextran, p values shown.

Discussion

Time-lapse imaging:

Unfortunately, the time-lapse imaging failed to demonstrate a difference between the asthmatic and healthy cell cultures' *intrinsic* rate of healing, as had been reported previously in the literature[93, 95, 360]. This study differed from those above however, in that media was not changed to 'starvation' media (lacking growth factors, EGF in particular) prior to experimentation (as occurred in the Freishtat paper, unclear methodology in the two other papers). This was based on preliminary experiments suggesting no significant difference with starvation (data not shown), and it may be that this supplemental EGF was sufficient to blur the distinctions between the two groups (when EGF was not removed from media in another wounding study, wounding increased consumption of EGF from the media, suggesting that it may play a role[361]). The other studies also involved larger numbers of subjects, particularly in the healthy arms, and given the degree of variability between subjects it may be that if increased numbers of healthy controls had been used a difference would have become more apparent.

The time-lapse imaging results do appear however to confirm that asthmatic cell cultures respond to KGF stimulation as well as normal cell cultures. Indeed, the effects of KGF seem to be more marked/only significant in the asthmatic group, although more numbers would have been helpful in the healthy control group. As expected and in concordance with previous studies, compression reduced wound healing as assessed by time-lapse imaging. Numbers were too small to separate out asthmatic from healthy cell cultures, whilst there was a suggestion that the asthmatic cultures were affected more, this was not significantly different. At the higher dose of KGF, wound repair in the compressed cells was statistically not dissimilar to the non-compressed, control cells i.e. KGF appeared sufficient to overcome the harmful effects of compression.

TER and FITC dextran results

As might be expected, given TER is felt to be more a measure of subtle permeability/tight junction integrity, the improvement in TER over the 48 hours was less marked than the imaging data i.e. it may be that a wound had fully/close to fully healed macroscopically, but that functional cell-cell junctions had not been formed/optimised. Baseline TER data showed a difference between the asthmatic and healthy groups as has been previous reported[76]. In the untreated (i.e. no KGF) control cells there was no significant improvement in TER (compared to post-wound values) over the 48 hour period, for either asthmatic or healthy cells, again reflecting a failure to detect an intrinsic difference in the healing between the two groups, and again possibly reflecting media choice as outlined above.

Treatment with KGF increased TER values in a dose-dependant fashion for both cohorts, and there was no significant difference seen between the asthmatic/healthy cohorts at each time point/treatment. Compression did not seem to affect the baseline recovery of the cultures (but as noted above this was poor anyway i.e. it may not have been able to drop any further), and whilst it did attenuate the response to KGF within the first 24 hours, this effect was lost by 48 hours. This likely indicates that the effect of the compression is only transient with this level/duration of compression.

In keeping with the TER results, the FITC dextran assay only started to show significant differences at the 48-hour time point (as presumably by then the epithelium had resealed the defect and started to form an effective barrier i.e. before then one might expect permeability to be equally impaired in all groups). As with the other data, we failed to detect a difference between the groups

Summary

In summary therefore these experiments appear to confirm KGF to be effective in the asthmatic cell cultures, increasing wound repair as assessed by time-lapse imaging of the wound, TEER measurement and FITC dextran permeability assay. Baseline TEER measurements were statistically different between asthmatic and healthy cultures, but in other measures (time-lapse imaging, TEER response over time, FITC dextran permeability) we were unable to discern a difference between asthmatic and healthy cell cultures as might have been expected from the literature – it is unclear whether this relates to the smaller numbers, or a methodological flaw such as not using starvation media, or indeed simply the correct results for our particular patient groups.

There are some limitations in the conclusions from the *in vitro* studies however. Firstly, it is not clear from the experiments what the mechanism of action was in relation to increased wound healing – was it simply increased proliferation, as is known as an effect of KGF[163], increased migration[164], or both? A straightforward way to help determine the proliferative effect would have been to quantify proliferation at various timepoints/conditions in the study, using static markers such as cell counts, mitotic index, or markers of proliferation, as is well reported[514]. This would be useful to narrow down the clinic effects/decide where best we would want a future drug's target of action to be i.e. if KGF proves only partially successful, would it be best to look at growth factors/drugs which boost proliferation, migration (or both).

Another limitation in the *in vitro* studies is the lack of a further control arm, wherein the effects of KGF are blocked, to further validate it's direct biological effect, and to demonstrate it's effects are

mediated via the KGFR (rather than a novel pathway). This could have been accomplished through use of a blocking antibody or interfering RNA, and has been reported in intestinal epithelial cells[515, 516].

Future Work

With increased time, it would have been preferential to look at several areas:

- Increasing numbers, particularly in the healthy cohort, for wound imaging, compression and FITC Dextran analysis.
- Immunostaining for tight junction proteins in cultures at different time points, to discover whether, as suspected, the formation of an effective epithelial barrier (with functional cell-cell contacts) takes longer to occur than the macroscopic closure of the mechanical wound. This was attempted, but initial experiments were unsuccessful, and would require further experimentation
- Exploration of the role of TGF-beta in wound repair, and its interaction with the restorative effects of KGF, specifically by using TGF-beta blocking antibodies as a variable to see if they reduce, improve or have no effect on wound closure.

With larger numbers still, it would also be of benefit to look at differing severities of asthma, and whether this in part reflects worsened intrinsic wound healing properties.

Section E : Imaging Study – Results and Discussion

Results

Patient demographics

Baseline subject characteristics are summarised in the table below, with statistical analysis:

	Healthy non-smokers (n = 3)	Non-smoking, controlled asthmatics (n = 3)	P value
Sex (M/F)	2/1	1/2	0.456
Age	22.3 (2.3)	32.3 (16.2)	0.029[1]
FEV_1% predicted	107 (8.7)	63.7 (10.1)	0.05[2]
FVC % predicted	106.3 (11)	86.7 (3.5)	0.05[2]
FEV_1/FVC % ratio	86 (7.2)	61.8 (5.9)	0.05[2]
Atopy (% total)	0	100	0.025[2]
eNO	24.6 (7.7)	26 (14.8)	0.83[2]
Reversibility	3 (2.5)	32.6 (25.9)	0.05[2]
Values shown are means with standard deviation in parentheses. P values are calculated from means using either [1]2 sample t test for normally distributed data or [2]Mann-Whitney u test for non-parametric data			

Table 36: Baseline characteristics for cohorts in radiological study.

Because the numbers in each group were small and the aim was to proceed to a larger sample, subjects were not chosen as matched pairs, and therefore the groups show differences e.g. in age. Also unsurprisingly, and as a reflection of the obstructive, variable nature of their disease, the asthmatic population showed significantly lower FEV_1 % predicted, FEV_1/FVC % ratio and FVC% predicted, with significantly higher reversibility. The healthy group were all non-atopic, whilst the asthmatic group were all atopic.

Safety of the procedure

As expected, nebulisation of the radiopharmaceuticals was well tolerated, with no significant changes in spirometry or pulse oximetery pre/post imaging. No adverse events were recorded for any subject.

Success of targeted deposition

Previous studies in this area have divided results into differing zones, namely 'central' and 'peripheral' areas of the lung (assessed using 2D planar images). These are thought to represent the 'airway' and 'alveolar' components of the lung (although clearly this is a simplification given the complex 3 dimensional nature of the lungs). There is a lack of agreement as to whether this separation then reveals proportional increased clearance in the peripheral regions vs. central regions [389, 423] or not [380, 382], regardless of whether asthma is present or not. Most studies have however therefore calculated the central:peripheral ratio to assess site of deposition. C/P ratios for our subjects, for each isotope, calculated using 2D data, were as follows:

	Healthy non-smokers (n = 3)	Non-smoking, controlled asthmatics (n = 3)
C/P ratio (technetium)	1.58 ± 0.46	2.01 ± 0.73
C/P ratio (Indium)	1.76 ± 0.03	1.84 ± 0.48
Values shown are mean ± SD		

Table 37: C/P ratios for each group, healthy and asthmatics, n= 3 for each group

These C/P ratios are slightly lower than those reported in the asthma permeability studies mentioned above (where reported)[393, 423], which tend to be closer to 3 rather than 2. This perhaps reflects the differing protocol used for inhalation (see section 5.3.4), with enhanced airways rather than alveolar deposition.

Clearance rates

As detailed above, clearance rates (measured as a % original count) were calculated at the 1- and 2-hour points for In-DTPA (reflecting MCC and epithelial permeability) and Tc-Nanocoll (reflecting MCC alone). These are shown in the table below.

Pt no.	Cohort	1 hr In-DTPA clearance (%)	2 hr In-DTPA clearance (%)	1 hr Tc-Nanocoll clearance (%)	2 hr Tc-Nanocoll clearance (%)
1	Healthy	26.2	46.7	13.7	23.7
2	Healthy	38.1	60.9	20.7	26.3
3	Healthy	34.5	57.3	20.5	32.5
6	Asthmatic	28.4	47.8	36.8	55.7
9	Asthmatic	28	48.1	1.4	-0.7
10	Asthmatic	30.2	50.7	13.9	20.9

Table 38: 1- and 2-hour clearance rates for technetium and indium, for each subject

Two potential problems were immediately apparent from the above results, affecting patients in the asthmatic cohort. Firstly, one would expect that for all patients, the indium (cleared by **both** mucociliary clearance and epithelial permeability) would clear more quickly than the technetium (cleared by mucociliary clearance **alone**). Whilst this was the case for the majority, the reverse occurred in PERM06, which does not make sense (see discussion for possible reasons). Secondly, the clearance for technetium in PERM09 was effectively zero i.e. no apparent mucociliary clearance (although when combined with the 24 hour data, there was a reduction in the count apparent over the longer time period i.e. a very slow mucociliary clearance). Whilst as discussed asthma can slow mucociliary clearance, this subject was not the most severe of the group in terms of physiological parameters, and a methodological flaw was suspected (again see discussion).

Epithelial permeability

Clearance curves for the technetium and indium were calculated for each subject and best fit curves plotted as detailed in section 5.3.5. From these curves, rate constants were determined for the technetium (K^{MCC}, as reflects mucociliary clearance alone) and the indium (K^{tot}, as reflects clearance by both MCC and permeability)). By subtracting K^{MCC} from K^{tot}, a rate constant for epithelial permeability alone, K^{perm}, was determined.

Participant no.	K^{tot} (Epithelial and mucociliary clearance)	K^{MCC} (Mucociliary clearance alone)	$K^{perm} = K^{tot} - K^{MCC}$ (Epithelial permeability alone)
1	0.005436	0.002052	0.0034
2	0.007645	0.001207	0.0064
3	0.007187	0.002719	0.0045
6	0.005287	0.005908	-0.0006
9	0.00544	-0.003575	0.0058
10	0.005792	0.001411	0.0044

Table 39: Rate constants calculated for technetium (K^{MCC}) and indium (K^{tot}) with subtraction of the former from the latter to give the rate constant reflecting epithelial permeability alone(K^{perm}).

As might be expected from the strange clearance rate results, the epithelial permeability results are somewhat confusing. For PERM06 (an asthmatic), mucociliary clearance appeared to be faster than total clearance, resulting in a negative permeability rate constant. Even if this patient is excluded, and allowing for the fact that the numbers are small, there does not immediately seem to be a difference between the two groups in terms of overall clearance (K^{tot}) i.e. assuming the Indium labelled DTPA analysis is correct (measuring both epithelial permeability and mucociliary clearance). For the healthy cohort (PERM 01/02/03) K^{tot} has a mean of 0.0068 (SD 0.0012) whereas for the asthmatics (PERM 09/10) K^{tot} has a mean of 0.0056 (SD 0.0002). This does not mean however, as stated above, that epithelial permeability could not be different – if the mucociliary clearance was significantly slower in the asthmatics, it would follow that the epithelial permeability might be significantly increased (so that total clearance was similar in the two groups). However we were unable to show this with the pilot data.

Discussion

The pilot study was stopped as planned after three patients in the two groups to allow interim analysis of the results to check the validity of the technique. As can be seen from the above results, something seemed to be going wrong with the methods employed, leading to strange results.

Initial suspicion focussed on the technetium-binded Nanocoll, as these results were both more variable within the two groups, and also more unexpected (for the asthmatic patient results i.e. a near zero mucociliary clearance for one patient, and a higher than total clearance for another). The most likely explanation relates to unbinding of the technetium. Mixtures of the two solutions had been made as part of the preliminary work however, and these had been stable on paper chromatographic analysis at both the 1- and 24-hour mark (data not shown). However these mixtures did not undergo nebulisation, which led to a hypothesis that unbinding was occuring with the physical act of nebulisation. This has been reported with technetium bound to **DTPA** (as opposed to Nanocoll in our study) in two studies[517, 518]. Whilst one study only found unbinding to occur after ultrasonic but *not* jet nebulisation[517] (our study used jet nebulisation), the other *did* see significant unbinding with jet nebulisation[518]. In this second study it was postulated that technetium binding in particular is susceptible as its binding is vulnerable to oxidation (as might be expected in the act of aerosolisation). Other studies have used jet nebulisation of technetium-labelled **Nanocoll** for measurement of mucociliary clearance with no reported problems previously, but it is unclear whether their reported low rate of unbinding was measured in samples that had undergone nebulisation[519, 520], and these studies were using single radiopharmaceuticals, rather than mixtures of two radiopharmaceuticals, which may greatly affect the pharmacokinetics.

If unbinding was occurring, the free (unbound) technetium might lead to a 'higher than actual' apparent clearance rate in single radioisotope studies[518], and this might explain the apparent discrepancies with patient PERM06 above, it would not then explain the slow mucociliary clearance in PERM09. What is not clear however is whether unbound technetium might then bind to the In-DTPA, forming a dual labelled molecule and further confusing the results.

The indium radiopharmaceutical seems less likely to be at fault with regards unbinding though - whilst the authors of the study above[518] also looked at Indium bound to DTPA, and did not perform analysis of unbinding of this compound, they felt this was less likely due to a higher intensity binding of the radioisotope, reflected in its relatively high log stability constant (a measure of the strength of binding) of 10^{29}[521].However the authors do note a possible

alternative flaw with the indium, namely that indium radiopharmaceuticals may exchange their metals to transferrin if the log stability constant is $<10^{30}$, and that transferrin may be found in macrophages which exist within the airway lumen. Whilst other studies have disputed the ability of human macrophages to synthesize transferrin[522], and lymphocytes may be the cellular source[523], regardless of cell type it is detectable in BAL fluid[524].

Further Work

As stated above, the pilot study produced conflicting results suggesting significant methodological flaws. Unfortunately due to funding and priority setting, it was decided not to pursue this study, either in working out where exactly the errors were occurring, or in an alternative form. This is a shame, as the results of investigations into the flaw would potentially apply to other studies in the future, using nebulised radioisotopes and/or mixtures of radioisotopes. If one wanted to pursue this however, the ideal plan would be to

a) Repeat chromatography studies on non-nebulised radioisotope-labelled compounds, both in isolation and in mixtures, to confirm our initial results suggesting stability.
b) Collection of nebulised solutions (again, in isolation and as a mixture), using a multistage-liquid impinger, and repeating the chromatographic analysis
c) Preferably in conjunction with this, would be to repeat the analysis in the same six subjects, but using Tc-Nanocoll/In-DTPA on different days, i.e. performed in isolation rather than as a mixture, and comparing the results to our results

Section F : Overall Discussion

Overall, whilst there are promising results from the work in this thesis, with regards the role of epithelial permeability in asthma, there is much that would need to be done to develop this further as a valid therapeutic target/hypothesis. With the benefit of hindsight/with the knowledge/experience gained from doing the thesis, there are a number of modifications that should have been made, or could be made with future work, to explore the effects of KGF on epithelial permeability and it's clinical effects in asthma, as follows:

A) Verification of a therapeutic effect of treatment with KGF and/or a similar agent.

The clinical trial was ambitious, and possibly over-reached, in an attempt to demonstrate both clinical and biological effects across multiple domains, over a prolonged period of time. A simpler, shorter trial may have been easier to recruit to, more cost-effective (and thus potentially involve larger numbers), and by concentrating on select areas.

For example, giving a single dose of KGF rather than 2 doses would have halved the main cost of the trial. This could have been combined with either BHR testing before and after, as in the current trial, or with inhalational allergen challenges, which have been well used in the development of various asthma drugs[525]. By increasing the number of participants, this may have allowed elimination of differences between the groups, and thus the ability to perform only a single bronchoscopy, post drug/placebo, and then comparing between the groups only. By limiting the trial to an objective test which will be positive i.e. rather than looking at asthma symptoms and quality of life as well, one could expand patient selection to include patients with controlled asthma (rather than uncontrolled as in the current trial), giving a larger pool of potential participants. The larger numbers would then (hopefully) allow demonstration of a biological effect of the drug within the lungs, as per comments in previous discussion, perhaps limiting biological sampling to bronchial biopsies only, and GMA-embedding only, to boost numbers of samples. The exploration of the biological effects *in vivo* would also benefit from greater knowledge/exploration of the *in vitro* effects (see next section) i.e. to know exactly which markers one should be analysing.

Once the above more limited trial had been done, this would have adequately established proof of concept, and would then lead to a larger, but still limited trial, now concentrating on the clinical outcomes more i.e. now looking at asthma control, quality of life, etc. Dependant on how the initial trial had gone, there would be the potential to add in elements to boost evidence supporting the effect of the drug i.e. if the biological effect was still in question, then a select number of the larger trial patients could be invited to have a bronchoscopy. Similarly, if biological

effects were not in question but the effect on response to BHR/inhalational allergen challenge was of borderline statistical significance, the relevant BHR/inhalational challenge could be built into the second trial. At a certain stage, it is likely that future trials would need to look at method of administration of the drug or alternative drugs to attempt to limit side effects, e.g. nebulisation. Lastly, assuming earlier trials had been successful, subsequent work would need to look at long-term treatment effects, to further verify safety and efficacy concerns.

B) Increased knowledge of mechanisms of epithelial dysfunction/KGF

There are differing theories as to the mechanism of epithelial dysfunction in asthma, and this area needs further clarification. Indeed, given the heterogeneity of asthma, it is likely that more than one process can result in epithelial damage/dysfunction, and it is not clear whether this would then affect the effectiveness of drugs targeting such epithelial damage, such as KGF. For example, the presence of the KGFR on asthmatic epithelium has only been verified immunohistochemically in unpublished data[216], and whilst in that study the receptor was present at similar levels to healthy controls, regardless of severity of asthma, one could postulate that abnormalities in expression of KGFR in certain populations of asthmatics could result in epithelial repair impairment and could be one mechanism of the 'chronic wound' epithelium. Similarly, whilst our knowledge of the downstream effects of KGFR activation, and how this translates into the cellular effects e.g. increased survival, is increasing[526], it is not known if these pathways are normal in the asthmatic epithelium; if one of the mechansims of the epithelial 'chronic wound' is a defect in the KGF signalling pathway, then the application of KGF as a therapy may be ineffective. The situation becomes more complex when we consider the potential interactions between KGF and other growth factors or cytokines, such as TGF-beta[117], and between KGF and glucocorticoids[156, 157]. Whilst complex, a full understanding is likely to be beneficial when considering further therapies to target the epithelial repair pathway.

The *in vitro* studies did not really advance our knowledge in this area, and were limited to showing a likely beneficial effect of KGF in the asthmatic epithelium to a wound model, and that this effect was reduced but not abolished by the effects of compression. Further work in this area, beyond that already discussed in the relevant chapter above, could also have involved demonstration of KGFR expression in asthmatic ALI cultures and biopsies, and an exploration of whether the elements of the KGFR signalling pathway (that we are aware of) were intact in the asthmatic epithelium.

C) Improved noninvasive techniques for epithelial permeability.

Whilst modification of epithelial permeability may prove effective across a large patient population, it can already be seen from this small study that some patients had a greater response than others. It would therefore be immensely helpful to have a non-invasive way of assessing permeability, both prior to treatment (to potentially identify who may benefit the most/at all) and as a measure of treatment response. Whilst the pilot study reported was essentially unsuccessful, imaging techniques are likely to be the best way of assessing this area, and the principles behind the technique, i.e. measurement of clearance of radiolabelled compounds from the lung, appear sound. Whilst it cannot be assumed that this technique will be sensitive enough to detect altered epithelial permeability in asthma, this does seem the best area to explore in future work. Another area, which may prove useful to look at however, would be the emerging role of exhaled breath condensate for monitoring airway inflammation/epithelial stress. Whilst this is still a technique in development, and may simply correlate with inflammation in asthma[527], rather than epithelial permeability per se, it may be useful to explore this as an outcome measure in future trials.

Section G: References

1. Marketos, S.G. and C.N. Ballas, *Bronchial asthma in the medical literature of Greek antiquity.* The Journal of asthma : official journal of the Association for the Care of Asthma, 1982. **19**(4): p. 263-9.
2. Karamanou, M. and G. Androutsos, *Aretaeus of Cappadocia and the first clinical description of asthma.* American journal of respiratory and critical care medicine, 2011. **184**(12): p. 1420-1.
3. Jackson, M., *Asthma: The Biography*. 1st ed. Vol. 1. 2009: Oxford University Press. 256.
4. Meyerhof, M., *Thirty-Three Clinical Observations by Rhazes (Circa 900 A.D.).* Isis, 1935. **23**(2): p. 51.
5. Sakula, A., *Sir John Floyer's A Treatise of the Asthma (1698).* Thorax, 1984. **39**(4): p. 248-54.
6. Fayssoil, A., *Rene Laennec (1781-1826) and the invention of the stethoscope.* The American journal of cardiology, 2009. **104**(5): p. 743-4.
7. Hutchinson, J., *On the capacity of the lungs, and on the respiratory functions, with a view of establishing a precise and easy method of detecting disease by the spirometer.* Medico-chirurgical transactions, 1846. **29**: p. 137-252.
8. Sakula, A., *Charcot-Leyden crystals and Curschmann spirals in asthmatic sputum.* Thorax, 1986. **41**(7): p. 503-7.
9. Salter, H., *On Asthma: Its Pathology and Treatment*, B.a. Lea, Editor. 1864: Philadelphia.
10. Stanworth, D.R., *The discovery of IgE.* Allergy, 1993. **48**(2): p. 67-71.
11. GINA, *Global Initiative for Asthma. Updated 2016. Global strategy for management and prevention.* N.H.National Institutes of Health, Lung and Blood Institute.NHLBI/WHO workshop report, 2016.
12. Kiley, J., R. Smith, and P. Noel, *Asthma phenotypes.* Current opinion in pulmonary medicine, 2007. **13**(1): p. 19-23.
13. Masoli, M., et al., *The global burden of asthma: executive summary of the GINA Dissemination Committee report.* Allergy, 2004. **59**(5): p. 469-78.
14. Simpson, C.R. and A. Sheikh, *Trends in the epidemiology of asthma in England: a national study of 333,294 patients.* Journal of the Royal Society of Medicine, 2010. **103**(3): p. 98-106.
15. Anandan, C., et al., *Is the prevalence of asthma declining? Systematic review of epidemiological studies.* Allergy, 2010. **65**(2): p. 152-67.
16. Bahadori, K., et al., *Economic burden of asthma: a systematic review.* BMC pulmonary medicine, 2009. **9**: p. 24.
17. Malone, D.C., K.A. Lawson, and D.H. Smith, *Asthma: an analysis of high-cost patients.* Pharmacy practice management quarterly, 2000. **20**(1): p. 12-20.
18. Woodcock, A. and A. Custovic, *Allergen avoidance: does it work?* British medical bulletin, 2000. **56**(4): p. 1071-86.
19. British Thoracic, S., *British Guideline on the Management of Asthma.* Thorax, 2008. **63**(Suppl_4): p. iv1-121.
20. Tovey, E.R. and G.B. Marks, *It's time to rethink mite allergen avoidance.* The Journal of allergy and clinical immunology, 2011. **128**(4): p. 723-727 e6.
21. British Thoracic, S. and N. Scottish Intercollegiate Guidelines, *British guideline on the management of asthma.* Thorax, 2014. **69 Suppl 1**: p. 1-192.
22. Strek, M.E., *Difficult asthma.* Proceedings of the American Thoracic Society, 2006. **3**(1): p. 116-23.
23. Barnes, P.J. and A.J. Woolcock, *Difficult asthma.* The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology, 1998. **12**(5): p. 1209-18.

24. Urso, D.L., et al., *Diagnosis and treatment of refractory asthma.* European review for medical and pharmacological sciences, 2008. **12**(5): p. 315-20.
25. Antonicelli, L., et al., *Asthma severity and medical resource utilisation.* The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology, 2004. **23**(5): p. 723-9.
26. Serra-Batlles, J., et al., *Costs of asthma according to the degree of severity.* The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology, 1998. **12**(6): p. 1322-6.
27. Gonem, S., et al., *Evidence for phenotype-driven treatment in asthmatic patients.* Current opinion in allergy and clinical immunology, 2011. **11**(4): p. 381-5.
28. Polosa, R. and T. Casale, *Monoclonal antibodies for chronic refractory asthma and pipeline developments.* Drug discovery today, 2012.
29. Jeffrey, P., *Microscopic structure of the lung*, in *Respiratory Medicine*, G.D. Gibson JG, Costabel U, Sterk P, Corrin B, Editor. 2003, Saunders. p. 48.
30. Godfrey, R.W., *Human airway epithelial tight junctions.* Microscopy research and technique, 1997. **38**(5): p. 488-99.
31. Farquhar, M.G. and G.E. Palade, *Junctional complexes in various epithelia.* The Journal of cell biology, 1963. **17**: p. 375-412.
32. Sawada, N., et al., *Tight junctions and human diseases.* Medical electron microscopy : official journal of the Clinical Electron Microscopy Society of Japan, 2003. **36**(3): p. 147-56.
33. Soini, Y., *Claudins in lung diseases.* Respiratory research, 2011. **12**: p. 70.
34. Chiba, H., et al., *Transmembrane proteins of tight junctions.* Biochimica et biophysica acta, 2008. **1778**(3): p. 588-600.
35. Proud, D. and R. Leigh, *Epithelial cells and airway diseases.* Immunological reviews, 2011. **242**(1): p. 186-204.
36. Tomee, J.F., et al., *Proteases from Aspergillus fumigatus induce release of proinflammatory cytokines and cell detachment in airway epithelial cell lines.* The Journal of infectious diseases, 1997. **176**(1): p. 300-3.
37. King, C., et al., *Dust mite proteolytic allergens induce cytokine release from cultured airway epithelium.* Journal of immunology, 1998. **161**(7): p. 3645-51.
38. Einarsson, O., et al., *Interleukin-11: stimulation in vivo and in vitro by respiratory viruses and induction of airways hyperresponsiveness.* The Journal of clinical investigation, 1996. **97**(4): p. 915-24.
39. Osterlund, C., et al., *Non-proteolytic aeroallergens from mites, cat and dog exert adjuvant-like activation of bronchial epithelial cells.* International archives of allergy and immunology, 2011. **155**(2): p. 111-8.
40. Churchill, L., et al., *Production of granulocyte-macrophage colony-stimulating factor by cultured human tracheal epithelial cells.* Immunology, 1992. **75**(1): p. 189-95.
41. van Wetering, S., et al., *Epithelial differentiation is a determinant in the production of eotaxin-2 and -3 by bronchial epithelial cells in response to IL-4 and IL-13.* Molecular immunology, 2007. **44**(5): p. 803-11.
42. Kidney, J.C. and D. Proud, *Neutrophil transmigration across human airway epithelial monolayers: mechanisms and dependence on electrical resistance.* American journal of respiratory cell and molecular biology, 2000. **23**(3): p. 389-95.
43. Dunnill, M.S., *The pathology of asthma, with special reference to changes in the bronchial mucosa.* Journal of clinical pathology, 1960. **13**: p. 27-33.
44. Houston, J.C., S. De Navasquez, and J.R. Trounce, *A clinical and pathological study of fatal cases of status asthmaticus.* Thorax, 1953. **8**(3): p. 207-13.
45. Chanez, P., et al., *Comparison between nasal and bronchial inflammation in asthmatic and control subjects.* American journal of respiratory and critical care medicine, 1999. **159**(2): p. 588-95.

46. Wardlaw, A.J., et al., *Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity.* The American review of respiratory disease, 1988. **137**(1): p. 62-9.
47. Beasley, R., et al., *Cellular events in the bronchi in mild asthma and after bronchial provocation.* The American review of respiratory disease, 1989. **139**(3): p. 806-17.
48. Laitinen, L.A., et al., *Damage of the airway epithelium and bronchial reactivity in patients with asthma.* The American review of respiratory disease, 1985. **131**(4): p. 599-606.
49. Jeffery, P.K., et al., *Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity.* The American review of respiratory disease, 1989. **140**(6): p. 1745-53.
50. Shahana, S., et al., *Ultrastructure of bronchial biopsies from patients with allergic and non-allergic asthma.* Respir Med, 2005. **99**(4): p. 429-443.
51. Fahy, J.V., *Remodeling of the airway epithelium in asthma.* American journal of respiratory and critical care medicine, 2001. **164**(10 Pt 2): p. S46-51.
52. Kirby, J.G., et al., *Bronchoalveolar cell profiles of asthmatic and nonasthmatic subjects.* The American review of respiratory disease, 1987. **136**(2): p. 379-83.
53. Van Vyve, T., et al., *Comparison between bronchial and alveolar samples of bronchoalveolar lavage fluid in asthma.* Chest, 1992. **102**(2): p. 356-61.
54. Foresi, A., et al., *Inflammatory markers in bronchoalveolar lavage and in bronchial biopsy in asthma during remission.* Chest, 1990. **98**(3): p. 528-35.
55. Montefort, S., et al., *The site of disruption of the bronchial epithelium in asthmatic and non-asthmatic subjects.* Thorax, 1992. **47**(7): p. 499-503.
56. Fahy, J.V., et al., *Cellular and biochemical analysis of induced sputum from asthmatic and from healthy subjects.* The American review of respiratory disease, 1993. **147**(5): p. 1126-31.
57. Woltmann, G., et al., *Objective quantitative analysis of eosinophils and bronchial epithelial cells in induced sputum by laser scanning cytometry.* Thorax, 1999. **54**(2): p. 124-30.
58. Boulet, L.P., et al., *Bronchial subepithelial fibrosis correlates with airway responsiveness to methacholine.* Chest, 1997. **112**(1): p. 45-52.
59. Lozewicz, S., et al., *Morphological integrity of the bronchial epithelium in mild asthma.* Thorax, 1990. **45**(1): p. 12-5.
60. Ordonez, C., et al., *Epithelial desquamation in asthma: artifact or pathology?* American journal of respiratory and critical care medicine, 2000. **162**(6): p. 2324-9.
61. Carroll, N., et al., *The structure of large and small airways in nonfatal and fatal asthma.* The American review of respiratory disease, 1993. **147**(2): p. 405-10.
62. Puddicombe, S.M., et al., *Involvement of the epidermal growth factor receptor in epithelial repair in asthma.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2000. **14**(10): p. 1362-74.
63. Crosby, L.M. and C.M. Waters, *Epithelial repair mechanisms in the lung.* American journal of physiology. Lung cellular and molecular physiology, 2010. **298**(6): p. L715-31.
64. Takeyama, K., J.V. Fahy, and J.A. Nadel, *Relationship of epidermal growth factor receptors to goblet cell production in human bronchi.* American journal of respiratory and critical care medicine, 2001. **163**(2): p. 511-6.
65. Karacsonyi, C., et al., *Epidermal growth factor receptor signaling mediates vesicant-induced airway epithelial secretion of interleukin-6 and production of mucin.* American journal of respiratory cell and molecular biology, 2012. **46**(2): p. 157-64.
66. Chaudhry, I.S., et al., *Short-term exposure to tobacco toxins alters expression of multiple proliferation gene markers in primary human bronchial epithelial cell cultures.* Journal of oncology, 2011. **2011**: p. 208563.

67. Chen, Y.T., et al., *Cigarette smoke induces epidermal growth factor receptor-dependent redistribution of apical MUC1 and junctional beta-catenin in polarized human airway epithelial cells.* The American journal of pathology, 2010. **177**(3): p. 1255-64.
68. Heijink, I.H., et al., *Cigarette smoke impairs airway epithelial barrier function and cell-cell contact recovery.* The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology, 2012. **39**(2): p. 419-28.
69. Tschumperlin, D.J., et al., *Mechanotransduction through growth-factor shedding into the extracellular space.* Nature, 2004. **429**(6987): p. 83-6.
70. Heijink, I.H., A. van Oosterhout, and A. Kapus, *Epidermal growth factor receptor signalling contributes to house dust mite-induced epithelial barrier dysfunction.* The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology, 2010. **36**(5): p. 1016-26.
71. Wadsworth, S.J., H.S. Nijmeh, and I.P. Hall, *Glucocorticoids increase repair potential in a novel in vitro human airway epithelial wounding model.* Journal of clinical immunology, 2006. **26**(4): p. 376-87.
72. Polosa, R., et al., *Expression of c-erbB receptors and ligands in the bronchial epithelium of asthmatic subjects.* The Journal of allergy and clinical immunology, 2002. **109**(1): p. 75-81.
73. Amishima, M., et al., *Expression of epidermal growth factor and epidermal growth factor receptor immunoreactivity in the asthmatic human airway.* American journal of respiratory and critical care medicine, 1998. **157**(6 Pt 1): p. 1907-12.
74. Fedorov, I.A., et al., *Epithelial stress and structural remodelling in childhood asthma.* Thorax, 2005. **60**(5): p. 389-94.
75. de Boer, W.I., et al., *Altered expression of epithelial junctional proteins in atopic asthma: possible role in inflammation.* Can.J Physiol Pharmacol., 2008. **86**(3): p. 105-112.
76. Xiao, C., et al., *Defective epithelial barrier function in asthma.* The Journal of allergy and clinical immunology, 2011. **128**(3): p. 549-56 e1-12.
77. Ahdieh, M., T. Vandenbos, and A. Youakim, *Lung epithelial barrier function and wound healing are decreased by IL-4 and IL-13 and enhanced by IFN-gamma.* Am J Physiol Cell Physiol, 2001. **281**(6): p. C2029-C2038.
78. Holgate, S.T., *The sentinel role of the airway epithelium in asthma pathogenesis.* Immunological reviews, 2011. **242**(1): p. 205-19.
79. Holgate, S.T., et al., *The role of the airway epithelium and its interaction with environmental factors in asthma pathogenesis.* Proceedings of the American Thoracic Society, 2009. **6**(8): p. 655-9.
80. Holgate, S.T., et al., *Epithelial-mesenchymal interactions in the pathogenesis of asthma.* The Journal of allergy and clinical immunology, 2000. **105**(2 Pt 1): p. 193-204.
81. Benard, A., et al., *Increased intestinal permeability in bronchial asthma.* J.Allergy Clin.Immunol., 1996. **97**(6): p. 1173-1178.
82. Hijazi, Z., et al., *Intestinal permeability is increased in bronchial asthma.* Archives of disease in childhood, 2004. **89**(3): p. 227-9.
83. Suzuki, Y., M. Kodama, and K. Asano, *Skin barrier-related molecules and pathophysiology of asthma.* Allergology international : official journal of the Japanese Society of Allergology, 2011. **60**(1): p. 11-5.
84. Linden, M.D., et al., *Clinical application of morphologic and immunocytochemical assessments of cell proliferation.* American journal of clinical pathology, 1992. **97**(5 Suppl 1): p. S4-13.
85. Bruno, S. and Z. Darzynkiewicz, *Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells.* Cell proliferation, 1992. **25**(1): p. 31-40.
86. Gerdes, J., et al., *Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67.* The American journal of pathology, 1991. **138**(4): p. 867-73.

87. Hassan, M., et al., *Airway smooth muscle remodeling is a dynamic process in severe long-standing asthma.* The Journal of allergy and clinical immunology, 2010. **125**(5): p. 1037-1045 e3.
88. Demoly, P., et al., *Cell proliferation in the bronchial mucosa of asthmatics and chronic bronchitics.* American journal of respiratory and critical care medicine, 1994. **150**(1): p. 214-7.
89. Benayoun, L., et al., *Regulation of peroxisome proliferator-activated receptor gamma expression in human asthmatic airways: relationship with proliferation, apoptosis, and airway remodeling.* American journal of respiratory and critical care medicine, 2001. **164**(8 Pt 1): p. 1487-94.
90. Cohen, L., et al., *Epithelial cell proliferation contributes to airway remodeling in severe asthma.* American journal of respiratory and critical care medicine, 2007. **176**(2): p. 138-45.
91. Hackett, T.L., et al., *Intrinsic phenotypic differences of asthmatic epithelium and its inflammatory responses to respiratory syncytial virus and air pollution.* American journal of respiratory cell and molecular biology, 2011. **45**(5): p. 1090-100.
92. Puddicombe, S.M., et al., *Increased expression of p21(waf) cyclin-dependent kinase inhibitor in asthmatic bronchial epithelium.* American journal of respiratory cell and molecular biology, 2003. **28**(1): p. 61-8.
93. Freishtat, R.J., et al., *Asthmatic airway epithelium is intrinsically inflammatory and mitotically dyssynchronous.* Am J Respir Cell Mol Biol, 2011. **44**(6): p. 863-9.
94. Kicic, A., et al., *Decreased fibronectin production significantly contributes to dysregulated repair of asthmatic epithelium.* American journal of respiratory and critical care medicine, 2010. **181**(9): p. 889-98.
95. Stevens, P.T., et al., *Dysregulated repair in asthmatic paediatric airway epithelial cells: the role of plasminogen activator inhibitor-1.* Clin.Exp.Allergy, 2008. **38**(12): p. 1901-1910.
96. Bucchieri, F., et al., *Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis.* American journal of respiratory cell and molecular biology, 2002. **27**(2): p. 179-85.
97. Bucchieri, F., et al., *Cigarette smoke causes caspase-independent apoptosis of bronchial epithelial cells from asthmatic donors.* PLoS One, 2015. **10**(3): p. e0120510.
98. Bayram, H., et al., *Effect of ozone and nitrogen dioxide on the permeability of bronchial epithelial cell cultures of non-asthmatic and asthmatic subjects.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2002. **32**(9): p. 1285-92.
99. Savla, U. and C.M. Waters, *Mechanical strain inhibits repair of airway epithelium in vitro.* Am J Physiol, 1998. **274**(6 Pt 1): p. L883-92.
100. Bossios, A., et al., *Rhinovirus infection induces cytotoxicity and delays wound healing in bronchial epithelial cells.* Respir Res, 2005. **6**: p. 114.
101. Dorscheid, D.R., et al., *Effects of corticosteroid-induced apoptosis on airway epithelial wound closure in vitro.* Am.J.Physiol Lung Cell Mol.Physiol, 2006. **291**(4): p. L794-L801.
102. Brandes, M.E., et al., *Type I transforming growth factor-beta receptors on neutrophils mediate chemotaxis to transforming growth factor-beta.* Journal of immunology, 1991. **147**(5): p. 1600-6.
103. Willis, B.C. and Z. Borok, *TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease.* American journal of physiology. Lung cellular and molecular physiology, 2007. **293**(3): p. L525-34.
104. Wahl, S.M. and W. Chen, *Transforming growth factor-beta-induced regulatory T cells referee inflammatory and autoimmune diseases.* Arthritis research & therapy, 2005. **7**(2): p. 62-8.

105. Minshall, E.M., et al., *Eosinophil-associated TGF-beta1 mRNA expression and airways fibrosis in bronchial asthma.* American journal of respiratory cell and molecular biology, 1997. **17**(3): p. 326-33.
106. Redington, A.E., et al., *Transforming growth factor-beta 1 in asthma. Measurement in bronchoalveolar lavage fluid.* American journal of respiratory and critical care medicine, 1997. **156**(2 Pt 1): p. 642-7.
107. Hoshino, M., Y. Nakamura, and J.J. Sim, *Expression of growth factors and remodelling of the airway wall in bronchial asthma.* Thorax, 1998. **53**(1): p. 21-7.
108. Redington, A.E., et al., *Co-localization of immunoreactive transforming growth factor-beta 1 and decorin in bronchial biopsies from asthmatic and normal subjects.* The Journal of pathology, 1998. **186**(4): p. 410-5.
109. Balzar, S., et al., *Increased TGF-beta2 in severe asthma with eosinophilia.* The Journal of allergy and clinical immunology, 2005. **115**(1): p. 110-7.
110. Howat, W.J., S.T. Holgate, and P.M. Lackie, *TGF-beta isoform release and activation during in vitro bronchial epithelial wound repair.* American journal of physiology. Lung cellular and molecular physiology, 2002. **282**(1): p. L115-23.
111. Lechapt-Zalcman, E., et al., *Transforming growth factor-beta1 increases airway wound repair via MMP-2 upregulation: a new pathway for epithelial wound repair?* American journal of physiology. Lung cellular and molecular physiology, 2006. **290**(6): p. L1277-82.
112. Zhang, M., et al., *TGF-beta1 induces human bronchial epithelial cell-to-mesenchymal transition in vitro.* Lung, 2009. **187**(3): p. 187-94.
113. Hackett, T.L., et al., *Induction of epithelial-mesenchymal transition in primary airway epithelial cells from patients with asthma by transforming growth factor-beta1.* Am J Respir Crit Care Med, 2009. **180**(2): p. 122-33.
114. Moheimani, F., et al., *Disruption of beta-catenin/CBP signaling inhibits human airway epithelial-mesenchymal transition and repair.* Int J Biochem Cell Biol, 2015. **68**: p. 59-69.
115. Borthwick, L.A., et al., *Inflammation and epithelial to mesenchymal transition in lung transplant recipients: role in dysregulated epithelial wound repair.* American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons, 2010. **10**(3): p. 498-509.
116. Neurohr, C., S.L. Nishimura, and D. Sheppard, *Activation of transforming growth factor-beta by the integrin alphavbeta8 delays epithelial wound closure.* American journal of respiratory cell and molecular biology, 2006. **35**(2): p. 252-9.
117. Zhang, F., et al., *Transforming growth factor-beta antagonizes alveolar type II cell proliferation induced by keratinocyte growth factor.* Am.J.Respir.Cell Mol.Biol., 2004. **31**(6): p. 679-686.
118. Chandel, N.S., et al., *Keratinocyte growth factor expression is suppressed in early acute lung injury/acute respiratory distress syndrome by smad and c-Abl pathways.* Critical care medicine, 2009. **37**(5): p. 1678-84.
119. Yi, E.S., et al., *Keratinocyte growth factor decreases pulmonary edema, transforming growth factor-beta and platelet-derived growth factor-BB expression, and alveolar type II cell loss in bleomycin-induced lung injury.* Inflammation, 1998. **22**(3): p. 315-325.
120. Loxham, M. and D.E. Davies, *Phenotypic and genetic aspects of epithelial barrier function in asthmatic patients.* J Allergy Clin Immunol, 2017. **139**(6): p. 1736-1751.
121. Sweerus, K., et al., *Claudin-18 deficiency is associated with airway epithelial barrier dysfunction and asthma.* J Allergy Clin Immunol, 2017. **139**(1): p. 72-81 e1.
122. Hristova, M., et al., *Airway epithelial dual oxidase 1 mediates allergen-induced IL-33 secretion and activation of type 2 immune responses.* J Allergy Clin Immunol, 2016. **137**(5): p. 1545-1556 e11.
123. Hallstrand, T.S., et al., *Airway epithelial regulation of pulmonary immune homeostasis and inflammation.* Clin Immunol, 2014. **151**(1): p. 1-15.

124. Angkasekwinai, P., et al., *Interleukin 25 promotes the initiation of proallergic type 2 responses.* J Exp Med, 2007. **204**(7): p. 1509-17.
125. Krishnan, K., B. Arnone, and A. Buchman, *Intestinal growth factors: potential use in the treatment of inflammatory bowel disease and their role in mucosal healing.* Inflammatory bowel diseases, 2011. **17**(1): p. 410-22.
126. Slonim, A.E., et al., *A preliminary study of growth hormone therapy for Crohn's disease.* The New England journal of medicine, 2000. **342**(22): p. 1633-7.
127. Sinha, A., et al., *Epidermal growth factor enemas with oral mesalamine for mild-to-moderate left-sided ulcerative colitis or proctitis.* The New England journal of medicine, 2003. **349**(4): p. 350-7.
128. Buchman, A.L., et al., *Teduglutide, a novel mucosally active analog of glucagon-like peptide-2 (GLP-2) for the treatment of moderate to severe Crohn's disease.* Inflammatory bowel diseases, 2010. **16**(6): p. 962-73.
129. Dieckgraefe, B.K. and J.R. Korzenik, *Treatment of active Crohn's disease with recombinant human granulocyte-macrophage colony-stimulating factor.* Lancet, 2002. **360**(9344): p. 1478-80.
130. Korzenik, J.R., et al., *Sargramostim for active Crohn's disease.* The New England journal of medicine, 2005. **352**(21): p. 2193-201.
131. Korzenik, J.R. and B.K. Dieckgraefe, *An open-labelled study of granulocyte colony-stimulating factor in the treatment of active Crohn's disease.* Alimentary pharmacology & therapeutics, 2005. **21**(4): p. 391-400.
132. Sandborn, W.J., et al., *Repifermin (keratinocyte growth factor-2) for the treatment of active ulcerative colitis: a randomized, double-blind, placebo-controlled, dose-escalation trial.* Alimentary pharmacology & therapeutics, 2003. **17**(11): p. 1355-64.
133. Han, D.S., et al., *Keratinocyte growth factor-2 (FGF-10) promotes healing of experimental small intestinal ulceration in rats.* American journal of physiology. Gastrointestinal and liver physiology, 2000. **279**(5): p. G1011-22.
134. Roth, L., et al., *Sargramostim (GM-CSF) for induction of remission in Crohn's disease.* Cochrane database of systematic reviews, 2011(11): p. CD008538.
135. Brown, G.L., et al., *Enhancement of wound healing by topical treatment with epidermal growth factor.* The New England journal of medicine, 1989. **321**(2): p. 76-9.
136. Cohen, I.K., et al., *Topical application of epidermal growth factor onto partial-thickness wounds in human volunteers does not enhance reepithelialization.* Plastic and reconstructive surgery, 1995. **96**(2): p. 251-4.
137. Lindsay, C.D., *Novel therapeutic strategies for acute lung injury induced by lung damaging agents: the potential role of growth factors as treatment options.* Human & experimental toxicology, 2011. **30**(7): p. 701-24.
138. Ware, L.B. and M.A. Matthay, *The acute respiratory distress syndrome.* The New England journal of medicine, 2000. **342**(18): p. 1334-49.
139. Haahtela, T., et al., *Comparison of a beta 2-agonist, terbutaline, with an inhaled corticosteroid, budesonide, in newly detected asthma.* The New England journal of medicine, 1991. **325**(6): p. 388-92.
140. Lundgren, R., et al., *Morphological studies of bronchial mucosal biopsies from asthmatics before and after ten years of treatment with inhaled steroids.* The European respiratory journal, 1988. **1**(10): p. 883-9.
141. Dorscheid, D.R., et al., *Apoptosis of airway epithelial cells induced by corticosteroids.* American journal of respiratory and critical care medicine, 2001. **164**(10 Pt 1): p. 1939-47.
142. White, S.R. and D.R. Dorscheid, *Corticosteroid-induced apoptosis of airway epithelium: a potential mechanism for chronic airway epithelial damage in asthma.* Chest, 2002. **122**(6 Suppl): p. 278S-284S.

143. Gospodarowicz, D., *Localisation of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth.* Nature, 1974. **249**(453): p. 123-7.
144. Beenken, A. and M. Mohammadi, *The FGF family: biology, pathophysiology and therapy.* Nature reviews. Drug discovery, 2009. **8**(3): p. 235-53.
145. Eswarakumar, V.P., I. Lax, and J. Schlessinger, *Cellular signaling by fibroblast growth factor receptors.* Cytokine & growth factor reviews, 2005. **16**(2): p. 139-49.
146. Trueb, B., *Biology of FGFRL1, the fifth fibroblast growth factor receptor.* Cellular and molecular life sciences : CMLS, 2011. **68**(6): p. 951-64.
147. Rubin, J.S., et al., *Purification and characterization of a newly identified growth factor specific for epithelial cells.* Proc.Natl.Acad.Sci.U.S.A, 1989. **86**(3): p. 802-806.
148. Smola, H., G. Thiekotter, and N.E. Fusenig, *Mutual induction of growth factor gene expression by epidermal-dermal cell interaction.* The Journal of cell biology, 1993. **122**(2): p. 417-29.
149. Winkles, J.A., et al., *Differential expression of the keratinocyte growth factor (KGF) and KGF receptor genes in human vascular smooth muscle cells and arteries.* Journal of cellular physiology, 1997. **173**(3): p. 380-6.
150. Boismenu, R. and W.L. Havran, *Modulation of epithelial cell growth by intraepithelial gamma delta T cells.* Science, 1994. **266**(5188): p. 1253-5.
151. Parrott, J.A., et al., *Expression and action of keratinocyte growth factor (KGF) in normal ovarian surface epithelium and ovarian cancer.* Molecular and cellular endocrinology, 2000. **167**(1-2): p. 77-87.
152. Ka, H., et al., *Keratinocyte growth factor: expression by endometrial epithelia of the porcine uterus.* Biology of reproduction, 2000. **62**(6): p. 1772-8.
153. Weng, J., et al., *Hepatocyte growth factor, keratinocyte growth factor, and other growth factor-receptor systems in the lens.* Investigative ophthalmology & visual science, 1997. **38**(8): p. 1543-54.
154. Brauchle, M., et al., *Large induction of keratinocyte growth factor expression by serum growth factors and pro-inflammatory cytokines in cultured fibroblasts.* Oncogene, 1994. **9**(11): p. 3199-204.
155. Chedid, M., et al., *Regulation of keratinocyte growth factor gene expression by interleukin 1.* The Journal of biological chemistry, 1994. **269**(14): p. 10753-7.
156. Chedid, M., et al., *Glucocorticoids inhibit keratinocyte growth factor production in primary dermal fibroblasts.* Endocrinology, 1996. **137**(6): p. 2232-7.
157. Brauchle, M., R. Fassler, and S. Werner, *Suppression of keratinocyte growth factor expression by glucocorticoids in vitro and during wound healing.* The Journal of investigative dermatology, 1995. **105**(4): p. 579-84.
158. Miki, T., et al., *Determination of ligand-binding specificity by alternative splicing: two distinct growth factor receptors encoded by a single gene.* Proceedings of the National Academy of Sciences of the United States of America, 1992. **89**(1): p. 246-50.
159. Finch, P.W., et al., *Pattern of keratinocyte growth factor and keratinocyte growth factor receptor expression during mouse fetal development suggests a role in mediating morphogenetic mesenchymal-epithelial interactions.* Developmental dynamics : an official publication of the American Association of Anatomists, 1995. **203**(2): p. 223-40.
160. Fehrenbach, H., et al., *Keratinocyte growth factor-induced hyperplasia of rat alveolar type II cells in vivo is resolved by differentiation into type I cells and by apoptosis.* Eur.Respir.J., 1999. **14**(3): p. 534-544.
161. Yano, T., et al., *KGF regulates pulmonary epithelial proliferation and surfactant protein gene expression in adult rat lung.* Am.J.Physiol Lung Cell Mol.Physiol, 2000. **279**(6): p. L1146-L1158.
162. Galiacy, S., et al., *Keratinocyte growth factor promotes cell motility during alveolar epithelial repair in vitro.* Experimental cell research, 2003. **283**(2): p. 215-29.

163. Michelson, P.H., et al., *Keratinocyte growth factor stimulates bronchial epithelial cell proliferation in vitro and in vivo.* Am.J.Physiol, 1999. **277**(4 Pt 1): p. L737-L742.
164. Waters, C.M. and U. Savla, *Keratinocyte growth factor accelerates wound closure in airway epithelium during cyclic mechanical strain.* J.Cell Physiol, 1999. **181**(3): p. 424-432.
165. Xu, X., et al., *KGF increases SP-A and SP-D mRNA levels and secretion in cultured rat alveolar type II cells.* Am.J.Respir.Cell Mol.Biol., 1998. **18**(2): p. 168-178.
166. Mouhieddine-Gueddiche, O.B., et al., *Dexamethasone potentiates keratinocyte growth factor-stimulated SP-A and SP-B gene expression in alveolar epithelial cells.* Pediatr.Res., 2003. **53**(2): p. 231-239.
167. Ikegami, M., A.H. Jobe, and A.M. Havill, *Keratinocyte growth factor increases surfactant pool sizes in premature rabbits.* Am.J.Respir.Crit Care Med., 1997. **155**(3): p. 1155-1158.
168. Sugahara, K., et al., *Keratinocyte growth factor increases mRNAs for SP-A and SP-B in adult rat alveolar type II cells in culture.* Am.J.Physiol, 1995. **269**(3 Pt 1): p. L344-L350.
169. Fehrenbach, H., et al., *Keratinocyte growth factor-induced proliferation of rat airway epithelium is restricted to Clara cells in vivo.* The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology, 2002. **20**(5): p. 1185-97.
170. Gesche, J., et al., *rhKGF stimulates lung surfactant production in neonatal rats in vivo.* Pediatric pulmonology, 2011. **46**(9): p. 882-95.
171. Guery, B.P., et al., *Keratinocyte growth factor increases transalveolar sodium reabsorption in normal and injured rat lungs.* American journal of respiratory and critical care medicine, 1997. **155**(5): p. 1777-84.
172. Wang, Y., et al., *Alveolar epithelial fluid transport can be simultaneously upregulated by both KGF and beta-agonist therapy.* J.Appl.Physiol, 1999. **87**(5): p. 1852-1860.
173. Takeoka, M., et al., *KGF facilitates repair of radiation-induced DNA damage in alveolar epithelial cells.* Am.J.Physiol, 1997. **272**(6 Pt 1): p. L1174-L1180.
174. Wu, K.I., et al., *Keratinocyte growth factor promotes alveolar epithelial cell DNA repair after H2O2 exposure.* Am.J.Physiol, 1998. **275**(4 Pt 1): p. L780-L787.
175. Buckley, S., et al., *Apoptosis and DNA damage in type 2 alveolar epithelial cells cultured from hyperoxic rats.* The American journal of physiology, 1998. **274**(5 Pt 1): p. L714-20.
176. Simonet, W.S., et al., *Pulmonary malformation in transgenic mice expressing human keratinocyte growth factor in the lung.* Proceedings of the National Academy of Sciences of the United States of America, 1995. **92**(26): p. 12461-5.
177. Tichelaar, J.W., W. Lu, and J.A. Whitsett, *Conditional expression of fibroblast growth factor-7 in the developing and mature lung.* The Journal of biological chemistry, 2000. **275**(16): p. 11858-64.
178. Shiratori, M., et al., *Keratinocyte growth factor and embryonic rat lung morphogenesis.* American journal of respiratory cell and molecular biology, 1996. **15**(3): p. 328-38.
179. Post, M., et al., *Keratinocyte growth factor and its receptor are involved in regulating early lung branching.* Development, 1996. **122**(10): p. 3107-15.
180. Chelly, N., et al., *Role of keratinocyte growth factor in the control of surfactant synthesis by fetal lung mesenchyme.* Endocrinology, 2001. **142**(5): p. 1814-9.
181. Miralles, F., et al., *Signaling through fibroblast growth factor receptor 2b plays a key role in the development of the exocrine pancreas.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(11): p. 6267-72.
182. Movassat, J., et al., *Keratinocyte growth factor and beta-cell differentiation in human fetal pancreatic endocrine precursor cells.* Diabetologia, 2003. **46**(6): p. 822-9.
183. Nguyen, H.Q., et al., *Expression of keratinocyte growth factor in embryonic liver of transgenic mice causes changes in epithelial growth and differentiation resulting in polycystic kidneys and other organ malformations.* Oncogene, 1996. **12**(10): p. 2109-19.

184. Baskin, L.S., et al., *Growth factors and receptors in bladder development and obstruction.* Laboratory investigation; a journal of technical methods and pathology, 1996. **75**(2): p. 157-66.
185. Tash, J.A., et al., *Fibroblast growth factor-7 regulates stratification of the bladder urothelium.* The Journal of urology, 2001. **166**(6): p. 2536-41.
186. Matsubara, Y., et al., *Stage-specific elevated expression of the genes for hepatocyte growth factor, keratinocyte growth factor, and their receptors during the morphogenesis and differentiation of rat stomach mucosa.* Biochemical and biophysical research communications, 1996. **222**(3): p. 669-77.
187. Chailler, P., et al., *Functional characterization of the keratinocyte growth factor system in human fetal gastrointestinal tract.* Pediatric research, 2000. **48**(4): p. 504-10.
188. Guo, L., Q.C. Yu, and E. Fuchs, *Targeting expression of keratinocyte growth factor to keratinocytes elicits striking changes in epithelial differentiation in transgenic mice.* The EMBO journal, 1993. **12**(3): p. 973-86.
189. Sugimura, Y., et al., *Keratinocyte growth factor (KGF) can replace testosterone in the ductal branching morphogenesis of the rat ventral prostate.* The International journal of developmental biology, 1996. **40**(5): p. 941-51.
190. Alarid, E.T., et al., *Keratinocyte growth factor functions in epithelial induction during seminal vesicle development.* Proceedings of the National Academy of Sciences of the United States of America, 1994. **91**(3): p. 1074-8.
191. Guo, L., L. Degenstein, and E. Fuchs, *Keratinocyte growth factor is required for hair development but not for wound healing.* Genes & development, 1996. **10**(2): p. 165-75.
192. Richardson, G.D., et al., *KGF and EGF signalling block hair follicle induction and promote interfollicular epidermal fate in developing mouse skin.* Development, 2009. **136**(13): p. 2153-64.
193. Werner, S., et al., *The function of KGF in morphogenesis of epithelium and reepithelialization of wounds.* Science, 1994. **266**(5186): p. 819-22.
194. Qiao, J., et al., *FGF-7 modulates ureteric bud growth and nephron number in the developing kidney.* Development, 1999. **126**(3): p. 547-54.
195. Lovicu, F.J., W.W. Kao, and P.A. Overbeek, *Ectopic gland induction by lens-specific expression of keratinocyte growth factor (FGF-7) in transgenic mice.* Mechanisms of development, 1999. **88**(1): p. 43-53.
196. Finch, P.W. and J.S. Rubin, *Keratinocyte growth factor/fibroblast growth factor 7, a homeostatic factor with therapeutic potential for epithelial protection and repair.* Advances in cancer research, 2004. **91**: p. 69-136.
197. Mason, I.J., et al., *FGF-7 (keratinocyte growth factor) expression during mouse development suggests roles in myogenesis, forebrain regionalisation and epithelial-mesenchymal interactions.* Mechanisms of development, 1994. **45**(1): p. 15-30.
198. Bellusci, S., et al., *Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung.* Development, 1997. **124**(23): p. 4867-78.
199. Thomson, A.A. and G.R. Cunha, *Prostatic growth and development are regulated by FGF10.* Development, 1999. **126**(16): p. 3693-701.
200. Werner, S., et al., *Large induction of keratinocyte growth factor expression in the dermis during wound healing.* Proceedings of the National Academy of Sciences of the United States of America, 1992. **89**(15): p. 6896-900.
201. Marchese, C., et al., *Modulation of keratinocyte growth factor and its receptor in reepithelializing human skin.* The Journal of experimental medicine, 1995. **182**(5): p. 1369-76.
202. Werner, S., et al., *Induction of keratinocyte growth factor expression is reduced and delayed during wound healing in the genetically diabetic mouse.* The Journal of investigative dermatology, 1994. **103**(4): p. 469-73.

203. Finch, P.W., et al., *Altered expression of keratinocyte growth factor and its receptor in psoriasis.* The American journal of pathology, 1997. **151**(6): p. 1619-28.
204. Kovacs, D., et al., *Immunohistochemical analysis of keratinocyte growth factor and fibroblast growth factor 10 expression in psoriasis.* Experimental dermatology, 2005. **14**(2): p. 130-7.
205. Baskin, L.S., et al., *Growth factors in bladder wound healing.* The Journal of urology, 1997. **157**(6): p. 2388-95.
206. Ichimura, T., et al., *Induction of FGF-7 after kidney damage: a possible paracrine mechanism for tubule repair.* The American journal of physiology, 1996. **271**(5 Pt 2): p. F967-76.
207. Brauchle, M., et al., *Keratinocyte growth factor is highly overexpressed in inflammatory bowel disease.* The American journal of pathology, 1996. **149**(2): p. 521-9.
208. Finch, P.W., et al., *Increased expression of keratinocyte growth factor messenger RNA associated with inflammatory bowel disease.* Gastroenterology, 1996. **110**(2): p. 441-51.
209. Bajaj-Elliott, M., et al., *Keratinocyte growth factor in inflammatory bowel disease. Increased mRNA transcripts in ulcerative colitis compared with Crohn's disease in biopsies and isolated mucosal myofibroblasts.* The American journal of pathology, 1997. **151**(5): p. 1469-76.
210. Salvati, V.M., et al., *Keratinocyte growth factor and coeliac disease.* Gut, 2001. **49**(2): p. 176-81.
211. Charafeddine, L., et al., *Hyperoxia increases keratinocyte growth factor mRNA expression in neonatal rabbit lung.* Am.J.Physiol, 1999. **276**(1 Pt 1): p. L105-L113.
212. Barton, C.C., et al., *Stimulated pulmonary cell hyperplasia underlies resistance to alpha-naphthylthiourea.* Toxicology, 2000. **143**(2): p. 167-81.
213. Adamson, I.Y. and J. Bakowska, *Relationship of keratinocyte growth factor and hepatocyte growth factor levels in rat lung lavage fluid to epithelial cell regeneration after bleomycin.* The American journal of pathology, 1999. **155**(3): p. 949-54.
214. Verghese, G.M., et al., *Hepatocyte growth factor and keratinocyte growth factor in the pulmonary edema fluid of patients with acute lung injury. Biologic and clinical significance.* American journal of respiratory and critical care medicine, 1998. **158**(2): p. 386-94.
215. Stern, J.B., et al., *Keratinocyte growth factor and hepatocyte growth factor in bronchoalveolar lavage fluid in acute respiratory distress syndrome patients.* Crit Care Med., 2000. **28**(7): p. 2326-2333.
216. Limbrey, R., *Epithelial repair in allergic airways tissue*, in *Faculty of Medicine, Health and Life Sciences, School of Medicine*. 2003, University of Southampton: Southampton.
217. Panos, R.J., et al., *Intratracheal instillation of keratinocyte growth factor decreases hyperoxia-induced mortality in rats.* J.Clin.Invest, 1995. **96**(4): p. 2026-2033.
218. Barazzone, C., et al., *Keratinocyte growth factor protects alveolar epithelium and endothelium from oxygen-induced injury in mice.* Am.J.Pathol., 1999. **154**(5): p. 1479-1487.
219. Yano, T., et al., *Keratinocyte growth factor reduces lung damage due to acid instillation in rats.* Am.J.Respir.Cell Mol.Biol., 1996. **15**(4): p. 433-442.
220. Guo, J., et al., *Intravenous keratinocyte growth factor protects against experimental pulmonary injury.* Am.J.Physiol, 1998. **275**(4 Pt 1): p. L800-L805.
221. Sugahara, K., et al., *Double intratracheal instillation of keratinocyte growth factor prevents bleomycin-induced lung fibrosis in rats.* J.Pathol., 1998. **186**(1): p. 90-98.
222. Yi, E.S., et al., *Keratinocyte growth factor ameliorates radiation- and bleomycin-induced lung injury and mortality.* Am.J.Pathol., 1996. **149**(6): p. 1963-1970.
223. Mason, C.M., et al., *Keratinocyte growth factor attenuates lung leak induced by alpha-naphthylthiourea in rats.* Critical care medicine, 1996. **24**(6): p. 925-31.

224. Lesur, O., et al., *Mechanical ventilation-induced pneumoprotein CC-16 vascular transfer in rats: effect of KGF pretreatment.* Am.J.Physiol Lung Cell Mol.Physiol, 2003. **284**(2): p. L410-L419.
225. Welsh, D.A., et al., *Keratinocyte growth factor prevents ventilator-induced lung injury in an ex vivo rat model.* American journal of respiratory and critical care medicine, 2000. **162**(3 Pt 1): p. 1081-6.
226. Oswari, J., M.A. Matthay, and S.S. Margulies, *Keratinocyte growth factor reduces alveolar epithelial susceptibility to in vitro mechanical deformation.* Am.J.Physiol Lung Cell Mol.Physiol, 2001. **281**(5): p. L1068-L1077.
227. Chapman, K.E., C.M. Waters, and W.M. Miller, *Continuous exposure of airway epithelial cells to hydrogen peroxide: protection by KGF.* J.Cell Physiol, 2002. **192**(1): p. 71-80.
228. Waters, C.M., U. Savla, and R.J. Panos, *KGF prevents hydrogen peroxide-induced increases in airway epithelial cell permeability.* Am.J.Physiol, 1997. **272**(4 Pt 1): p. L681-L689.
229. Tillie-Leblond, I., et al., *Keratinocyte growth factor improves alterations of lung permeability and bronchial epithelium in allergic rats.* European Respiratory Journal, 2007. **30**(1): p. 31-39.
230. Ptitsyn, L.R., et al., *[Production of recombinant human keratinocyte growth factor in Escherichia coli cells].* Bioorganicheskaia khimiia, 1998. **24**(7): p. 523-9.
231. Luo, Y., et al., *Improved production of recombinant fibroblast growth factor 7 (FGF7/KGF) from bacteria in high magnesium chloride.* Protein expression and purification, 2004. **33**(2): p. 326-31.
232. Biovitrium, *KGF Prescribing information*. 2010.
233. Spielberger, R., et al., *Palifermin for oral mucositis after intensive therapy for hematologic cancers.* The New England journal of medicine, 2004. **351**(25): p. 2590-8.
234. Meropol, N.J., et al., *Randomized phase I trial of recombinant human keratinocyte growth factor plus chemotherapy: potential role as mucosal protectant.* J.Clin.Oncol., 2003. **21**(8): p. 1452-1458.
235. Stiff, P.J., et al., *Palifermin reduces patient-reported mouth and throat soreness and improves patient functioning in the hematopoietic stem-cell transplantation setting.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2006. **24**(33): p. 5186-93.
236. Rosen, L.S., et al., *Palifermin reduces the incidence of oral mucositis in patients with metastatic colorectal cancer treated with fluorouracil-based chemotherapy.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2006. **24**(33): p. 5194-200.
237. Brizel, D.M., et al., *Phase II study of palifermin and concurrent chemoradiation in head and neck squamous cell carcinoma.* Journal of clinical oncology : official journal of the American Society of Clinical Oncology, 2008. **26**(15): p. 2489-96.
238. Zia-Amirhosseini, P., et al., *Pharmacokinetics of palifermin administered as the standard dose and as a collapsed dose in patients with hematologic malignancies.* Pharmacotherapy, 2007. **27**(10): p. 1353-1360.
239. Jagasia, M.H., et al., *Palifermin for the reduction of acute GVHD: a randomized, double-blind, placebo-controlled trial.* Bone marrow transplantation, 2012.
240. Fitzpatrick, A.M., et al., *Heterogeneity of severe asthma in childhood: confirmation by cluster analysis of children in the National Institutes of Health/National Heart, Lung, and Blood Institute Severe Asthma Research Program.* The Journal of allergy and clinical immunology, 2011. **127**(2): p. 382-389 e1-13.
241. Gibson, P.G., J.L. Simpson, and N. Saltos, *Heterogeneity of airway inflammation in persistent asthma : evidence of neutrophilic inflammation and increased sputum interleukin-8.* Chest, 2001. **119**(5): p. 1329-36.

242. Jarjour, N.N., et al., *Severe asthma: lessons learned from the National Heart, Lung, and Blood Institute Severe Asthma Research Program.* American journal of respiratory and critical care medicine, 2012. **185**(4): p. 356-62.
243. Sterk, P.J., *Bronchial hyperresponsiveness: definition and terminology.* Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology, 1996. **7**(9 Suppl): p. 7-9.
244. Al-Muhsen, S., J.R. Johnson, and Q. Hamid, *Remodeling in asthma.* The Journal of allergy and clinical immunology, 2011. **128**(3): p. 451-62; quiz 463-4.
245. Busse, W.W., *The relationship of airway hyperresponsiveness and airway inflammation: Airway hyperresponsiveness in asthma: its measurement and clinical significance.* Chest, 2010. **138**(2 Suppl): p. 4S-10S.
246. O'Byrne, P.M., G.M. Gauvreau, and J.D. Brannan, *Provoked models of asthma: what have we learnt?* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2009. **39**(2): p. 181-92.
247. Cockcroft, D.W., *Allergen-induced increase in nonallergic airway responsiveness: a citation classic revisited.* Canadian respiratory journal : journal of the Canadian Thoracic Society, 2000. **7**(2): p. 182-7.
248. Kariyawasam, H.H., et al., *Remodeling and airway hyperresponsiveness but not cellular inflammation persist after allergen challenge in asthma.* American journal of respiratory and critical care medicine, 2007. **175**(9): p. 896-904.
249. Joos, G.F., et al., *Indirect airway challenges.* The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology, 2003. **21**(6): p. 1050-68.
250. Eggleston, P.A., A. Kagey-Sobotka, and L.M. Lichtenstein, *A comparison of the osmotic activation of basophils and human lung mast cells.* The American review of respiratory disease, 1987. **135**(5): p. 1043-8.
251. Brannan, J.D., et al., *Evidence of mast cell activation and leukotriene release after mannitol inhalation.* European Respiratory Journal, 2003. **22**(3): p. 491-496.
252. Brannan, J.D., et al., *Nedocromil sodium inhibits responsiveness to inhaled mannitol in asthmatic subjects.* Am.J.Respir.Crit Care Med., 2000. **161**(6): p. 2096-2099.
253. Anderson, S.D., et al., *Sodium cromoglycate alone and in combination with montelukast on the airway response to mannitol in asthmatic subjects.* The Journal of asthma : official journal of the Association for the Care of Asthma, 2010. **47**(4): p. 429-33.
254. Brannan, J.D., et al., *Fexofenadine decreases sensitivity to and montelukast improves recovery from inhaled mannitol.* American journal of respiratory and critical care medicine, 2001. **163**(6): p. 1420-5.
255. Singapuri, A., et al., *Mannitol and AMP do not induce bronchoconstriction in eosinophilic bronchitis: further evidence for dissociation between airway inflammation and bronchial hyperresponsiveness.* Respirology, 2010. **15**(3): p. 510-5.
256. Anderson, S.D., *Indirect challenge tests: Airway hyperresponsiveness in asthma: its measurement and clinical significance.* Chest, 2010. **138**(2 Suppl): p. 25S-30S.
257. du Toit, J.I., et al., *Airway responsiveness in asthma: bronchial challenge with histamine and 4.5% sodium chloride before and after budesonide.* Allergy and asthma proceedings : the official journal of regional and state allergy societies, 1997. **18**(1): p. 7-14.
258. Benckhuijsen, J., et al., *Differences in the effect of allergen avoidance on bronchial hyperresponsiveness as measured by methacholine, adenosine 5'-monophosphate, and exercise in asthmatic children.* Pediatric pulmonology, 1996. **22**(3): p. 147-53.
259. Doull, J., et al., *Differential inhibitory effect of regular inhaled corticosteroid on airway responsiveness to adenosine 5' monophosphate, methacholine, and bradykinin in symptomatic children with recurrent wheeze.* Pediatric pulmonology, 1997. **23**(6): p. 404-11.

260. Leff, J.A., et al., *Montelukast, a leukotriene-receptor antagonist, for the treatment of mild asthma and exercise-induced bronchoconstriction.* The New England journal of medicine, 1998. **339**(3): p. 147-52.
261. Weersink, E.J., et al., *Fluticasone propionate, salmeterol xinafoate, and their combination in the treatment of nocturnal asthma.* American journal of respiratory and critical care medicine, 1997. **155**(4): p. 1241-6.
262. Leuppi, J.D., J.D. Brannan, and S.D. Anderson, *Bronchial provocation tests: the rationale for using inhaled mannitol as a test for airway hyperresponsiveness.* Swiss medical weekly, 2002. **132**(13-14): p. 151-8.
263. Cockcroft, D.W., *Direct challenge tests: Airway hyperresponsiveness in asthma: its measurement and clinical significance.* Chest, 2010. **138**(2 Suppl): p. 18S-24S.
264. Verma, V.K., D.W. Cockcroft, and J.A. Dosman, *Airway responsiveness to inhaled histamine in chronic obstructive airways disease. Chronic bronchitis vs emphysema.* Chest, 1988. **94**(3): p. 457-61.
265. Swystun, V.A., et al., *Comparison of 3 different doses of budesonide and placebo on the early asthmatic response to inhaled allergen.* The Journal of allergy and clinical immunology, 1998. **102**(3): p. 363-7.
266. Reddel, H.K., et al., *An official American Thoracic Society/European Respiratory Society statement: asthma control and exacerbations: standardizing endpoints for clinical asthma trials and clinical practice.* American journal of respiratory and critical care medicine, 2009. **180**(1): p. 59-99.
267. Juniper, E.F., et al., *Development and validation of the Mini Asthma Quality of Life Questionnaire.* Eur Respir J, 1999. **14**(1): p. 32-8.
268. Juniper, E.F., et al., *Evaluation of impairment of health related quality of life in asthma: development of a questionnaire for use in clinical trials.* Thorax, 1992. **47**(2): p. 76-83.
269. Juniper, E.F., et al., *Determining a minimal important change in a disease-specific Quality of Life Questionnaire.* J Clin Epidemiol, 1994. **47**(1): p. 81-7.
270. Juniper, E.F., E. Ståhl, and P.M. O'Byrne, *Minimal important difference for the Asthma Control Questionnaire.* Am J Respir Crit Care Med, 2001. **163**: p. A642.
271. Taylor, D.R., et al., *Exhaled nitric oxide measurements: clinical application and interpretation.* Thorax, 2006. **61**(9): p. 817-27.
272. Massaro, A.F., et al., *Expired nitric oxide levels during treatment of acute asthma.* American journal of respiratory and critical care medicine, 1995. **152**(2): p. 800-3.
273. Schatz, M., et al., *Relationships among quality of life, severity, and control measures in asthma: an evaluation using factor analysis.* The Journal of allergy and clinical immunology, 2005. **115**(5): p. 1049-55.
274. Juniper, E.F., et al., *Asthma quality of life during 1 year of treatment with budesonide with or without formoterol.* The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology, 1999. **14**(5): p. 1038-43.
275. Osborne, M.L., et al., *Assessing future need for acute care in adult asthmatics: the Profile of Asthma Risk Study: a prospective health maintenance organization-based study.* Chest, 2007. **132**(4): p. 1151-61.
276. LaFemina, M.J., et al., *Keratinocyte growth factor enhances barrier function without altering claudin expression in primary alveolar epithelial cells.* American journal of physiology. Lung cellular and molecular physiology, 2010. **299**(6): p. L724-34.
277. Vignola, A.M., et al., *Airway inflammation in mild intermittent and in persistent asthma.* American journal of respiratory and critical care medicine, 1998. **157**(2): p. 403-9.
278. Djukanovic, R., et al., *Bronchial mucosal manifestations of atopy: a comparison of markers of inflammation between atopic asthmatics, atopic nonasthmatics and healthy controls.* The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology, 1992. **5**(5): p. 538-44.

279. Djukanovic, R., et al., *Quantitation of mast cells and eosinophils in the bronchial mucosa of symptomatic atopic asthmatics and healthy control subjects using immunohistochemistry.* The American review of respiratory disease, 1990. **142**(4): p. 863-71.
280. Bradding, P., et al., *Interleukin-4, -5, and -6 and tumor necrosis factor-alpha in normal and asthmatic airways: evidence for the human mast cell as a source of these cytokines.* American journal of respiratory cell and molecular biology, 1994. **10**(5): p. 471-80.
281. Brown, J.L., et al., *Lower airways inflammation in allergic rhinitics: a comparison with asthmatics and normal controls.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2007. **37**(5): p. 688-95.
282. Braunstahl, G.J., et al., *Mucosal and systemic inflammatory changes in allergic rhinitis and asthma: a comparison between upper and lower airways.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2003. **33**(5): p. 579-87.
283. Djukanovic, R., et al., *Montelukast added to fluticasone propionate does not alter inflammation or outcomes.* Respiratory medicine, 2010. **104**(10): p. 1425-35.
284. Wallin, A., et al., *Effect of inhaled fluticasone with and without salmeterol on airway inflammation in asthma.* The Journal of allergy and clinical immunology, 2003. **112**(1): p. 72-8.
285. Wenzel, S.E., et al., *Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics.* American journal of respiratory and critical care medicine, 1999. **160**(3): p. 1001-8.
286. Amin, K., et al., *Inflammation and structural changes in the airways of patients with atopic and nonatopic asthma. BHR Group.* American journal of respiratory and critical care medicine, 2000. **162**(6): p. 2295-301.
287. Ramsay, C.F., et al., *Oral montelukast in acute asthma exacerbations: a randomised, double-blind, placebo-controlled trial.* Thorax, 2011. **66**(1): p. 7-11.
288. Balbi, B., et al., *Bronchoalveolar lavage, sputum and exhaled clinically relevant inflammatory markers: values in healthy adults.* The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology, 2007. **30**(4): p. 769-81.
289. Bousquet, J., et al., *Eosinophilic inflammation in asthma.* The New England journal of medicine, 1990. **323**(15): p. 1033-9.
290. Booth, H., et al., *Effect of high dose inhaled fluticasone propionate on airway inflammation in asthma.* American journal of respiratory and critical care medicine, 1995. **152**(1): p. 45-52.
291. Adelroth, E., et al., *Inflammatory cells and eosinophilic activity in asthmatics investigated by bronchoalveolar lavage. The effects of antiasthmatic treatment with budesonide or terbutaline.* The American review of respiratory disease, 1990. **142**(1): p. 91-9.
292. Macedo, P., et al., *Inflammatory biomarkers in airways of patients with severe asthma compared with non-severe asthma.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2009. **39**(11): p. 1668-76.
293. Brightling, C.E., et al., *Mast-cell infiltration of airway smooth muscle in asthma.* The New England journal of medicine, 2002. **346**(22): p. 1699-705.
294. De Monchy, J.G., et al., *Bronchoalveolar eosinophilia during allergen-induced late asthmatic reactions.* The American review of respiratory disease, 1985. **131**(3): p. 373-6.
295. Liu, M.C., et al., *Immediate and late inflammatory responses to ragweed antigen challenge of the peripheral airways in allergic asthmatics. Cellular, mediator, and permeability changes.* The American review of respiratory disease, 1991. **144**(1): p. 51-8.
296. Martin, R.J., et al., *Airways inflammation in nocturnal asthma.* The American review of respiratory disease, 1991. **143**(2): p. 351-7.

297. Wenzel, S.E., *Abnormalities of cell and mediator levels in bronchoalveolar lavage fluid of patients with mild asthma.* The Journal of allergy and clinical immunology, 1996. **98**(5 Pt 2): p. S17-21; discussion S33-40.
298. Wenzel, S.E., et al., *Effect of 5-lipoxygenase inhibition on bronchoconstriction and airway inflammation in nocturnal asthma.* American journal of respiratory and critical care medicine, 1995. **152**(3): p. 897-905.
299. Wilson, J.W. and X. Li, *The measurement of reticular basement membrane and submucosal collagen in the asthmatic airway.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 1997. **27**(4): p. 363-71.
300. Hoshino, M., et al., *Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation.* The Journal of allergy and clinical immunology, 1998. **102**(5): p. 783-8.
301. Benayoun, L., et al., *Airway structural alterations selectively associated with severe asthma.* American journal of respiratory and critical care medicine, 2003. **167**(10): p. 1360-8.
302. Payne, D.N., et al., *Early thickening of the reticular basement membrane in children with difficult asthma.* American journal of respiratory and critical care medicine, 2003. **167**(1): p. 78-82.
303. Phipps, S., et al., *Acute allergen-induced airway remodeling in atopic asthma.* American journal of respiratory cell and molecular biology, 2004. **31**(6): p. 626-32.
304. Shiba, K., et al., *Structural changes of the airway wall impair respiratory function, even in mild asthma.* Chest, 2002. **122**(5): p. 1622-6.
305. Milanese, M., et al., *On the functional consequences of bronchial basement membrane thickening.* Journal of applied physiology, 2001. **91**(3): p. 1035-40.
306. Tsurikisawa, N., et al., *Bronchial reactivity to histamine is correlated with airway remodeling in adults with moderate to severe asthma.* The Journal of asthma : official journal of the Association for the Care of Asthma, 2010. **47**(8): p. 841-8.
307. Grainge, C.L., et al., *Effect of bronchoconstriction on airway remodeling in asthma.* The New England journal of medicine, 2011. **364**(21): p. 2006-15.
308. Durrani, S.R., R.K. Viswanathan, and W.W. Busse, *What effect does asthma treatment have on airway remodeling? Current perspectives.* The Journal of allergy and clinical immunology, 2011. **128**(3): p. 439-48; quiz 449-50.
309. Pavord, I.D., et al., *Airway inflammation in patients with asthma with high-fixed or low-fixed plus as-needed budesonide/formoterol.* The Journal of allergy and clinical immunology, 2009. **123**(5): p. 1083-9, 1089 e1-7.
310. Erpenbeck, V.J., N. Krug, and J.M. Hohlfeld, *Therapeutic use of surfactant components in allergic asthma.* Naunyn-Schmiedeberg's archives of pharmacology, 2009. **379**(3): p. 217-24.
311. Forbes, L.R. and A. Haczku, *SP-D and regulation of the pulmonary innate immune system in allergic airway changes.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2010. **40**(4): p. 547-62.
312. Cheng, G., et al., *Increased levels of surfactant protein A and D in bronchoalveolar lavage fluids in patients with bronchial asthma.* The European respiratory journal, 2000. **16**(5): p. 831-5.
313. van de Graaf, E.A., et al., *Surfactant protein A in bronchoalveolar lavage fluid.* The Journal of laboratory and clinical medicine, 1992. **120**(2): p. 252-63.
314. Wright, S.M., et al., *Altered airway surfactant phospholipid composition and reduced lung function in asthma.* Journal of applied physiology, 2000. **89**(4): p. 1283-92.
315. Erpenbeck, V.J., et al., *Surfactant protein levels in bronchoalveolar lavage after segmental allergen challenge in patients with asthma.* Allergy, 2006. **61**(5): p. 598-604.

316. Deb, R., et al., *Major house dust mite allergens Dermatophagoides pteronyssinus 1 and Dermatophagoides farinae 1 degrade and inactivate lung surfactant proteins A and D.* The Journal of biological chemistry, 2007. **282**(51): p. 36808-19.
317. Mouhieddine-Gueddiche, O.B., et al., *Dexamethasone potentiates keratinocyte growth factor-stimulated SP-A and SP-B gene expression in alveolar epithelial cells.* Pediatric research, 2003. **53**(2): p. 231-9.
318. Vignola, A.M., et al., *Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis.* American journal of respiratory and critical care medicine, 1997. **156**(2 Pt 1): p. 591-9.
319. Hollander, C., et al., *Serum and bronchial lavage fluid concentrations of IL-8, SLPI, sCD14 and sICAM-1 in patients with COPD and asthma.* Respiratory medicine, 2007. **101**(9): p. 1947-53.
320. Bosson, J., et al., *Ozone-induced bronchial epithelial cytokine expression differs between healthy and asthmatic subjects.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2003. **33**(6): p. 777-82.
321. Pepe, C., et al., *Differences in airway remodeling between subjects with severe and moderate asthma.* The Journal of allergy and clinical immunology, 2005. **116**(3): p. 544-9.
322. Howarth, P.H., et al., *Tumour necrosis factor (TNFalpha) as a novel therapeutic target in symptomatic corticosteroid dependent asthma.* Thorax, 2005. **60**(12): p. 1012-8.
323. Hackett, T.L., et al., *Caveolin-1 Controls Airway Epithelial Barrier Function: Implications for Asthma.* American journal of respiratory cell and molecular biology, 2013.
324. Wu, H., et al., *Keratinocyte growth factor augments pulmonary innate immunity through epithelium-driven, GM-CSF-dependent paracrine activation of alveolar macrophages.* The Journal of biological chemistry, 2011. **286**(17): p. 14932-40.
325. Hardyman, M.A., et al., *TNF-alpha-mediated bronchial barrier disruption and regulation by src-family kinase activation.* The Journal of allergy and clinical immunology, 2013.
326. Peebles, R.S., Jr., et al., *Allergen-induced changes in airway responsiveness are not related to indices of airway edema.* The Journal of allergy and clinical immunology, 2001. **107**(5): p. 805-11.
327. Kim, C.K., et al., *Bronchoalveolar lavage eosinophil cationic protein and interleukin-8 levels in acute asthma and acute bronchiolitis.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2005. **35**(5): p. 591-7.
328. Snijders, D., et al., *Markers of eosinophilic and neutrophilic inflammation in bronchoalveolar lavage of asthmatic and atopic children.* Allergy, 2010. **65**(8): p. 978-85.
329. von Ungern-Sternberg, B.S., et al., *Value of eosinophil cationic protein and tryptase levels in bronchoalveolar lavage fluid for predicting lung function impairment in anaesthetised, asthmatic children.* Anaesthesia, 2006. **61**(12): p. 1149-54.
330. Olivieri, D., et al., *Effect of short-term treatment with low-dose inhaled fluticasone propionate on airway inflammation and remodeling in mild asthma: a placebo-controlled study.* American journal of respiratory and critical care medicine, 1997. **155**(6): p. 1864-71.
331. Balzar, S., et al., *Mast cell phenotype, location, and activation in severe asthma. Data from the Severe Asthma Research Program.* American journal of respiratory and critical care medicine, 2011. **183**(3): p. 299-309.
332. Polosa, R., et al., *Release of mast-cell-derived mediators after endobronchial adenosine challenge in asthma.* American journal of respiratory and critical care medicine, 1995. **151**(3 Pt 1): p. 624-9.
333. Kraft, M., et al., *Airway tissue mast cells in persistent asthma: predictor of treatment failure when patients discontinue inhaled corticosteroids.* Chest, 2003. **124**(1): p. 42-50.

334. Ali, F.R., A.B. Kay, and M. Larche, *Airway hyperresponsiveness and bronchial mucosal inflammation in T cell peptide-induced asthmatic reactions in atopic subjects.* Thorax, 2007. **62**(9): p. 750-57.
335. Borish, L., et al., *Interleukin-10 regulation in normal subjects and patients with asthma.* The Journal of allergy and clinical immunology, 1996. **97**(6): p. 1288-96.
336. Batra, V., et al., *Bronchoalveolar lavage fluid concentrations of transforming growth factor (TGF)-beta1, TGF-beta2, interleukin (IL)-4 and IL-13 after segmental allergen challenge and their effects on alpha-smooth muscle actin and collagen III synthesis by primary human lung fibroblasts.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2004. **34**(3): p. 437-44.
337. Bossley, C.J., et al., *Pediatric severe asthma is characterized by eosinophilia and remodeling without T(H)2 cytokines.* The Journal of allergy and clinical immunology, 2012. **129**(4): p. 974-82 e13.
338. Van Vyve, T., et al., *Protein content in bronchoalveolar lavage fluid of patients with asthma and control subjects.* The Journal of allergy and clinical immunology, 1995. **95**(1 Pt 1): p. 60-8.
339. Van de Graaf, E.A., et al., *Respiratory membrane permeability and bronchial hyperreactivity in patients with stable asthma. Effects of therapy with inhaled steroids.* The American review of respiratory disease, 1991. **143**(2): p. 362-8.
340. Marguet, C., T.P. Dean, and J.O. Warner, *Soluble intercellular adhesion molecule-1 (sICAM-1) and interferon-gamma in bronchoalveolar lavage fluid from children with airway diseases.* American journal of respiratory and critical care medicine, 2000. **162**(3 Pt 1): p. 1016-22.
341. Lee, Y.C., K.T. Cheon, and Y.K. Rhee, *Changes of soluble ICAM-1 levels in serum and bronchoalveolar lavage fluid from patients with atopic bronchial asthma after allergen challenge.* The Journal of asthma : official journal of the Association for the Care of Asthma, 1997. **34**(5): p. 405-12.
342. Takahashi, N., et al., *Soluble intracellular adhesion molecule 1 in bronchoalveolar lavage fluid of allergic subjects following segmental antigen challenge.* American journal of respiratory and critical care medicine, 1994. **150**(3): p. 704-9.
343. Kim, J.S., et al., *Expression of nerve growth factor and matrix metallopeptidase-9/tissue inhibitor of metalloproteinase-1 in asthmatic patients.* The Journal of asthma : official journal of the Association for the Care of Asthma, 2013.
344. Tang, L.F., et al., *Levels of matrix metalloproteinase-9 and its inhibitor in bronchoalveolar lavage cells of asthmatic children.* Fetal and pediatric pathology, 2006. **25**(1): p. 1-7.
345. Ko, F.W., et al., *A comparison of airway and serum matrix metalloproteinase-9 activity among normal subjects, asthmatic patients, and patients with asthmatic mucus hypersecretion.* Chest, 2005. **127**(6): p. 1919-27.
346. Mattos, W., et al., *Matrix metalloproteinase-9 expression in asthma: effect of asthma severity, allergen challenge, and inhaled corticosteroids.* Chest, 2002. **122**(5): p. 1543-52.
347. Adler, K.B., P.W. Cheng, and K.C. Kim, *Characterization of guinea pig tracheal epithelial cells maintained in biphasic organotypic culture: cellular composition and biochemical analysis of released glycoconjugates.* American journal of respiratory cell and molecular biology, 1990. **2**(2): p. 145-54.
348. Whitcutt, M.J., K.B. Adler, and R. Wu, *A biphasic chamber system for maintaining polarity of differentiation of cultured respiratory tract epithelial cells.* In vitro cellular & developmental biology : journal of the Tissue Culture Association, 1988. **24**(5): p. 420-8.
349. Kondo, M., W.E. Finkbeiner, and J.H. Widdicombe, *Simple technique for culture of highly differentiated cells from dog tracheal epithelium.* The American journal of physiology, 1991. **261**(2 Pt 1): p. L106-17.

350. Yamaya, M., et al., *Differentiated structure and function of cultures from human tracheal epithelium.* The American journal of physiology, 1992. **262**(6 Pt 1): p. L713-24.
351. Pezzulo, A.A., et al., *The air-liquid interface and use of primary cell cultures are important to recapitulate the transcriptional profile of in vivo airway epithelia.* American journal of physiology. Lung cellular and molecular physiology, 2011. **300**(1): p. L25-31.
352. Kikuchi, T., et al., *Differentiation-dependent responsiveness of bronchial epithelial cells to IL-4/13 stimulation.* American journal of physiology. Lung cellular and molecular physiology, 2004. **287**(1): p. L119-26.
353. Cozens, A.L., et al., *CFTR expression and chloride secretion in polarized immortal human bronchial epithelial cells.* American journal of respiratory cell and molecular biology, 1994. **10**(1): p. 38-47.
354. Brightling, C.E., S. Saha, and F. Hollins, *Interleukin-13: prospects for new treatments.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2010. **40**(1): p. 42-9.
355. Venkayya, R., et al., *The Th2 lymphocyte products IL-4 and IL-13 rapidly induce airway hyperresponsiveness through direct effects on resident airway cells.* Am.J.Respir.Cell Mol.Biol., 2002. **26**(2): p. 202-208.
356. Yang, M., et al., *Interleukin-13 mediates airways hyperreactivity through the IL-4 receptor-alpha chain and STAT-6 independently of IL-5 and eotaxin.* American journal of respiratory cell and molecular biology, 2001. **25**(4): p. 522-30.
357. Whittaker, L., et al., *Interleukin-13 mediates a fundamental pathway for airway epithelial mucus induced by CD4 T cells and interleukin-9.* Am.J.Respir.Cell Mol.Biol., 2002. **27**(5): p. 593-602.
358. Wan, H., et al., *Der p 1 facilitates transepithelial allergen delivery by disruption of tight junctions.* J Clin Invest, 1999. **104**(1): p. 123-133.
359. Lordan, J.L., et al., *Cooperative effects of Th2 cytokines and allergen on normal and asthmatic bronchial epithelial cells.* Journal of immunology, 2002. **169**(1): p. 407-14.
360. Kicic, A., et al., *Intrinsic biochemical and functional differences in bronchial epithelial cells of children with asthma.* American journal of respiratory and critical care medicine, 2006. **174**(10): p. 1110-8.
361. Arold, S.P., N. Malavia, and S.C. George, *Mechanical compression attenuates normal human bronchial epithelial wound healing.* Respir Res, 2009. **10**: p. 9.
362. Schnackenberg, B.J., et al., *The beta-agonist isoproterenol attenuates EGF-stimulated wound closure in human airway epithelial cells.* American journal of physiology. Lung cellular and molecular physiology, 2006. **290**(3): p. L485-91.
363. Davies, D.E., *The role of the epithelium in airway remodeling in asthma.* Proceedings of the American Thoracic Society, 2009. **6**(8): p. 678-82.
364. Gomperts, B.N., et al., *Keratinocyte growth factor improves repair in the injured tracheal epithelium.* American journal of respiratory cell and molecular biology, 2007. **37**(1): p. 48-56.
365. Vermeer, P.D., et al., *Segregation of receptor and ligand regulates activation of epithelial growth factor receptor.* Nature, 2003. **422**(6929): p. 322-6.
366. Wan, H., et al., *Tight junction properties of the immortalized human bronchial epithelial cell lines Calu-3 and 16HBE14o.* The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology, 2000. **15**(6): p. 1058-68.
367. Park, J.A. and D.J. Tschumperlin, *Chronic intermittent mechanical stress increases MUC5AC protein expression.* American journal of respiratory cell and molecular biology, 2009. **41**(4): p. 459-66.
368. Tschumperlin, D.J., et al., *Mechanical stress triggers selective release of fibrotic mediators from bronchial epithelium.* Am.J.Respir.Cell Mol.Biol., 2003. **28**(2): p. 142-149.

369. Ludwig, M.S., et al., *Mechanical strain enhances proteoglycan message in fibroblasts from asthmatic subjects.* Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology, 2004. **34**(6): p. 926-30.
370. Grainge, C.L., Dennison, P., Davies D., Howarth, P.. *Compressive forces stimulate release of TGFß2 from differentiated bronchial epithelial cell cultures from asthmatic but not normal donors*, in *ERS Congress 2011*. 2011, European Respiratory Society: Amsterdam.
371. Hollander, D., *Intestinal permeability, leaky gut, and intestinal disorders.* Current gastroenterology reports, 1999. **1**(5): p. 410-6.
372. O'Brodovich, H. and G. Coates, *Pulmonary clearance of 99mTc-DTPA: a noninvasive assessment of epithelial integrity.* Lung, 1987. **165**(1): p. 1-16.
373. Kohn, H., et al., *Urine excretion of inhaled technetium-99m-DTPA: an alternative method to assess lung epithelial transport.* J.Nucl.Med., 1990. **31**(4): p. 441-449.
374. Braude, S., et al., *Comparison of lung vascular and epithelial permeability indices in the adult respiratory distress syndrome.* Am.Rev.Respir.Dis., 1986. **133**(6): p. 1002-1005.
375. Todisco, T., et al., *99mTc-DTPA-surfactant inhalation in adult respiratory distress syndrome (ARDS): a new diagnostic-therapeutic tool.* J.Aerosol Med., 1992. **5**(2): p. 113-122.
376. Chinet, T., et al., *Lung function declines in patients with pulmonary sarcoidosis and increased respiratory epithelial permeability to 99mTc-DTPA.* Am.Rev.Respir.Dis., 1990. **141**(2): p. 445-449.
377. Wells, A.U., et al., *Clearance of inhaled 99mTc-DTPA predicts the clinical course of fibrosing alveolitis.* Eur.Respir.J., 1993. **6**(6): p. 797-802.
378. Antoniou, K.M., et al., *Clearance of technetium-99m-DTPA and HRCT findings in the evaluation of patients with Idiopathic Pulmonary Fibrosis.* BMC.Pulm.Med., 2006. **6**: p. 4.
379. Mason, G.R., et al., *Rapidly reversible alterations of pulmonary epithelial permeability induced by smoking.* Chest, 1983. **83**(1): p. 6-11.
380. O'Byrne, P.M., et al., *Lung epithelial permeability: relation to nonspecific airway responsiveness.* Journal of Applied Physiology, 1984. **57**(1): p. 77-84.
381. Kennedy, S.M., et al., *Increased airway mucosal permeability of smokers. Relationship to airway reactivity.* Am.Rev.Respir.Dis., 1984. **129**(1): p. 143-148.
382. Taylor, R.G., et al., *Respiratory epithelial permeability is unrelated to bronchial reactivity and small airway function in young smokers and nonsmokers.* Eur.Respir.J., 1988. **1**(4): p. 319-323.
383. Morrison, D., et al., *Epithelial permeability, inflammation, and oxidant stress in the air spaces of smokers.* Am.J.Respir.Crit Care Med., 1999. **159**(2): p. 473-479.
384. Minty, B.D., C. Jordan, and J.G. Jones, *Rapid improvement in abnormal pulmonary epithelial permeability after stopping cigarettes.* Br.Med.J.(Clin.Res.Ed), 1981. **282**(6271): p. 1183-1186.
385. Boucher, R.C., et al., *The effect of cigarette smoke on the permeability of guinea pig airways.* Lab Invest, 1980. **43**(1): p. 94-100.
386. Olivera, D.S., et al., *Cellular mechanisms of mainstream cigarette smoke-induced lung epithelial tight junction permeability changes in vitro.* Inhal.Toxicol., 2007. **19**(1): p. 13-22.
387. Elwood, R.K., et al., *Respiratory mucosal permeability in asthma.* Am.Rev.Respir.Dis., 1983. **128**(3): p. 523-527.
388. Chan, T.B., *Pulmonary epithelial permeability in normal individuals and asthmatic patients.* Ann.Acad.Med.Singapore, 1985. **14**(3): p. 462-464.
389. Del, D.M., et al., *Lung epithelial permeability and bronchial responsiveness in subjects with stable asthma.* Chest, 1997. **111**(5): p. 1255-1260.
390. Lemarchand, P., et al., *Bronchial clearance of DTPA is increased in acute asthma but not in chronic asthma.* Am.Rev.Respir.Dis., 1992. **145**(1): p. 147-152.

391. Chen, A.C., et al., *Simultaneously evaluating the effects of one-week fluticasone propionate inhalation therapy on lung ventilation and permeability in children with asthma.* Lung, 2003. **181**(5): p. 283-289.
392. Del, D.M., et al., *The effect of inflammation on mucociliary clearance in asthma: an overview.* Chest, 2000. **118**(4): p. 1142-1149.
393. Ilowite, J.S., et al., *Permeability of the bronchial mucosa to 99mTc-DTPA in asthma.* Am.Rev.Respir.Dis., 1989. **139**(5): p. 1139-1143.
394. Bennett, W.D., *Controlled inhalation of aerosolised therapeutics.* Expert Opinion on Drug Delivery, 2005. **2**(4): p. 763-767.
395. Tossici-Bolt, L., et al., *An analytical technique to recover the third dimension in planar imaging of inhaled aerosols--2 estimation of the deposition per airway generation.* J.Aerosol Med., 2007. **20**(2): p. 127-140.
396. Hankinson, J.L., J.R. Odencrantz, and K.B. Fedan, *Spirometric reference values from a sample of the general U.S. population.* American journal of respiratory and critical care medicine, 1999. **159**(1): p. 179-87.
397. Pellegrino, R., et al., *Interpretative strategies for lung function tests.* The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology, 2005. **26**(5): p. 948-68.
398. Eliasson, O. and A.C. Degraff, Jr., *The use of criteria for reversibility and obstruction to define patient groups for bronchodilator trials. Influence of clinical diagnosis, spirometric, and anthropometric variables.* The American review of respiratory disease, 1985. **132**(4): p. 858-64.
399. Brand, P.L., et al., *Interpretation of bronchodilator response in patients with obstructive airways disease. The Dutch Chronic Non-Specific Lung Disease (CNSLD) Study Group.* Thorax, 1992. **47**(6): p. 429-36.
400. Ulrik, C.S. and V. Backer, *Nonreversible airflow obstruction in life-long nonsmokers with moderate to severe asthma.* The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology, 1999. **14**(4): p. 892-6.
401. Miller, M.R., et al., *Standardisation of spirometry.* The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology, 2005. **26**(2): p. 319-38.
402. Juniper, E.F., et al., *Identifying 'well-controlled' and 'not well-controlled' asthma using the Asthma Control Questionnaire.* Respiratory Medicine, 2006. **100**(4): p. 616-621.
403. Juniper, E.F., et al., *Measurement properties and interpretation of three shortened versions of the asthma control questionnaire.* Respiratory Medicine, 2005. **99**(5): p. 553-558.
404. Jarvis, D. and P. Burney, *ABC of allergies. The epidemiology of allergic disease.* BMJ, 1998. **316**(7131): p. 607-10.
405. Bryant, D.H., M.W. Burns, and L. Lazarus, *The correlation between skin tests, bronchial provocation tests and the serum level of IgE specific for common allergens in patients with asthma.* Clin Allergy, 1975. **5**(2): p. 145-57.
406. Reddel, H.K., et al., *Which index of peak expiratory flow is most useful in the management of stable asthma?* Am J Respir Crit Care Med, 1995. **151**(5): p. 1320-5.
407. Matsunaga, K., et al., *Peak expiratory flow variability adjusted by forced expiratory volume in one second is a good index for airway responsiveness in asthmatics.* Intern Med, 2008. **47**(12): p. 1107-12.
408. Chai, H., et al., *Standardization of bronchial inhalation challenge procedures.* The Journal of allergy and clinical immunology, 1975. **56**(4): p. 323-7.
409. Crapo, R.O., et al., *Guidelines for methacholine and exercise challenge testing - 1999.* Am J Respir Crit Care Med, 2000. **161**: p. 309-329.

410. *ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005.* Am J Respir Crit Care Med, 2005. **171**(8): p. 912-30.
411. Pisi, R., et al., *Measurement of fractional exhaled nitric oxide by a new portable device: comparison with the standard technique.* J Asthma, 2010. **47**(7): p. 805-9.
412. Chen, W., et al., *Niox and Niox Mino: comparison of exhaled NO in grass pollen allergic adult volunteers.* Allergy, 2007. **62**(5): p. 571-2.
413. *British Thoracic Society guidelines on diagnostic flexible bronchoscopy.* Thorax, 2001. **56 Suppl 1**: p. i1-21.
414. Britten, K.M., P.H. Howarth, and W.R. Roche, *Immunohistochemistry on resin sections: a comparison of resin embedding techniques for small mucosal biopsies.* Biotech Histochem, 1993. **68**(5): p. 271-80.
415. Gray, T.E., et al., *Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells.* Am.J.Respir.Cell Mol.Biol., 1996. **14**(1): p. 104-112.
416. Sanchez-Crespo, A., et al., *A novel quantitative dual-isotope method for simultaneous ventilation and perfusion lung SPET.* Eur J Nucl Med Mol Imaging, 2002. **29**(7): p. 863-75.
417. Kobayashi, H., et al., *Scatter correction by two-window method standardizes cardiac I-123 MIBG uptake in various gamma camera systems.* Ann Nucl Med, 2003. **17**(4): p. 309-13.
418. Thompson, P.J., *Drug delivery to the small airways.* Am J Respir Crit Care Med, 1998. **157**(5 Pt 2): p. S199-202.
419. Dolovich, M.A., *Influence of inspiratory flow rate, particle size, and airway caliber on aerosolized drug delivery to the lung.* Respir Care, 2000. **45**(6): p. 597-608.
420. Sarangapani, R. and A.S. Wexler, *The role of dispersion in particle deposition in human airways.* Toxicol Sci, 2000. **54**(1): p. 229-36.
421. Bhure, U.N., et al., *Lung epithelial permeability and inhaled furosemide: added dimensions in asthmatics.* Ann.Nucl.Med., 2009. **23**(6): p. 549-557.
422. Rees, P.J., et al., *Effects of histamine on lung permeability in normal and asthmatic subjects.* Thorax, 1985. **40**(8): p. 603-606.
423. Bennett, W.D. and J.S. Ilowite, *Dual pathway clearance of 99mTc-DTPA from the bronchial mucosa.* Am.Rev.Respir.Dis., 1989. **139**(5): p. 1132-1138.
424. IRCP, *2. Morphometric model.* Annals of the ICRP, 1994. **24**(1-3): p. 8-21.
425. Tatsis, G., K. Horsfield, and G. Cumming, *Distribution of dead space volume in the human lung.* Clin Sci (Lond), 1984. **67**(5): p. 493-7.
426. Usmani, O.S., M.F. Biddiscombe, and P.J. Barnes, *Regional lung deposition and bronchodilator response as a function of beta2-agonist particle size.* Am J Respir Crit Care Med, 2005. **172**(12): p. 1497-504.
427. Brand, P., et al., *Total deposition of therapeutic particles during spontaneous and controlled inhalations.* J Pharm Sci, 2000. **89**(6): p. 724-31.
428. Porsbjerg, C., et al., *Relationship between airway responsiveness to mannitol and to methacholine and markers of airway inflammation, peak flow variability and quality of life in asthma patients.* Clin Exp Allergy, 2008. **38**(1): p. 43-50.
429. Brannan, J.D., et al., *Budesonide reduces sensitivity and reactivity to inhaled mannitol in asthmatic subjects.* Respirology., 2002. **7**(1): p. 37-44.
430. Baraket, M., et al., *Is low dose inhaled corticosteroid therapy as effective for inflammation and remodeling in asthma? A randomized, parallel group study.* Respiratory research, 2012. **13**: p. 11.
431. O'Connor, B.J., S.L. Aikman, and P.J. Barnes, *Tolerance to the nonbronchodilator effects of inhaled beta 2-agonists in asthma.* The New England journal of medicine, 1992. **327**(17): p. 1204-8.

432. Koskela, H., et al., *Sensitivity and validity of three bronchial provocation tests to demonstrate the effect of inhaled corticosteroids in asthma.* Chest, 2003. **124**(4): p. 1341-1349.
433. Vickers, A.J. and D.G. Altman, *Statistics notes: Analysing controlled trials with baseline and follow up measurements.* BMJ, 2001. **323**(7321): p. 1123-4.
434. Clearie, K.L., et al., *Fluticasone/Salmeterol combination confers benefits in people with asthma who smoke.* Chest, 2012. **141**(2): p. 330-8.
435. Cockcroft, D.W. and B.E. Davis, *The bronchoprotective effect of inhaling methacholine by using total lung capacity inspirations has a marked influence on the interpretation of the test result.* J Allergy Clin Immunol, 2006. **117**(6): p. 1244-1248.
436. Prieto, L., et al., *Effect of challenge method on sensitivity, reactivity, and maximal response to methacholine.* Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology, 2006. **97**(2): p. 175-81.
437. Popa, V., *ATS guidelines for methacholine and exercise challenge testing.* American journal of respiratory and critical care medicine, 2001. **163**(1): p. 292-3.
438. Wiebicke, W., R. Jorres, and H. Magnussen, *Comparison of the effects of inhaled corticosteroids on the airway response to histamine, methacholine, hyperventilation, and sulfur dioxide in subjects with asthma.* The Journal of allergy and clinical immunology, 1990. **86**(6 Pt 1): p. 915-23.
439. Freezer, N.J., et al., *Effect of regular inhaled beclomethasone on exercise and methacholine airway responses in school children with recurrent wheeze.* The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology, 1995. **8**(9): p. 1488-93.
440. Corsico, A., et al., *Effects of inhaled steroids on methacholine-induced bronchoconstriction and gas trapping in mild asthma.* The European respiratory journal : official journal of the European Society for Clinical Respiratory Physiology, 2000. **15**(4): p. 687-92.
441. Booms, P., et al., *Protective effect of inhaled budesonide against unlimited airway narrowing to methacholine in atopic patients with asthma.* The Journal of allergy and clinical immunology, 1997. **99**(3): p. 330-7.
442. Kidney, J.C., et al., *Evaluation of single-dose inhaled corticosteroid activity with an allergen challenge model.* The Journal of allergy and clinical immunology, 1997. **100**(1): p. 65-70.
443. Vathenen, A.S., et al., *Time course of change in bronchial reactivity with an inhaled corticosteroid in asthma.* The American review of respiratory disease, 1991. **143**(6): p. 1317-21.
444. Joyce, D.P., et al., *The placebo effect in asthma drug therapy trials: a meta-analysis.* The Journal of asthma : official journal of the Association for the Care of Asthma, 2000. **37**(4): p. 303-18.
445. Kemeny, M.E., et al., *Placebo response in asthma: a robust and objective phenomenon.* The Journal of allergy and clinical immunology, 2007. **119**(6): p. 1375-81.
446. Wise, R.A., et al., *Randomized trial of the effect of drug presentation on asthma outcomes: the American Lung Association Asthma Clinical Research Centers.* The Journal of allergy and clinical immunology, 2009. **124**(3): p. 436-44, 444e1-8.
447. Gelb, A.F., et al., *Role of spirometry and exhaled nitric oxide to predict exacerbations in treated asthmatics.* Chest, 2006. **129**(6): p. 1492-9.
448. Kitch, B.T., et al., *A single measure of FEV1 is associated with risk of asthma attacks in long-term follow-up.* Chest, 2004. **126**(6): p. 1875-82.
449. Fuhlbrigge, A.L., et al., *FEV(1) is associated with risk of asthma attacks in a pediatric population.* The Journal of allergy and clinical immunology, 2001. **107**(1): p. 61-7.
450. Sharek, P.J., et al., *Agreement among measures of asthma status: a prospective study of low-income children with moderate to severe asthma.* Pediatrics, 2002. **110**(4): p. 797-804.

451. Moy, M.L., et al., *Clinical predictors of health-related quality of life depend on asthma severity.* American journal of respiratory and critical care medicine, 2001. **163**(4): p. 924-9.
452. Lehtimaki, L., et al., *Peripheral inflammation in patients with asthmatic symptoms but normal lung function.* The Journal of asthma : official journal of the Association for the Care of Asthma, 2005. **42**(7): p. 605-9.
453. Szefler, S.J., et al., *Asthma outcomes: biomarkers.* The Journal of allergy and clinical immunology, 2012. **129**(3 Suppl): p. S9-23.
454. Warke, T.J., et al., *Exhaled nitric oxide correlates with airway eosinophils in childhood asthma.* Thorax, 2002. **57**(5): p. 383-7.
455. Jatakanon, A., et al., *Correlation between exhaled nitric oxide, sputum eosinophils, and methacholine responsiveness in patients with mild asthma.* Thorax, 1998. **53**(2): p. 91-5.
456. Payne, D.N., et al., *Relationship between exhaled nitric oxide and mucosal eosinophilic inflammation in children with difficult asthma, after treatment with oral prednisolone.* American journal of respiratory and critical care medicine, 2001. **164**(8 Pt 1): p. 1376-81.
457. Lim, S., et al., *Relationship between exhaled nitric oxide and mucosal eosinophilic inflammation in mild to moderately severe asthma.* Thorax, 2000. **55**(3): p. 184-8.
458. Dweik, R.A., et al., *An official ATS clinical practice guideline: interpretation of exhaled nitric oxide levels (FENO) for clinical applications.* American journal of respiratory and critical care medicine, 2011. **184**(5): p. 602-15.
459. Jindal, S.K., A.N. Aggarwal, and D. Gupta, *Diurnal variability of peak expiratory flow.* The Journal of asthma : official journal of the Association for the Care of Asthma, 2002. **39**(5): p. 363-73.
460. Nguyen, B.P., S.R. Wilson, and D.F. German, *Patients' perceptions compared with objective ratings of asthma severity.* Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology, 1996. **77**(3): p. 209-15.
461. Troyanov, S., et al., *Comparison of circadian variations using FEV1 and peak expiratory flow rates among normal and asthmatic subjects.* Thorax, 1994. **49**(8): p. 775-80.
462. Khoo, K.T. and C.K. Connolly, *A comparison of three methods of measuring broncholability in asthmatics, bronchitic cigarette smokers and normal subjects.* Respiration; international review of thoracic diseases, 1984. **45**(3): p. 219-24.
463. Ryan, G., et al., *Bronchial responsiveness to histamine: relationship to diurnal variation of peak flow rate, improvement after bronchodilator, and airway calibre.* Thorax, 1982. **37**(6): p. 423-9.
464. Oosterhoff, Y., et al., *Circadian variation in airway responsiveness to methacholine, propranolol, and AMP in atopic asthmatic subjects.* The American review of respiratory disease, 1993. **147**(3): p. 512-7.
465. Grootendorst, D.C., et al., *Comparison of inflammatory cell counts in asthma: induced sputum vs bronchoalveolar lavage and bronchial biopsies.* Clin Exp Allergy, 1997. **27**(7): p. 769-79.
466. Anderson, J.T., et al., *Elevated levels of NO are localized to distal airways in asthma.* Free Radic Biol Med, 2011. **50**(11): p. 1679-88.
467. Gorska, K., et al., *Relationship between airway inflammation and remodeling in patients with asthma and chronic obstructive pulmonary disease.* Eur J Med Res, 2009. **14 Suppl 4**: p. 90-6.
468. Najafi, N., et al., *Differential cytology of bronchoalveolar lavage fluid in asthmatic children.* Pediatr Pulmonol, 2003. **35**(4): p. 302-8.
469. Keatings, V.M., et al., *Cellular profiles in asthmatic airways: a comparison of induced sputum, bronchial washings, and bronchoalveolar lavage fluid.* Thorax, 1997. **52**(4): p. 372-4.

470. Oddera, S., et al., *Airway eosinophilic inflammation, epithelial damage, and bronchial hyperresponsiveness in patients with mild-moderate, stable asthma.* Allergy, 1996. **51**(2): p. 100-7.
471. Yildirim, A.O., et al., *Palifermin induces alveolar maintenance programs in emphysematous mice.* American journal of respiratory and critical care medicine, 2010. **181**(7): p. 705-17.
472. Baba, Y., et al., *Keratinocyte growth factor gene transduction ameliorates acute lung injury and mortality in mice.* Human gene therapy, 2007. **18**(2): p. 130-41.
473. Turcotte, H., et al., *Variability of inflammatory cell counts on bronchial biopsies of normal subjects.* Lung, 2003. **181**(1): p. 9-21.
474. Sont, J.K., et al., *Repeatability of measures of inflammatory cell number in bronchial biopsies in atopic asthma.* The European respiratory journal, 1997. **10**(11): p. 2602-8.
475. Boulet, L.P., et al., *Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma. Influence of inhaled corticosteroids.* American journal of respiratory and critical care medicine, 2000. **162**(4 Pt 1): p. 1308-13.
476. Ward, C., et al., *Airway inflammation, basement membrane thickening and bronchial hyperresponsiveness in asthma.* Thorax, 2002. **57**(4): p. 309-16.
477. Sont, J.K., et al., *Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. The AMPUL Study Group.* American journal of respiratory and critical care medicine, 1999. **159**(4 Pt 1): p. 1043-51.
478. Trigg, C.J., et al., *Placebo-controlled immunopathologic study of four months of inhaled corticosteroids in asthma.* American journal of respiratory and critical care medicine, 1994. **150**(1): p. 17-22.
479. Berger, P., et al., *Assessment of bronchial inflammation using an automated cell recognition system based on colour analysis.* The European respiratory journal, 1999. **14**(6): p. 1394-402.
480. ten Hacken, N.H., et al., *Submucosa 1.0 x 0.1 mm in size is sufficient to count inflammatory cell numbers in human airway biopsy specimens.* Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc, 1998. **11**(3): p. 292-4.
481. Jeffery, P., S. Holgate, and S. Wenzel, *Methods for the assessment of endobronchial biopsies in clinical research: application to studies of pathogenesis and the effects of treatment.* American journal of respiratory and critical care medicine, 2003. **168**(6 Pt 2): p. S1-17.
482. Sullivan, P., et al., *Variation in the measurements of basement membrane thickness and inflammatory cell number in bronchial biopsies.* The European respiratory journal, 1998. **12**(4): p. 811-5.
483. Carroll, M.L., N.G. Carroll, and A.L. James, *Do bronchial biopsies represent mast cell density in airways? A stereological study.* The European respiratory journal, 2006. **28**(3): p. 612-21.
484. Carroll, M.L., et al., *Mast cell densities in bronchial biopsies and small airways are related.* Journal of clinical pathology, 2011. **64**(5): p. 394-8.
485. Smith, D.L. and R.D. Deshazo, *Bronchoalveolar lavage in asthma. An update and perspective.* The American review of respiratory disease, 1993. **148**(2): p. 523-32.
486. Djukanovic, R., et al., *The safety aspects of fiberoptic bronchoscopy, bronchoalveolar lavage, and endobronchial biopsy in asthma.* Am Rev Respir Dis, 1991. **143**(4 Pt 1): p. 772-7.
487. Facciolongo, N., et al., *Incidence of complications in bronchoscopy. Multicentre prospective study of 20,986 bronchoscopies.* Monaldi Arch Chest Dis, 2009. **71**(1): p. 8-14.

488. Torrego, A., et al., *Expression and activation of TGF-beta isoforms in acute allergen-induced remodelling in asthma.* Thorax, 2007. **62**(4): p. 307-13.
489. Al-Alawi, M., T. Hassan, and S.H. Chotirmall, *Transforming growth factor beta and severe asthma: a perfect storm.* Respir Med, 2014. **108**(10): p. 1409-23.
490. Lee, C.G., et al., *Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury.* Annu Rev Physiol, 2011. **73**: p. 479-501.
491. Chupp, G.L., et al., *A chitinase-like protein in the lung and circulation of patients with severe asthma.* N Engl J Med, 2007. **357**(20): p. 2016-27.
492. Leonardi, S., et al., *Serum Ykl-40 in Children with Asthma.* J Biol Regul Homeost Agents, 2015. **29**(2 Suppl 1): p. 114-9.
493. Lai, T., et al., *YKL-40 is correlated with FEV1 and the asthma control test (ACT) in asthmatic patients: influence of treatment.* BMC Pulm Med, 2015. **15**: p. 1.
494. Specjalski, K., M. Chelminska, and E. Jassem, *YKL-40 protein correlates with the phenotype of asthma.* Lung, 2015. **193**(2): p. 189-94.
495. Hakonarson, H., et al., *Autocrine role of interleukin 1beta in altered responsiveness of atopic asthmatic sensitized airway smooth muscle.* J Clin Invest, 1997. **99**(1): p. 117-24.
496. Borish, L., et al., *Detection of alveolar macrophage-derived IL-1 beta in asthma. Inhibition with corticosteroids.* J Immunol, 1992. **149**(9): p. 3078-82.
497. Sousa, A.R., et al., *Expression of interleukin-1 beta (IL-1beta) and interleukin-1 receptor antagonist (IL-1ra) on asthmatic bronchial epithelium.* Am J Respir Crit Care Med, 1996. **154**(4 Pt 1): p. 1061-6.
498. Lilly, C.M., et al., *Expression of eotaxin by human lung epithelial cells: induction by cytokines and inhibition by glucocorticoids.* J Clin Invest, 1997. **99**(7): p. 1767-73.
499. Broide, D.H., et al., *Inhibition of eosinophilic inflammation in allergen-challenged, IL-1 receptor type 1-deficient mice is associated with reduced eosinophil rolling and adhesion on vascular endothelium.* Blood, 2000. **95**(1): p. 263-9.
500. Schmitz, N., M. Kurrer, and M. Kopf, *The IL-1 receptor 1 is critical for Th2 cell type airway immune responses in a mild but not in a more severe asthma model.* Eur J Immunol, 2003. **33**(4): p. 991-1000.
501. He, Y., et al., *Association between polymorphism of interleukin-1 beta and interleukin-1 receptor antagonist gene and asthma risk: a meta-analysis.* ScientificWorldJournal, 2015. **2015**: p. 685684.
502. Hosoki, K., et al., *Analysis of a Panel of 48 Cytokines in BAL Fluids Specifically Identifies IL-8 Levels as the Only Cytokine that Distinguishes Controlled Asthma from Uncontrolled Asthma, and Correlates Inversely with FEV1.* PLoS One, 2015. **10**(5): p. e0126035.
503. Shyamsundar, M., et al., *Keratinocyte growth factor promotes epithelial survival and resolution in a human model of lung injury.* Am J Respir Crit Care Med, 2014. **189**(12): p. 1520-9.
504. Farber, J.M., *Mig and IP-10: CXC chemokines that target lymphocytes.* J Leukoc Biol, 1997. **61**(3): p. 246-57.
505. Sauty, A., et al., *The T cell-specific CXC chemokines IP-10, Mig, and I-TAC are expressed by activated human bronchial epithelial cells.* J Immunol, 1999. **162**(6): p. 3549-58.
506. Fulkerson, P.C., et al., *Negative regulation of eosinophil recruitment to the lung by the chemokine monokine induced by IFN-gamma (Mig, CXCL9).* Proc Natl Acad Sci U S A, 2004. **101**(7): p. 1987-92.
507. Hartl, D., et al., *Pulmonary chemokines and their receptors differentiate children with asthma and chronic cough.* J Allergy Clin Immunol, 2005. **115**(4): p. 728-36.
508. Brasier, A.R., et al., *Molecular phenotyping of severe asthma using pattern recognition of bronchoalveolar lavage-derived cytokines.* J Allergy Clin Immunol, 2008. **121**(1): p. 30-37 e6.

509. Machura, E., et al., *Selected CC and CXC chemokines in children with atopic asthma.* Postepy Dermatol Alergol, 2016. **33**(2): p. 96-101.
510. Goss, V., A.N. Hunt, and A.D. Postle, *Regulation of lung surfactant phospholipid synthesis and metabolism.* Biochim Biophys Acta, 2013. **1831**(2): p. 448-58.
511. Wright, S.M., et al., *Altered airway surfactant phospholipid composition and reduced lung function in asthma.* J Appl Physiol (1985), 2000. **89**(4): p. 1283-92.
512. Athar, U. and T.C. Gentile, *Keratinocyte growth factor.* Expert Opin Biol Ther, 2009. **9**(6): p. 779-87.
513. She, J., et al., *KGF-2 targets alveolar epithelia and capillary endothelia to reduce high altitude pulmonary oedema in rats.* J Cell Mol Med, 2012. **16**(12): p. 3074-84.
514. Goodlad, R.A., *Quantification of epithelial cell proliferation, cell dynamics, and cell kinetics in vivo.* Wiley Interdiscip Rev Dev Biol, 2017. **6**(4).
515. Cai, Y.J., et al., *Keratinocyte growth factor up-regulates Interleukin-7 expression following intestinal ischemia/reperfusion in vitro and in vivo.* Int J Clin Exp Pathol, 2012. **5**(6): p. 569-80.
516. Visco, V., et al., *Human colon fibroblasts induce differentiation and proliferation of intestinal epithelial cells through the direct paracrine action of keratinocyte growth factor.* J Cell Physiol, 2009. **220**(1): p. 204-13.
517. Waldman, D.L., et al., *Chemical breakdown of technetium-99m DTPA during nebulization.* Journal of nuclear medicine : official publication, Society of Nuclear Medicine, 1987. **28**(3): p. 378-82.
518. Huchon, G.J., et al., *Respiratory clearance of aerosolized radioactive solutes of varying molecular weight.* Journal of nuclear medicine : official publication, Society of Nuclear Medicine, 1987. **28**(5): p. 894-902.
519. Bondesson, E., et al., *Site of deposition and absorption of an inhaled hydrophilic solute.* British journal of clinical pharmacology, 2007. **63**(6): p. 722-31.
520. Bondesson, E., et al., *Effects of sodium taurocholate on the absorption of inhaled 99mTc-DTPA.* Pharmaceutical research, 2006. **23**(9): p. 2122-8.
521. Moerlein, S.M. and M.J. Welch, *The chemistry of gallium and indium as related to radiopharmaceutical production.* International journal of nuclear medicine and biology, 1981. **8**(4): p. 277-87.
522. Djeha, A., et al., *Transferrin synthesis by macrophages: up-regulation by gamma-interferon and effect on lymphocyte proliferation.* FEMS microbiology immunology, 1992. **5**(5-6): p. 279-82.
523. Djeha, A., et al., *Cytokine-mediated regulation of transferrin synthesis in mouse macrophages and human T lymphocytes.* Blood, 1995. **85**(4): p. 1036-42.
524. Mateos, F., J.H. Brock, and J.L. Perez-Arellano, *Iron metabolism in the lower respiratory tract.* Thorax, 1998. **53**(7): p. 594-600.
525. Cockcroft, D.W., et al., *Understanding allergic asthma from allergen inhalation tests.* Can Respir J, 2007. **14**(7): p. 414-8.
526. Ray, P., *Protection of epithelial cells by keratinocyte growth factor signaling.* Proc Am Thorac Soc, 2005. **2**(3): p. 221-5.
527. Konstantinidi, E.M., et al., *Exhaled Breath Condensate: Technical and Diagnostic Aspects.* ScientificWorldJournal, 2015. **2015**: p. 435160.

Section G: Appendices

Name:_____ Subject no:

Asthma symptom diary card

Morning diary

Please do the breathing test and fill in the diary before taking your medication. Please write in the number that best describes how your asthma has been during the night and this morning (think about how your asthma has been since you filled in this diary last night).

DATE															
Peak expiratory flow rate Please record the best of three blows before you take any asthma medication															
How often were you woken by your asthma during the night? 0 not woken at all 1 once 2 a few times 3 several times 4 many times 5 a great many times 6 awake all night															
How bad were your asthma symptoms when you woke up this 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															

Bedtime diary

Please write in the number that best describes how your asthma has been during the day today (think about how your asthma has been since you filled in this diary this morning).

DATE															
Peak expiratory flow rate Please record the best of three blows before you take any asthma medication															
How limited were you in your activities today 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															
How much shortness of breath did you experience today? 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															
How much of the time did you wheeze today? 0 not at all 1 hardly any of the time 2 a little of the time 3 a moderate amount of time 4 a lot of the time 5 most of the time 6 all of the time															
Please record the total number of puffs/inhalations of bronchodilator (_____) you have used in the past 24 hrs															

Figure 39: Example Diary card for KGF clinical study